

UC Santa Barbara

UC Santa Barbara Electronic Theses and Dissertations

Title

Measurement and Control of Crossed Reduction Potentials in a Flavoprotein

Permalink

<https://escholarship.org/uc/item/7bw5x81d>

ISBN

9798186386612

Author

Jones, Benjamin Joseph

Publication Date

2026-06-15

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Santa Barbara

Measurement and Control of Crossed Reduction Potentials in a Flavoprotein

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Chemistry

by

Benjamin Joseph Jones

Committee in charge:

Professor Brandon Greene, Chair

Professor Lior Sepunaru

Professor Alison Butler

Professor Arnab Mukherjee

June 2026

The dissertation of Benjamin Joseph Jones is approved.

Lior Sepunaru

Alison Butler

Arnab Mukherjee

Brandon Greene, Committee Chair

May 2026

Measurement and Control of Crossed Reduction Potentials in a Flavoprotein

Copyright © 2026

by

Benjamin Joseph Jones

ACKNOWLEDGEMENTS

I want to thank my PhD advisor, Professor Brandon Greene, for his support and guidance over the past six years of my graduate career. I feel fortunate to have worked with a mentor whose intelligence, patience, and passion for science shaped my own development as a scientist. I would also like to acknowledge the other members of my PhD committee, Professors Alison Butler, Arnab Mukherjee, and Lior Sepunaru for the additional assistance and guidance they provided in our yearly meetings regarding my project. In particular, I would like to thank Professor Sepunaru and his former graduate student Dr. Brian Roerich who, together, taught me everything I know about electrochemical measurements. To this day, I maintain that Professor Sepunaru's class on electrochemistry was the most useful class that I have taken during my PhD.

This work would not have been possible without the contribution of the undergraduate students that I have had the privilege to mentor over these last six years. I thank Brett Haynes, Chloe Reavill, Ian O'Connor, Collin Origer, Solenn Matsuka, Sierra Wong, and Nadine Malhis for their assistance with this research. Working alongside them and watching them grow as scientists was among the most fulfilling aspects of my PhD. Teaching them how to conduct experiments in a professional research laboratory has further helped me grow as a mentor.

I also want to thank my fellow graduate students who worked in the Greene Lab. In particular, I want to acknowledge Mary Lerner who helped to establish this project and who provided early insight for the direction of our work. This project has come a long way since those early days, and none of it would have been possible without Mary's courage and

willingness to take on a new and untested project in a first-year lab. Additionally, I also want to thank Dr. Juan Carlos Cáceres for teaching me how to work in anaerobic conditions and how to conduct EPR measurements at cryogenic temperatures, both of which became critical components of this work. I thank Sichu Wang, who will continue to carry on this work when I leave. I have confidence that he will one day materialize our ambitious goal of constructing an artificial electron-bifurcating flavoprotein.

I am grateful to my collaborators Sarah Elhajj and Professor Samer Gozem at Georgia State University who provided computational results that proved invaluable to interpret the experimental results in this work.

I want to thank the many friends that I made here in Santa Barbara who have made my life outside of work enjoyable and my girlfriend Marianna Karagiannis who has stood alongside me for the adventures and hurdles I overcame in these last few years.

Lastly, I want to thank my family who raised me and encouraged me to pursue this path in life. They taught me the value of hard work and critical thinking early on. Above all, I thank them for the emotional support they provided throughout the many challenges and setbacks that I encountered during this work.

VITA OF BENJAMIN JOSEPH JONES

May 2026

EDUCATION

Bachelor of Science in Biochemistry with a Minor in Chemistry, University of California, Riverside, June 2020 (with Highest Honors)

Doctor of Philosophy in Chemistry and Biochemistry, University of California, Santa Barbara, June 2026 (expected)

PROFESSIONAL EMPLOYMENT

2025W-2026S Graduate Student Researcher

2024F CHEM 110L | Introductory Biochemistry Laboratory | Teaching Assistant

2024S CHEM 1CL | General Chemistry Laboratory | Teaching Assistant

2023W-2024W Graduate Student Researcher

2022F CHEM 110L | Introductory Biochemistry Laboratory | Teaching Assistant

2022S CHEM 1CL | General Chemistry Laboratory | Teaching Assistant

2022WCHEM 125 | Laboratory Techniques in Biochemistry | Teaching Assistant

2021F CHEM 110L | Introductory Biochemistry Laboratory | Teaching Assistant

2021S CHEM CL | General Chemistry Laboratory | Teaching Assistant

2021WCHEM BL | General Chemistry Laboratory | Teaching Assistant

2020F CHEM AL | General Chemistry Laboratory | Teaching Assistant

PUBLICATIONS

6. Wang, S.; **Jones, B. J.**; Greene, B. L. Site-specific Tuning of Flavin Reduction Potentials via N3: Insights into Aromaticity Assisted Hydrogen Bonding. (*in preparation*).
5. Jones, **B. J.**; Wang, S.; Greene, B. L. Site-specific Control of Reorganization Energy in a Crossed Potential Flavoprotein. (*in preparation*).
4. **Jones, B. J.**; Wang, S.; Greene, B. L. Modulation of N1 Increases the Energetic Span of Reduction Potentials in a Crossed Flavoprotein. (*in preparation*).
3. **Jones, B. J.**; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.
2. **Jones, B. J.**; Greene, B. L. Singlet Oxygen-Mediated Photochemical Cross-Linking of an Engineered Fluorescent Flavoprotein iLOV. *J. Biol. Chem.* **2024**, 300 (11). <https://doi.org/10.1016/j.jbc.2024.107845>.

1. Liu, S.; Yeh, C.; Reavill, C.; **Jones, B. J.**; Zou, Y.; Hai, Y. Molecular and Structural Basis for C γ -C Bond Formation by PLP-Dependent Enzyme Fub7. *Angew. Chem. Int. Ed Engl.* **2024**, 63 (13), e202317161. <https://doi.org/10.1002/anie.202317161>.

AWARDS AND FELLOWSHIPS

2025 ExFAB Fellowship

2025 B.R. Baker Memorial Award

2021 Department of Chemistry and Biochemistry Chair's Fellowship

CONFERENCE POSTERS

2025 Metals in Biology, Gordon Research Conference (GRC)

MENTORSHIP

Undergraduate Students: Brett Haynes, Chloe Reavill, Ian O'Connor, Collin Origer, Solenn Matsuka, Sierra Wong, and Nadine Malhis

ABSTRACT

Measurement and Control of Crossed Reduction Potentials in a Flavoprotein

by

Benjamin Joseph Jones

Flavoproteins comprise an estimated 1-3% of all eukaryotic and prokaryotic proteins, functioning as light-, oxygen-, and voltage-sensors, photosensitizers, catalysts, and electron-transfer reagents. These proteins contain a yellow redox-active flavin prosthetic group in the form of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), derived from riboflavin (vitamin B2). Flavins contain an aromatic isoalloxazine ring system whose redox properties are highly sensitive to the characteristics of the surrounding protein medium. Thus, they are highly versatile, as their reduction potentials can be finely tuned to match those of various substrates determined by the specific protein in which they are bound.

Depending on the specific thermodynamic arrangement of a flavin's two one-electron reduction potentials, they can be coaxed to participate in either one- or two-electron transfer reactions. While the mechanisms that flavoproteins use to modulate the reduction potentials of flavins that undergo one-electron transfer have been investigated, the thermodynamic control of flavins that preferentially mediate two-electron transfer is less clear. This is because the measurement of each of the two one-electron potentials requires the detection of a highly unstable radical called the flavin "semiquinone". Furthermore, the proteins that

contain these flavins often possess other features that make these measurements difficult, including the presence of other, often oxygen sensitive, cofactors that may interfere with measurements, low purification yields, low flavin binding, substrate-effector binding effects, and the presence of other protein subunits. In this work, to circumvent the problems that plague the measurement of natural flavoproteins, I leverage the simple engineered flavoprotein called “iLOV” to be used as a model to investigate how flavin reduction potentials are tuned more broadly. iLOV contains two glutamine residues that are hydrogen-bonded to two separate nitrogens of FMN which become protonated over the course of reduction. Through site-directed mutagenesis, various mutants were generated with different thermodynamic properties. The reduction potentials of these mutants were measured through potentiometric titrations, spectrophotometric comproportionation, and quantitative electron paramagnetic resonance (EPR). This work demonstrates that hydrogen bonding interactions at these two separate sites have distinct effects on the flavin’s two one-electron reduction potentials, laying a foundation for understanding the mechanisms that natural flavoproteins manipulate the cofactor.

TABLE OF CONTENTS

I. Flavins and Flavoproteins: The Challenge of Measuring Reduction Potential.....	1
Abstract.....	1
Tuning Flavins for Metabolic Function.....	2
Difficulties in Measuring Reduction Potentials in Crossed Flavins	7
Quantum Mechanics/Molecular Mechanics Modelling and the Pitfalls of Simulated Potentials	13
iLOV and a Better Path Forward	15
References.....	19
II. Making iLOV Work: Singlet Oxygen Mediated Cross-linking and the Myth of a Simple Model	25
Abstract.....	25
Introduction.....	26
Materials and Methods	28
Materials	28
Construction of pSUMO-iLOV Plasmid Vector	29
Site-directed Mutagenesis (SDM) of iLOV Mutants.....	30
Growth and Purification of iLOV	30
ULP1 Digestion and Loading of FMN	32
SDS-PAGE	33
Photochemical cross-linking of iLOV Mutants	34
MALDI-ToF	35

Detection of $^1\text{O}_2$	35
Polyacrylamide Gel Extraction.....	36
Peptide Mapping by UPLC-MS/MS: iLOV Exposed to Ambient Light.....	37
Peptide Mapping by UPLC-MS/MS: iLOV Actively Illuminated with Blue Light.....	40
Fluorescence Lifetime Measurements	43
Molecular Dynamics Simulations.....	44
Results.....	45
Discussion.....	54
Conclusion	58
References.....	60
Supplementary Material.....	66
III. The N5 Site: Modulating iLOV's OX/NSQ Redox Couple	84
Abstract.....	84
Introduction.....	85
Materials and Methods	86
Materials	86
Production of iLOV Mutants.....	88
Site-Directed Mutagenesis of iLOV	88
Quantum Yields, Excitation, and Emission Spectra.....	88
Photoluminescence Lifetimes.....	89
Preparation of Anaerobic iLOV HQ and OX States.....	91
Anaerobic UV-vis Measurements.....	92

Measurement of the NSQ Molar Absorptivity	93
Purifying and Characterizing Isomers of Safranine.....	94
Measurement of iLOV $E^{\circ}_{\text{OX/HQ}}$	95
Analysis of Redox Titration Spectra.....	96
Comproportionation of iLOV HQ and OX.....	98
Electron Paramagnetic Resonance (EPR).....	100
Molecular Dynamics, Hybrid Quantum Mechanical/Molecular Mechanical (QM/MM), and Alchemical Free Energy Calculations	101
Results.....	104
Discussion.....	121
Conclusion	130
References.....	131
Supplemental Information	138
IV. The N1 Site: Modulating iLOV's NSQ/NHQ Redox Couple	166
Abstract.....	166
Introduction.....	166
Materials and Methods	167
Materials	167
Production of iLOV Mutants.....	169
Site-Directed Mutagenesis of iLOV	169
Preparation of Anaerobic iLOV HQ and OX States, Anaerobic UV-vis Measurements, and Measurement of the NSQ Molar Absorptivity ..	169

Purifying and Characterizing Isomers of Phenosafranine and Anthraquinone-2-Sulfonate (AQ2S).....	169
Measurement of iLOV $E^{\circ}_{\text{OX/HQ}}$	170
Analysis of Redox Titration Spectra.....	171
Comproportionation of iLOV HQ and OX, and Quantitative Electron Paramagnetic Resonance (EPR)	171
Results.....	172
Discussion.....	180
Conclusion	182
References.....	184
Supplemental Information	187

LIST OF FIGURES

Figure I-1. Flavin redox chemistry	2
Figure I-2. The span of reduction potentials in flavoproteins	6
Figure I-3. The flavin binding pocket of iLOV	16
Figure II-1. Recombinant expression, purification, and MALDI-ToF analysis of cross-linked iLOV.	45
Figure II-2. Photochemical dimerization of iLOV	47
Figure II-3. Cross-linking of iLOV in reducing or anaerobic conditions.	48
Figure II-4. iLOV-mediated photochemical $^1\text{O}_2$ generation.	49
Figure II-5. The role of histidine in iLOV cross-linking.	51
Figure II-6. Predicted O_2 diffusion into iLOV and role of A_{40} in access to FMN cofactor ...	53
Figure III-1. Thermodynamic cycle used to computationally calculate $\Delta G^{\circ}_{\text{OX/NSQ}}$ for Q_{103}X mutants via alchemical transformations.	102
Figure III-2. Effect of Q_{103}X mutations on optical properties of iLOV	105
Figure III-3. Spectrophotometric redox titrations of iLOV	107
Figure III-4. Comproportionation and characterization of the NSQ	111
Figure III-5. Water dynamics throughout MD simulations with alchemical transformation	116
Figure IV-1. Normalized UV-vis absorption spectra of iLOV and Q_{44}X mutants.	174
Figure IV-2. Spectrophotometric redox titrations of iLOV and Q_{44}X Mutants	177
Figure IV-3. Comproportionation and characterization of the NSQ	179

LIST OF SCHEMES

Scheme I-1. A proposed redox cycle for flavin-based electron bifurcation.	5
Scheme III-1. Proposed role of hydrogen bonding to the isoalloxazine ring affecting the FMN $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ for free FMN, Q _{103A} , and Q _{103D}	122

LIST OF EQUATIONS

Equation I-1. Fermi's Golden Rule	10
Equation I-2. Classical Marcus Rate Equation	10
Equation I-3. Page-Moser-Dutton Equation	11
Equation III-1. Formula for the Calculation of Emission Quantum Yield	89
Equation III-2. Fitted Equation for Fluorescence Lifetimes	90
Equation III-3. Standard cell potential for the iLOV half-reaction in reductive titrations	96
Equation III-4. Standard cell potential for the redox indicator dye half-reaction in reductive titrations	96
Equation III-5. Linearized form of the Nernst equation used to fit reductive titration data...	97
Equation III-6. Definition of $E^{\text{iLOV}}_{\text{rel}}$	97
Equation III-7. Definition of $E^{\text{dye}}_{\text{app}}$	97
Equation III-8. Equilibrium Constant for Comproportionation Experiments	98
Equation III-9. Equation for ΔE using the Equilibrium Constant of Comproportionation	99
Equation III-10. Definition of ΔE	99
Equation III-11. $E^{\circ}_{\text{OX/NHQ}}$ as the average of the two one-electron potentials	99
Equation III-12. Definition of Reduction Potential	102

LIST OF REACTIONS

Reaction III-1. Proton-coupled half-cell reaction of iLOV in reductive titrations	96
Reaction III-2. Proton-coupled half-cell reactions for redox indicator in reductive titrations	96
Reaction III-3. Modified proton-coupled half-cell reaction of iLOV in reductive titrations .	96
Reaction III-4. Modified proton-coupled half-cell reaction for redox indicator in reductive titrations	96
Reaction III-5. Comproportionation of iLOV HQ and OX	98
Reaction III-6. Proton-coupled half-cell reaction of iLOV OX reduction in comproportionation reaction.....	99
Reaction III-7. Proton-coupled half-cell reaction of iLOV HQ oxidation in comproportionation reaction.....	99

LIST OF TABLES

Table III-1. Reduction potentials, pH dependence, and electron equivalents with the mediator of iLOV and Q _{103X} mutants determined from reductive titrations.....	108
Table III-2. ΔE , $E^{\circ}_{OX/NSQ}$, and $E^{\circ}_{NSQ/NHQ}$ values for iLOV and Q _{103X} mutants from spectrophotometric FMN _{OX} and FMN _{NHQ} comproportionation at pH 7.9.	112
Table III-3. A comparison of simulated and experimental redox potentials	119
Table IV-1. Reduction potentials, pH dependence, and electron equivalents with the indicator of iLOV and Q _{44X} mutants determined from reductive titrations.	178
Table IV-2. ΔE , $E^{\circ}_{OX/NSQ}$, and $E^{\circ}_{NSQ/NHQ}$ values for iLOV and Q _{44X} mutants from spectrophotometric FMN _{OX} and FMN _{NHQ} comproportionation at pH 7.0.	181

LIST OF SUPPLEMENTARY FIGURES

Figure II-S1. Total ion chromatograms from tryptic digests of 13 and 26 kDa bands extracted from an SDS-PAGE iLOV purification gel resolved by UPLC MS1 chromatograms.	66
Figure II-S2. UPLC-MS/MS evidence of a +32 Da modification of peptide 94-110 in the 4- hour illuminated dimeric gel extracted iLOV band.....	71
Figure II-S3. Fluorescence lifetime measurements of iLOV and iLOV ^{ΔYW} and photochemical cross-linking behavior.....	72
Figure II-S4. Investigation of •O ²⁻ contribution to photochemical cross-linking	74
Figure II-S5. SDS-PAGE analysis of illumination time-dependent photochemical cross- linking for iLOV and various histidine mutants	75
Figure II-S6. Total ion chromatograms of tryptic digests of iLOV ^{ΔH} with and without illumination.....	76
Figure II-S7. Representative UPLC-MS/MS spectra of tryptic W-peptides from the 30 min illuminated iLOV ^{ΔH}	78
Figure II-S8. RMSD of backbone atoms in iLOV over the course of 5 molecular dynamics simulations.....	79
Figure II-S9. UV-vis spectra of reconstituted iLOV, iLOV ^{ΔH} , and iLOV ^{ΔYW}	83
Figure III-S1. Purification and purity SDS-PAGE gels of iLOV and Q ₁₀₃ X variants.	139
Figure III-S2. Determination of the Q ₁₀₃ D NSQ extinction coefficient at 616 nm.	141

Figure III-S3. ^1H NMR spectra and resonance assignments of safranine isomers in d6-DMSO in the aromatic region.....	142
Figure III-S4. Pourbaix analysis of safranine isomer 3	144
Figure III-S5. Statistical clustering analysis in MD Simulations.	145
Figure III-S6. Emission-normalized absorption spectra of iLOV and Q _{103X} mutants..	146
Figure III-S7. Fluorescent lifetimes of iLOV, Q _{103X} mutants, and riboflavin.	147
Figure III-S8. Initial attempts to measure $E^{\circ}_{\text{OX/NHQ}}$ for iLOV variants using protein film voltammetry	149
Figure III-S9. Xanthine/xanthine oxidase redox titration assay to measure the $E^{\circ}_{\text{OX/NHQ}}$ couple.....	150
Figure III-S10. Inset from Figure III-3A of the main text reproduced for closer inspection.	151
Figure III-S11. Redox titrations of iLOV Q _{103X} mutants.	152
Figure III-S12. Oxidative redox titrations of iLOV with ferricyanide.	153
Figure III-S13. Radical quantification by EPR.....	154
Figure III-S14. UV-vis quantitation of NSQ formation during the time course of comproportionation experiments for iLOV and Q _{103X} mutants.	156
Figure III-S15. EPR spectrum of Q _{103D} at pH 9.25	157
Figure III-S16. Continuous wave X-band EPR spectra of the NSQ radical of each iLOV mutant.	158
Figure III-S17. MD simulations of iLOV in the OX and NSQ state.	165
Figure III-S18. Structures obtained by clustering analysis for the mutants shown in the OX state	166

Figure IV-S1. Purification and purity SDS-PAGE gels of iLOV and Q ₄₄ X variants.	189
Figure IV-S2. A cyclic voltammogram of commercially obtained phenosafranine.....	191
Figure IV-S3. Cyclic voltammograms and resultant Pourbaix diagrams from redox indicators phenosafranine and AQ2S.	192
Figure IV-S4. ¹ H NMR spectra and resonance assignments of phenosafranine and AQ2S.	193
Figure IV-S5. Redox titrations of iLOV Q ₄₄ X mutants..	195
Figure IV-S6. UV-Vis based comproportionation experiments detecting the concentration of NSQ via the absorbance at 616 nm.....	196
Figure IV-S7. Continuous wave X-band EPR spectra of the NSQ radical of each iLOV mutant.	198

LIST OF SUPPLEMENTARY TABLES

Table II-S1. Tryptic peptides of iLOV detected by UPLC-MS/MS from the SDS-PAGE gel extracted <i>monomeric</i> iLOV band following purification using Water's PLGS software.	67
Table II-S2. Tryptic peptides of iLOV detected by UPLC-MS/MS from the SDS-PAGE gel extracted <i>dimeric</i> iLOV band following purification using Water's PLGS software. ...	68
Table II-S3. Tryptic peptides of non-illuminated iLOV detected by UPLC-MS/MS from a single SDS-PAGE gel extracted <i>monomeric</i> iLOV band using the FragPipe software package.	69

Table II-S4. Tryptic peptides of 4 hr illuminated iLOV detected by UPLC-MS/MS from a single SDS-PAGE gel extracted <i>dimeric</i> iLOV band using the FragPipe software package..	70
Table II-S5. Fluorescence decay lifetimes (τ) for iLOV mutants for which tyrosines and tryptophans had been mutated to phenylalanine.....	73
Table II-S6. Tryptic peptides detected within a MS2 fragmentation range of 685-730 m/z derived from the SDS PAGE gel extracted monomeric iLOV ^{ΔH} band.	77
Table II-S7. Primers that were used in the assembly of the pSUMO (H) ₆ -SUMO-iLOV	80
Table II-S8. Primers used for site-directed mutagenesis of pSUMO (H) ₆ -SUMO-iLOV	81
Table II-S9. As purified and reconstituted FMN loading of iLOV ^{ΔYW} and iLOV ^{ΔH} variants.	82
Table III-S1. As-purified and reconstituted FMN loading of iLOV Q ₁₀₃ X variants.	138
Table III-S2. Site directed mutagenesis primer sequences for iLOV Q ₁₀₃ X variants.....	140
Table III-S3. One-dimensional ¹ H NMR spectral assignments of safranine isomers.....	143
Table III-S4. Fluorescence decay lifetimes, quantum yields, and emission maxima of iLOV and Q ₁₀₃ X variants.....	148
Table III-S5. ΔE values for each mutant using both the UV-Vis estimated extinction coefficient and using the extinction coefficient estimated using EPR radical counting for the NSQ radical.....	155
Table III-S6. EPR simulation parameters for iLOV and Q ₁₀₃ X NSQ spectra, and associated fit 68% confidence interval.....	159
Table III-S7. Free energies of mutation computed for the oxidized and neutral semiquinone states of iLOV from different replicas.....	160

Table III-S8: Reduction potentials computed for Q103X mutations using different binning for the replicas obtained in Table III-S7.....	162
Table IV-S1. As-purified and reconstituted FMN loading of iLOV Q ₄₄ X variants.....	188
Table IV-S2. Site directed mutagenesis primer sequences for iLOV variants	190
Table IV-S3. One-dimensional ¹ H NMR spectral assignments of phenosafranine and AQ2S.	194
Table IV-S4. EPR simulation parameters for iLOV and Q ₄₄ X NSQ spectra, and associated fit 68% confidence interval.	197
Table IV-S5. ΔE values for each mutant determined via comproportionation experiments monitored by UV-Vis.	199

I. Flavins and Flavoproteins: The Challenge of Measuring Reduction Potential

Part of the content of this chapter was published in Jones, B. J.; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.

ABSTRACT

Flavins are ubiquitous redox-active biomolecules serving a diverse assortment of functions in cellular metabolism⁷. All flavins, including riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), possess a characteristic conjugated isoalloxazine ring containing nitrogen and oxygen heteroatoms (**Figure I-1A**). Typically housed inside a parent “flavo”-protein as a prosthetic group, the non-covalent interactions between the polypeptide matrix and the heteroatoms of the flavin’s isoalloxazine ring give rise to the flavin’s versatility¹. With different interactions and electrostatic nature of the surrounding binding pocket, certain flavin redox states can be raised or lowered in energy relative to each other, allowing reduction-potentials to be finely tuned to match those of various substrates. Furthermore, depending on the arrangement of these potentials, a flavin can be coaxed to mediate both one- and two-electron transfer reactions^{2,3}. Because the energetics of these molecules are highly malleable, they can facilitate a wide-variety of functions including as redox-active enzyme cofactors, sensing or regulatory modules, and electron-transfer reagents⁴⁻⁶.

TUNING FLAVINS FOR METABOLIC FUNCTION

Flavins in the oxidized (OX) state can be reduced by one electron to form a stable radical semiquinone (SQ) state. This can then be reduced sequentially to form the fully reduced hydroquinone (HQ) state. Alternatively, if the SQ state is unstable, the OX state can accept an electron pair simultaneously and transition directly to HQ. Each of these three redox states have an associated pK_a , resulting in anionic (A) or neutral (N) forms that respond differently to the surrounding dielectric. Thus, depending on the pH, electron transfer between states may or may not be proton-coupled, giving rise to differences in pH-dependent reduction potentials from flavoprotein to flavoprotein (**Figure I-1A**). When in aqueous solution at neutral pH, “free” FMN exhibits what is often referred to as “crossed” or “inverted” potentials. This is an arrangement in which the OX/SQ reduction potential ($E^{\circ}_{OX/NSQ} = -238 \text{ mV}$ vs NHE) is lower than the SQ/HQ potential ($E^{\circ}_{NSQ/AHQ} = -172 \text{ mV}$

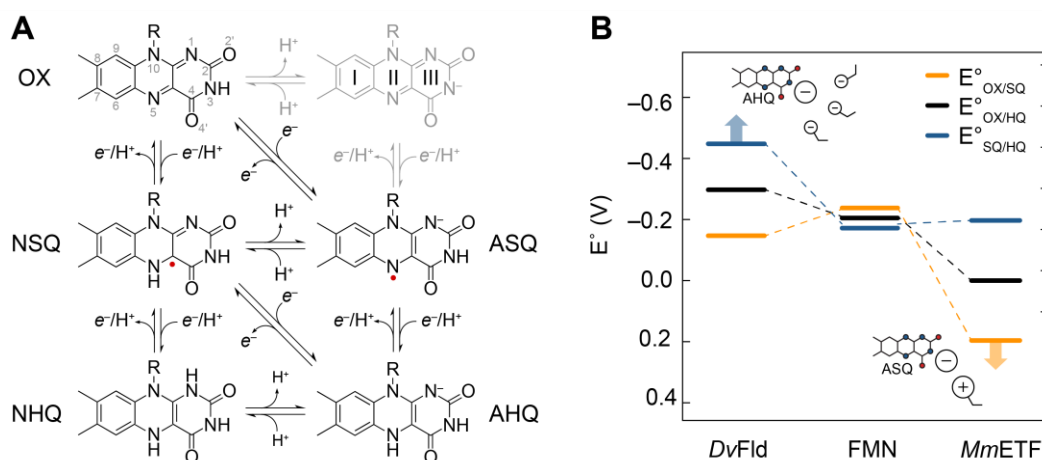


Figure I-1. Flavin redox chemistry. (A) Redox and protonation states of the flavin isoalloxazine ring. (B) A comparison of the reduction potentials of *D. vulgaris* Fld and *M. methylotrophus* ETF. Through coulombic interactions with the flavin, different redox states can be stabilized or destabilized to achieve similar ΔE but with different reduction potentials

vs NHE), and electrons are generally accepted as a pair⁸. The potential “crossing” ($\Delta E = E^{\circ}_{\text{OX/SQ}} - E^{\circ}_{\text{SQ/HQ}}$) is a modest -66 mV. Energetics of this kind result when the SQ radical is elevated in energy relative to OX and HQ (or conversely if OX and HQ are lowered in energy relative to SQ).

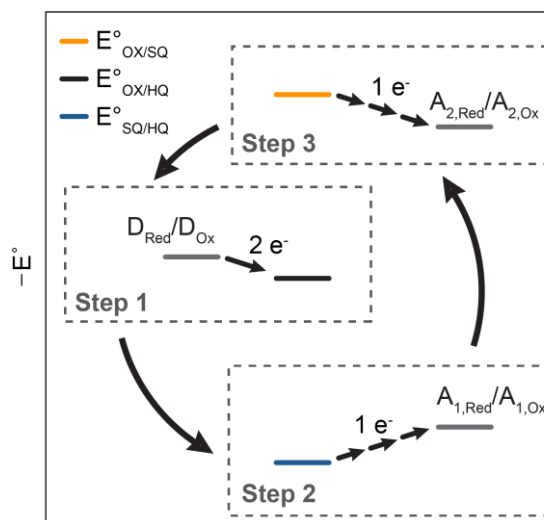
Through interactions with the polypeptide scaffold, the pK_a and energy of each redox state can be dramatically altered according to the needs of the enzymes they inhabit. In some cases, this results in a stable SQ radical. For example, the *Methylophilus methylotrophus* electron transfer flavoprotein (ETF) functions as a shuttle for a single electron between trimethylamine dehydrogenase and the electron transport chain⁹. As a result, it exhibits “normally” ordered potentials: the $E^{\circ}_{\text{OX/ASQ}}$ is higher at $+196$ mV than the $E^{\circ}_{\text{ASQ/AHQ}}$ at -197 mV and the associated ΔE is $+393$ mV. The unusually high potential of the OX/ASQ couple is matched to the iron sulfur cluster of trimethylamine dehydrogenase at $+79$ mV allowing for fluid one-electron transfer between substrates, and the lower ASQ/AHQ couple is metabolically irrelevant¹⁰. It is thought that electrostatic effects in the vicinity of the flavin binding pocket are responsible for the unique redox properties of the flavin observed in this protein. In one study, mutation of a positively charged arginine residue to a nonpolar alanine on the *si* face of the flavin isoalloxazine ring lowered the $E^{\circ}_{\text{OX/ASQ}}$ by over ~ 200 mV¹¹. The presence of this positive charge is thought to stabilize the negatively charged ASQ, lowering its energy relative to the neutral OX state.

By comparison, the *Desulfovibrio vulgaris* flavodoxin (Fld) possesses more negative reduction potentials, yet it too sports a “normal” arrangement ($E^{\circ}_{\text{OX/NSQ}} = -148$ mV and $E^{\circ}_{\text{NSQ/AHQ}} = -443$ mV with a ΔE of $+295$ mV)¹². Again, this configuration of potentials serves the flavoprotein’s purpose as a one-electron shuttle in sulfur-reducing bacteria,

transporting electrons between a hydrogenase in the outer membrane and a sulfur-reducing system¹³. In this system, mutagenesis studies have demonstrated that an asymmetric distribution of acidic residues, some of which are quite distant (>12 Å) from the flavin, play an important role in preferentially lowering the reduction potential of the NSQ/AHQ transition over that of the OX/NSQ couple¹². Under standard conditions, it is thought that these acidic residues possess a negative charge that destabilize the NSQ/AHQ transition, which is not proton-coupled and thus involves a decrease in charge. Likewise, these residues do not seem to affect the OX/NSQ couple, which is proton-coupled and charged balanced. While coulombic interactions are at play for both *D. vulgaris* Fld and *M. methylotrophus* ETF, the exact redox species being affected differs. In *D. vulgaris* Fld, the charge unbalanced AHQ is *destabilized* relative to NSQ, and in *M. methylotrophus* ETF the charge balanced ASQ is *stabilized* relative to OX (**Figure I-1B**). Through opposing methods, a similar positive ΔE is achieved to serve their purposes as one-electron shuttles, yet the exact potentials differ to match their respective substrates.

Of particular interest to my research are flavin-based electron bifurcating enzymes (FBEs), including the bifurcating electron transfer flavoproteins (Etf, descended from non-bifurcating ETF's), NADPH-dependent ferredoxin: NAD⁺ oxidoreductases (Nfn), heterodisulfide reductases (Hdr), and the “Bfu” class (formerly called “Hd”). Electron bifurcation is a process in which the oxidation of a two-electron donor at a moderate energy is coupled to the simultaneous reduction of two one-electron acceptors^{14–16}. Relative to the donor, one reduction reaction is exergonic, while the other is endergonic. Free energy released by the downhill reaction is repurposed by the enzyme to drive the uphill reaction

forward, conserving energy and producing high energy reductants necessary for metabolism. In flavin-based electron bifurcation (FBEB), the site at which electrons are “split” is a flavin cofactor. Unlike the one-electron shuttle flavoproteins mentioned above, bifurcating flavins are thought to possess potential arrangements that are *more* crossed than free flavin¹⁷. This assumption, which is mostly theoretical with some experimental backing, is motivated by the logic of a highly unstable SQ. In a scheme based on



Scheme I-1. A proposed redox cycle for flavin-based electron bifurcation. This typical bifurcation scheme is designed around the logic of crossed potentials and a highly unstable semiquinone. First, the 2-electron reduction of the OX state produces HQ. Next, the the one electron reduction of the exergonic acceptor (here depicted slightly uphill as proposed by Beretan et. al), produces a highly unstable SQ. Finally, the unstable SQ has sufficient energy to reduce the endergonic acceptor.

the Q-cycle of quinone-based electron bifurcation (QBEB, **Scheme I-1**)^{17,18}, FBEBases are theorized to operate by first accepting an electron pair from the initial donor during an OX to HQ transition. Then, a single electron is transferred to the exergonic path causing a transition from HQ to the highly unstable SQ. Dictated by the highly crossed arrangement of potentials, the SQ to OX transition should be so favorable that the endergonic acceptor spontaneously becomes reduced. Several theories, ranging from conformational gating¹⁹ to Boltzmann occupancy¹⁷, have been proposed to explain how FBEBases maintain the fidelity of electron transfer down the appropriate channels and avoid “short-circuiting”, a non-productive scenario in which the high energy electron meant for the endergonic path instead travels to the exergonic acceptor. In Etf’s, it has even been proposed that conformational

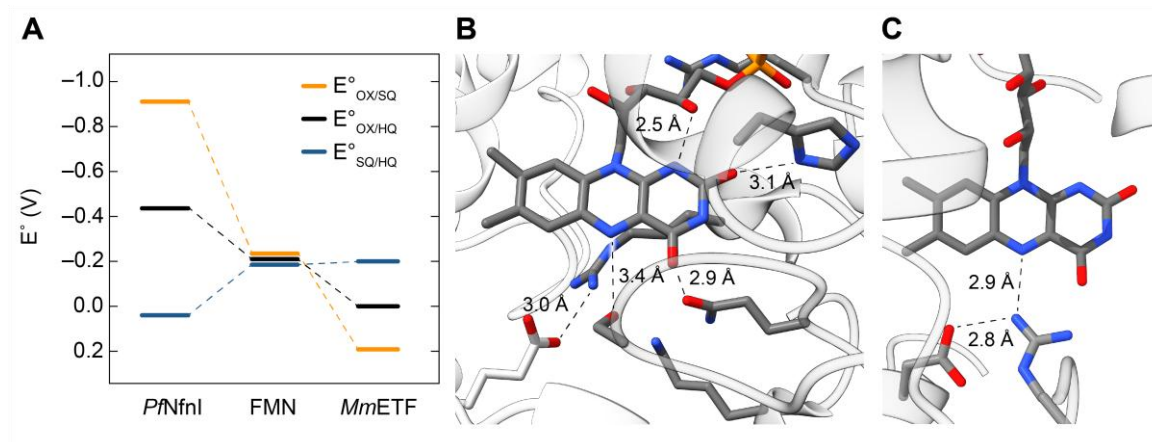


Figure I-2. The span of reduction potentials in flavoproteins. (A) A comparison of the reduction potentials of *P. furiosus* NfnI, a bifurcating flavoprotein with highly crossed potentials, and *M. methylotrophus* ETF, a one-electron shuttle with normally ordered potentials. Potentials of *M. methylotrophus* ETF are reproduced from Figure I-1B. (B) Interactions of the *Pf*NfnI (PDB ID: 5JFC) FAD cofactor with the protein environment. The N3 of *Pf*NfnI is hydrogen bonded to a valine backbone carbonyl and N1 is hydrogen bonded to the substrate nicotinamide amide oxygen of NADPH (3.2 Å, not shown). (C) Interactions of the *Mm* ETF (PDB ID: 1O96) FAD cofactor with the protein environment. Both proteins have a D/E→R salt bridge where an R guanidinium nitrogen is within 4.5 Å of the flavin N5

changes cause the reduction potentials of the bifurcating flavin to switch between crossed and uncrossed configurations over the course of catalysis^{20,21}. While the necessity of crossed potentials has been called into question²², particularly in the Bfu class of FBEBases²³, the logic of crossed potentials remains a dominant view among authors publishing on FBEB²⁴⁻²⁶.

Few potentials have been measured in FBEBases, but one study estimated that the bifurcating FAD in *Pyrococcus furiosus* NfnI, which catalyzes the NADPH-dependent reduction of both NAD⁺ (exergonic) and ferredoxin (endergonic), exhibits a ΔE of roughly -951 mV (**Figure 1.3**)^{27,28}. It's not immediately obvious how these enzymes are able to tune their flavins to such extreme lengths, but clues might be found in the flavin binding pocket. In the three classes of FBEBase which are thought to operate with crossed potential flavins, a conserved arginine or lysine residue is found in close proximity (<3.5 Å) to the N5

position of the flavin's isoalloxazine ring²⁹ and has been proposed to inhibit the formation of an NSQ³⁰, but it's unclear what role, if any, this plays in tuning the potentials of the flavin. In one mutagenesis study in an Etf-class FBEBase, it was shown that mutation of the mysterious N5 cationic residue caused either loss of flavin binding or total unfolding of the protein leaving the true role of this position ambiguous³¹. Due to difficulties in handling FBEBases and measuring the one-electron potentials of crossed flavins, the mechanisms by which FBEBases achieve their hyper-crossed potential arrangement remain a mystery.

In general, flavoproteins employ multiple mechanisms to tune the potentials of their cofactor. While I have emphasized here the role that electrostatic effects have on altering the energies of transitions that involve a change in electric charge, flavoproteins are also known to leverage sterics^{32,33}, hydrogen bonding^{34,35}, and aromaticity^{35,36} to achieve their desired effects. These alternative tools may be particularly important in tuning the potentials of flavin redox couples that involve proton transfer and do not involve changes in electric charge. In all cases, the underlying principle remains the same: a redox potential can be adjusted by raising or lowering the energy of one redox state relative to the others.

DIFFICULTIES IN MEASURING REDUCTION POTENTIALS IN CROSSED FLAVINS

Flavoprotein reduction potentials are generally measured by one of two methods: reductive titrations in the presence of a redox standard, or by protein film voltammetry (PFV). In the classic former method, the analyte of interest is mixed with an indicator of a known reduction potential to be used as a standard reference. Then, electron equivalents are titrated to the solution either by the manual addition of a strong reductant^{37,38} or by the slow enzymatic oxidation of an organic substrate such as xanthine via xanthine oxidase^{39,40}. As

the solution potential evolves, the ratio of reduced-to-oxidized forms of the analyte and indicator change. Usually, these ratios are experimentally determined by measuring the concentration of each species via UV-vis spectroscopy. Depending on the relationship between these two ratios and the known reduction potential of the indicator, the reduction potential of the analyte can be extracted. For flavoproteins with normally ordered potentials that are highly separated, the bulk ensemble of flavins will transition from OX to SQ in one phase of the titration, and then from SQ to HQ in the second phase of the titration. This is because as the solution potential evolves, the first reduction potential encountered is that of the OX/SQ transition, followed later by the second SQ/HQ couple. Consequently, both the OX/SQ and SQ/HQ couples can be measured. By contrast, in a flavin with highly crossed potentials, the SQ is unstable and two-electron transfer is favored. The first potential that would be encountered over the titration is that of the SQ/HQ transition, but for this transition to occur, some OX must have already converted to SQ. Thus, one phase representing the direct transition from OX to HQ is observed since reduction from SQ to HQ occurs rapidly after the reduction of OX to SQ for any given flavin molecule. The overall two-electron potential (the average of the two one-electron potentials) is the only value that can be extracted. If the equilibrium concentration of SQ can be measured, it is theoretically possible to extract values for the one-electron potentials of crossed flavins over the titration, but this redox species is usually only present in miniscule quantities and its concentration is often too low to measure accurately.

In the second method, PFV, the flavoprotein of interest is immobilized on a working electrode which is then immersed in an electrolyte solution^{41,42}. A potential is applied to the working electrode via a potentiostat. This working electrode potential is then scanned from

one potential to another and then back again using a standard electrode as a reference producing a cyclic voltammogram (CV). Changes in current detected from the electrode are associated with reduction and oxidation of the flavoprotein of interest and can be used to determine to reduction potential. This method offers some advantages over reductive titration such as the speed and ease at which a potential can be measured and avoids certain issues such as the need for unique spectral wavelengths with which to monitor different species in a titration, but it also introduces other problems such as uncertainty whether the measured potential reflects the true solution-state potential.⁴³ However, similar to the classical method of reductive titration, this method can only measure the one-electron potentials of normally-ordered flavins. As before, if the flavin is crossed, the reduction of SQ to HQ occurs simultaneously with the reduction of OX to SQ, and no bulk conversion to SQ is observed⁴⁴. A single two-electron peak is observed in the CV corresponding to the average of the two one-electron potentials.

Consequently, measuring the one-electron potentials of crossed flavins remains exceedingly difficult. Doing so would require one to measure the equilibrium concentrations of each redox species in solution at a given solution potential. As a flavin becomes more crossed, the equilibrium concentration of SQ shrinks. For most crossed flavoproteins, this lies far beyond the limit of detection by most methods such as UV-vis or continuous-wave EPR, even at solution potentials that favor the maximum quantity of SQ. While the mechanisms that flavoproteins use to tune normally ordered flavins are well-studied, difficulties in measuring the potentials of crossed flavins have prevented a wholistic understanding of flavin redox tuning.

Nonetheless, a few clever experiments have been designed to estimate the potentials of crossed flavins. As mentioned earlier, the ΔE of *P. furiosus* NfnI was estimated to be roughly -951 mV.^{27,28} This measurement was performed by Lubner et. al leveraging the relationship between electron-transfer kinetics and reaction thermodynamics predicted by Marcus Theory. To understand the details of Lubner's method, it is instructive to briefly highlight the quantum mechanical basis for this measurement. Fermi's Golden Rule (**equation I-1**) arises from quantum mechanical perturbation theory and states that the rate of tunneling, or electron transfer, can be represented as a product of two terms: an electronic coupling term and the Franck-Condon weighted probability of states.⁴⁵

$$k_{et} = \frac{2\pi}{\hbar} |\langle f | H' | i \rangle|^2 \times p(E_f) \quad (\text{Eq. I-1})$$

The electronic coupling term, " $|\langle f | H' | i \rangle|^2$ ", is the quantum-mechanical matrix element coupling reactant and product electronic wavefunctions, " $\langle i |$ " and " $| f \rangle$ " respectively. It depends on wavefunction overlap and decays exponentially with distance according to a decay constant called the "tunneling constant", which is proportional to the barrier height and is unique to the tunneling medium (e.g. the protein matrix). The latter term, the Franck-Condon weighted probability of states or " $p(E_f)$ ", was classically modelled by Marcus who treated solvent molecules as simple harmonic oscillators in a Boltzmann distribution.^{46,47} The mathematical result represents this term as a Gaussian containing the reorganization energy and the overall driving force for the reaction, ΔG . With some substitution, Fermi's Golden Rule can be rewritten such that the logarithm of the rate of electron transfer is directly proportional to the tunneling constant, the distance between electron transferring species, and the square of the driving force (**equation I-2**).^{45,48}

$$\ln k_{et}^{ex} = \ln \left(\frac{2\pi}{\hbar} \right) + [-\beta R \ln V_o^2] + \left[\ln \left(\frac{1}{\sqrt{4\pi\lambda kT}} \right) - \frac{(-\Delta G - \lambda)^2}{4\lambda kT} \right] \quad (\text{Eq. I-2})$$

Here, “ $\hbar$ ” is the reduced Planck’s constant, “ β ” represents the tunneling constant, “ R ” is the edge-to-edge electron transfer distance between cofactors, “ V_o^2 ” represents the quantum mechanical matrix element coupling reactant and product electronic wavefunctions *under maximal overlap*, “ ΔG ” is the free energy change of the reaction (also called the “driving-force”), “ k ” is Boltzmann’s constant, “ T ” is the temperature, and “ λ ” is the solvent reorganization energy.

In the study by Lubner *et al.*, the distance between the crossed flavin of *P. furiosus* NfnI and its electron transferring partner, a nearby iron-sulfur cluster, were known. Furthermore, the reduction potential of this proximal cluster was measured by PFV. The flavin was then photo-reduced to the ASQ state, and the rate of electron-transfer to the iron-sulfur cluster was measured. The authors then used the Page–Moser–Dutton equation (**equation I-3**), which is a version of **equation I-2** fitted to data from a survey of photosynthetic proteins with known driving forces and distances between electron-transferring cofactors⁴⁸.

$$\log_{10} k_{et}^{ex} = 13.0 - (1.2 - 0.8\rho)(R - 3.6) - \frac{3.1(\Delta G + \lambda)^2}{\lambda} \quad (Eq. I-3)$$

In this equation, “ ρ ” represents the “packing-density” of atoms as defined in the original paper by Page *et al.* and is used to estimate a tunneling constant unique to a given electron transfer pathway. With this model, the difference in energy between the iron-sulfur cluster and the one-electron OX/ASQ potential of the flavin was extracted. Knowing the 2-electron potential of the flavin from PFV, both the OX/ASQ and ASQ/AHQ potentials were estimated and thus the ΔE .

The measurement of *P. furiosus* NfnI is one of only a few examples in which the one-electron potentials of a crossed flavin have been measured. While this method is both brilliant and creative, it contains several pitfalls. In order to obtain this value, several

assumptions must be made. The Page–Moser–Dutton model requires the reorganization of energy to be known, but this value is often difficult to obtain. Instead, the authors arbitrarily assumed a value for the reorganization energy of 0.7 eV, which might be reasonable for electron-transferring cofactors in photosynthetic proteins from which the model was fit, but may not necessarily translate to flavins. For example, the reorganization energy for flavoproteins has been estimated by QM/MM simulations to be between 0.96 and 1.31 eV.⁴⁹ Furthermore, the model approximates the electronic coupling term as a decaying exponential but does not incorporate wavefunction overlap which would vary significantly depending on the relative orientation of electron transferring cofactors. If cofactors are not maximally coupled as assumed, the measured rate of electron transfer would be slower than the theoretically maximal rate, meaning that the measured ΔE would be of lower magnitude than the true value. Additionally, the equation uses the “atom packing density” along a presumed tunneling pathway to estimate a tunneling constant. This is treated as a weighted average of a maximally packed medium and a vacuum. In reality, the presence of certain secondary structures such as α -helices along the tunneling path are known to influence the rate of electron transfer significantly⁵⁰ and these effects are averaged out in this fitted model. Even if one accepts the assumptions made from the fitted theoretical model, the complexity of the experimental measurement creates additional problems. *P. furiosus* NfnI contains two FADs, and in order to deconvolute signals from the non-bifurcating FAD, the entire system was partially reduced prior to the illumination. As a result, it’s unclear whether the charge transfer event that was observed truly occurred between the iron sulfur cluster and the FAD of interest. Additionally, since this reaction was prompted by photoreduction, it’s not certain whether electron-transfer occurred through the ground state NSQ as assumed, or if this

species remained in a vibrationally excited state resulting from the excitation method. If this is true, then the reduction potentials extracted would not be representative of the ground state flavin. These issues are further exacerbated by the fact that the system never fully returned to its original state prior to illumination. Taken together, while this measurement was certainly valuable as one of the few recorded measurements of a crossed flavin, it is built upon several shaky assumptions and thus remains a rough approximation.

Given the difficulties described above, it should be clear that the mechanisms that highly crossed flavoproteins such as FBEases use to manipulate the potentials of their prosthetic group is not well understood nor studied. The fundamental instability of the SQ radical that arises from the crossed arrangement of potentials requires highly sensitive instruments to detect and presents a fundamental barrier that must be overcome when obtaining these measurements. To add to this problem, these proteins often contain multiple cofactors (including other flavins) which are difficult to deconvolute in electrochemical and UV-vis measurements, they are usually multimeric meaning that measurements are often performed on incomplete enzymes, they sometimes contain specialized cofactors that are sensitive to oxygen, and, as in the case of ETF's, the flavin binding pocket of the crossed flavin is not often amenable to mutagenesis. Clearly, another approach is needed.

QUANTUM MECHANICS/MOLECULAR MECHANICS MODELLING AND THE PITFALLS OF SIMULATED POTENTIALS

Given modern advancements in our understanding of quantum mechanics, one might ask why we cannot simply calculate the reduction potentials of crossed flavoproteins from first principles. Indeed, this has been the goal of many computational chemists employing hybrid

quantum mechanical/molecular mechanical (QM/MM) dynamics simulations, but this method is not as simple as it might seem. A protein structure, as determined by methods such as X-ray crystallography, contains a defined set of 3-dimensional coordinates for each atom in a protein. This might lead one to the misunderstanding that proteins are a static and unmoving set of atoms with which one could model molecular orbitals and their associated energies. In truth, proteins are dynamic and will explore countless different conformations as they collide with nearby solvent molecules and randomly walk about the surrounding medium. The reduction potentials of the flavin are not dependent on just one single structure, but arise out of the ensemble of structures that exist in bulk solution.^{51,52} If one is to estimate these potentials, they need to sufficiently and accurately sample the many possible conformations that a protein can occupy and model the molecular orbitals of relevant cofactors in this sample.

Modelling the electrons and orbitals of one single protein structure with thousands of atoms to any degree of accuracy is already a complex multi-bodied problem that is computationally difficult.⁵³ Accurately modelling the dynamics of this protein with just a small sample of several thousand different structures would be virtually impossible. In practice, to simulate the dynamics of protein motion with acceptable accuracy and model the reduction potentials of interesting prosthetic groups, a hybrid classical/quantum mechanical approach is taken.^{54,55} Here, only the valence orbitals of the redox active group and the atoms in the immediate vicinity are modelled under quantum mechanics, while the vast majority of protein atoms and bonds are modelled as simple-harmonic oscillators under the influence of simple electrostatics. Even with these simplifying assumptions, modelled proteins often need to be small and structured enough for high performance computers to

simulate an acceptably large sample of structures.⁵³ The calculated reduction potentials from these QM/MM simulations usually have high error and low precision when compared to measured potentials of proteins.^{49,56} This is because the simulated reduction potentials are highly sensitive to a number of factors that are difficult to model accurately. Wide amplitude motion of charged and dipolar residues can give rise to dramatic fluctuations in the electrostatic potential at the active site.⁵⁷ This necessitates larger sampling of the protein dynamics, or alternative methods entirely. The pK_a s and thus the protonation states of nearby residues can dramatically influence the potentials of the flavin, and are difficult to predict accurately.⁵⁸ Furthermore, in simulations by our collaborators, described in detailed in Chapter III, the intermittent penetration of water molecules into the flavin binding pocket was shown to dramatically increase the error in initial measurements necessitating careful binning of various dynamical behaviors with respect to water molecules.⁵⁹ In order to estimate the potentials of flavoproteins accurately, computational chemists need to develop newer methods that must be benchmarked with experimentally derived reduction potentials. This returns us back to the original problem: how can we better approach the problem of experimentally measuring the potentials of crossed flavins?

ILOV AND A BETTER PATH FORWARD

For my project, we began addressing these problems by considering a “bottom-up” approach. Instead of juggling the sensitivities and complexities of a real FBEBase, we wondered if it were possible to recreate their theorized properties in a much simpler non-bifurcating model flavoprotein, thus circumventing the obstacles that typically plague their study. Specifically, we sought to find a small, well-studied flavoprotein containing a moderately crossed flavin, with which we could mutate residues within hydrogen bonding

distance of relevant heteroatoms of the isoalloxazine ring. From this platform we could measure the potentials of various mutants, especially those with unique interactions to sites proposed to be important for bifurcation, and test existing theories via “gain-of-function” mutations. By choosing a moderately crossed flavoprotein with a single cofactor, we would be able to measure the unstable SQ by traditional UV-vis spectroscopy and employ additional methods such as quantitative EPR to probe the radical.

Our chosen platform for these studies is the “improved Light, Oxygen, and Voltage sensing” protein, “iLOV”. iLOV is an FMN-binding fluorescent protein (FbFP) that was initially engineered as an oxygen-independent alternative to green-fluorescent protein (GFP) for use as a small, genetically encoded fluorescent tag in anaerobic imaging and microscopy.⁶⁰ Originally derived from the LOV2 domain of *Arabidopsis thaliana* phototropin 2 via truncation, iLOV lacks a characteristic photoreactive cysteine residue (C40), a modification which prevents its natural function as a light, oxygen, and voltage sensor but enhances its optical capabilities. Despite lacking any natural substrates or unique catalytic capabilities, the small size and simplicity of this flavoprotein offers a convenient model from which to test theories of reduction potential tuning in flavoproteins more broadly and avoids additional problems that plague the study of more complex natural flavoenzymes. Additionally, the crystal structure of iLOV reveals a rich assortment of flavin-protein

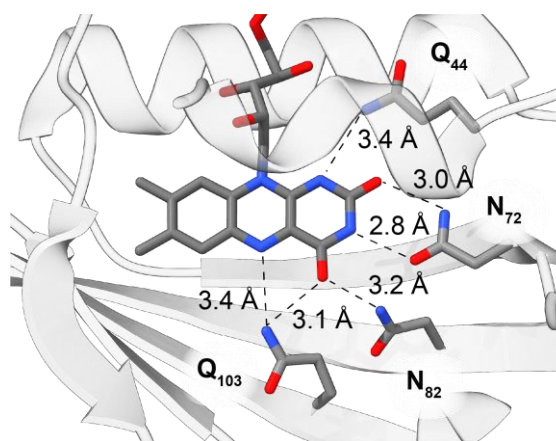


Figure I-3. The flavin binding pocket of iLOV. Hydrogen bonding interactions of iLOV FMN cofactor (PDB ID: 4EES) with the protein environment are shown. The breadth of flavin-protein hydrogen bonding interactions in iLOV make it an ideal subject for mutagenesis studies.

interactions that can be investigated through mutagenesis (**Figure I-3**).⁶¹ Our early exploratory experiments further showed that it had moderately crossed potentials with an NSQ just above the limit of detection for UV-vis spectroscopy (see Chapter III).

The following work seeks to demonstrate iLOV's utility as a model for reduction potential tuning in a crossed flavoprotein. In chapter II, the difficulties that came with managing this deceptively simple flavoprotein are highlighted. Through these struggles, we discovered unexpected side-reactions that cause undesirable aggregation when iLOV is exposed to both light and oxygen. The results of these studies allowed us to design new protocols tailored to iLOV's unique photosensitivity which are certainly relevant to those using iLOV as a fluorescent reporter. Furthermore, this work has broader appeal as one of a growing number of examples of histidine modification in the presence of reactive oxygen species (ROS).⁶² Next, after addressing these problems, we used site-directed mutagenesis to alter a glutamine residue, which forms a hydrogen bond to the flavin N5/O4' in the oxidized state, which is described in Chapter III. By measuring the potentials of a series of mutants, we were able to observe a decrease in the extent of crossing primarily caused by tuning of the OX/SQ redox couple. QM/MM simulations by our collaborators helped illuminate the mechanism of this tuning as occurring via hydrogen-bonding interactions to invasive water molecules from the surrounding solvent. Finally in Chapter IV, we migrate to a new site, N1, and investigate the effect that mutation of a second glutamine residue has on the SQ/HQ redox couple. We show that mutation of this site results in mutants with more extreme ΔE 's than that exhibited by the wild-type iLOV, necessitating a different approach to measuring SQ concentrations. These and future studies using iLOV as a model flavoprotein provide

invaluable insights to crossed-flavin redox tuning and may eventually assist our long-term goal of replicating the properties of electron bifurcation in a synthetic system.

REFERENCES

1. Stankovich, M. T. Redox Properties of Flavins and Flavoproteins. In *Chemistry and Biochemistry of Flavoenzymes*; CRC Press, 1991.
2. Walsh, C. Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry. *Acc. Chem. Res.* **1980**, *13* (5), 148–155. <https://doi.org/10.1021/ar50149a004>.
3. Massey, V. The Chemical and Biological Versatility of Riboflavin. *Biochem. Soc. Trans.* **2000**, *28* (4), 283–296. <https://doi.org/10.1042/bst0280283>.
4. Hemmerich, P.; Nagelschneider, G. Chemistry and Molecular Biology of Flavins and Flavoproteins. *FEBS Lett.* **1970**, *8* (2), 69–83. [https://doi.org/10.1016/0014-5793\(70\)80229-0](https://doi.org/10.1016/0014-5793(70)80229-0).
5. Singer, T. P.; Edmondson, D. E. Flavoproteins (Overview). *Methods Enzymol.* **1978**, *53*, 397–418. [https://doi.org/10.1016/s0076-6879\(78\)53045-0](https://doi.org/10.1016/s0076-6879(78)53045-0).
6. Fagan, R. L.; Palfey, B. A. Flavin-Dependent Enzymes. In *Comprehensive Natural Products II*; Elsevier, 2010; pp 37–113. <https://doi.org/10.1016/B978-008045382-8.00135-0>.
7. Macheroux, P.; Kappes, B.; Ealick, S. E. Flavogenomics – a Genomic and Structural View of Flavin-Dependent Proteins. *FEBS J.* **2011**, *278* (15), 2625–2634. <https://doi.org/10.1111/j.1742-4658.2011.08202.x>.
8. Draper, R. D.; Ingraham, L. L. A Potentiometric Study of the Flavin Semiquinone Equilibrium. *Arch. Biochem. Biophys.* **1968**, *125* (3), 802–808. [https://doi.org/10.1016/0003-9861\(68\)90517-1](https://doi.org/10.1016/0003-9861(68)90517-1).
9. Byron, Colleen M.; Stankovich, Marian T.; Husain, Mazhar; Davidson, Victor L.; Unusual redox properties of electron-transfer flavoprotein from *Methylophilus methylotrophus*. *Biochemistry* 1989 *28* (21), 8582–8587. DOI: 10.1021/bi00447a047
10. Davidson, V. L.; Husain, M.; Neher, J. W. Electron Transfer Flavoprotein from *Methylophilus Methylotrophus*: Properties, Comparison with Other Electron Transfer Flavoproteins, and Regulation of Expression by Carbon Source. *J. Bacteriol.* **1986**, *166* (3), 812–817. <https://doi.org/10.1128/jb.166.3.812-817.1986>.
11. Talfournier, F.; Munro, A. W.; Basran, J.; Sutcliffe, M. J.; Daff, S.; Chapman, S. K.; Scrutton, N. S. α Arg-237 in *Methylophilus Methylotrophus* (Sp. W3A1) Electron-Transferring Flavoprotein Affords ~200-Millivolt Stabilization of the FAD Anionic Semiquinone and a Kinetic Block on Full Reduction to the Dihydroquinone*. *J. Biol. Chem.* **2001**, *276* (23), 20190–20196. <https://doi.org/10.1074/jbc.M010853200>.
12. Zhou, Z.; Swenson, R. P. Electrostatic Effects of Surface Acidic Amino Acid Residues on the Oxidation-Reduction Potentials of the Flavodoxin from *Desulfovibrio Vulgaris*

- (Hildenborough). *Biochemistry* **1995**, *34* (10), 3183–3192.
<https://doi.org/10.1021/bi00010a007>.
13. Hsieh, Y.-C.; Chia, T. S.; Fun, H.-K.; Chen, C.-J. Crystal Structure of Dimeric Flavodoxin from *Desulfovibrio Gigas* Suggests a Potential Binding Region for the Electron-Transferring Partner. *Int. J. Mol. Sci.* **2013**, *14* (1), 1667–1683.
<https://doi.org/10.3390/ijms14011667>.
 14. Peters, J. W.; Miller, A.-F.; Jones, A. K.; King, P. W.; Adams, M. W. Electron Bifurcation. *Curr. Opin. Chem. Biol.* **2016**, *31*, 146–152.
<https://doi.org/10.1016/j.cbpa.2016.03.007>.
 15. Buckel, W.; Thauer, R. K. Flavin-Based Electron Bifurcation, A New Mechanism of Biological Energy Coupling. *Chem. Rev.* **2018**, *118* (7), 3862–3886.
<https://doi.org/10.1021/acs.chemrev.7b00707>.
 16. Zhang, P.; Yuly, J. L.; Lubner, C. E.; Mulder, D. W.; King, P. W.; Peters, J. W.; Beratan, D. N. Electron Bifurcation: Thermodynamics and Kinetics of Two-Electron Brokering in Biological Redox Chemistry. *Acc. Chem. Res.* **2017**, *50* (9), 2410–2417.
<https://doi.org/10.1021/acs.accounts.7b00327>.
 17. Yuly, J. L.; Zhang, P.; Lubner, C. E.; Peters, J. W.; Beratan, D. N. Universal Free-Energy Landscape Produces Efficient and Reversible Electron Bifurcation. *Proc. Natl. Acad. Sci.* **2020**, *117* (35), 21045–21051. <https://doi.org/10.1073/pnas.2010815117>.
 18. Bergdoll, L.; ten Brink, F.; Nitschke, W.; Picot, D.; Baymann, F. From Low- to High-Potential Bioenergetic Chains: Thermodynamic Constraints of Q-Cycle Function. *Biochim. Biophys. Acta BBA - Bioenerg.* **2016**, *1857* (9), 1569–1579.
<https://doi.org/10.1016/j.bbabi.2016.06.006>.
 19. Chowdhury, N. P.; Mowafy, A. M.; Demmer, J. K.; Upadhyay, V.; Koelzer, S.; Jayamani, E.; Kahnt, J.; Hornung, M.; Demmer, U.; Ermler, U.; Buckel, W. Studies on the Mechanism of Electron Bifurcation Catalyzed by Electron Transferring Flavoprotein (Etf) and Butyryl-CoA Dehydrogenase (Bcd) of *Acidaminococcus Fermentans**. *J. Biol. Chem.* **2014**, *289* (8), 5145–5157. <https://doi.org/10.1074/jbc.M113.521013>.
 20. Vigil, W.; Tran, J.; Niks, D.; Schut, G. J.; Ge, X.; Adams, M. W. W.; Hille, R. The Reductive Half-Reaction of Two Bifurcating Electron-Transferring Flavoproteins: Evidence for Changes in Flavin Reduction Potentials Mediated by Specific Conformational Changes. *J. Biol. Chem.* **2022**, *298* (6), 101927.
<https://doi.org/10.1016/j.jbc.2022.101927>.
 21. Menjivar, K.; Tran, J.; Niks, D.; Ge, X.; Schut, G. J.; Adams, M. W. W.; Hille, R. Flavin Redox Tuning and Ferredoxin-Dependent Kinetics in the Electron-Bifurcating EtfABCX Complex from *Thermotoga Maritima*. *J. Biol. Chem.* **2026**, 111442.
<https://doi.org/10.1016/j.jbc.2026.111442>.

22. Yuly, J. L.; Zhang, P.; Ru, X.; Terai, K.; Singh, N.; Beratan, D. N. Efficient and Reversible Electron Bifurcation with Either Normal or Inverted Potentials at the Bifurcating Cofactor. *Chem* **2021**, *7* (7), 1870–1886. <https://doi.org/10.1016/j.chempr.2021.03.016>.
23. Schut, G. J.; Haja, D. K.; Feng, X.; Poole, F. L.; Li, H.; Adams, M. W. W. An Abundant and Diverse New Family of Electron Bifurcating Enzymes With a Non-Canonical Catalytic Mechanism. *Front. Microbiol.* **2022**, *13*, 946711. <https://doi.org/10.3389/fmicb.2022.946711>.
24. Nitschke, W.; Russell, M. J. Redox Bifurcations: Mechanisms and Importance to Life Now, and at Its Origin. *BioEssays* **2012**, *34* (2), 106–109. <https://doi.org/10.1002/bies.201100134>.
25. Baymann, F.; Schoepp-Cothenet, B.; Duval, S.; Guiral, M.; Brugna, M.; Baffert, C.; Russell, M. J.; Nitschke, W. On the Natural History of Flavin-Based Electron Bifurcation. *Front. Microbiol.* **2018**, *9*. <https://doi.org/10.3389/fmicb.2018.01357>.
26. L. Yuly, J.; E. Lubner, C.; Zhang, P.; N. Beratan, D.; W. Peters, J. Electron Bifurcation: Progress and Grand Challenges. *Chem. Commun.* **2019**, *55* (79), 11823–11832. <https://doi.org/10.1039/C9CC05611D>.
27. Lubner, C. E.; Jennings, D. P.; Mulder, D. W.; Schut, G. J.; Zadvornyy, O. A.; Hoben, J. P.; Tokmina-Lukaszewska, M.; Berry, L.; Nguyen, D. M.; Lipscomb, G. L.; Bothner, B.; Jones, A. K.; Miller, A.-F.; King, P. W.; Adams, M. W. W.; Peters, J. W. Mechanistic Insights into Energy Conservation by Flavin-Based Electron Bifurcation. *Nat. Chem. Biol.* **2017**, *13* (6), 655–659. <https://doi.org/10.1038/nchembio.2348>.
28. Wise, C. E.; Ledinina, A. E.; Mulder, D. W.; Chou, K. J.; Peters, J. W.; King, P. W.; Lubner, C. E. An Uncharacteristically Low-Potential Flavin Governs the Energy Landscape of Electron Bifurcation. *Proc. Natl. Acad. Sci.* **2022**, *119* (12), e2117882119. <https://doi.org/10.1073/pnas.2117882119>.
29. Lerner, M. Designer Flavoproteins for the Elucidation of the Fundamental Mechanism of Potential Inversion, UC Santa Barbara, 2022. <https://escholarship.org/uc/item/23v9h8qj> (accessed 2026-05-01).
30. Liang, J.; Huang, H.; Wang, S. Distribution, Evolution, Catalytic Mechanism, and Physiological Functions of the Flavin-Based Electron-Bifurcating NADH-Dependent Reduced Ferredoxin: NADP⁺ Oxidoreductase. *Front. Microbiol.* **2019**, *10*. <https://doi.org/10.3389/fmicb.2019.00373>.
31. Mohamed-Raseek, N.; Miller, A.-F. Contrasting Roles for Two Conserved Arginines: Stabilizing Flavin Semiquinone or Quaternary Structure, in Bifurcating Electron Transfer Flavoproteins. *J. Biol. Chem.* **2022**, *298* (4). <https://doi.org/10.1016/j.jbc.2022.101733>.

32. Wessiak, A.; Schopfer, L. M.; Yuan, L. C.; Bruice, T. C.; Massey, V. Use of Riboflavin-Binding Protein to Investigate Steric and Electronic Relationships in Flavin Analogs and Models. *Proc. Natl. Acad. Sci.* **1984**, *81* (14), 4246–4249. <https://doi.org/10.1073/pnas.81.14.4246>.
33. Umhau, S.; Pollegioni, L.; Molla, G.; Diederichs, K.; Welte, W.; Pilone, M. S.; Ghisla, S. The X-Ray Structure of d-Amino Acid Oxidase at Very High Resolution Identifies the Chemical Mechanism of Flavin-Dependent Substrate Dehydrogenation. *Proceedings of the National Academy of Sciences* **2000**, *97* (23), 12463–12468. <https://doi.org/10.1073/pnas.97.23.12463>.
34. Das, D.; Miller, A.-F. A Single Hydrogen Bond That Tunes Flavin Redox Reactivity and Activates It for Modification. *Chem. Sci.* **2024**, *15* (20), 7610–7622. <https://doi.org/10.1039/D4SC01642D>.
35. Kakeshpour, T.; Van Wiemeersch, A.; Jackson, J. E. Redox Potential Tuning in Bio-Relevant Heterocycles via (Anti)Aromaticity Modulated H-Bonding (AMHB). *Can. J. Chem.* **2020**, *98* (7), 337–346. <https://doi.org/10.1139/cjc-2019-0410>.
36. Swenson, R. P.; Krey, G. D. Site-Directed Mutagenesis of Tyrosine-98 in the Flavodoxin from *Desulfovibrio Vulgaris* (Hildenborough): Regulation of Oxidation-Reduction Properties of the Bound FMN Cofactor by Aromatic, Solvent, and Electrostatic Interactions. *Biochemistry* **1994**, *33* (28), 8505–8514. <https://doi.org/10.1021/bi00194a015>.
37. Minnaert, K. Measurement of the Equilibrium Constant of the Reaction between Cytochrome c and Cytochrome a. *Biochim. Biophys. Acta* **1965**, *110* (1), 42–56. [https://doi.org/10.1016/s0926-6593\(65\)80093-5](https://doi.org/10.1016/s0926-6593(65)80093-5).
38. Bonomi, F.; Iametti, S. Redox Titration of Flavoproteins: An Overview. In *Flavins and Flavoproteins: Methods and Protocols*; Barile, M., Ed.; Springer US: New York, NY, 2021; pp 119–133. https://doi.org/10.1007/978-1-0716-1286-6_8.
39. Massey, V. A Simple Method for the Determination of Redox Potentials. In *Flavins and Flavoproteins 1990: Proceedings of the Tenth International Symposium, Como, Italy, July 15–20, 1990*; Curti, B., Ronchi, S., Zanetti, G., Eds.; De Gruyter, 2019; pp 59–66. <https://doi.org/10.1515/9783110855425-012>.
40. Miller, A.-F.; Duan, H. D.; Varner, T. A.; Mohamed Raseek, N. Chapter Fourteen - Reduction Midpoint Potentials of Bifurcating Electron Transfer Flavoproteins. In *Methods in Enzymology*; Palfey, B. A., Ed.; New Approaches for Flavin Catalysis; Academic Press, 2019; Vol. 620, pp 365–398. <https://doi.org/10.1016/bs.mie.2019.03.017>.
41. Léger, C.; Elliott, S. J.; Hoke, K. R.; Jeuken, L. J. C.; Jones, A. K.; Armstrong, F. A. Enzyme Electrokinetics: Using Protein Film Voltammetry To Investigate Redox Enzymes and Their Mechanisms. *Biochemistry* **2003**, *42* (29), 8653–8662. <https://doi.org/10.1021/bi034789c>.

42. Armstrong, F. A.; Cheng, B.; Herold, R. A.; Megarity, C. F.; Siritanaratkul, B. From Protein Film Electrochemistry to Nanoconfined Enzyme Cascades and the Electrochemical Leaf. *Chem. Rev.* **2023**, *123* (9), 5421–5458. <https://doi.org/10.1021/acs.chemrev.2c00397>.
43. Hirst, J. Elucidating the Mechanisms of Coupled Electron Transfer and Catalytic Reactions by Protein Film Voltammetry. *Biochim. Biophys. Acta BBA - Bioenerg.* **2006**, *1757* (4), 225–239. <https://doi.org/10.1016/j.bbabbio.2006.04.002>.
44. Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications*, 2nd ed.; John Wiley & Sons, Ltd: New York, NY, 2001.
45. Moser, C., Keske, J., Warncke, K. et al. Nature of biological electron transfer. *Nature* **355**, 796–802 (1992). <https://doi.org/10.1038/355796a0>
46. Marcus, R. A.; Sutin, N. Electron Transfers in Chemistry and Biology. *Biochim. Biophys. Acta BBA - Rev. Bioenerg.* **1985**, *811* (3), 265–322. [https://doi.org/10.1016/0304-4173\(85\)90014-X](https://doi.org/10.1016/0304-4173(85)90014-X).
47. Marcus, R. A. On the Theory of Oxidation-Reduction Reactions Involving Electron Transfer. I. *J. Chem. Phys.* **1956**. <https://doi.org/10.1063/1.1742723>.
48. Page, C. C.; Moser, C. C.; Chen, X.; Dutton, P. L. Natural Engineering Principles of Electron Tunnelling in Biological Oxidation–Reduction. *Nature* **1999**, *402* (6757), 47–52. <https://doi.org/10.1038/46972>.
49. Kılıç, M.; Ensing, B. Redox Properties of Flavin in BLUF and LOV Photoreceptor Proteins from Hybrid QM/MM Molecular Dynamics Simulation. *J. Phys. Chem. B* **2024**, *128* (13), 3069–3080. <https://doi.org/10.1021/acs.jpcc.3c06245>.
50. Langen, R.; Chang, I.-J.; Germanas, J. P.; Richards, J. H.; Et., A. Electron Tunneling in Proteins: Coupling Through a β Strand. *Science* **1995**. <https://doi.org/10.1126/science.7792598>.
51. Allison, J. R. Computational Methods for Exploring Protein Conformations. *Biochem. Soc. Trans.* **2020**, *48* (4), 1707–1724. <https://doi.org/10.1042/BST20200193>.
52. Maity, S.; Sarangi, R.; Acharya, A. Effects of Conformational Sampling on Computing Redox Properties Using Linear Response Approach. *J. Phys. Chem. B* **2025**, *129* (29), 7465–7474. <https://doi.org/10.1021/acs.jpcc.5c01121>.
53. Chen, C. G.; Nardi, A. N.; Amadei, A.; D'Abramo, M. Theoretical Modeling of Redox Potentials of Biomolecules. *Molecules* **2022**, *27* (3), 1077. <https://doi.org/10.3390/molecules27031077>.
54. Case, D. A.; Cerutti, D. S.; Cruzeiro, V. W. D.; Darden, T. A.; Duke, R. E.; Ghazimirsaeed, M.; Giambaşu, G. M.; Giese, T. J.; Götz, A. W.; Harris, J. A.; Kasavajhala, K.; Lee, T.-S.; Li, Z.; Lin, C.; Liu, J.; Miao, Y.; Salomon-Ferrer, R.; Shen,

- J.; Snyder, R.; Swails, J.; Walker, R. C.; Wang, J.; Wu, X.; Zeng, J.; Cheatham III, T. E.; Roe, D. R.; Roitberg, A.; Simmerling, C.; York, D. M.; Nagan, M. C.; Merz, K. M. Jr. Recent Developments in Amber Biomolecular Simulations. *J. Chem. Inf. Model.* **2025**, *65* (15), 7835–7843. <https://doi.org/10.1021/acs.jcim.5c01063>.
55. Brooks, B. R.; Brooks, C. L.; MacKerell, A. D.; Nilsson, L.; Petrella, R. J.; Roux, B.; Won, Y.; Archontis, G.; Bartels, C.; Boresch, S.; Caflisch, A.; Caves, L.; Cui, Q.; Dinner, A. R.; Feig, M.; Fischer, S.; Gao, J.; Hodoscek, M.; Im, W.; Kuczera, K.; Lazaridis, T.; Ma, J.; Ovchinnikov, V.; Paci, E.; Pastor, R. W.; Post, C. B.; Pu, J. Z.; Schaefer, M.; Tidor, B.; Venable, R. M.; Woodcock, H. L.; Wu, X.; Yang, W.; York, D. M.; Karplus, M. CHARMM: The Biomolecular Simulation Program. *J. Comput. Chem.* **2009**, *30* (10), 1545–1614. <https://doi.org/10.1002/jcc.21287>.
 56. Li, G.; Zhang, X.; Cui, Q. Free Energy Perturbation Calculations with Combined QM/MM Potentials Complications, Simplifications, and Applications to Redox Potential Calculations. *J. Phys. Chem. B* **2003**, *107* (33), 8643–8653. <https://doi.org/10.1021/jp034286g>.
 57. Blumberger, J. Recent Advances in the Theory and Molecular Simulation of Biological Electron Transfer Reactions. *Chem. Rev.* **2015**, *115* (20), 11191–11238. <https://doi.org/10.1021/acs.chemrev.5b00298>.
 58. Stier, M.; Kästner, J. Reliable Redox-Potential Simulations of Proteins. *J. Chem. Theory Comput.* **2026**, *22* (1), 334–357. <https://doi.org/10.1021/acs.jctc.5c01656>.
 59. Jones, B. J.; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.
 60. Chapman, S.; Faulkner, C.; Kaiserli, E.; Garcia-Mata, C.; Savenkov, E. I.; Roberts, A. G.; Oparka, K. J.; Christie, J. M. The Photoreversible Fluorescent Protein iLOV Outperforms GFP as a Reporter of Plant Virus Infection. *Proc. Natl. Acad. Sci.* **2008**, *105* (50), 20038–20043. <https://doi.org/10.1073/pnas.0807551105>.
 61. Christie, J. M.; Hitomi, K.; Arvai, A. S.; Hartfield, K. A.; Mettlen, M.; Pratt, A. J.; Tainer, J. A.; Getzoff, E. D. Structural Tuning of the Fluorescent Protein iLOV for Improved Photostability. *J. Biol. Chem.* **2012**, *287* (26), 22295–22304. <https://doi.org/10.1074/jbc.M111.318881>.
 62. Wang, R.; Ingle, R. G.; Xie, H.-F.; Wu, S.-Y.; Fang, W.-J. Chemical Degradation of Histidine Residues and Histidine as an Excipient in Protein-Based Biopharmaceuticals. *Int. J. Pharm.* **2026**, *697*, 126842. <https://doi.org/10.1016/j.ijpharm.2026.126842>.

II. Making iLOV Work: Singlet Oxygen Mediated Cross-linking and the Myth of a Simple Model

Part of the content of this chapter was published in Jones, Benjamin J.; Greene, Brandon L.; Singlet oxygen-mediated photochemical cross-linking of an engineered fluorescent flavoprotein iLOV. *J. Biol. Chem.*, Volume 300, Issue 11, 2024, 107845, ISSN 0021-9258. <https://doi.org/10.1016/j.jbc.2024.107845>.

ABSTRACT

Genetically-encoded photoactive proteins are integral tools in modern biochemical and molecular biological research. Within this toolbox, truncated variants of the phototropin 2 light-oxygen-voltage (LOV) flavoprotein have been developed for various applications, particularly as fluorescent tags and as photosensitizers of singlet oxygen ($^1\text{O}_2$). Due to its extensive prior characterization and established use as a fluorescent tag, we selected one such variant, iLOV, for our flavoprotein model to test reduction potential tuning. At the onset of this work, iLOV had not been reported as among those LOV protein's capable of photochemical $^1\text{O}_2$ generation, nor had the effect of $^1\text{O}_2$ on these genetically encoded photosensitizers been explored. In this chapter, we demonstrate that iLOV is capable of photochemical $^1\text{O}_2$ generation, a characteristic that complicates its use as a model flavoprotein. Once generated, $^1\text{O}_2$ induces protein oligomerization via covalent cross-linking. The molecular targets of protein oligomerization by cross-linking are not endogenous tryptophans or tyrosines, but rather primarily histidines. Substitution of surface-

exposed histidines for serine or glycine residues effectively eliminates protein cross-linking. When used in biochemical applications, such protein-protein cross-links may interfere with native biological responses to $^1\text{O}_2$, which can be ameliorated by substitution of the surface exposed histidines of iLOV or other $^1\text{O}_2$ -generating flavoproteins.

INTRODUCTION

The reactivity of molecular oxygen (O_2) presents a challenge to both aerobic life and life at the aerobic-anaerobic interface due to the production of reactive oxygen species (ROS) from O_2 .¹⁻³ These ROS, such as singlet oxygen ($^1\text{O}_2$), superoxide ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet\text{OH}$), are capable of oxidatively damaging biomolecules, which disrupt downstream cellular functions, leading to oxidative stress, tumorigenesis, and cell death.⁴⁻⁶ On the other hand, ROS also play a crucial role in signal transduction and post-translational enzyme processing.^{3, 7, 8} The interplay between detrimental and beneficial contributions of ROS to biological function remains unclear, and thus, tools that selectively generate ROS under defined conditions, which do not interfere with the biological response under study, would be of value.

Among the ROS family, $^1\text{O}_2$ is the only species that is not generated by electron transfer, but rather by energy transfer in a triplet-triplet annihilation reaction between the ground state triplet $^3\text{O}_2$ and another triplet species, or the oxidative decomposition of peroxides.⁹ In the biological context, energy transfer partner triplets are typically relaxation products of chromophore electronic excited singlet states. The ability to generate $^1\text{O}_2$ photochemically has motivated the development of genetically encodable photoactive flavoproteins based on the *Arabidopsis thaliana* light-oxygen-voltage (LOV) sensing domain of phototropin 2.^{10, 11}

These proteins bind an FMN cofactor and photochemically generate $^1\text{O}_2$, but the quantum yield of $^1\text{O}_2$ generation and the distribution of other ROS products (e.g. $\bullet\text{O}_2^-$) remain unsettled.^{10, 12, 13}

By genetically tagging $^1\text{O}_2$ -generating proteins to specific tissue-localized genes, LOV-based proteins can, in principle, be used to examine the effect of $^1\text{O}_2$ on biomolecules and biomolecular pathways, but the reactivity of $^1\text{O}_2$ with the LOV photosensitizer itself has been unexplored. The reactivity of $^1\text{O}_2$ towards common biomolecules is distinct from other ROS, where it acts as an electrophile and a Diels-Alder reactant, targeting nucleoside bases, unsaturated lipids, and amino acid residues histidine (H), cysteine (C), methionine (M), tyrosine (Y), and tryptophan (W).¹⁴ All currently known $^1\text{O}_2$ -generating flavoproteins contain at least three of these reactive amino acids, and thus could directly react with photogenerated $^1\text{O}_2$.

In this chapter we demonstrate that iLOV, which is highly homologous to two previously reported $^1\text{O}_2$ generating proteins: “mini $^1\text{O}_2$ generator” (miniSOG) and $^1\text{O}_2$ -producing protein (SOPP)¹⁵, is capable of photochemically generating $^1\text{O}_2$, a function that it was not intentionally designed to perform. Next, we investigate the reactivity of photogenerated $^1\text{O}_2$ with iLOV. We find that photogenerated $^1\text{O}_2$ reacts with the protein to form iLOV-iLOV cross-links, a process that is mediated by surface exposed histidines. This observation is consistent with previous studies, and we propose a mechanism of cross-linking involving a histidine attack of a 2-oxo-histidine product of $^1\text{O}_2$ and another histidine N ϵ from a second iLOV.¹⁶ These findings suggest that such protein-based $^1\text{O}_2$ -generating photosensitizers may, themselves, engage in *in vivo* reactivity, which could affect subsequent $^1\text{O}_2$ responses *in vivo*. Additionally, these results highlight the need to develop

special methods of handling iLOV variants to minimize photochemical crosslinking for its use as a flavoprotein model in subsequent chapters.

MATERIALS AND METHODS

Materials

The iLOV gene was obtained as a gracious gift from the Mukherjee lab, inside a pQE80L vector backbone.³⁸ The 6× polyhistidine-tagged ubiquitin like protease 1 (ULP1) was expressed and purified as previously described.^{39, 40} All DNA oligomers used as primers for PCR were ordered from Integrated DNA Technologies. DpnI, Phusion HF polymerase, chemically competent DH5- α and BL21 (DE3) *E. coli*, the Qiagen QIAprep Spin Miniprep and Gel Extraction Kits, and the NEBuilder HiFi DNA Assembly kit were purchased from New England Biolabs (NEB). Isopropyl- β -d-1-thiogalactopyranoside (IPTG) was purchased from Merck. Sephadex® G-25 fine resin, LB Miller broth, NaH₂PO₄ (P_i), iodoacetamide, trifluoroacetic acid (TFA), DTT, formic acid, urea, phenylmethylsulfonyl fluoride (PMSF), bovine SOD, bovine XO, xanthine, and Amicon® Ultra centrifugal filter units were purchased from Millipore Sigma. FMN sodium salt, HisPur™ Ni-NTA resin, Pierce 660nm protein assay kit, SOSG, and Imperial protein stain were obtained from ThermoFisher Scientific. Bradford reagent and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from VWR. Mini-PROTEAN TGX-Precast protein gels containing a polyacrylamide gradient from 4-20%, 2x Laemmli sample buffer, and Precision Plus Protein™ Standards were purchased from BioRad. The single-color, cold, visible, mounted, light-emitting diode (LED) with a wavelength of 450 nm and a bandwidth of 18 nm was

obtained from ThorLabs. The MALDI steel target plates, Sinapinic acid matrix, and MALDI MS calibration standards (Protein Standards I and II) were obtained from Bruker.

Bisacrylamide and kanamycin sulfate were obtained from Genesee Scientific. Millipore water was obtained by flowing deionized water through a 4-module Barnstead E-Pure ultrapure water system until a resistivity of $>17 \text{ M}\Omega/\text{cm}$ was reached.

Construction of pSUMO-iLOV Plasmid Vector

The iLOV gene was cloned into the pET backbone of pHYRSF53 (AddGene #64696) via Gibson assembly. The resulting inducible expression gene codes for iLOV N-terminally tagged with a $6 \times$ polyhistidine tag and the small ubiquitin-like modifying protein (SUMO) with flexible serine and glycine residues in between fragments, denoted (H)₆-SUMO-iLOV. Fragments for this Gibson assembly were generated by polymerase chain reaction (PCR) using primers with sequences shown in the **Table II-S7**. The fragments were gel-purified using a Qiagen Gel Extraction kit and assembled using an NEBuilder HiFi DNA Assembly kit. The assembled product was used to transform chemically competent DH5- α E. coli which were then plated onto agar plates containing LB (Miller, pH 7) and 50 $\mu\text{g/mL}$ Kanamycin as the selection antibiotic. After growth over-night, single colonies were picked and used to inoculate a small culture in 5 mL LB media with 50 $\mu\text{g/mL}$ Kanamycin in 15 mL sterile culture tubes. This culture was allowed to grow for 18-20 hours at 37 °C shaking at 220 rpm. The plasmid was purified from these cells using a Qiagen QIAprep Spin Miniprep Kit. The purified plasmids were sent to the UC Berkeley DNA Sequencing Facility to undergo full plasmid nanopore sequencing. After validation of the correct sequences, the plasmids were used as templates for subsequent rounds of mutagenesis described in the following section.

Site-directed Mutagenesis (SDM) of iLOV Mutants

Site-directed mutagenesis (SDM) was carried out using PCR primers containing nucleotide mismatches consisting of a codon for the appropriate amino acid replacement (**Table II-S8**). The pSUMO-iLOV assembled in the previous section was used as a plasmid template for PCR using Phusion HF polymerase. The resulting PCR product was digested with DpnI to remove methylated template DNA and transformed into DH5- α cells. Plasmid DNA was purified and sequence verified as previously described. After validation of the correct mutation(s), the product plasmids were used as templates for subsequent rounds of mutagenesis for double, triple, and quadruple mutants.

Growth and Purification of iLOV

The pSUMO-iLOV plasmid was used to transform BL21 (DE3) *E. coli* which were then plated on LB agar plates with Kanamycin and grown overnight. A single colony was picked and grown in 5 mL LB with 50 μ g/mL Kanamycin overnight. From this overnight seed culture, 750 μ L were added to each of two sterile 250 mL Erlenmeyer flasks containing 75 mL LB with Kanamycin. These flasks were shaken at 200 rpm at 37 °C until the cells reached an OD₆₀₀ of 0.6. At this point small aliquots of cells were collected, pelleted, flash frozen, and stored in a -80 °C freezer for future growths. Simultaneously, to one of the remaining 75-mL cultures, a final concentration of 20 μ M of IPTG was added to induce protein expression. The two flasks were left to grow for another 5 hours before samples of each were removed for SDS-PAGE to confirm expression of protein at the appropriate molecular weight.

After confirmation of protein expression, large scale cultures were prepared. About 15 mL of cultured cells from the uninduced flasks were used to inoculate 1.5 L of LB,

supplemented with 50 $\mu\text{g/mL}$ Kanamycin. These cultures were allowed to grow and expression was induced as previously described. After growth, the cells were pelleted at $3,000 \times g$ for 10 min at 4 $^{\circ}\text{C}$. The yellow pellet was then flash frozen in liquid nitrogen and stored at -80°C for purification at a later date.

To purify (H)₆-SUMO-iLOV, the cell pellet was placed on ice and allowed to thaw slowly. This pellet was resuspended in 200 mM NaCl, 50 mM Pi, and 1.5 mM DTT at pH 7.50 (Buffer A) supplemented with 1 mM PMSF to a volume of 5 mL per gram of cell paste and homogenized with a glass homogenizer. The cells were then lysed by passing the mixture 5-6 times through a French press homogenizer operating at 14,500 psi. The resulting lysate was then placed into 50 mL centrifuge tubes and pelleted at $14,000 \times g$ for 10 min at 4 $^{\circ}\text{C}$ to remove insoluble debris. The supernatant was placed in a beaker with a stir bar on ice, while a 1:4 (v/v) ratio of 6% (w/v) streptomycin sulfate was added dropwise. The solution was allowed to stir on ice for 10 minutes, then the insoluble material was pelleted at $29,000 \times g$ for 30 minutes. The resulting supernatant was combined in a 1:1 ratio with Buffer A supplemented with 15 mM imidazole and applied to a HisPurTM Ni-NTA resin, equilibrated with the same buffer. The column was then washed with 30 column volumes of Buffer A supplemented with 50 mM imidazole. The protein was eluted with Buffer A supplemented with 250 mM imidazole. Fractions were pooled based on either yellow color or positive reactions with Bradford reagent. The eluted (H)₆-SUMO-iLOV was then dialyzed in the dark overnight at 4 $^{\circ}\text{C}$ against 1,000-2,000 $\times$ (v/v) Buffer A. The pure (H)₆-SUMO-iLOV was either used immediately or supplemented with 10% (v/v) glycerol, flash frozen in liquid nitrogen, and stored at -80°C .

ULP1 Digestion and Loading of FMN

The recombinantly expressed and purified (H)₆-SUMO-iLOV was first digested with ULP1 to cleave the N-terminal (H)₆-SUMO fragment then reconstituted with FMN before separating the (H)₆-SUMO fragment from iLOV via Ni-NTA chromatography. The resulting cleaved protein contains the additional (–3)S-H-M-M(0) N-terminal amino acids, relative to the original iLOV (15). In all steps, the protein quantitation of iLOV variants were measured via the bicinchoninic acid-based Pierce 660TM assay kit.⁴¹ The FMN content was determined by the absorbance at 448 nm with an estimated extinction coefficient of 14,800 M^{–1} cm^{–1} for the protein-bound cofactor.⁴²

To cleave the (H)₆-SUMO fragment, 30-40 mg of (H)₆-SUMO-iLOV, or variants, were mixed in a 1:500 molar ratio of ULP1 to (H)₆-SUMO-iLOV and incubated at room temperature for <2 hours. To reconstitute iLOV, free FMN was then added to this mixture at 50× molar excess of iLOV from a saturated solution of FMN in 200 mM NaCl, 50 mM P_i at pH 8.0. The actual concentration of FMN was determined by measuring the absorbance of FMN at 450 nm using a molar extinction coefficient of 12,500 M^{–1} cm^{–1} for the free cofactor.⁴³ The mixture was then heated to 60 °C for 15 minutes, 5 °C below the unfolding temperature of the apo protein, then cooled back to room temperature.

The reconstituted iLOV in the FMN, (H)₆-SUMO, and ULP1 mixture was then passed through a second Ni-NTA column equilibrated with Buffer A supplemented with 25 mM imidazole to remove the cleaved (H)₆-SUMO fragment, and the flow through was collected. The resin was further washed with Buffer A supplemented with 25 mM imidazole at pH 7.50 until the characteristic yellow color of iLOV was no longer visible on the column. The eluted iLOV and FMN solution was then concentrated in a 10 kDa molecular weight cutoff

Amicon® Ultra centrifugal filter. This concentrated solution was buffer-exchanged using a Sephadex® G-25 desalting column into 200 mM NaCl, 50 mM P_i at pH 7.50 (Buffer B), which clearly resolved free FMN and reconstituted iLOV. Initial and post-reconstitution FMN/protein content is provided in **Table II-S9**, and the corresponding FMN UV-vis spectra are reported in **Figure II-S9**. Pure, reconstituted iLOV was then flash frozen in liquid nitrogen, and stored at –80 °C for later use. The final yield of iLOV after purification, SUMO cleavage, reconstitution, and buffer exchange, routinely ranged from 10-20 mg iLOV/g wet cell paste.

SDS-PAGE

For visualization of protein samples by SDS-PAGE, purified iLOV samples were diluted to 33 µM (0.43 mg/mL) into phosphate buffer while impure protein samples were diluted to about 0.7 mg/mL of total protein determined by the aforementioned Pierce 660 assay. Equal volumes of diluted protein and 2 × Laemmli sample buffer containing 5% (v/v) 2-mercaptoethanol were mixed and heated to 98°C for 5-10 minutes. Denatured samples were then either loaded into homemade 16% bisacrylamide polyacrylamide gels containing a 9.3% stacking layer or into premanufactured gels containing a polyacrylamide gradient from 4-20%. Additionally pre-stained protein standards were added alongside samples to estimate molecular weights. A voltage of 90 V was applied for 15 minutes before switching to 180 V for approximately 45 minutes or until the pre-stained ladders migrated across the gel. The gels were then washed in water for 5 minutes before being bathed in Imperial protein stain for several hours or overnight. The gels were then destained in water for several hours or overnight before either imaging or gel extraction.

Photochemical cross-linking of iLOV Mutants

To initiate photochemistry, we used a 450 nm LED, mounted onto a home-made illumination apparatus delivering 3 W of power. The apparatus positions the light 1.6 cm above the center of a ring of six sample holders made to fit 1.5 mL plastic microcentrifuge tubes (ca. 1 mW/mm² irradiance). A solution of 100 µL of purified and reconstituted iLOV was prepared to a final concentration of 100 µM and placed into open opaque amber 1.5 mL microcentrifuge tubes. The tubes were then illuminated for various times. At specified time points, 10 µL was removed and combined with 20 µL water and 30 µL 2 × Laemmli buffer with 5% v/v 2-mercaptoethanol and flash frozen in liquid nitrogen until all time points were collected. Aliquots were also collected for UV-vis absorption analysis at 4 hours of illumination, diluted 10-fold into Buffer B and measured on a Cary 60 Agilent UV-vis absorption spectrometer. The samples were then thawed, heated to 98 °C for 5 minutes, then resolved by SDS-PAGE. For experiments performed in the presence of reductant, DTT was added to concentrations of 10 mM. For experiments performed with SOD, lyophilized SOD was dissolved in water and added to the illumination mixture in various concentrations.

For experiments performed in the dark with xanthine oxidase, xanthine was dissolved to a concentration of 10 mM in 0.1 M NaOH while lyophilized XO was dissolved in water. Xanthine and XO were then added to the mixture to final concentrations of 400 µM and 20 U/L respectively. Samples with xanthine oxidase were kept in the dark, and no LED illumination was performed.

For anaerobic illumination, protein samples were degassed on a Schlenk line prior to illumination. Next, the sealed, anaerobic samples would be removed from the Schlenk line to be placed into a Vacuum Atmospheres glovebox (<1 ppm O₂). Illumination of the

degassed samples was performed inside the glovebox via the apparatus used aerobically described above with identical settings.

MALDI-ToF

Samples of iLOV that were exposed to ambient light over a two-day purification process in the absence of a reductant were dialyzed overnight against $2000\text{--}3000 \times (\text{v/v})$ of 5 mM ammonium bicarbonate to remove salts. The dialysis was repeated in fresh buffer for a few more hours. The samples were then diluted to 10–20 μM of protein in 5 mM ammonium bicarbonate. A saturated solution of sinapinic acid matrix in a 30:70 (v/v) mixture of acetonitrile and 0.1% TFA in water served as the matrix. Equal volumes of protein and matrix were mixed and 1 μL was applied to a MALDI steel target plate and allowed to dry at room temperature. MALDI-ToF mass spectra were collected on a Bruker Microflex LRF MALDI ToF (ONR N00014-12-1-0792) in linear mode configuration at ca. 70% laser power. For mass calibration, protein standards I and II, mixed together, were prepared in the same way as iLOV samples. Peaks were picked automatically by the Bruker PolyTools software to generate the polynomial calibration and verified manually.

Detection of $^1\text{O}_2$

SOSG was used to quantify $^1\text{O}_2$ generation. Standard solutions of 100 μg SOSG in 33 μL of methanol were prepared immediately before use. Samples of 50 μL of 116 μM iLOV were mixed with 1.5 μL of the SOSG stock solution in open opaque amber colored plastic microcentrifuge tubes and illuminated as described previously for 1 hour, or non-illuminated in control experiments. For negative controls, SOSG in Buffer B was used in the absence of iLOV. After illumination, 1.450 mL of Buffer B was added to each sample to dilute the

fluorescent SOSG and minimize inner filter effects, and the sample was loaded into a fluorescence quartz cuvette. The emission spectra of SOSG between 520 and 700 nm was collected in a Duetta Spectrometer (Horiba Scientific). The excitation wavelength was 505 nm with a bandpass of 3 nm. Samples were temperature-controlled at 25 °C during data acquisition. For the construction of bar charts, triplicate samples were illuminated and prepared. Triplicate emission spectra were integrated in MATLAB and normalized to the intensity of the triplicate average of iLOV^{WT} samples. Error bars were constructed from the standard deviation of each triplicate. Statistical calculation of p-values demonstrating a statistical difference between iLOV^{WT} and mutant SOSG responses to ¹O₂ yield was performed using a t-test. This was calculated using the "ttest2" function as part of the Statistics and Machine Learning toolbox add-on to MATLAB.

Polyacrylamide Gel Extraction of Purified iLOV Exposed to Ambient Light

Initially, tryptic peptides of gel-extracted iLOV samples exposed only to ambient light used for **Supplementary Figure II-S1** and **Tables II-S1** and **II-S2** were extracted from SDS-PAGE gels using the following procedure. Initially, pure samples of iLOV containing both dimeric and monomeric forms were resolved by electrophoresis on a 16% bisacrylamide gel. Bands of interest were excised using a razor blade and minced to ~1 mm³ pieces. The minced gel sample was mixed with 150 µL 50 mM ammonium bicarbonate and then was pelleted via centrifugation at 3,000 × g and the supernatant was discarded three times. The washed, gel extract samples were subsequently condensed and washed with 100-150 µL of acetonitrile three times. After washing, the acrylamide entrapped protein samples were resuspended in aqueous 50 mM ammonium bicarbonate supplemented with 10 mM DTT, and incubated at 56 °C for 30 minutes. The sample was pelleted via centrifugation and

supernatant was discarded. The gel pieces were, again, shrunk by washing 2-3 times with ACN. To the washed 55 mM IAA in 50 mM ammonium bicarbonate was added and the samples were incubated at room temperature in the dark for 1 hour. The sample was pelleted via centrifugation and supernatant was discarded. The pieces were then washed in 150-200 μ L 50 mM ammonium bicarbonate two more times and then dried in a SpeedVac. Enough 50 mM ammonium bicarbonate to just barely cover the gel pieces (30 -50 μ L) was added along with 1 μ L of 0.5 mg/mL trypsin. This was incubated at room temperature for 10 minutes. Next, 50-100 μ L of 50 mM ammonium bicarbonate was added and the sample was incubated at 50 °C for 1-4 hours. After digestion, 5 μ L of 10% formic acid was added and the sample was centrifuged for 5 minutes. The supernatant was collected into a fresh microcentrifuge tube and set aside for analysis. The remaining gel pieces were mixed with 60% ACN and 0.1% TFA to cover the gel followed by sonication for 10 minutes. The gel pieces were centrifuged for 10 minutes and the supernatant was combined with the previously collected supernatant. The volume of was then reduced to a final volume of about 10 μ L using a SpeedVac. These samples were then flash frozen in liquid nitrogen and stored at -80°C until analysis by HPLC MS/MS as described below on a later date.

Peptide Mapping of Purified iLOV Exposed to Ambient Light by UPLC-MS/MS

The extracted samples were then injected into the UPLC C18 column and resolved and resolved by UPLC-MS/MS similar to previous reports with minor modifications⁴⁴. The digested samples were centrifuged for 30 minutes at 20,000 $\times$ g, then 2.5 μ L of the gel extracted peptides were injected onto a Waters Acquity H-class UPLC system coupled with a Waters Xevo G2-XS quadrupole Time-of-Flight (qToF) Mass Spectrometer. The UPLC was equipped with a Waters BEH C18 column with dimensions of 50 mm $\times$ 2.1 mm with

1.7 μm bead diameter, equilibrated with aqueous 0.1% (v/v) formic acid (Solution A) and resolved in the UPLC against an isocratic gradient from 0-45% (v/v) of 1% formic acid in acetonitrile (Solution B) over 75 min at a flow rate of 0.2 mL/min at 60 °C. A solution of 150 pg/ μL Leu-enkephalin and 100 fmol/ μL Glu-fibrinopeptide B prepared in 25:75 acetonitrile:H₂O with 0.1% v/v formic acid was used as a lock mass, using the average of 3 scans injected every 30 s. Eluent from the LC was injected directly into the qToF. The mass spectrometer settings were as follow: source capillary voltage 2.8 kV, sampling cone voltage 20 V, source offset 20 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow was 50 L/h and desolvation gas flow was 800 L/h. The spectrometer was set in MS/MS detection mode in positive polarity in the resolution regime, detecting from 50 to 2000 Da, with a scan time of 3 s. No restrictions were placed on the masses of precursors to be fragmented in MS² (MSE detection mode).

MS data used for the construction of Supplementary Information **Figures II-S1** and **Tables II-S1** and **II-S2** were analyzed using Water's ProteinLynx Global Server (PLGS) v3.0.3. This preliminary analysis of tryptic peptides, derived from two SDS-PAGE gel-purified bands of pure iLOV protein exposed to ambient light, was based on measurements from a single replicate. The construction of peak lists is incorporated into the analysis workflow. The PLGS software uses a deconvolution algorithm that collapses the several MS peaks of single peptides into single exact mass-retention time (EMRT) entities. This algorithm, described in detail in PLGS user manual, takes into account properties of the peaks detected including elution time, integrated intensity, charge and isotopic distributions. For data curation in PLGS, a low energy threshold was set to 500.0 counts while an elevated energy threshold was set to 25.0 counts. A sequence database was constructed to include the

Uniprot database in addition to the FASTA sequences for iLOV and (H)₆-SUMO-iLOV (569,340 entries). Peptides were generated by “digesting” the sequences with trypsin (cleaves at the C-terminus of K/R except when preceded by P) with a maximum of 1 missed cleavage. Only one fixed modification was used - carbamidomethyl on cysteine (+57.02146 Da). The only variable modifications considered was oxidation on methionine with a mass shift of 15.99491. The mass tolerance for both precursors and fragment ions were set to the default setting in PLGS which automatically calculates a tolerance to match intact peptide and fragment masses. A false discovery rate was set to 4%. No contaminants were excluded during the analysis (with both trypsin and keratin being detected – data not shown). Statistical tests are applied when PLGS produces scores which is fully described in the PLGS user manual⁵²⁻⁵⁴.

Polyacrylamide Gel Extraction of iLOV Actively Illuminated with Blue Light

After identifying the heavy molecular weight band as dimeric iLOV, the gel extraction procedure for iLOV samples was modified to increase peptide yields by excluding the reaction with iodoacetamide (iLOV lacks cysteine residues, so this omission decreases the number of washing steps required). Gel extraction of iLOV monomer and dimer bands reported in **Figure II-S2** was performed as follows: illuminated samples of iLOV were resolved by electrophoresis on a 16% bisacrylamide gel. The monomer and dimer bands of interest were excised using a razor blade and minced to ~1 mm³ pieces. The minced gel sample was mixed 3 × with 150 µL 50 mM ammonium bicarbonate and then was pelleted via centrifugation at 3,000 × g and supernatant was discarded. Following this initial wash, the gel pieces were incubated in 500 µL acetonitrile for 10 minutes at room temperature. This acetonitrile was removed and the pieces were allowed to air-dry for 15 minutes at room

temperature. At this point, 30 μ L of 50 μ g/mL trypsin in 50 mM ammonium bicarbonate was added to the gel pieces to re-swell them. The samples were incubated on ice for 20 minutes. Once, the gel pieces had resumed their former size, enough 50 mM ammonium bicarbonate was added to just cover the gel pieces. The samples were incubated at 37°C overnight with shaking. The digestion mixture was then moved to a new clean test tube. The same volume of 1% formic acid was added to the gel pieces and incubated for 15 minutes at room temperature. This extraction solution was removed and added to the digestion mixture. To extract hydrophobic peptides, 1% formic acid in 75% acetonitrile were added to the gel pieces following incubation for 15 min. This was added to the digestion/extraction mixture. The digestion/extraction mixture was placed on a SpeedVac to dry completely. At this point, about 30 μ L of 1% formic acid in 30% acetonitrile were added to resolubilize digested peptides. These samples were then flash frozen in liquid nitrogen and stored at -80°C until analysis by HPLC MS/MS as described below on a later date.

Peptide Mapping of iLOV Actively Illuminated with Blue Light by UPLC-MS/MS

Tryptic peptides of gel-extracted iLOV samples intentionally illuminated with blue light (**Figures II-2B, II-S2, II-S6, II-S7, and Tables II-S3, II-S4, and II-S6**) were injected into a Shimadzu 9030 ultrahigh performance liquid chromatography-tandem ToF/quadrupole mass spectrometer system (UPLC-MS/MS) equipped with a C18 column with dimensions of 150 mm x 2.0 mm i.d. (2.2 μ m particle size) equilibrated with aqueous 0.1% (v/v) formic acid (Solution A) and resolved in the UPLC against an isocratic gradient from 0-60% (v/v) of acetonitrile with 0.1% (v/v) formic acid (Solution B) over 90 minutes at a flow rate of 0.2 mL/min. For samples with iLOV ^{Δ H}, the gradient was changed to 0-55% over 25 minutes. The eluent was injected into the qTOF mass spectrometer calibrated with sodium iodide.

Mass spectra were collected in data-dependent acquisition (DDA) mode with MS1 spectra collected over a range of 200-2500 m/z (or 200-1500 m/z for iLOV^{ΔH},samples). Five dependent events were chosen for fragmentation to create MS2 spectra of precursor peptides with m/z values of 400 to 1500. An event time of 0.2 s, a base peak chromatogram intensity threshold ion count of 200, and a collision energy of 35 V was used. To identify potential W oxidation products in iLOV^{ΔH} the range in which precursor peptides were selected to fragment was narrowed to 685 to 730 corresponding to the mass range of the single peptide containing W81 (residues 80-90). In order to observe the effect this illumination had on another peptide of iLOV^{ΔH}, also formerly containing histidine (residues 94-110), this run was repeated with a fragmentation range of 910-960. However, no significant differences between the illuminated and non-illuminated spectra were detected for this run. The MS1 spectra of these two otherwise identical replicates showed consistent results in terms of both the number of peaks in the chromatograms and the observed MS1 m/z values. MS2 spectra covered a mass range of 200-2500 m/z (or 200-1500 m/z for iLOV^{ΔH},samples).

Data used for the construction of **Figures II-2B, II-S2, II-S6, II-S7, and Tables II-S3, II-S4, and II-S6** were analyzed using FragPipe v.22.0⁵⁵⁻⁵⁸. The data used for this analysis consisted of tryptic peptides originating from SDS page gel-purified bands intentionally illuminated with blue light. Positive controls included iLOV bands that were not illuminated by light. The data for **Figure II-2B** originated from a single illumination injected in triplicate under identical MS parameters. The data in **Figures II-S6, II-S7, and Table II-S6** are the results of a single illumination, injected in duplicate, where each replicate was run with different MS2 parameters targeting specific precursor peptides as described above. The

data contained in each figure or table are a representative result from one of the replicates under the conditions previously specified.

Peak lists for data analyzed in FragPipe were exported directly from Shimadzu's LabSolutions Postrun software in the form of .mzML files with centroid peaks. Analysis of data presented in **Figure II-2B** was performed with a sequence database composed of the Uniprot database accessed on 12/6/2022 in addition to the FASTA sequences for iLOV and cleaved (H)₆-SUMO added manually for a total of 569,340 entries. Decoy peptides were generated by FragPipe with reverse sequences of each entry as a negative control for the analysis. For data presented in **Figures II-S6, II-S7** and **Table II-S6** the goal was to observe any modifications on specific precursors rather than for the identification of the protein species. As such, the sequence "database" only included the sequence for iLOV^{ΔH} along with its associated reverse sequence decoy added by the FragPipe software. Peptides from the database were generated by "digesting" the sequences with trypsin (cleaves C-terminally of lysine/arginine except when preceded by proline) with no allowed missed cleavages. No fixed modifications were used (no iodoacetamide was used to prepare these samples as described in the Methods section titled "Polyacrylamide Gel Extraction"), but variable modifications included oxidation on methionine (+15.9949 Da), N-terminal acetylation (+42.0106), oxidation on histidine (+31.9988 Da and +15.9994 Da), and oxidation on tryptophan (+31.9988 Da and +3.9994 Da). The mass tolerance for precursor peptides was set to a range of 40 ppm while a mass tolerance for fragment ions was set to 2 Da. Statistical tests are employed by FragPipe's MSFragger module to generate PeptideProphet Probabilities as previously described.⁵⁵ Within MSFragger MS2 analysis, unfragmented precursor peaks were ignored (within an m/z range of -1.5 to 1.5 Da) and spectral

processing identified the top 150 peaks from each spectrum then removed peaks with relative intensities of ≤ 0.01 . In the analysis, no decoy peptides were detected.

Fluorescence Lifetime Measurements

Fluorescence life-time measurements were performed using Time-Correlated Single Photon Counting (TCSPC) technique.⁴⁵ An approximately 100 femtosecond (fs) excitation pulses with wavelength of 400 nm was generated by doubling the fundamental frequency of a Ti:Sapphire laser (SpectraPhysics Tsunami) in a commercial optical harmonics generator (Inrad/Coherent). The laser repetition rate was reduced by a home-made acousto-optical pulse picker in order to avoid saturation of the chromophore. The TCSPC detection system is equipped with a single-photon counting avalanche photodiode module (Photon Micro Devices) and electronics board (Becker & Hickl SPC-630) and has an instrument response time of less than 30 picoseconds. The triggering signal for the TCSPC board was generated by sending a small fraction of the laser beam onto a fast (400 MHz bandwidth) Si photodiode (Thorlabs Inc.). Fluorescence signal was collected in 90 degrees geometry and dispersed in Acton Research SPC-500 monochromator after passing through a pump blocking, long wavelength-pass, autofluorescence-free, interference filter (Omega Filters, ALP series). The monochromator was equipped with a CCD camera (Roper Scientific PIXIS-400) allowing for monitoring of the fluorescence spectrum. Fluorescence transients were not deconvolved with the instrument response function since their characteristic decay time-constants were much longer than the width of the system response to the excitation pulse.

Molecular Dynamics Simulations.

Molecular dynamics (MD) modelling of O₂ diffusion into iLOV was performed similarly as previously described with minor modifications using the visual molecular dynamics (VMD) software.^{28, 46} The crystal structure (PDB ID: 4EES) was used as the starting coordinates for heavy atoms of FMN and protein.¹⁵ Ten O₂ molecules were placed close to the protein surface randomly. Hydrogen atoms were added assuming the conventional protonation states of ionizable groups at pH 7.00, while histidine residues were modelled as neutral. Water molecules resolved in the crystal structure were not removed, and a solvation box was generated with an edge width of 96.497 Å. Sodium and chloride ions were added to a final concentration of 0.15 M. The additive force field CHARMM36 was employed to model iLOV and waters were modeled with the TIP3P intermolecular potential function.^{47, 48} Force field parameters for oxidized FMN were obtained from a prior study⁴⁹ as well as those used for O₂.⁵⁰

Classical MD simulations were performed using the NAMD 3.0 software package.⁵¹ An isothermal-isobaric (NPT) ensemble was maintained at 300 K and 1.01325 atm with Nosé–Hoover Langevin piston pressure control and Langevin dynamics. Periodic boundary conditions were employed and a Particle Mesh-Ewald (PME) algorithm was used to calculate long-range interactions. A cutoff for non-bonded interactions was set to 12 Å. The SHAKE algorithm is used to constrain bonds involving hydrogen atoms. An integration time step of 2 femtoseconds was used and a total of 1425.15 ns were simulated in five independent simulations. RMSD plots of backbone atoms in iLOV over the course of each ~285 ns simulation are shown in **Figure II-S8**.

RESULTS

Following purification of iLOV, we consistently observe two species by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, despite extensive effort to optimize purification, small ubiquitin-like modifier protein (SUMO) cleavage, FMN reconstitution, and iLOV isolation (**Figure II-1A**). The most intense band in the SDS-PAGE of the purified iLOV product exhibits an apparent molecular weight of 13 kDa, similar to the expected molecular weight of iLOV of 13,232 Da (isotopic average mass), while a fainter band of variable intensity exhibits an apparent molecular weight of 26 kDa. The minor species is similar in electrophoretic mobility to the non-cleaved (H)₆-SUMO-iLOV (25,360 Da) iLOV parent protein, but is also the expected molecular weight of a purified iLOV dimer that cannot be chemically separated by denaturation and reduction with 2-mercaptoethanol.

We turned to mass spectrometry to determine the nature of this minor protein species. Using matrix-assisted laser desorption ionization-time-of-flight (MALDI-ToF) mass spectrometry, we identified four peaks with apparent mass-to-charge (m/z) values of 13,230, 26,460, 39,760, and 53,140 with progressively

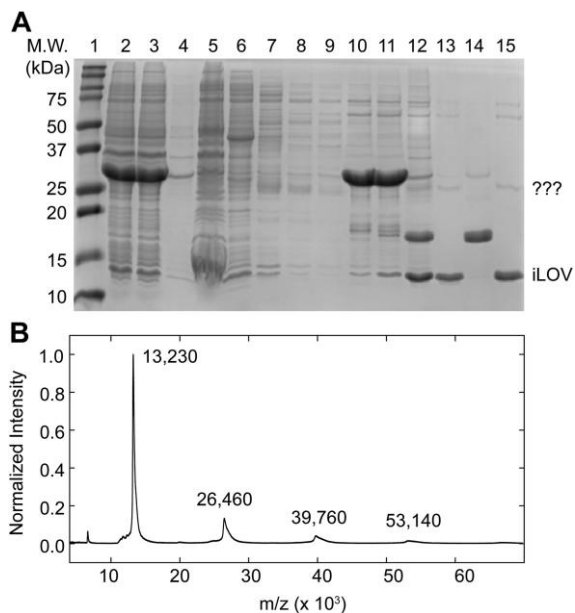


Figure II-1. Recombinant expression, purification, and MALDI-ToF analysis of cross-linked iLOV. **A**, SDS-PAGE gel depicting the purification of iLOV. Lanes: one molecular weight marker, 2 *Escherichia coli* cell lysate, three soluble lysate, four cell debris, five immediate Ni-NTA flow-through, 6 to 9 Ni-NTA flow-through during the imidazole wash, 10 Ni-NTA eluted (H)₆-SUMO-iLOV, 11 desalted (H)₆-SUMO-iLOV, 12 ULP1-digestion product, 13 ULP1-digested Ni-NTA flow-through, 14 ULP1-digestion Ni-NTA elution, 15 desalted ULP1-digested Ni-NTA flow-through. **B**, MALDI-ToF mass spectrum of purified iLOV suggests oligomerization of WT iLOV.

lower intensity (**Figure II-1B**). Based on the similarity of the 13,230 m/z MALDI peak to the expected mass of iLOV (0.02% mass difference) and the observed 26,460 m/z of the second peak, this higher molecular weight species is most consistent with an iLOV dimer (26,462 Da, assuming a bond is formed between the two proteins via formerly R–H groups, 0.01% mass difference), as opposed to a potentially non-cleaved (H)₆-SUMO-iLOV (1% mass difference). Similarly, the 39,760 m/z peak appears consistent with an iLOV trimer with an expected molecular weight of 39,692 Da (0.2% mass difference) and the peak at 53,140 m/z is similar in mass to an iLOV tetramer at 52,922 Da (0.4% mass difference).

To complement the MALDI analysis, we also gel-extracted both the predicted iLOV monomer and potential dimer bands from the SDS-PAGE gel, performed tryptic digestions and subjected them to ultra-performance liquid chromatography tandem ToF/quadrupole mass spectrometry (UPLC-MS/MS) analysis (**Figure II-S1**). In both the lower and higher apparent molecular weight gel bands several unique ions were observed by both their primary mass spectrum (MS1) m/z and their ion fragments in the secondary mass spectrum (MS2) to be derived from iLOV, with 86% of the iLOV sequence identified. A complete list of the identified peptides, measured and calculated masses, and fragment ions identified for both the monomer and dimer bands are provided in **Table II-S1** and **II-S2**, respectively. Some of the expected (H)₆-SUMO tryptic peptides were observed in the UPLC-MS/MS analysis of the digested monomer band at low intensities, but none were observed in the dimer band. Collectively, the mass spectrometry and SDS-PAGE analysis support the assignment of the higher molecular weight species in purified iLOV samples as homomeric cross-links of iLOV.

We suspected the origin of iLOV cross-linking was due to photochemical reactions mediated by the protein-bound FMN cofactor. To investigate this hypothesis, we performed time-dependent illuminations of iLOV and analyzed the products by SDS-PAGE (**Figure II-2A**). Following illumination of iLOV we observe the accumulation of the 26 kDa band with a concomitant diminished intensity of the monomer 13 kDa band. This effect can be seen after as little as 30 seconds of illumination, while after 2 hours of illumination the original monomer had nearly disappeared. In non-illuminated samples, little change in the ratio of dimer to monomer band intensities occurred over the course of the experiment. Analysis of

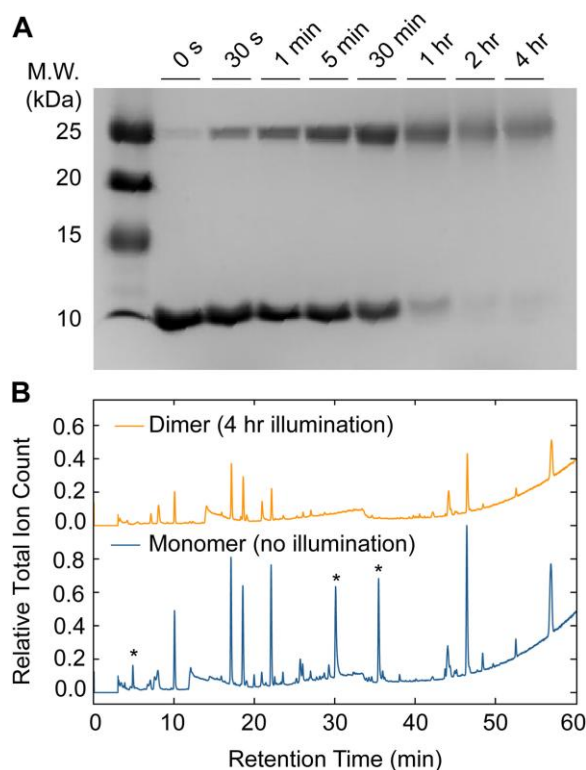


Figure II-2. Photochemical dimerization of iLOV. **A**, SDS-PAGE analysis of illumination time-dependent dimerization of iLOV. **B**, UPLC-MS analysis of total ion counts of trypsin digested SDS-PAGE gel-excised monomer (navy) and dimer (orange) bands. Peptides lost upon illumination in the dimer band are indicated by asterisks.

each gel extracted, trypsin digest of the illuminated dimer and non-illuminated monomer by UPLC-MS/MS revealed a loss of three peptide fragments, residues –3-3, 80-90, and 94-110 (**Figure II-2B**, **Table II-S3**, and **II-S4**). These peptides contain amino acids with known reactivity towards $^1\text{O}_2$, including histidine, methionine, and tryptophan, but only histidine is contained in all of the peptides. We also searched for potential post-translational modifications that may be expected following a reaction with $^1\text{O}_2$. We identified a +32 Da modification of the C-terminal peptide composed of residues 94-110 (**Figure II-S2**). Based on the

fragmentation pattern of this peptide in the MS2 spectrum, the +32 Da modification is C-terminal to L₁₀₄ within the amino acid sequence -DGSDHV-COOH.

Photochemical protein modifications are commonly associated with O₂ and ROS. Given that all the prior investigations were conducted aerobically, we sought to test whether O₂ and ROS were involved in iLOV cross-linking. We repeated the photochemical cross-linking experiment anaerobically and analyzed the photoproducts by SDS-PAGE (**Figure II-3A**). Over the course of the illumination, we observed little change in the ratio of the

iLOV monomer and dimer, in contrast to the same experiment performed aerobically in otherwise identical conditions. We then repeated the photochemical cross-linking experiments aerobically, supplemented with 10 mM of the sacrificial reductant dithiothreitol (DTT), which does not readily react with ³O₂, but does effectively scavenge the ROS ¹O₂ and other oxidized biomolecules (**Figure II-3B**).¹⁴ Again, we observed a substantial suppression of photochemical dimerization, compared to control experiments where DTT was omitted. The cumulative suppression of cross-linking in either O₂-free or DTT

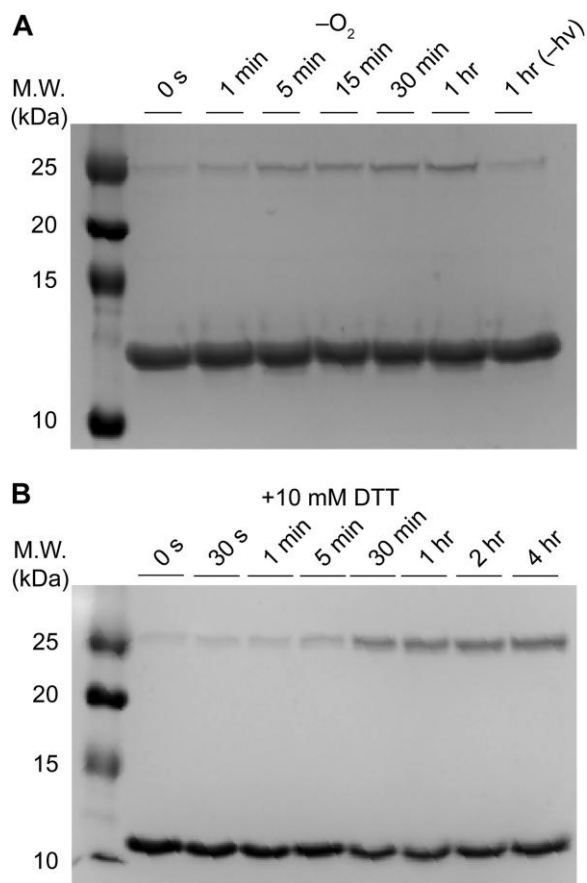


Figure II-3. Cross-linking of iLOV in reducing or anaerobic conditions. **A**, illumination of anaerobic iLOV. **B**, illumination of an aerobic samples of iLOV in the presence of 10 mM DTT.

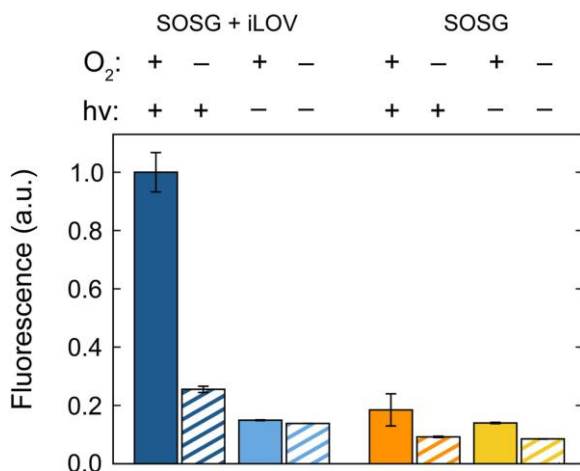


Figure II-4. iLOV-mediated photochemical ¹O₂ generation, observed through SOSG fluorescence in the presence (+) and absence (-) of O₂ and illumination (hν). The fluorescence response in all conditions is normalized to the SOSG signal of iLOV illumination aerobically (navy). Anaerobic SOSG responses shown in stripped bars. Photochemical response of SOSG alone is shown in orange and yellow.

supplemented conditions supports a role of O₂-mediated generation of ROS that cross-link iLOV.

Given the known reactivity of LOV-based flavoproteins towards O₂, with both ¹O₂ and •O₂⁻ being reported as photochemical products of reactions with

³O₂, we sought to identify the ROS responsible for iLOV cross-linking.^{11, 13, 17}

The singlet oxygen sensor green (SOSG) fluorophore is a ¹O₂ sensor which generates

an increase in fluorescence following reaction with ¹O₂ and is non-reactive with other ROS such as H₂O₂ and •O₂⁻.^{18, 19} Illumination of iLOV in the presence of SOSG results in an increase in fluorescence of SOSG (**Figure II-4**). In the absence of ³O₂, no production of ¹O₂ is observed by iLOV. The generation of ¹O₂ was also dependent on iLOV; no fluorescent SOSG was observed in the absence of iLOV in the presence or absence of ³O₂. These results suggest that ¹O₂ is a photoproduct of iLOV in the presence of ³O₂.

Regardless of the identity of the ROS, the molecular target of ROS cross-linking reactivity in iLOV must be identified if this chemistry is to be controlled. Several amino acids are susceptible to reactivity with diverse ROS, primarily W, Y, C, M, and H. The iLOV protein has four redox-active aromatic amino acid residues, one tryptophan and three tyrosines residues. In model studies, both tryptophan and tyrosine have been shown to covalently cross-link following exposure to ROS.^{14, 20} The sole tryptophan residue of iLOV

has been proposed to be an electron donor in photochemical electron transfer, generating a neutral $W^\bullet$ and a neutral FMN semiquinone (NSQ). This photochemical generation of an NSQ in iLOV has only been demonstrated in an iLOV mutant, Q₁₀₃D¹⁷, but the possibility of this reactivity in authentic iLOV could function as a route to FMN reduction and $\bullet O_2^-$ production. Alternatively, tyrosine and tryptophan also react with 1O_2 to form reactive intermediates that may chemically cross-link.¹⁴

To examine the role of tyrosine and tryptophan amino acids in protein cross-linking, we mutated each of these residues for the redox-inactive amino acid phenylalanine (F), denoted hereafter as iLOV ^{Δ YW}. Initially, we examined the excited state dynamics of the iLOV FMN via fluorescence lifetime measurements of the reconstituted protein. The FMN singlet excited state ($^1\text{FMN}^*$) lifetime was unchanged by the substitution of any one redox-active amino acid or the concerted substitution of all four of them, with iLOV $^1\text{FMN}^*$ fluorescence decay lifetimes of 4.43 (0.01) ns for iLOV and 4.42 (0.01) ns in iLOV ^{Δ YW} (**Figure II-S3A, SII-S3B** and **Table II-S5**). The lack of difference in the $^1\text{FMN}^*$ lifetime between iLOV and iLOV ^{Δ YW} does not rule out a role of the $^3\text{FMN}^*$ state in photochemical cross-linking via tyrosine or tryptophan photooxidation. To address the role of $^3\text{FMN}^*$ -mediated aromatic amino acid oxidation and iLOV cross-linking, we measured the cross-linking propensity of iLOV ^{Δ YW}. The iLOV ^{Δ YW} protein exhibited similar or increased levels of photochemical cross-linking, determined by SDS-PAGE, as the parent iLOV (**Figure II-S3C**). Thus, another amino acid residue enables photochemical cross-linking of iLOV.

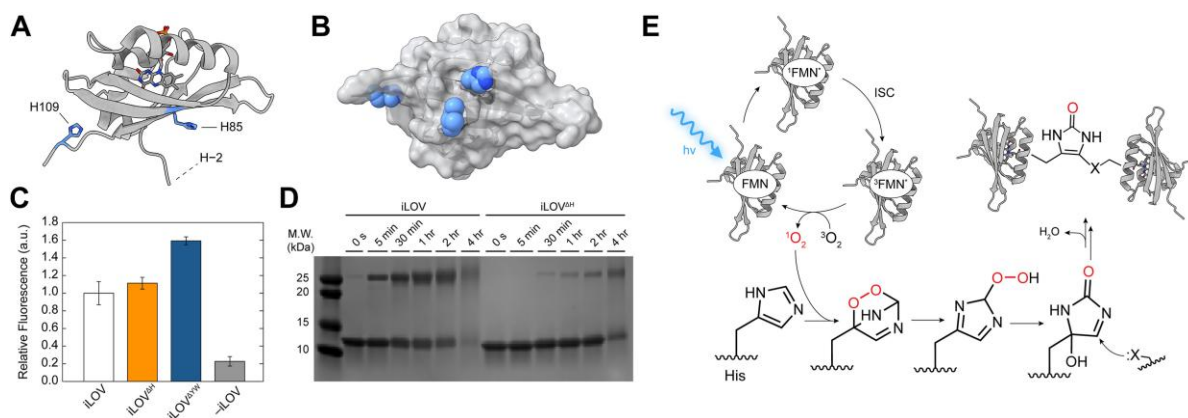


Figure II-5. The role of histidines in iLOV cross-linking. **A**, an X-ray crystal structure of iLOV (PDB: 4EES) depicting the locations of each histidine residue. H₋₂ is not present in the crystal structure as it was introduced only in this work. **B**, the same crystal structure depicting the van der Waals protein surface. The native histidine residues and the N-terminal I₁ (as a proxy for the N-terminal H₋₂) are depicted in blue spheres. **C**, SOSG response following 30 min illumination of iLOV (white), iLOV^{ΔH} (orange, p-value of 0.21), iLOV^{ΔYW} (navy, p value of 0.00015), and a -iLOV control (gray). **D**, effect of histidine residues on photochemical cross-linking measured by illumination time-dependent SDS-PAGE. **E**, the proposed mechanism of ¹O₂/histidine-mediated protein cross-linking.

To further test the potential role of $\bullet\text{O}_2^-$ in photochemical cross-linking we compared the ¹O₂ generation efficiency of iLOV and iLOV^{ΔYW}, as the two ROS are generated from the same triplet flavin photoproduct. Using SOSG as a fluorescent reporter for ¹O₂ (**Figure II-5**), we observed that the iLOV^{ΔYW} exhibited a larger SOSG response than the wild-type protein (p-value of 0.00015), suggesting $\bullet\text{O}_2^-$ formation is suppressed in the iLOV^{ΔYW} mutant, resulting in a greater quantum yield of ¹O₂ production from the flavin triplet. Illumination of samples of iLOV supplemented by large quantities of superoxide dismutase (SOD) did not affect the propensity for cross-linking and even resulted in a distribution of cross-linked products between iLOV and SOD as evidenced by SDS-PAGE (**Figure II-S4A**). The reaction of xanthine oxidase with ³O₂ and xanthine is known to produce $\bullet\text{O}_2^-$ and H₂O₂, but not ¹O₂, in a light-independent reaction, allowing the interrogation of $\bullet\text{O}_2^-$ -induced protein cross-linking^{21, 22}. Incubation of iLOV with xanthine oxidase (XO) and

xanthine in the dark did not result in detectable cross-linking (**Fig II-S4B**), again supporting the unique role of $^1\text{O}_2$.

The evidence presented herein is consistent with $^1\text{O}_2$ -mediated photochemical cross-linking of iLOV via an amino acid other than tyrosine or tryptophan. The known reactivity of $^1\text{O}_2$ with histidine, and the potential of these reactive products towards covalent cross-linking is well documented.^{23–25} There are three histidine residues in our iLOV protein (**Figure II-5A and II-5B**), two associated with the source phototropin 2 LOV protein, and one introduced during engineering. We mutated each H₈₅ and H₁₀₉ to serine (S) and H₂ to glycine (G), individually and collectively (iLOV^{ΔH}), to determine their role in photochemical cross-linking. None of the single histidine iLOV mutants exhibited significant attenuation of photochemical cross-linking by SDS-PAGE analysis (**Figure II-S5**). On the other hand, significantly less cross-linking was observed for the iLOV^{ΔH} with statistical change in the $^1\text{O}_2$ generation efficiency relative to the wild-type protein (**Figure II-5C and II-5D**). To examine potential post-translational modifications in the iLOV^{ΔH} protein following illumination, we performed tryptic digest of gel-extracted iLOV^{ΔH} pre- and post-illumination. After 30 minutes of illumination, two new peptides were identified with MS1 and MS2 masses consistent with the oxidation of W₈₁ (+32 Da and +4 Da) with similar, but distinct retention times to the parent non-oxidized W peptide (**Figure II-S6, II-S7 and Table II-S6**). Neither of these W oxidation photoproducts were observed in the parent iLOV after illumination, suggesting this is a minor modification when histidines are present. We also observed a loss of a mass associated with the N-terminal –3-3 peptide, despite the H₂G mutation. This peptide also contains two methionine residues, which we suspect are potential sites of post-translational modification in the absence of histidine.

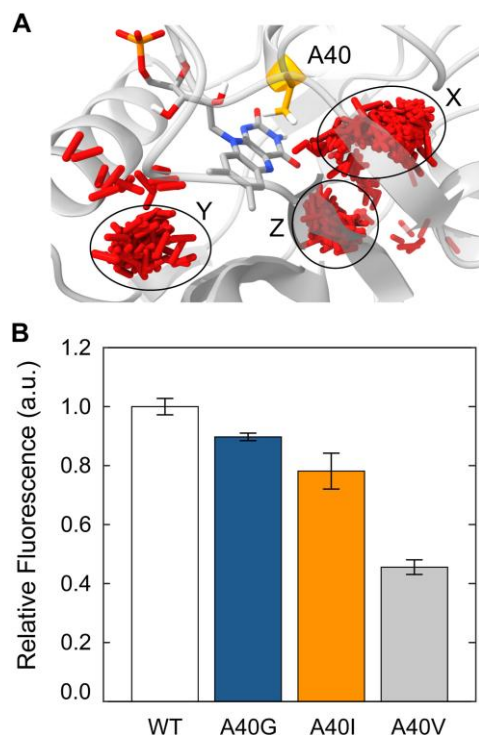


Figure II-6. Predicted O₂ diffusion into iLOV and role of A40 in access to FMN cofactor.

A, molecular dynamics simulations predict three sites occupied by O₂ within iLOV labeled “X,” “Y,” and “Z” near A₄₀. **B**, ¹O₂ generation by iLOV and various A₄₀ mutants determined by SOSG fluorescence after illumination. *p* values, calculated using a *t* test against parental iLOV were 0.0043, 0.000095, and 0.0048 for A40G, A40V, and A40I mutants, respectively. FMN, flavin mononucleotide; iLOV, improved light-oxygen-voltage; SOSG, singlet oxygen sensor *green*.

Understanding the nature of the photochemical ROS product(s) as well as the mechanism of generation is essential for rational engineering of iLOV photochemical properties. If ¹O₂ generation by iLOV mediates protein cross-linking via surface exposed histidine residues, limiting ¹O₂ generation via triplet-triplet energy transfer should suppress cross-linking activity. Energy transfer is a donor-acceptor distance-dependent process, and thus, the proximity of ³FMN* and ³O₂ may dictate ¹O₂ generation efficiency via a “type II” process.^{13, 27} To address the potential for O₂ to approach the FMN cofactor we performed five independent molecular

dynamics (MD) trajectory simulations of iLOV in the presence of O₂ over a collective 1.4 μs. Each simulation exhibited average protein backbone atom root-mean square deviations (RMSD) <2 Å, with only transient excursions >2 Å (**Figure II-S8**). In each of five independent MD trajectory simulations O₂ penetrated into the protein structure, occupying at least one of three sites, denoted “X,” “Y,” and “Z,” within 10 Å of the FMN cofactor (**Figure II-6A**).

The O₂ binding site X, identified in previous MD simulations, is lined with the alanine (A) residue A₄₀, which is a glycine (G) in the ¹O₂-generating LOV protein “miniSOG”^{10, 28, 29}. To determine whether steric access to this site was impactful on ¹O₂ generation, we mutated the A₄₀ residue in iLOV to either phenylalanine, isoleucine (I), valine (V), and glycine, and quantitated their respective ¹O₂ generation efficiency by reaction with SOSG via illumination-dependent fluorescence response. While A₄₀F exhibited weak binding of the FMN cofactor after reconstitution, A₄₀I, A₄₀V, and A₄₀G all exhibited varying degrees of diminished ¹O₂ yields after illumination, as determined by the SOSG fluorescent ¹O₂ reporter (**Figure II-6B**).

DISCUSSION

The redox environment of cells in aerobic conditions is dictated, in part, through ROS. The effects of ROS are complex, involving both the control of essential cellular functions as well as detrimental damage to biomolecular species such as proteins, nucleic acids, and lipids.^{14, 30} Genetically-encodable flavoproteins based on the LOV domain have been employed to spatio-temporally perturb the redox environment via photochemical generation of ¹O₂ or other ROS in cellular environments.^{10, 31–35} In these studies, the LOV proteins are assumed to be non-interacting with photo-generated ¹O₂ or other ROS sources, such that downstream cellular responses can be investigated with fidelity to the ROS, yet the reactivity of LOV-based proteins with ¹O₂ has not been thoroughly investigated.

In this study, we find that iLOV is a member of the LOV proteins that photochemically generate ¹O₂ and reacts directly with ¹O₂ to form homo-oligomeric protein covalent cross-links, evidenced by SDS-PAGE and mass spectrometry. The ¹O₂-mediated protein cross-linking is light- and ³O₂-dependent, and can be mitigated by thiol-based ¹O₂ scavengers.

Oligomerization proceeds through sequential steps of addition (i.e. monomer to dimer to trimer, etc.), shown by the MALDI intensity profile and SDS-PAGE analysis, but whether a previously oligomerized iLOV can serve as a photochemical $^1\text{O}_2$ generator remains to be determined. Using site-directed mutagenesis targeting the redox-active aromatic residues tryptophan and tyrosine in iLOV, we see an enhanced capacity for $^1\text{O}_2$ generation and photochemical cross-linking. We ascribe this phenomenon to the suppression of $^3\text{FMN}^*$ -W/Y electron transfer, eliminating a competing pathway for $^3\text{FMN}^*$ quenching, and therefore increasing the yield of $^1\text{O}_2$. This implies that $\bullet\text{O}_2^-$ is a photoproduct of iLOV, in addition to $^1\text{O}_2$. Even concentrations of SOD as high as 100 μM did not suppress photochemical cross-linking, supporting the assertion that $\bullet\text{O}_2^-$ is not responsible for the observed cross-linking, but we acknowledge that $^1\text{O}_2$ is a known inhibitor of SOD which could complicate the interpretation of these results.^{26,36} The role of $\bullet\text{O}_2^-$ is more directly demonstrated in the absence of observable cross-linking in the presence of xanthine and xanthine oxidase, a system known to produce $\bullet\text{O}_2^-$ but not $^1\text{O}_2$. Together with our estimation of $^1\text{O}_2$ by SOSG for iLOV and iLOV $^{\Delta\text{YW}}$, these results suggest that $\bullet\text{O}_2^-$ generation is a competing pathway for photochemical reactivity with $^3\text{O}_2$, but $\bullet\text{O}_2^-$ generation does not result in protein cross-linking. Conversely, mutation of surface exposed histidines to serine or glycine resulted in the ablation of photochemical cross-linking, while $^1\text{O}_2$ generation was maintained (**Figure II-S4A**). Whether as a product of photooxidation or ground-state reactivity with $^1\text{O}_2$, W₈₁ appears to be a secondary degradation target of iLOV, with +32 Da and +4 Da modifications which we tentatively assign to dioxyindolalanine or N-formylkynurenine and kynurenine respectively. A role for H_2O_2 must also be considered. While H_2O_2 is not a known photoproduct of either miniSOG or iLOV, the $\bullet\text{O}_2^-$ produced by

iLOV would be disproportionated to O₂ and H₂O₂ by SOD. We see no evidence for increased cross-linking of iLOV in the presence of SOD, again suggesting ¹O₂ initiates iLOV cross-linking.

Based on the evidence presented herein and literature precedent, we propose a mechanism for photochemical cross-linking described in **Figure II-5**.^{16, 37} The protein structure and dynamics facilitate ³O₂ penetration into iLOV's core where it resides for extended periods of time in one of three pockets, identified by MD simulations. These sites poise ³O₂ for triplet-triplet annihilation with the ³FMN* photoproduct of FMN excitation, following ¹FMN* intersystem crossing. While distinct O₂ binding sites are supported by our MD simulations, a series of mutant proteins designed to test the role of steric access to one of these binding sites revealed no correlation between steric bulk and ¹O₂ yield (**Figure II-6**), suggesting the exact nature of the encounter complex, and the potentially pleiotropic role of these mutations remain unresolved, particularly in relation to flavin-binding, as evidenced by the significantly reduced FMN binding in the A₄₀F mutant (**Table II-S9**). Once generated, ¹O₂ diffuses out of the protein and reacts with surface-exposed histidine residues in iLOV, resulting in an endoperoxide Diels-Alder adduct intermediate. This assertion is supported by the MS2 identification of a +32 Da modification of the C-terminal peptide fragment -DGSDHV-COOH of illuminated dimeric iLOV. This unstable species quickly rearranges to form a hydroperoxide intermediate, and later a reactive urea Schiff base species. This species is then vulnerable to nucleophilic attack by several possible nucleophilic residues on the protein surface. Based on the loss of histidine-containing peptides in the tryptic digest mass spectrometry analysis of dimerized proteins, we propose

histidine-histidine cross-links as the most common. The similarities between all phototropin 2-based LOV proteins suggest that $^1\text{O}_2$ -mediated cross-linking is a common phenomenon.

Our results demonstrate that, at least in the *in vitro* conditions examined in this study, iLOV photochemically cross-links to form oligomers via histidine residues. The implications of iLOV photochemical cross-linking on *in vivo* studies involving the use of genetically encoded $^1\text{O}_2$ generators remains to be determined. Several differences between the conditions investigated herein and those that may be expected *in vivo* will likely diminish iLOV cross-linking including decreased $^1\text{O}_2$ quantum yield due to light scattering and inner filter effects of competing chromophores, competing reactions such as $^1\text{O}_2$ reduction by cellular small molecules, enzymes or nucleic acids, or limited O_2 availability in microaerobic or anaerobic conditions. Nevertheless, given the success of prior studies in generating $^1\text{O}_2$ *in vivo*, the potential for iLOV heter- or homo-oligomeric cross-linking deserves consideration.^{10, 14} Given the potential for spatio and temporally resolved $^1\text{O}_2$ generation by this genetically encoded flavoprotein, $^1\text{O}_2$ generation and cross-linking properties of iLOV could be utilized for *in vivo* affinity labeling and pull-down of proteins reactive to ROS in a spatio-temporal manner via iLOV-binding antibodies or other similar technologies. For example, genetically fusing iLOV to one of two proteins suspected of forming a protein-protein complex would place the site of $^1\text{O}_2$ generation in close proximity to the protein-protein complex of interest. Upon illumination, iLOV-generated $^1\text{O}_2$ could attack histidines on the surface of one of the target proteins, forming a reactive electrophile, and subsequently generating cross-links with the proximal protein-protein complex partner. The iLOV fluorescence or affinity to antibodies could then be used to identify and enrich the protein-protein complex for identification. Alternatively, $^1\text{O}_2$ -mediated protein cross-linking,

particularly between iLOV and other proteins involved in the ROS response pathway, could complicate interpretation of experimental results. This can be controlled for by ablation of the iLOV histidines, which we show successfully inhibits iLOV cross-linking, limiting $^1\text{O}_2$ chemistry to cross-linking other proteins in the biological milieu or alternative pathways of $^1\text{O}_2$ quenching detailed above.

For our intended purpose of using iLOV as a model flavoprotein to test theories on flavin reduction potential tuning, these results suggest that special care must be taken when handling iLOV to avoid undesirable damage to the protein. Prior to this work, purification of iLOV was performed aerobically without the presence of thiol-based reductants such as DTT. Typically, DTT is added to protein purifications to mimic the reducing conditions inside the cell, preventing the formation of disulfide bridges between and within proteins via cysteine. iLOV contains no cysteine residues, which initially led us to believe that this addition was not necessary. Furthermore, no special care was taken to protect iLOV from ambient light, further exacerbating the problem of $^1\text{O}_2$ -mediated crosslinking via histidine. With this knowledge in mind, all aerobic purifications of iLOV in future chapters were performed mostly in the dark and in the presence of DTT to mitigate oxidative damage that might interfere with the interpretation of downstream results.

CONCLUSION

In this chapter, we have demonstrated that the iLOV protein photochemically generates $^1\text{O}_2$, which causes protein cross-linking in vitro. Based on our results from mutagenesis studies, the photochemical cross-linking chemistry can be rationally modulated to either bias cross-linking or ameliorate it for specified applications in vitro and in vivo. We hope these results will inform biotechnological development of genetically encoded fluorescent

tools and provide insight into the role of $^1\text{O}_2$ reactivity in biological systems with potential therapeutic applications. Furthermore, these results inform the creation of special protocols tailored to iLOV intended to prevent oxidative damage in the presence of ambient light during aerobic purifications.

REFERENCES

1. Fridovich, I. (1999) Fundamental Aspects of Reactive Oxygen Species, or What's the Matter with Oxygen? *Ann. N. Y. Acad. Sci.* 893, 13–18
2. Khademian, M., and Imlay, J. A. (2020) Do reactive oxygen species or does oxygen itself confer obligate anaerobiosis? The case of *Bacteroides thetaiotaomicron*. *Mol. Microbiol.* 114, 333–347
3. Zuo, L., Zhou, T., Pannell, B. K., Ziegler, A. C., and Best, T. M. (2015) Biological and physiological role of reactive oxygen species – the good, the bad and the ugly. *Acta Physiol.* 214, 329–348
4. Nathan, C., and Cunningham-Bussel, A. (2013) Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat. Rev. Immunol.* 13, 349–361
5. Feig, D. I., Reid, T. M., and Loeb, L. A. (1994) Reactive Oxygen Species in Tumorigenesis. *Cancer Res.* 54, 1890s–1894s
6. Dixon, S. J., and Stockwell, B. R. (2014) The role of iron and reactive oxygen species in cell death. *Nat. Chem. Biol.* 10, 9–17
7. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 10, 2247–2258
8. Greene, B. L., Kang, G., Cui, C., Bennati, M., Nocera, D. G., Drennan, C. L. et al. (2020) Ribonucleotide Reductases: Structure, Chemistry, and Metabolism Suggest New Therapeutic Targets. *Annu. Rev. Biochem.* 89, 45–75
9. Foote, C. S. (1968) Photosensitized oxygenations and the role of singlet oxygen. *Acc. Chem. Res.* 1, 104–110
10. Shu, X., Lev-Ram, V., Deerinck, T. J., Qi, Y., Ramko, E. B., Davidson, M. W., et al. (2011) A Genetically Encoded Tag for Correlated Light and Electron Microscopy of Intact Cells, Tissues, and Organisms. *PLOS Biol.* 9, e1001041
11. Westberg, M., Holmegaard, L., Pimenta, F. M., Etzerodt, M., and Ogilby, P. R. (2015) Rational Design of an Efficient, Genetically Encodable, Protein-Encased Singlet Oxygen Photosensitizer. *J. Am. Chem. Soc.* 137, 1632–1642

12. Ruiz-González, R., Cortajarena, A. L., Mejias, S. H., Agut, M., Nonell, S., and Flors, C. (2013) Singlet Oxygen Generation by the Genetically Encoded Tag miniSOG. *J. Am. Chem. Soc.* 135, 9564–9567
13. Pimenta, F. M., Jensen, R. L., Breitenbach, T., Etzerodt, M., and Ogilby, P. R. (2013) Oxygen-Dependent Photochemistry and Photophysics of “MiniSOG,” a Protein-Encased Flavin. *Photochem. Photobiol.* 89, 1116–1126
14. Di Mascio, P., Martinez, G. R., Miyamoto, S., Ronsein, G. E., Medeiros, M. H. G., and Cadet, J. (2019) Singlet Molecular Oxygen Reactions with Nucleic Acids, Lipids, and Proteins. *Chem. Rev.* 119, 2043–2086
15. Christie, J. M., Hitomi, K., Arvai, A. S., Hartfield, K. A., Mettlen, M., Pratt, A. J., et al. (2012) Structural Tuning of the Fluorescent Protein iLOV for Improved Photostability. *J. Biol. Chem.* 287, 22295–22304
16. Liu, M., Zhang, Z., Cheetham, J., Ren, D., and Zhou, Z. S. (2014) Discovery and Characterization of a Photo-Oxidative Histidine-Histidine Cross-Link in IgG1 Antibody Utilizing ¹⁸O-Labeling and Mass Spectrometry. *Anal. Chem.* 86, 4940–4948
17. Kopka, B., Magerl, K., Savitsky, A., Davari, M. D., Röllen, K., Bocola, M., et al. (2017) Electron transfer pathways in a light, oxygen, voltage (LOV) protein devoid of the photoactive cysteine. *Sci. Rep.* 7, 13346
18. Tanaka, K., Miura, T., Umezawa, N., Urano, Y., Kikuchi, K., Higuchi, T., et al. (2001) Rational Design of Fluorescein-Based Fluorescence Probes. Mechanism-Based Design of a Maximum Fluorescence Probe for Singlet Oxygen. *J. Am. Chem. Soc.* 123, 2530–2536
19. Singlet Oxygen Sensor Green Reagent (Invitrogen, Eugene, 2004) pp 1–2
20. Ludvíková, L., Štacko, P., Sperry, J., and Klán, P. (2018) Photosensitized Cross-Linking of Tryptophan and Tyrosine Derivatives by Rose Bengal in Aqueous Solutions. *J. Org. Chem.* 83, 10835–10844
21. De Pascali, F.; Hemann, C.; Samons, K.; Chen, C.-A.; Zweier, J. L. (2014) Hypoxia and Reoxygenation Induce Endothelial Nitric Oxide Synthase Uncoupling in Endothelial Cells through Tetrahydrobiopterin Depletion and S-Glutathionylation. *Biochemistry* 53, 3679–3688.
22. Lee, M.-C.; Velayutham, M.; Komatsu, T.; Hille, R.; Zweier, J. L. Measurement and Characterization of Superoxide Generation from Xanthine Dehydrogenase: (2014) A

Redox-Regulated Pathway of Radical Generation in Ischemic Tissues. *Biochemistry* 53, 6615–6623.

23. Marques, E. F., Medeiros, M. H. G., and Di Mascio, P. (2019) Singlet oxygen-induced protein aggregation: Lysozyme crosslink formation and nLC-MS/MS characterization. *J. Mass Spectrom.* 54, 894–905
24. Davies, M. J. (2003) Singlet oxygen-mediated damage to proteins and its consequences. *Biochem. Biophys. Res. Commun.* 305, 761–770
25. Davies, M. J. (2004) Reactive species formed on proteins exposed to singlet oxygen. *Photochem. Photobiol. Sci.* 3, 17–25
26. Kim, S. Y.; Kwon, O. J.; Park, J.-W. Inactivation of Catalase and Superoxide Dismutase by Singlet Oxygen Derived from Photoactivated Dye. *Biochimie* 2001, 83 (5), 437–444. [https://doi.org/10.1016/S0300-9084\(01\)01258-5](https://doi.org/10.1016/S0300-9084(01)01258-5).
27. Schweitzer, C., and Schmidt, R. (2003) Physical Mechanisms of Generation and Deactivation of Singlet Oxygen. *Chem. Rev.* 103, 1685–1758
28. Polyakov, I., Kulakova, A., and Nemukhin, A. (2023) Computational Modeling of the Interaction of Molecular Oxygen with the miniSOG Protein—A Light Induced Source of Singlet Oxygen. *Biophysica.* 3, 252–262
29. Pietra, F. (2014) Molecular Dynamics Simulation of Dioxygen Pathways through Mini Singlet Oxygen Generator (miniSOG), a Genetically Encoded Marker and Killer Protein. *Chem. Biodivers.* 11, 1883–1891
30. Sies, H., Berndt, C., and Jones, D. P. (2017) Oxidative Stress. *Annu. Rev. Biochem.* 86, 715–748
31. Xu, S., and Chisholm, A. D. (2016) Highly efficient optogenetic cell ablation in *C. elegans* using membrane-targeted miniSOG. *Sci. Rep.* 6, 21271
32. Westberg, M., Bregnhøj, M., Etzerodt, M., and Ogilby, P. R. (2017) No Photon Wasted: An Efficient and Selective Singlet Oxygen Photosensitizing Protein. *J. Phys. Chem. B.* 121, 9366–9371
33. Makhijani, K., To, T.-L., Ruiz-González, R., Lafaye, C., Royant, A., and Shu, X. (2017) Precision Optogenetic Tool for Selective Single- and Multiple-Cell Ablation in a Live Animal Model System. *Cell Chem. Biol.* 24, 110–119

34. Westberg, M., Etzerodt, M., and Ogilby, P. R. (2019) Rational design of genetically encoded singlet oxygen photosensitizing proteins. *Curr. Opin. Struct. Biol.* 57, 56–62
35. Trewin, A. J., Berry, B. J., Wei, A. Y., Bahr, L. L., Foster, T. H., and Wojtovich, A. P. (2018) Light-induced oxidant production by fluorescent proteins. *Free Radic. Biol. Med.* 128, 157–164
36. Escobar, J. A.; Rubio, M. A.; Lissi, E. A. SOD and Catalase Inactivation by Singlet Oxygen and Peroxyl Radicals. *Free Radic. Biol. Med.* 1996, 20 (3), 285–290.
[https://doi.org/10.1016/0891-5849\(95\)02037-3](https://doi.org/10.1016/0891-5849(95)02037-3).
37. Méndez-Hurtado, J., López, R., Suárez, D., and Menéndez, M. I. (2012) Theoretical Study of the Oxidation of Histidine by Singlet Oxygen. *Chem. – Eur. J.* 18, 8437–8447
38. Anderson, N. T., Weyant, K. B., and Mukherjee, A. (2020) Characterization of flavin binding in oxygen-independent fluorescent reporters. *AIChE J.* 66, e17083
39. Guerrero, F., Ciragan, A., and Iwaï, H. (2015) Tandem SUMO fusion vectors for improving soluble protein expression and purification. *Protein Expr. Purif.* 116, 42–49
40. Cáceres, J. C., Bailey, C. A., Yokoyama, K., and Greene, B. L. (2022) Selenocysteine substitutions in thiyl radical enzymes. *Methods Enzymol.* 662, 119–141
41. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76–85
42. Davari, M. D., Kopka, B., Wingen, M., Bocola, M., Drepper, T., Jaeger, K.-E., et al. (2016) Photophysics of the LOV-Based Fluorescent Protein Variant iLOV-Q489K Determined by Simulation and Experiment. *J. Phys. Chem. B.* 120, 3344–3352
43. van den Berg, P. A. W., Widengren, J., Hink, M. A., Rigler, R., and Visser, A. J. W. G. (2001) Fluorescence correlation spectroscopy of flavins and flavoenzymes: photochemical and photophysical aspects. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* 57, 2135–2144
44. Cáceres, J. C., Dolmatch, A., and Greene, B. L. (2023) The Mechanism of Inhibition of Pyruvate Formate Lyase by Methacrylate. *J. Am. Chem. Soc.* 145, 22504–22515
45. Becker, W. (2005) *Advanced Time-Correlated Single Photon Counting Techniques*, Springer Science & Business Media

46. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics. *J. Mol. Graph.* 14, 33–38
47. Best, R. B., Zhu, X., Shim, J., Lopes, P. E. M., Mittal, J., Feig, M., and MacKerell, A. D. Jr. (2012) Optimization of the Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling of the Backbone ϕ , ψ and Side-Chain χ_1 and χ_2 Dihedral Angles. *J. Chem. Theory Comput.* 8, 3257–3273
48. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 79, 926–935
49. Aleksandrov, A. (2019) A Molecular Mechanics Model for Flavins. *J. Comput. Chem.* 40, 2834–2842
50. Wang, S., Hou, K., and Heinz, H. (2021) Accurate and Compatible Force Fields for Molecular Oxygen, Nitrogen, and Hydrogen to Simulate Gases, Electrolytes, and Heterogeneous Interfaces. *J. Chem. Theory Comput.* 17, 5198–5213
51. Phillips, J. C., Hardy, D. J., Maia, J. D. C., Stone, J. E., Ribeiro, J. V., Bernardi, R. C., et al. (2020) Scalable molecular dynamics on CPU and GPU architectures with NAMD. *J. Chem. Phys.* 153, 044130
52. ProteinLynx Global SERVER Version 2.2.5, Revision A. Waters Corp., 2006, pp. B1-B7. Accessed: July. 16, 2024. [Online]. Available: <https://www.waters.com/webassets/cms/support/docs/71500125602ra.pdf>
53. Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. *Anal. Chem.* 2003, 75 (17), 4646–4658. <https://doi.org/10.1021/ac0341261>.
54. Li, G.; Vissers, J. P. C.; Silva, J. C.; Golick, D.; Gorenstein, M. V.; Geromanos, S. J. Database Searching and Accounting of Multiplexed Precursor and Product Ion Spectra from the Data Independent Analysis of Simple and Complex Peptide Mixtures. *Proteomics* 2009, 9 (6), 1696–1719. <https://doi.org/10.1002/pmic.200800564>.
55. Kong, A. T.; Leprevost, F. V.; Avtonomov, D. M.; Mellacheruvu, D.; Nesvizhskii, A. I. MSFragger: Ultrafast and Comprehensive Peptide Identification in Shotgun Proteomics. *Nat Methods* 2017, 14 (5), 513–520. <https://doi.org/10.1038/nmeth.4256>.
56. Yu, F., Teo, G. C., Kong, A. T., Haynes, S. E., Avtonomov, D. M., Geiszler, D. J., & Nesvizhskii, A. I. (2020). Identification of modified peptides using localization-aware open search. *Nature Communications*, 11(1), 1-9.

57. Yu, F., Haynes, S. E., Teo, G. C., Avtonomov, D. M., Polasky, D. A., & Nesvizhskii, A. I. (2020). Fast quantitative analysis of timsTOF PASEF data with MSFragger and IonQuant. *Molecular & Cellular Proteomics*, 10(9), 1575-1585.
58. Teo, G. C., Polasky, D. A., Yu, F., Nesvizhskii, A. I. (2020). A fast deisotoping algorithm and its implementation in the MSFragger search engine. *Journal of Proteome Research*, 20(1), 498-505.

SUPPLEMENTARY MATERIAL

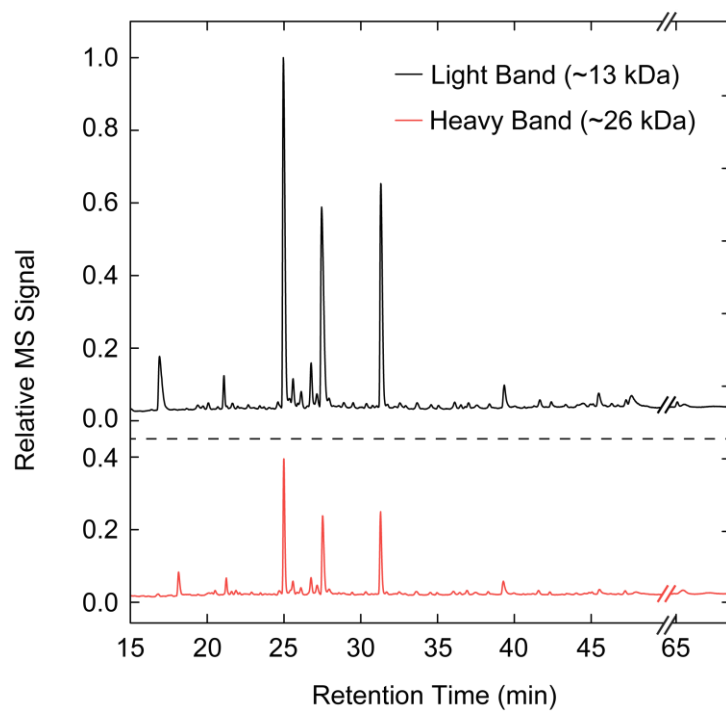


Figure II-S1. Total ion chromatograms from tryptic digests of 13 and 26 kDa bands extracted from an SDS-PAGE iLOV purification gel resolved by UPLC MS1 chromatograms. Both extractions exhibited the same major peptides identified from fragmentation data as originating from iLOV.

Table II-S1. Tryptic peptides of iLOV detected by UPLC-MS/MS from the SDS-PAGE gel extracted monomeric iLOV band following purification using Water's ProteinLynx Global Server (PLGS) software. The iLOV protein was detected with an “OK” designation, signifying >95% probability, a PLGS scores of 11,188.0, and 84.2% sequence coverage.

Coverage:

SHMMIEKNFVITDPRLPDNPIIFASDGFLTEYSREEILGRnarFLQGPETDQATVQkirdairdqrET
TVQLINYTKsgkKFWNLLHLQPVRdqkGELQYFIGVQLDGS DHV. Capital letters indicate amino acids detected in tryptic peptides.

Peptide Residues	Sequences	Relative Intensity	Observed Peptide MH ⁺ (Da)	Expected Peptide MH ⁺ (Da)	MH ⁺ Error (ppm)	Avg. Charge	PLGS Score	Fragmentation Products Detected
-3-3	SHMMIEK	0.0142	875.4119	875.4114	0.6509	1.99	8.0001	b2, b3, b4, b5, b6, y1, y2, y3, y4, y5, y6, y7
4-11	NFVITDPR	0.5564	961.5161	961.5102	6.2278	1.8	7.354	b1, b2, b3, b4, b5, b6, b8, y1, y2, y6, y7, y8
12-32	LPDNPIIFASDGFLTEYSR	0.0066	2397.188	2397.1973	3.8924	2.44	8.0644	b2, b3, b4, b5, b6, b7, b8, b9, b10, b11, b12, b13, b14, b16, b17, b18, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y14, y15
33-38	EEILGR	0.1210	716.3931	716.3937	0.8657	1.33	8.117	b1, b2, b3, b4, b5, b6, y1, y2, y3, y4, y5
42-55	FLQGPETDQATVQK	1.0000	1561.7892	1561.7856	2.2687	2.04	8.4694	b3, b4, b5, b6, b8, b9, b10, b11, b13, y1, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13
65-75	ETTVQLINYTK	0.6205	1309.7028	1309.6998	2.2334	1.93	8.4951	b1, b2, b3, b5, b6, b7, b8, b9, b10, b11, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10
79-90	KFWNLLHLQPV R	0.0020	1550.894	1550.8954	0.9358	3.08	7.219	b1, b3, b4, y2, y3, y5, y6, y8, y9
80-90	FWNLLHLQPVR	0.0393	1422.7986	1422.8005	1.3094	2.89	8.0217	b2, b3, b4, b5, b6, b7, b8, b11, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11
94-110	GELQYFIGVQLDGS DHV	0.0007	1876.9077	1876.9076	0.0834	2.36	7.9295	b2, b4, b5, b6, b7, b8, b9, b17, y2, y3, y5, y7, y8, y10, y12

Table II-S2. Tryptic peptides of iLOV detected by UPLC-MS/MS from an SDS-PAGE gel extracted dimeric iLOV band using Water's ProteinLynx Global Server (PLGS) software. The iLOV protein was detected with an "OK" designation, signifying >95% probability, a PLGS scores of 8901.957, and sequence coverage of 86.0%.

Coverage:

SHMMIEKNFVITDPRLPDNPIIFASDGFLELTEYSREEILGRnarFLQGPETDQATVQKIRdairdqrET
TVQLINYTKsgkKFWNLLHLQPVRdqkGELQYFIGVQLDGS DHV. Capital letters indicate amino acids detected in tryptic peptides.

Peptide Residues	Sequences	Relative Intensity	Observed Peptide MH ⁺ (Da)	Expected Peptide MH ⁺ (Da)	MH ⁺ Error (ppm)	Avg. Charge	PLGS Score	Fragmentation Products Detected
-3-3	SHMMIEK	0.0142	875.4119	875.4114	0.6509	1.99	8.0001	b2, b3, b4, b5, b6, y1, y2, y3, y4, y5, y6, y7
4-11	NFVITDP R	0.5564	961.5161	961.5102	6.2278	1.8	7.354	b1, b2, b3, b4, b5, b6, b8, y1, y2, y6, y7, y8
12-32	LPDNPIIF ASDGFLELTE YSR	0.0066	2397.188	2397.1973	3.8924	2.44	8.0644	b2, b3, b4, b5, b6, b7, b8, b9, b10, b11, b12, b13, b14, b16, b17, b18, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y14, y15
33-38	EEILGR	0.1210	716.3931	716.3937	0.8657	1.33	8.117	b1, b2, b3, b4, b5, b6, y1, y2, y3, y4, y5
42-55	FLQGPET DQATVQK	1.0000	1561.7892	1561.7856	2.2687	2.04	8.4694	b3, b4, b5, b6, b8, b9, b10, b11, b13, y1, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13
65-75	ETTVQLI NYTK	0.6205	1309.7028	1309.6998	2.2334	1.93	8.4951	b1, b2, b3, b5, b6, b7, b8, b9, b10, b11, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10
79-90	KFWNLL HLQPVR	0.0020	1550.894	1550.8954	0.9358	3.08	7.219	b1, b3, b4, y2, y3, y5, y6, y8, y9
80-90	FWNLLHL QPVR	0.0393	1422.7986	1422.8005	1.3094	2.89	8.0217	b2, b3, b4, b5, b6, b7, b8, b11, y1, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11
94-110	GELQYFI GVQLDGS DH V	0.0007	1876.9077	1876.9076	0.0834	2.36	7.9295	b2, b4, b5, b6, b7, b8, b9, b17, y2, y3, y5, y7, y8, y10, y12

Table II-S3. Tryptic peptides of non-illuminated iLOV detected by UPLC-MS/MS from a single SDS-PAGE gel extracted monomeric iLOV band using the FragPipe software package. The iLOV protein was detected with protein probability of 1.0000, and a sequence coverage of 71.9%.

Coverage:

SHMMIEKnfvitdprLPDNPIIFASDGFLELTEYSReeilgrnarFLQGPETDQATVQKirdairdqrETTVQLIN
YTKsgkKFWNLLHLQPVRdqkGELQYFIGVQLDGSDHV. Capital letters indicate amino acids detected in tryptic peptides.

Peptide Residues	Peptide Sequence	Observed Precursor m/z	Expected Peptide m/z	m/z Error (ppm)	z	Spectral Count	PeptideProphet Probability	Fragmentation Products Detected
-3-3	SHMMIEK	875.4119	875.4113	-0.72	1.0	2	0.7898	b2, b3, b4, b5, b6, y2, y4, y5
12-32	LPDNPIIF ASDGFLELTE YSR	799.0659	799.7372	-0.79	3.0	35	0.9975	b2, b3, b4, b5, b6, b7, b8, b9, b10, b11, b12, b13, b14, b15, b16, b17, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13, y14, y18
12-32	LPDNPIIF ASDGFLELTE YSR	1198.599	1199.102	-0.53	2.0	6	0.9975	b2, b3, b4, b5, b6, b7, b8, b9, b11, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13, y14, y15, y17, y18, y20
42-55	FLQGPET DQATVQK	520.5954	521.2667	-1.08	3.0	12	0.9666	b2, b3, b4, b5, b6, b7, b8, b9, y2, y3, y4, y5, y6, y7, y8, y9, y10
42-55	FLQGPET DQATVQK	780.8931	781.3964	-0.74	2.0	4	0.9604	b2, b3, b4, b6, b7, b10, b11, b12, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13
65-75	ETTVQLI NYTK	654.8502	655.3535	-0.89	2.0	2	0.8991	b2, b4, y2, y3, y4, y5, y6, y7, y8, y9
79-90	KFWNLL HLQPVR	516.9653	517.6366	-1.15	3.0	2	0.8542	b3, b6, b7, b10, y3, y4, y5, y6, y7, y8, y9, y10, y11
80-90	FWNLLHLQ PVR	474.267	474.9383	-1.19	. 0	15	0.9604	b2, b3, b4, y2, y3, y4, y5, y6, y7, y8, y9, y10
80-90	FWNLLHLQ PVR	711.4005	711.9038	-0.81	. 0	3	0.8542	b2, b3, b4, b6, b8, y3, y4, y5, y6, y7, y8, y9, y10
94-110	GELQYFIGV QLDGSDHV	625.636	626.3073	-0.90	. 0	12	0.9840	b3, b4, b5, b6, b7, b8, b9, b10, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13, y15
94-110	GELQYFIGV QLDGSDHV	938.454	938.9573	-0.62	. 0	7	0.8801	b3, b5, b6, b7, b9, b11, y2, y5, y6, y7, y8, y9, y10, y11, y12, y13, y15

Table II-S4. Tryptic peptides of 4 hr illuminated iLOV detected by UPLC-MS/MS from a single SDS-PAGE gel extracted dimeric iLOV band using the FragPipe software package. The iLOV protein was detected with protein probability of 1.0000, and a sequence coverage of 62.3%.

Coverage:

shmmiekNFVITDPRLPDNPIIFASDGFLELTEYSReeilgrnarFLQGPETDQATVQKirdairdqrETTVQL
INYTKsgkkfwnllhlqpvrdaqkGELQYFIGVQLDGS DH[+32]V. Capital letters indicate amino acids detected in tryptic peptides.

Peptide Residues	Peptide Sequence	Observed Precursor m/z	Expected Peptide m/z	m/z Error (ppm)	z	Spectral Count	PeptideProphet Probability	Fragmentation Products Detected
4-11	NFVITDP R	961.5101	961.5107	-0.62	1	1	0.8501	b2, b3, b4, y2
4-11	NFVITDP R	481.2587	481.2593	-1.14	2	3	0.8449	b2, b3, y2, y4, y5, y6
12-32	LPDNPIIF ASDGFLELTE YSR	1199.102	1199.103	-0.50	2	20	0.9929	b2, b3, b4, b5, b6, b7, b8, b9, b10, b11, b13, b15, b16, b18, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12, y13, y14, y15, y16, y17, y18, y19, y20
12-32	LPDNPIIF ASDGFLELTE YSR	799.7372	799.7378	-0.75	3	34	0.9929	b2, b3, b4, b5, b6, b7, b8, b9, b10, b11, b12, b13, b14, b15, b16, b17, b19, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y14
42-55	FLQGPET DQATVQK	521.2667	521.2672	-1.02	3	29	0.9929	b2, b3, b4, b5, b6, b7, b8, b9, b10, y2, y3, y4, y5, y6, y7, y8, y9, y10, y11
65-75	ETTVQLI NYTK	655.3535	655.3541	-0.84	2	4	0.8870	b2, b3, b4, b5, b6, b7, b8, b9, y2 y3, y4
94-110	GELQYFI GVQLDGS DH[+32]V	954.9580	954.9573	-0.73	2.0	3	0.8519	b ⁵ , b ⁶ , b ⁷ , y ⁶ , y ⁷ , y ¹⁰

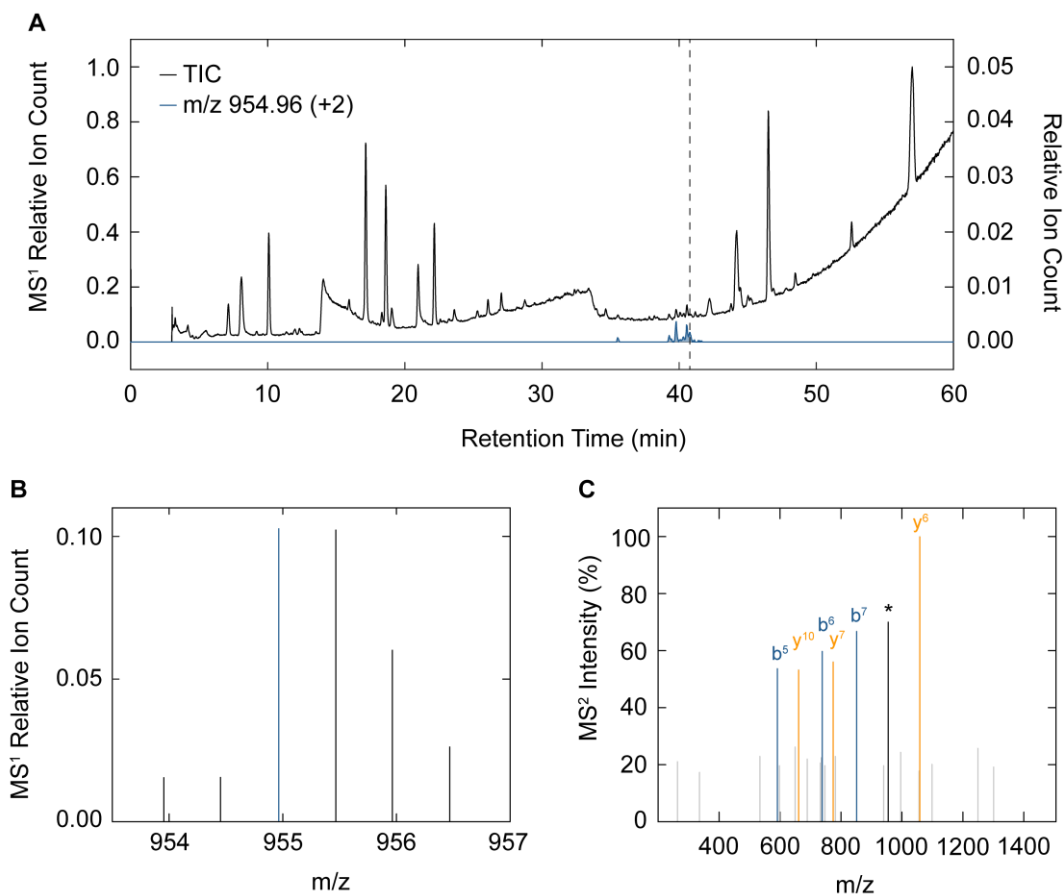


Figure II-S2. UPLC-MS/MS evidence of a +32 Da modification of peptide 94-110 H₃N⁺-GELQYFIGVQLDGSDHV-COOH in the 4-hour illuminated dimeric gel extracted iLOV band. **A UPLC-MS¹ total ion chromatogram (black) and +2 peptide +32 Da (954.96 m/z, navy). **B** MS¹ spectrum at retention time indicated by the dashed line in panel **A**. The navy peak was fragments in the MS² spectrum shown in **C**. Identified y (orange) and b (navy) ions are annotated, and the precursor peptide is shown in black with an asterisk. All y and b ions were consistent with a +32 Da modification to an amino acid N-terminal to L104 (sequence underlined above).**

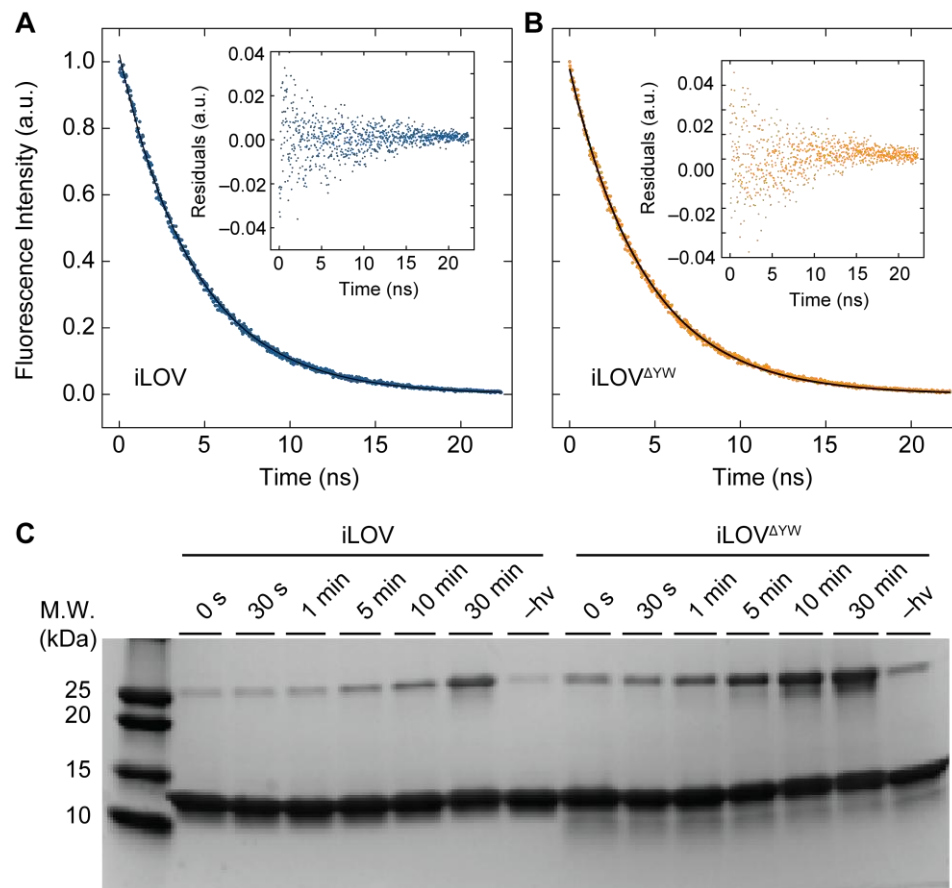


Figure II-S3. Fluorescence lifetime measurements of iLOV and iLOV^{ΔYW} and photochemical cross-linking behavior. **A** Normalized fluorescence lifetime measurement of iLOV (navy circles) and corresponding single-exponential fit to experimental data (black line). Inset shows experimental residuals to the fit. **B** Normalized fluorescence lifetime measurement of iLOV^{ΔYW} (orange circles) and corresponding single-exponential fit to experimental data (black line). Inset shows experimental residuals to the fit. **C** Illumination time-dependent SDS-PAGE analysis of iLOV and iLOV^{ΔYW} exposed under identical conditions.

Table II-S5. Fluorescence decay lifetimes (τ) for iLOV mutants for which tyrosines and tryptophans had been mutated to phenylalanine. Error is represented by 95 % confidence intervals determined from an exponential fit of the raw fluorescence data. Representative raw data, fits, and residuals for iLOV and iLOV $^{\Delta YW}$ are shown in Figure S2.

iLOV Variant	τ (ns)
WT	4.43 ± 0.01
Y30F	4.44 ± 0.01
Y73F	4.46 ± 0.02
Y98F	4.40 ± 0.02
Y30F/Y98F	4.43 ± 0.02
Y30F/Y72F/Y98F	4.40 ± 0.02
ΔYW (Y30F/Y72F/Y98F/W81F)	4.42 ± 0.01

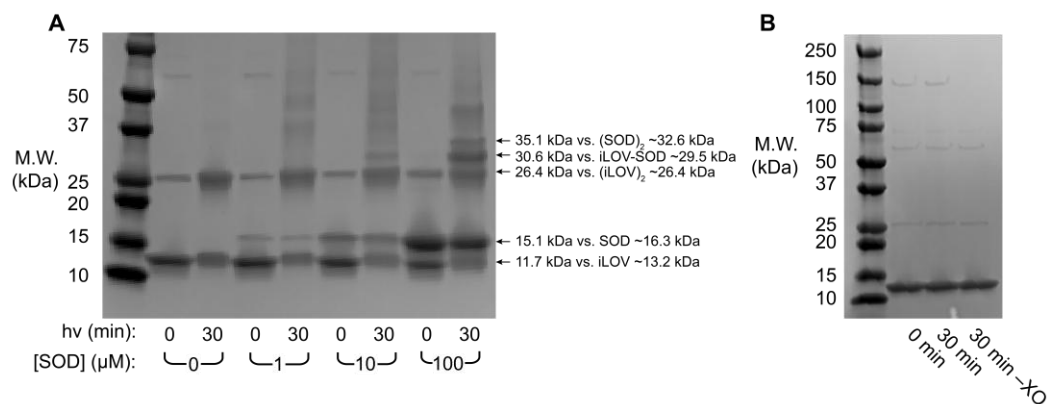


Figure II-S4. Investigation of $\bullet\text{O}_2^-$ contribution to photochemical cross-linking. **A** SDS-PAGE analysis of iLOV photochemical cross-linking in the presence or absence of superoxide dismutase (SOD). Samples were illuminated for 0 or 30 mins. The molecular weight standards were used for a semi-log regression analysis to calculate apparent molecular weights of various protein cross-link products. **B** SDS-PAGE analysis of non-illuminated iLOV in the presence or absence of the xanthine-xanthine oxidase (XO) system.

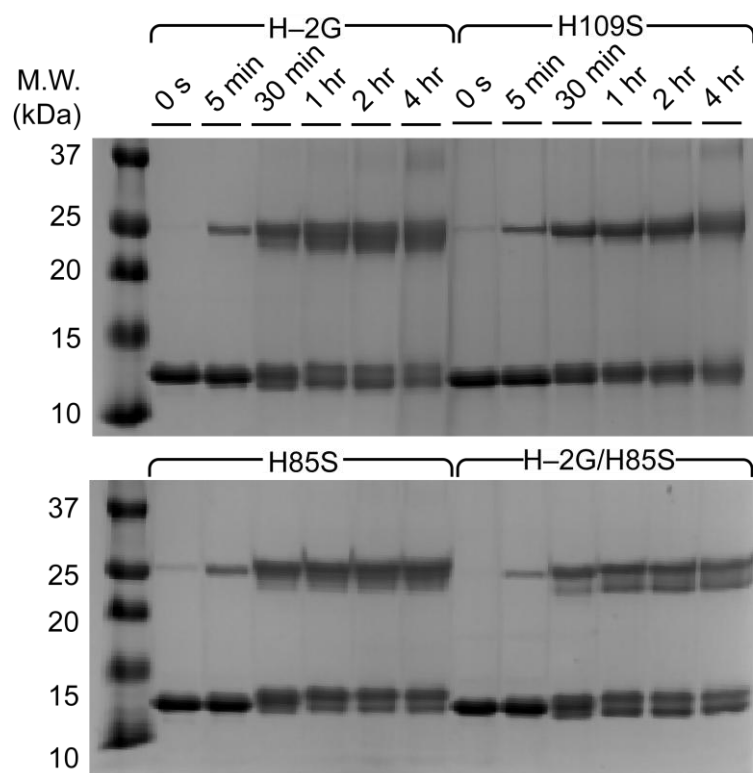


Figure II-S5. SDS-PAGE analysis of illumination time-dependent photochemical cross-linking for iLOV and various histidine mutants. Apparent band “doubling” observed in H-2G, H85S, and H-2G/H85S appears to be an artifact of the home-made SDS-PAGE gels, and was not reproducible in separate experiments with Bio-Rad pre-cast 4-20% gradient gels.

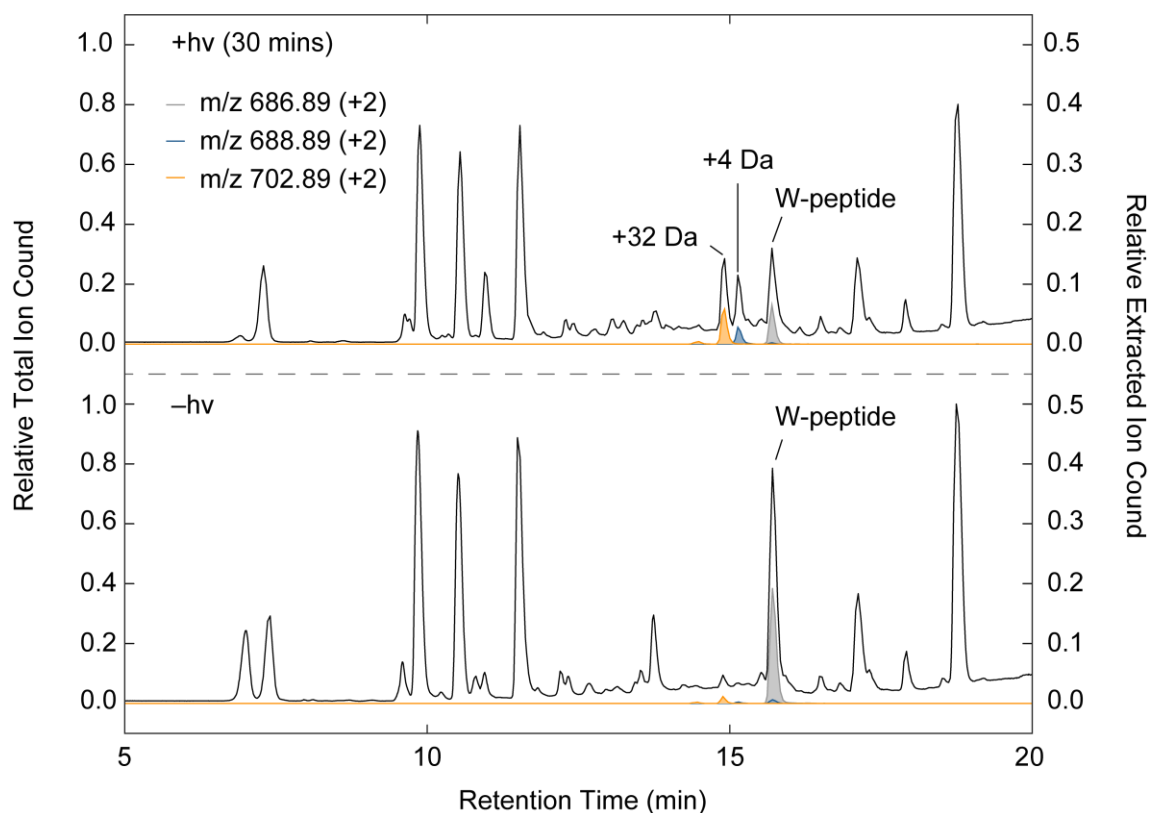


Figure II-S6. Total ion chromatograms of tryptic digests of iLOV Δ^H with (30 mins, top) and without (bottom) illumination. The iLOV Δ^H peptide fragment (80-90) containing the sole W residue is indicated, as well as the corresponding +4 and +32 Da peptides identified in the illuminated samples. Extracted ion chromatograms of the unmodified peptide (gray), the +4 Da modified peptide (navy), and the +32 Da modified peptide (orange) are also shown. Each chromatogram is scaled to the maximum ion count of the total ion chromatogram of the non-illuminated sample.

Table II-S6. Tryptic peptides detected within a MS² fragmentation range of 685-730 m/z derived from the SDS PAGE gel extracted monomeric iLOV^{ΔH} band. Peaks were detected using FragPipe. This sample was actively illuminated for 30 minutes. Sequence indicates site of H85S mutation (underlined bold). The y and b ions which demonstrate the localization of the +32 or +4 Da modifications of W81 are indicated with “*”.

Peptide	Sequence	Observed Precursor m/z	Expected Peptide m/z	m/z Error (ppm)	z	Spectral Count	PeptideProphet Probability	Fragmentation Products Detected
80-90	FWNLL <u>S</u> LQPVR	686.8903	686.8908	0.728	2.0	4	1.0000	b2, b3, b4, b5, b6, b7, b8, y2, y3, y4, y5, y6, y7, y8, y9, y10
80-90	FW[+32] NLL <u>S</u> LQPVR	702.8897	702.8857	5.691	2.0	3	1.0000	b2*, b3*, b4*, b5*, b8*, b9*, y2, y3, y4, y5, y6, y7, y8, y9
80-90	FW[+4]N LL <u>S</u> LQPVR	688.8900	688.8882	2.613	2.0	6	1.0000	b2*, b3*, b4*, b5 8 b8*, y3, y4, y5, y6, y7, y8, y9

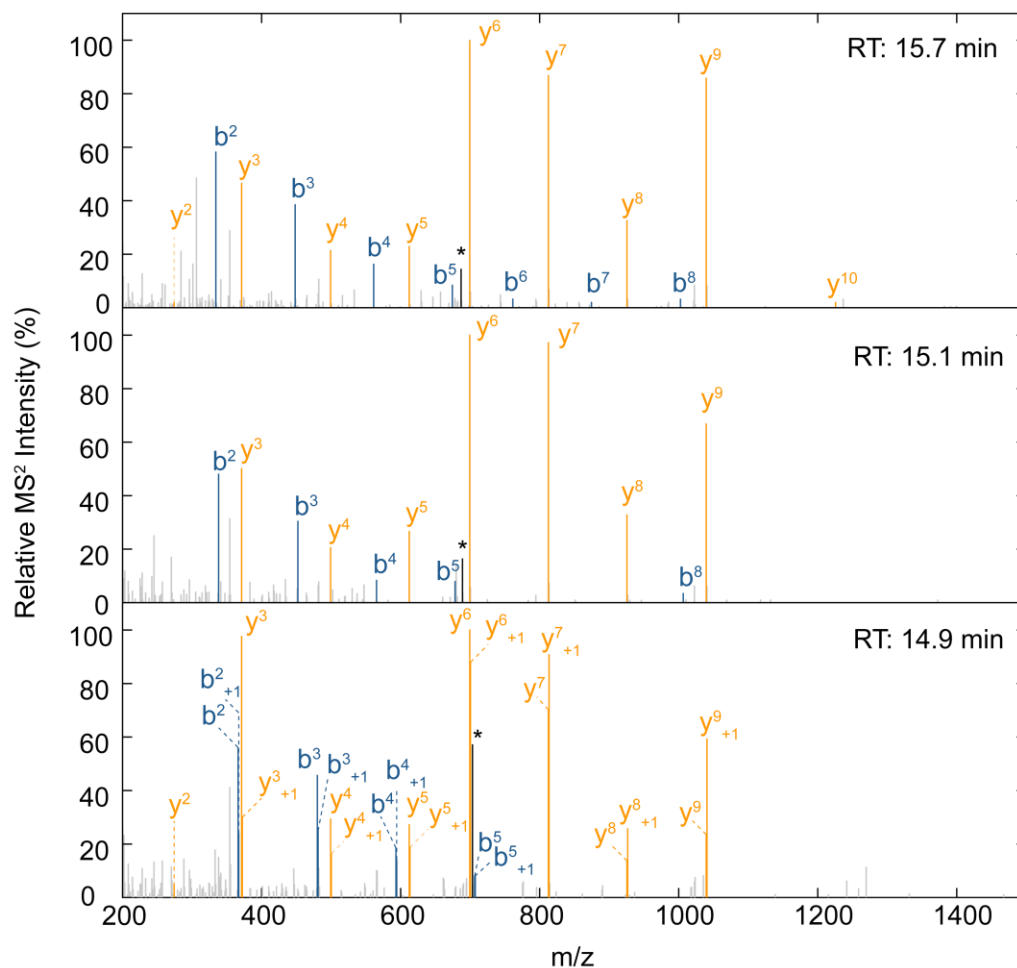


Figure II-S7. Representative UPLC-MS/MS spectra of tryptic W-peptides from the 30 min illuminated iLOV^{ΔH}. The peptide sequence is H₃N⁺-FWNLLSLQPVR-COOH, where the underlined W is the site of post-translational modification. Retention times correspond to peaks in **Figure S2.6**. Identified +1 y (orange) and b (navy) ions are annotated and summarized in **Table S6**. The parental ion is shown in black with “*”. For the peptide at retention time 14.9 min, the +1 Da isotope peak was fragmented by the data-dependent acquisition parameters (although the monoisotopic peak was observed in the MS¹ spectra)resulting in a mixture of both y/b and y/b+1 ions.

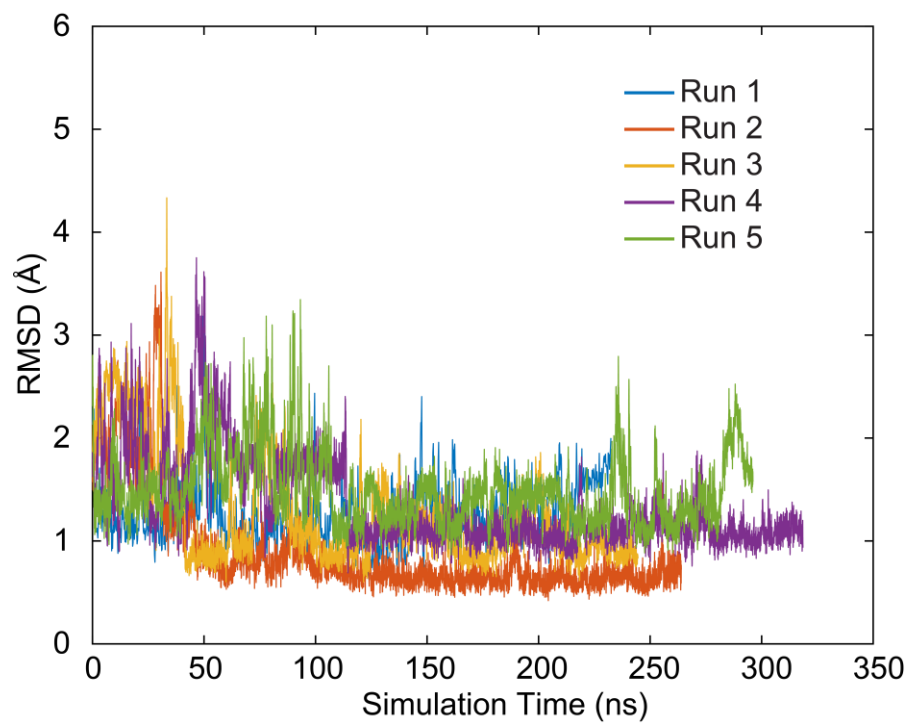


Figure II-S8. RMSD of backbone atoms in iLOV over the course of 5 molecular dynamics simulations of approximately 285 ns using the average structure as a reference molecule. Calculations were performed in the RMSD Trajectory plugin for Visual Molecular Dynamics (VMD) software.

Table II-S7. Primers that were used in the assembly of the pSUMO (H)₆-SUMO-iLOV.

Primer	Sequence
iLOV forward	5'-agattggtggatcccatatgatgattgaaaaaactttgtgattaccgacccg-3'
iLOV reverse	5'-gcggtggcagcagcctactcgagttatacgtggtcagaaccatccag-3'
pSUMO forward	5'-atggttctgaccacgtataactcgagtaggctgctgccaccgc-3'
pSUMO reverse	5'-acaaagtttttcaatcatcatatgggatccaccaatctgttctct-3'

Table II-S8. Primers used for site-directed mutagenesis (SDM) of pSUMO (H)₆-SUMO-iLOV.

Primer	Sequence
Y30F forward	5'-cctggaactgactgagtttagccgtg-3'
Y30F reverse	5'-ggattttctcacggctaaactcag-3'
Y73F forward	5'-cgactgtgcagctgattaactttaccaa-3'
Y73F reverse	5'-ccgctcttggtaaagtaatcagc-3'
W81F forward	5'-caagagcggtaaaaaattcttaacctgctgc-3'
W81F reverse	5'-cggctgcaggtgcagcaggttaagaattttt-3'
Y98F forward	5'-gaaaggcgagctgcagttttcatcggtgttc-3'
Y98F reverse	5'-ccatccagctgaacaccgatgaaaaactg-3'
A40G forward	5'-gcaatggctgttttctgcagggcccg-3'
A40G reverse	5'-gcagaaaacgaccattgcggcccaggatttc-3'
A40F forward	5'-gcaattttcgttttctgcagggcccg-3'
A40F reverse	5'-gcagaaaacgaaaattgcggcccagg-3'
A40I forward	5'-gcaatattcgttttctgcagggcccg-3'
A40I reverse	5'-gcagaaaacgaatattgcggcccagg-3'
A40V forward	5'-gcaatgttcgttttctgcagggcc-3'
A40V reverse	5'-cagaaaacgaacattgcggcccagg-3'
H-2G forward	5'-ggtggatccggtatgatgattgaaaaaactttgtgattaccgaccc-3'
H-2G reverse	5'-catcataccggatccaccaatctgttctctgtgagcctc-3'
H85S forward	5'-cctgctgtccctgcagccggtccgtg-3'
H85S reverse	5'-gcagggacagcaggttcagaatttttaccgc-3'
H109S forward	5'-gttctgactccgtataactcgagtagtaggctgctgc-3'
H109S reverse	5'-gttatacggagtcagaacctccagctgaacacc-3'

Table II-S9. As purified and reconstituted FMN loading of iLOV^{ΔYW} and iLOV^{ΔH} variants. Flavin concentrations were determined via UV-vis absorption at 448 nm using an extinction coefficient of 14,800 M⁻¹ cm⁻¹. Protein molarity was determined via the Pierce 660 assay. Initial FMN:iLOV ratios prior to flavin reconstitution are based on single measurements. Reconstituted FMN:iLOV ratios and uncertainty are based on the average and standard deviation of triplicate measurements.

iLOV Variant	As purified FMN:iLOV	Reconstituted FMN:iLOV
WT	0.21	1.05 ± 0.02
Y30F	0.33	1.05 ± 0.02
Y73F	0.34	0.98 ± 0.02
Y98F	0.30	1.12 ± 0.02
W81F	0.36	1.09 ± 0.02
Y30F/Y98F	0.31	1.16 ± 0.02
Y30F/Y72F/Y98F	0.15	0.80 ± 0.02
ΔYW (Y30F/Y72F/Y98F/W81F)	0.00	0.65 ± 0.03
A40G	0.21	0.92 ± 0.01
A40V	0.38	0.95 ± 0.01
A40I	0.11	0.94 ± 0.01
A40F	0.00	0.26 ± 0.04
H-2G	0.34	1.19 ± 0.02
H85S	0.54	1.16 ± 0.02
H109S	0.42	1.17 ± 0.02
H-2G/H85S	0.64	1.25 ± 0.02
ΔH (H-2G/H85S/H109S)	0.50	1.37 ± 0.02

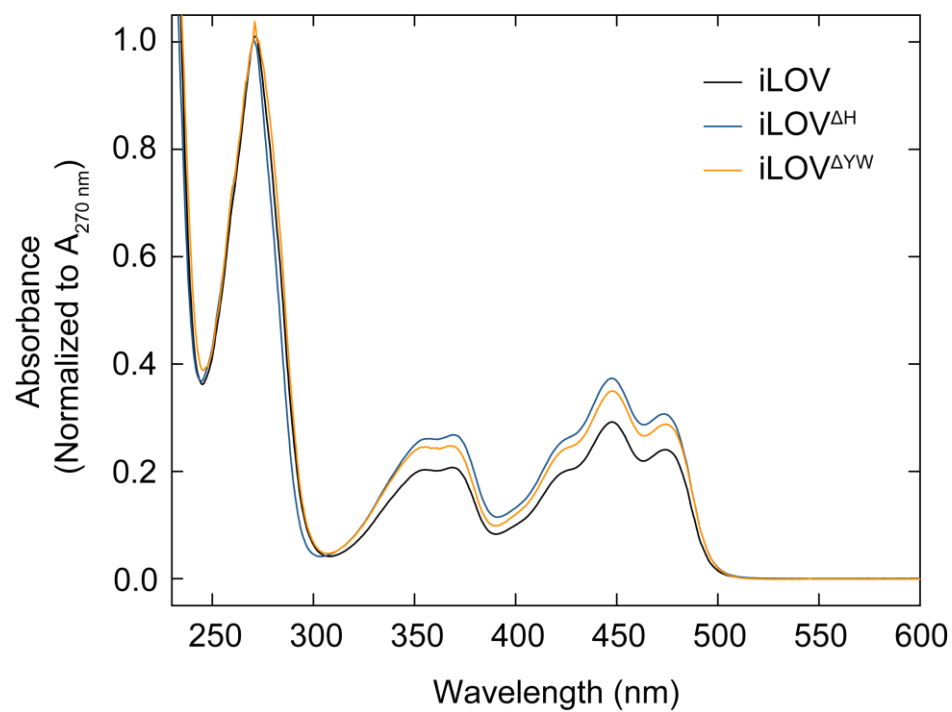


Figure II-S9. UV-vis spectra of reconstituted iLOV, iLOV Δ^H , and iLOV Δ^{YW} . Spectra were normalized to the absorbance at 270 nm.

III. The N5 Site: Modulating iLOV's OX/NSQ Redox Couple

Part of the content of this chapter was published in Jones, B. J.; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.

ABSTRACT

In this chapter, we investigate how the flavin N5 environment influences the redox properties of the flavin mononucleotide cofactor in iLOV using site-directed mutagenesis and redox titrations. Mutating the residue Q₁₀₃, which interacts with the flavin N5 and O4 atoms in the X-ray crystallographic structure, exerts a modest <35 mV effect on the overall two-electron reduction potential, but significantly alters the potential separation of the two one-electron couples (potential crossing) by up to 168 mV. QM/MM and free energy calculations conducted by our collaborators reveal that water penetration into the flavin-binding pocket near N5 and O4 largely explains the trend in reduction potentials among the mutants. The results suggest a molecular mechanism of flavin tuning in which hydrogen bonding to the neutral semiquinone, either directly by the side-chain or a protein-penetrating water, contributes significantly to the potential crossing. These findings establish quantitative experimental benchmarks for theoretical models and advance a molecular mechanism for redox tuning in flavoproteins.

INTRODUCTION

In a crossed flavin, the two-electron reduction potential ($E_{OX/HQ}$) arises from the average of its two one-electron potentials ($E_{OX/SQ}$ and $E_{SQ/HQ}$), a natural consequence of the law of conservation of energy. The pH-dependence of each redox couple is determined by whether protonation accompanies the transfer of the electron, which further depends on the solution pH and the pK_a of the participating redox species.^{1,2} Because pK_a is highly dependent on the local flavin binding pocket, the pH dependence of flavin reduction potentials can vary from protein to protein (**equation III-11**).^{3,4} In the subset of flavoproteins that undergo the proton-coupled reduction of flavin OX by one electron to produce NSQ, the N5 atom becomes protonated, transforming it from a hydrogen bond acceptor to a donor. Similarly, during the proton-coupled reduction by a second electron, NSQ may be protonated at the N1 position to form NHQ, while the protonation state of N5 remains unchanged (see **Figure I-1A**).^{5,6} These changes in protonation state affect the strength and character of hydrogen bonds that can be made to the flavin at these sites, in turn affecting the energetics of each redox state. The extent to which a particular redox state will be stabilized depends on the ΔpK_a of the donor and acceptor atoms, the orientation of the hydrogen bond participants, and the hydrogen bond distance.⁷ Thus, we hypothesized that by changing the identity of the residue or species that participates in hydrogen bonding at N5, we would preferentially affect $E_{OX/NSQ}$ while minimally affecting the $E_{NSQ/HQ}$ couple. By asymmetrically biasing one couple over the other, we anticipated that the overall two electron couple ($E_{OX/HQ}$) to be moderately affected, while the extent of potential crossing (ΔE) will either be lowered or increased. Based on the conserved presence of positive lysine (K) or arginine (R) nearby N5 in most evolutionarily distinct FBEBases we further anticipated that the introduction of a

positive charge near this site might change the ionization state of the SQ radical from neutral to anionic and increase the extent of reduction potential crossing.⁸

Here, we leverage the iLOV as a model flavoprotein to dissect the role of the protein scaffold near the flavin N5 in controlling redox behavior of a crossed potential flavin (**Figure I-3**).^{9,10} Using site-directed mutagenesis, we altered residue Q₁₀₃ (residue 489 in the original *A. thaliana* phototropin 2 numbering scheme), which forms a hydrogen bond with the flavin N5 and O4 in the oxidized state.¹⁰ Potentiometric titrations, spectrophotometric comproportionation, and electron paramagnetic resonance (EPR) spectroscopy demonstrate that Q₁₀₃ variants primarily shift $E^{\circ}_{\text{OX/NSQ}}$, while minimally affecting $E^{\circ}_{\text{NSQ/NHQ}}$, thereby altering ΔE . Surprisingly, all mutants, including Q₁₀₃K, exhibited a decrease in potential crossing and did not follow expected trends based on the pK_a of the introduced residue. Hybrid quantum mechanical molecular mechanical (QM/MM) and classical alchemical free energy simulations reproduce these experimental results and provide a mechanistic basis; hydrogen bonding to N5 and O4 by penetrating water molecules from the solvent stabilizes the NSQ state, but not the OX state, increasing $E^{\circ}_{\text{OX/NSQ}}$. Taken together, these results establish that the protein scaffold is capable of independent tuning of flavin one-electron chemistry by modulating N5 protonation and N5 or O4 hydrogen bonding, offering new strategies for rational design and mechanistic interpretation of flavoprotein redox behavior.

MATERIALS AND METHODS

Materials

The iLOV gene within a pQE80L vector backbone was obtained from the Mukherjee lab and cloned into a pSUMO vector as reported previously^{11,12}. The 6× polyhistidine-tagged

ubiquitin-like protease 1 (ULP1) was expressed and purified as reported previously.^{13,14} Primers for PCR were ordered from Integrated DNA Technologies (IDT). DpnI, Phusion HF polymerase, chemically competent DH5- α and BL21 (DE3) *Escherichia coli*, the Qiagen QIAprep Spin Miniprep and Gel Extraction kits, and the NEBuilder HiFi DNA Assembly kit were purchased from New England Biolabs (NEB). IPTG was purchased from Merck. Sephadex G-25 fine resin, LB Miller broth, NaH₂PO₄ (P_i), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), bovine xanthine oxidase (XO), xanthine, 4-hydroxy-(2,2,6,6-tetramethylpiperidin-1-yl)oxyl (4-hydroxy-TEMPO), sodium hydrosulfite (dithionite, NaDT), and Amicon Ultra centrifugal filters were purchased from Millipore Sigma. FMN sodium salt, HisPur nickel nitrilotriacetic acid (Ni-NTA) resin, bicine, safranin O, methyl viologen (MV²⁺), 96-well plates, Pierce 660 nm protein assay kit, amber NMR tubes, and Imperial protein stain were obtained from Thermo Fisher Scientific. Bradford reagent was purchased from VWR. Kanamycin sulfate was obtained from Genesee Scientific. Riboflavin was obtained from Acros Organics. Microseal “B” 96-well plate seals were obtained from BioRad. The single-color, cold, mounted, light-emitting diode (LED) with a wavelength of 450 nm and a bandwidth of 18 nm, KG-1, and KG-2 glass filters were obtained from ThorLabs. The pyrolytic graphite edge-plane (PGE) electrode, platinum wire counter electrode, Ag/AgCl (3M) reference electrode, and saturated calomel reference electrode (SCE) were obtained from Bioanalytical Systems (BASi) research products. The SP-200 single channel potentiostat and FC-45 Faraday cage were obtained from BioLogic. Alumina polishing pads and 0.05, 0.3, 1.0 μ m sized alumina were obtained from Buehler. Precision quartz (0.40 mm wall) EPR tubes were obtained from SP Wilmad. Nanopure water

was obtained via a Barnstead E-Pure ultrapure water system ($>17\text{ M}\Omega/\text{cm}$) and was used for all solutions.

Production of iLOV Mutants

Construction of the pSUMO-iLOV plasmid vector, growth and aerobic purification of (H)₆-SUMO-iLOV and mutants, ULP1 digestion, purification of iLOV, and loading and quantitation of FMN were conducted as described in Chapter II.¹² Associated FMN content before and after purification is provided in **Table III-S1** and protein purity gels are shown in **Figure III-S1**.

Site-Directed Mutagenesis of iLOV

Mutagenic primers used for site-directed mutagenesis to generate Q₁₀₃X ($X = \text{G, A, R, K, E and D}$) iLOV are reported in **Table III-S2**, and mutagenesis was carried out via the polymerase chain reaction (PCR) per the NEB protocol. The PCR products were transformed into *E. coli* DH5 α and the cells were plated onto LB/agar plates supplemented with 50 mg/L kanamycin and grown overnight. The following day, colonies were picked for growth in small 5 mL cultures. Mutant plasmids were purified by DNA miniprep and sent for nanopore sequencing at UC Berkeley's DNA sequencing facility. Upon confirmation of the correct sequences, mutant plasmids were used to transform *E. coli* BL21(DE3) cells for protein expression and growth as described previously.¹²

Quantum Yields, Excitation, and Emission Spectra

Samples of iLOV were prepared in 50 mM P_i 200 mM NaCl at pH 7.50 to an absorbance of 0.05 at 450 nm to minimize inner filter effects, and verified on an Agilent Cary 60 UV-vis spectrophotometer in triplicate. The emission spectra of each variant between 470 and

900 nm was collected in a Duetta Spectrometer (Horiba Scientific) using an excitation wavelength of 450 nm and samples were thermostated at 25 °C. An excitation bandpass was set to 5 nm, while the emission bandpass was set to 1 nm with an emission increment of 0.5 nm. A total of 50 detector accumulations each with an integration time of 300 ms were averaged to obtain final emission spectra. The quantum yield of the sample was calculated using **equation III-1**.

$$\Phi_S = \Phi_R \cdot \frac{I_S}{I_R} \cdot \frac{1 - 10^{-A_R}}{1 - 10^{-A_S}} \quad (\text{Eq. III-1})$$

Here, Φ_S is the quantum yield of the sample unknown (iLOV and Q_{103X} mutants), Φ_R is the quantum yield of some reference, (in this case riboflavin with a reported quantum yield of 0.26),¹⁵ I_S and I_R represent the integrated emission intensity of the sample and reference, respectively, and A_S and A_R represent the absorbance of the sample and reference, respectively. The standard deviations of the Φ_S were propagated by both the standard deviation of the absorbance measurement and the integrated emission intensity.

Excitation spectra were collected on the same instrument scanning excitation wavelengths from 300 to 500 nm with an excitation step of 1 nm. The emission wavelength was fixed at 520 nm. Both the excitation and emission bandpass were set to 5 nm with an integration time of 50 ms. A total of 10 detector accumulations were used for each data point.

Photoluminescence Lifetimes

Samples of iLOV and Q_{103X} mutants were diluted to an absorbance of 0.05 at 400 nm to minimize inner filter effects. Fluorescence lifetime measurements were performed using

time-correlated single photon counting (TCSPC). An approximately 100 fs excitation pulse with wavelength of 400 nm was generated by doubling the fundamental frequency of Ti:sapphire laser (Spectraphysics Tsunami) pulses in a commercial optical harmonics generator (Inrad/Coherent). The laser repetition rate was reduced by a homemade acousto-optical pulse picker in order to avoid saturation of the chromophore. The TCSPC detection system was equipped with a single-photon counting avalanche photodiode module (Photon Micro Devices) and electronics board (PicoQuant PicoHarp 300) with an instrument response time <50 ps. A triggering signal for the TCSPC board was generated by sending a small fraction of the excitation beam onto a fast (400 MHz bandwidth) Si photodiode (Thorlabs Inc.). The fluorescence signal was collected in a 90° geometry and dispersed in Acton Research SPC-500 monochromator after passing through a pump blocking, long wavelength-pass, autofluorescence-free, interference filter (Omega Filters, ALP series). The monochromator was equipped with a CCD camera (Roper Scientific PIXIS-400) allowing for monitoring of the fluorescence spectrum. Fluorescence transients were not deconvolved with the instrument response function since their fluorescent decay lifetimes were much longer than the system response to the excitation pulse. The resulting fluorescent lifetime traces were fit to a single exponential (**equation 3.2**)

$$I(t) = I_0 \cdot e^{\frac{-t}{\tau}} + C \quad (\text{Eq. III-2})$$

where $I(t)$ is the time-dependent intensity, I_0 is the initial emission intensity, t is time, τ is the emission lifetime, and C is a constant baseline offset, using MATLAB's "fitype" function. Standard deviations in τ were extracted from the fit using the 68% confidence interval as reported by MATLAB's "confint" command.

Preparation of Anaerobic iLOV HQ and OX States

Samples of ~300 μ M purified and reconstituted iLOV or Q₁₀₃X mutants in the flavin OX state were degassed on a Schlenk line before being brought into a COY anaerobic glove box (< 20 ppm O₂). Simultaneously, solid samples of NaDT and MV²⁺ were brought into the glove box and separately dissolved in anaerobic water to final concentration of 1 M NaDT and 0.1 M MV²⁺. MV²⁺ was quantitated by reduction with excess NaDT and measuring the absorbance at 606 nm of the cation radical (MV^{•+}), with an extinction coefficient of 13,700 M⁻¹ cm⁻¹.¹⁶ The degassed OX iLOV was then split into two equal volumes. To prepare the HQ iLOV, NaDT and MV²⁺ were added to 100-fold and 10-fold molar excess over iLOV, respectively, to fully reduce the flavin over 15 min. MV²⁺/MV^{•+} was necessary to kinetically accelerate flavin reduction over NaDT alone, and also served as a visual indicator to ensure NaDT and MV²⁺/MV^{•+} were subsequently removed during buffer exchange of the iLOV HQ via a Sephadex G25-desalting column.

After reduction, both OX (unreacted) and HQ iLOV samples were buffer exchanged into 20 mM NaCl and 5 mM P_i at pH 7.90 via a Sephadex G25 desalting column. The eluted protein samples were then concentrated via Amicon centrifugal filters (10 kDa MWCO) to a volume of about 300 μ L before placing them in glass crimp vials and moved to a second VAC Atmospheres glovebox (< 2 ppm O₂) for further analysis.

In the second glovebox, the OX and HQ protein samples were diluted 20-fold in 200 mM NaCl, 50 mM P_i at pH 7.90 and placed in a quartz UV-vis cuvette with a rubber septum. The cuvettes were brought out of the glovebox so that the UV-vis spectra of both the iLOV HQ and OX samples were recorded. The HQ sample was then exposed to air for 2 min with intermittent manual mixing to reoxidize the sample before again collecting a

spectrum. Concentrations of the initial HQ and OX iLOV samples were then determined by their OX flavin absorbances (448 nm for iLOV OX, 445 nm for Q_{103D} OX, or 440 nm for all other mutants). An extinction coefficient of 14,800 M⁻¹ cm⁻¹ was used for wild-type, while 16,100 M⁻¹ cm⁻¹ was used for blue-shifted mutants.¹⁷ Protein samples were used immediately after preparation.

Anaerobic UV-vis Measurements

Anaerobic UV-vis measurements were carried out as described previously¹⁸ with minor modifications as described here. Spectra were collected using a custom fiber optic-connected Ocean Optics QEPro spectrometer and DH-2000-BAL light source. The sample cuvette was mounted in a custom cuvette holder inside the glovebox. The cuvette holder includes fiber optic connectors, a slot for variable sized pinholes at the light-incident face to control light exposure, and a slot for colored glass filters on the light-exiting face (KG-1 or KG-2). The purpose of the pinhole is to narrow the beam of light to reproducibly fit within the confinement of the window of a low-volume 585.3-Q-10 quartz flow cell from Starna Cells. In addition, we have observed that illumination of iLOV OX in anaerobic conditions results in the formation of small amounts of NSQ. Thus, the pinhole, in concert with automated shutter control on the light source set only to open when a measurement is being collected, minimizes light exposure and light-induced accumulation of NSQ. Control experiments where the frequency of the measurement was doubled showed no effect on NSQ accumulation, indicating photochemical SQ formation is indeed minimized by this approach. The use of the flow cell, while not actually being used to flow liquids during measurements, allows for very small sample volumes (70-80 μ L) to be used. The purpose of the glass filter is to improve the balance of the light source output across the region of the

spectrum where OX and NSQ absorb (450–650 nm), improving the signal-to-noise ratio across this region. A buffer blank was used to establish zero absorbance and a linear baseline correction was used by subtracting a least-squares linear regression fit calculated between 680 and 900 nm—where no species exhibits absorption—and extrapolated across the entire wavelength range. Linear baseline shifts were observed with even minor mechanical adjustments to the fiber cables, but otherwise does not affect the spectrum.

Measurement of the NSQ Molar Absorptivity

To quantify NSQ formation by comproportionation (*vida infra*) we measured the extinction coefficient (ϵ) of the NSQ at its maximal absorbance (λ_{max}) at the red-shifted absorption feature at 616 nm (ϵ_{616}). The iLOV Q₁₀₃D mutant is light-sensitive and undergoes quantitative photo-reduction to the NSQ state when exposed to 450 nm light, making it an ideal candidate for estimation of the ϵ_{616} , which was assumed to be identical across the iLOV variants in this study.¹⁹ Samples of OX Q₁₀₃D were brought into a VAC glovebox after degassing, as described above. A quartz cuvette with an open top was prepared containing 400 μL of roughly 25 μM protein in a buffer composed of 200 mM NaCl and 100 mM MES at pH 6.50. As the sample sat in a cuvette holder, it was illuminated directly from above with a 2 W 450 nm LED, and photoreduction was monitored spectrophotometrically. The increase in sample absorbance at 616 nm indicated the generation of NSQ, which reached equilibrium after 10 minutes **Figure III-S2**. At this point the LED was turned off to remove interference from the excitation light, and the final spectrum of the semiquinone species was obtained. The absorptivity of the NSQ was estimated from the ratio of the absorbance at 445 nm in the original OX state (which we assumed had an absorptivity of

16,100 M⁻¹ cm⁻¹ from the similarly blue-shifted Q₁₀₃K mutant with λ_{max} of 440 nm)¹⁷ and the absorbance at 616 nm in the photoreduced NSQ state with ϵ_{616} of 8,200 $\pm$ 100 M⁻¹ cm⁻¹.

Purifying and Characterizing Isomers of Safranin.

Commercially available safranin O contains several isomers with distinct optical and redox properties.²⁰ To ensure reliable redox titrations with a dye indicator of a known reduction potential and extinction coefficient, we purified the isomers of commercially available safranin O using a Shimadzu Nexera preparative high-performance liquid chromatography (HPLC) system with an inline single quadrupole mass spectrometer (MS) detector and UV-vis detector. The isomers were resolved on a 19 mm x 150 mm XBridge BEH C18 OBD column with 130 Å pore size and 5 μ m particle size, using a stepwise elution gradient, with acetonitrile concentrations increasing from 12.5% to 13.5% over 25 minutes, and then 13.5% to 20% over 2 min. Three different species were separated, labelled “1”, “2”, and “3”. Isomer 1 was not sufficiently resolved from isomer 2 to be used, but isomers 2 and 3 fractions were sufficiently pure for analysis and use. Both isomers were analyzed by ¹H NMR in *d*₆-DMSO on a 500 MHz Bruker Avance NEO spectrometer. Amber NMR tubes were used to protect the sample from light. Structural assignments of isomer 2 and 3 are reported in Supplemental Information **Figure III-S3** and **Table III-S3**. The reduction potential of isomer 3 (1 mM) in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 18 mM K₂PO₄) was measured as a function of pH by cyclic voltammetry with a pyrolytic graphite (edge plane) working electrode, a platinum wire counter electrode, and a Ag/AgCl (3 M NaCl) reference electrode (**Figure III-S4**). Potentials were scanned from -0.2 V to -1 V with a scan speed of 100 mV/s. The Ag/AgCl reference electrode potential was calibrated against a saturated calomel electrode (SCE)

using a multimeter and the resulting potentials were adjusted to be relative to the standard hydrogen electrode (SHE). Based on the close match in E° of the safranine isomer 3 and iLOV, this isomer was used for all redox titration experiments and is simply referred to as “safranine,” henceforth.

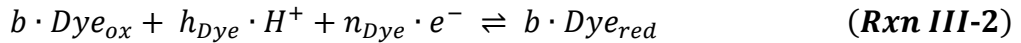
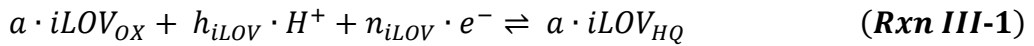
Measurement of iLOV $E^\circ_{OX/HQ}$

To measure the equilibrium two-electron $E^\circ_{OX/HQ}$ potential, we developed a method, based on previous work,^{21–23} to perform redox titrations using a 96-well plate format and a BMG VANTASTAR microplate reader housed inside a Coy anaerobic chamber (<20 ppm of O_2). To prepare the plate, a master mix containing 3× Buffer C (600 mM NaCl, 150 mM Pi at pH 7.9) or 3× Buffer D (600 mM NaCl, 300 mM Bicine at pH 8.9), 180 μ M iLOV OX, 90 μ M of MV^{2+} , and 240 μ M safranine were aliquoted into several wells of a 96-well plate at a volume of 50 μ L. Different ratios of water and a 700 μ M NaDT stock were combined into each well to bring the final volume to 150 μ L. At least one well contained no reductant, and one well contained excess (3.3-fold molar equivalents) reductant to serve as reference spectra for 100% OX and 100% HQ respectively. In wells in which excess NaDT was added, an authentic spectrum of $MV^{+\bullet}$ mediator was subtracted from the resultant spectra using the absorbance at 606 nm. In these wells with low solution potential, it can be assumed that the concentration of SQ is too low to interfere with the absorbance from $MV^{+\bullet}$ at this wavelength. A typical plate measurement contained 14 different reducing conditions, 3 different replicates, and 2 different pH conditions using a total of 84 wells. Remaining wells were used for blanks and standard spectra of methyl viologen in 1× Buffer C to be subtracted during the analysis. The 96-well plate was then sealed and placed into the plate reader, and spectra from 300 to 900 nm with a resolution of 5 nm were collected from each

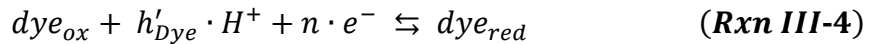
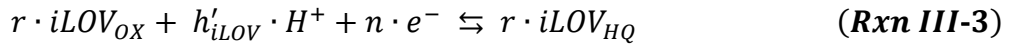
well, averaging a total of 22 flashes each, every 30 min for 5 to 12 h, though samples often reached equilibrium after about 5 h. Finally, the redox titration UV-vis data was analyzed similarly to previous treatments,^{22,23} with some notable differences as described below.

Analysis of Redox Titration Spectra.

The proton-coupled half-cell reactions for the unknown analyte (iLOV) and the standard indicator dye (safranin) are represented by **reactions III-1** and **III-2**,



where a, b, h, and n are the stoichiometric coefficients for iLOV, safranin, H^+ , and e^- , respectively. These two reactions can be scaled by dividing reaction **III-1** by n_{iLOV} and multiplying by n_{dye}/b and dividing **reaction III-2** by b , such that the same number of electrons transferred in both reactions are equal via **reactions III-3** and **III-4**.



Here, the new stoichiometric coefficients, r , h'_{iLOV} , h'_{dye} , and n are related to the original stoichiometric coefficients by $r = (a/n_{iLOV})/(b/n_{dye})$, $h'_{iLOV} = (h_{iLOV}/n_{iLOV})/(b/n_{dye})$, $h'_{dye} = (h_{dye}/b)$, and $n = n_{dye}/b$. If the two reactions are in equilibrium, within the same reaction vessel at the same pH, and assuming an activity coefficient of unity, then the standard cell potential for each half-reaction (E°) is related to the solution potential (E_{sol}) by **equation III-3** and **III-4**.

$$E_{sol} = E_{iLOV}^0 - \left(h'_{iLOV} \cdot \frac{2.303 \cdot RT}{nF} \cdot pH \right) - \frac{RT}{nF} \cdot \ln \left(\frac{[iLOV_{NHQ}]^r}{[iLOV_{OX}]^r} \right) \quad (\text{Eq. III-3})$$

$$E_{sol} = E_{dye}^o - \left(h'_{dye} \cdot \frac{2.303 \cdot RT}{nF} \cdot pH \right) - \frac{RT}{nF} \cdot \ln \left(\frac{[dye_{red}]}{[dye_{ox}]} \right) \quad (\text{Eq. III-4})$$

At a given point in the titration, E_{sol} dictates the ratio oxidized and reduced states of the standard dye indicator (safranine) and the unknown analyte (iLOV) based on their respective E° . Setting these equations equal to one another at $T = 298$ K, and $n_{iLOV}/a = n_{dye}/b = 2$, such that $r = 1$, $n = 2$, and $h'_{iLOV} = h_{iLOV}/a$, and then rearranging into a linear format, we obtain **equation III-5**.

$$E_{app}^{dye} = -r \cdot E_{rel}^{iLOV} + E_{iLOV}^o - \left(\frac{h'_{iLOV}}{n} \cdot 59.16 \text{ mV} \cdot pH \right) \quad (\text{Eq. III-5})$$

where E_{app}^{dye} and E_{rel}^{iLOV} are defined by **equation III-6** and **III-7**.

$$E_{rel}^{iLOV} = \frac{RT}{nF} \cdot \ln \left(\frac{[iLOV_{NHQ}]}{[iLOV_{OX}]} \right) \quad (\text{Eq. III-6})$$

$$E_{app}^{dye} = E_{dye}^o - \left(\frac{h'_{dye}}{n} \cdot \frac{2.303 \cdot RT}{F} \cdot pH \right) - \frac{RT}{nF} \cdot \ln \left(\frac{[dye_{red}]}{[dye_{ox}]} \right) \quad (\text{Eq. III-7})$$

We determined the pH dependent reduction potential of safranine E_{app}^{dye} directly from the Pourbaix diagram in supplemental **Figure III-S4**. Thus, linear plots of E_{app}^{dye} vs. E_{rel}^{iLOV} , generated by spectrophotometric measurement of $\ln([dye_{red}]/[dye_{ox}])$ and $\ln([iLOV_{HQ}]/[iLOV_{OX}])$, one obtains a slope of r (1 for stoichiometric reduction of dye and iLOV by two electrons), and a y-intercept equal to the $E_{OX/NHQ}^\circ$ of iLOV at a given pH.

Using the absorption of iLOV OX on the blue edge of the $S_0 \rightarrow S_1$ absorption band (420 nm) to minimize interference from safranine, and 520 nm for oxidized safranine, the concentration of the oxidized species was determined and converted to fraction oxidized and fraction reduced to calculate $\ln([x_{red}]_{frac}/[x_{ox}]_{frac})$, where $[x_{ox}]_{frac} = [x_{ox}]_x/[x_{ox}]_i$, and $[x_{red}]_{frac} = I - [x_{ox}]_{frac}$. These relationships hold for situations where both the iLOV and safranine equilibria are composed of only the fully reduced or oxidized states, simplifying the

analysis. However, in the Q₁₀₃K and Q₁₀₃D iLOV, appreciable NSQ accumulates during these titrations, and must also be accounted for, but the E°_{OX/HQ} midpoint potential for OX/HQ is still defined as the solution potential at which the concentration of OX and HQ are equal. To quantitate NSQ contributions to the iLOV population during redox titrations, and therefore accurately estimate HQ, the concentration of NSQ was estimated using the absorbance at 616 nm and the aforementioned extinction coefficient of 8,200 M⁻¹ cm⁻¹ for each well. Next, an authentic NSQ spectrum collected as described above, was scaled to the absorbance at 616 nm and subtracted from each spectrum to obtain the concentration of OX at 420 nm without convolution with NSQ. From this, $[X_{red}]_{frac} = 1 - [X_{ox}]_{frac} - [X_{nsq}]_{frac}$ was used to estimate HQ. Data points in the final plot outside the range of $0.05 < [x]_{frac} < 0.95$ were ignored in the linear regression. The error at each point was determined from the standard deviation of triplicate measurements. Replicates with obvious aberrations (such as resulting from a bubble within a well) were discarded as outliers.

Comproportionation of iLOV HQ and OX

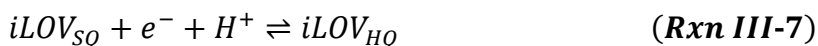
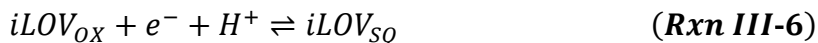
When equimolar concentrations of OX and HQ are mixed (that is, initially, $[OX]_i = [HQ]_i$), they will comproportionate to an equilibrium concentration of $[SQ]_{eq}$, $[OX]_{eq}$, and $[HQ]_{eq}$ via reaction **III-5**,



with an associated equilibrium constant (K_{eq} , **equation III-8**),

$$K_{eq} = \frac{[SQ]_{eq}^2}{[OX]_{eq}[HQ]_{eq}} = \frac{[SQ]_{eq}^2}{\left([OX]_i - \frac{[SQ]_{eq}}{2}\right)^2} \quad (\text{Eq. III-8})$$

where $[X]_{eq}$ is the equilibrium concentration and $[X]_i$ is the initial concentration of species X . **Equation III-8** is equivalent to the product of the K_{eq} 's for its respective one-electron half **reactions III-6** and **III-7**,



meaning that the Nernstian potential derived from K_{eq} corresponds to difference between the two one-electron couples, $E^\circ_{OX/SQ}$ and $E^\circ_{SQ/HQ}$, by **equation III-9**,

$$\Delta E = \frac{R \cdot T}{n \cdot F} \cdot \ln(K_{eq}) \quad (\text{Eq. III-9})$$

where ΔE is the relative electrochemical potential separation of $E^\circ_{OX/SQ}$ and $E^\circ_{SQ/HQ}$ couples, R is the gas constant, T is the temperature in Kelvin, n is the number of electrons (1 for the comproportionation reaction), and F is Faraday's constant. In our convention, ΔE is equivalent to the extent of potential "crossing", where negative values are crossed, and positive values are "normally-ordered" potentials. If the absolute midpoint potential for the two-electron process ($E^\circ_{OX/NHQ}$) is known, it becomes straightforward to calculate the values of the two one-electron reduction potentials from ΔE by **equations III-10** and **III-11**.

$$\Delta E = E^\circ_{OX/SQ} - E^\circ_{SQ/HQ} \quad (\text{Eq. III-10})$$

$$E^\circ_{OX/NHQ} = \frac{E^\circ_{OX/SQ} + E^\circ_{SQ/HQ}}{2} \quad (\text{Eq. III-11})$$

To measure ΔE , pure samples of iLOV or Q103X mutants in the OX and HQ state were prepared and quantified as described earlier inside a VAC Atmospheres glove box (< 0.2 ppm). These two species were combined to a final concentration of 50 μ M of OX and HQ in 200 mM NaCl and 50 mM Pi at pH 7.90, mixed, then quickly loaded into the flow cell and placed into the cuvette holder for a kinetics measurement. About 10-15 s passed between

mixing the sample and beginning the kinetics measurement. The resulting time-dependent increase in NSQ concentration was monitored by the absorbance at 616 nm until it reached equilibrium (5-24 h), fit to a single exponential, and the exponential function pre-factor was used to quantify NSQ based on the ϵ_{616} of $8,200 \text{ M}^{-1} \text{ cm}^{-1}$. The value of K_{eq} was then calculated using **equation III-8** with $[iLOV_{OX}]_i = 50 \text{ }\mu\text{M}$ and ΔE was extracted from **equation III-9**. Values of $E^{\circ}_{OX/NSQ}$ and $E^{\circ}_{NSQ/NHQ}$ were obtained by respectively adding and subtracting $\Delta E/2$ from the previously measured value of $E_{OX/HQ}$. Each mutant was measured in triplicate at pH 7.90.

Electron Paramagnetic Resonance (EPR)

As the value of ΔE was being measured optically for each mutant, an EPR sample was produced simultaneously with equimolar quantities of OX and HQ between 200 and 400 μM total protein and a final volume of 300 μL with 10% glycerol and was kept in the dark. When the low concentration samples monitored by UV-vis spectroscopy reached equilibrium, the EPR sample was placed into a high-precision quartz EPR tube and flash frozen in liquid N_2 -cooled isopentane ($<-130 \text{ }^{\circ}\text{C}$) cold well within the glovebox. The sample was then removed from the glovebox and placed into a storage dewar in liquid N_2 before analysis by X-band EPR spectroscopy on a Bruker EMXplus EPR spectrometer. Because the Q103D iLOV variant has the least crossed potentials the radical concentrations were large enough to be quantified and measured in triplicate. X-band EPR and radical quantitation was performed as described previously.¹⁸ The spectra were simulated using EasySpin 6.0 in MATLAB, and spin quantitation was performed by double integration of the EPR signal, divided by the Q-value, and comparison to three 4-hydroxy-TEMPO standards. The

concentration of the 4-hydroxy-TEMPO standards were determined by the absorbance of a stock solution using the reported extinction coefficient at 428 nm of $12.4 \text{ M}^{-1} \text{ cm}^{-1}$.²⁴

Molecular Dynamics, Hybrid Quantum Mechanical/Molecular Mechanical (QM/MM), and Alchemical Free Energy Calculations

Our collaborators, Sarah Elhajj and Samer Gozem, conducted classical molecular dynamics, QM/MM, and alchemical free energy calculations on iLOV Q₁₀₃X mutants. A full description of their methods is described in the original source publication listed at the beginning of this chapter. Below, I have listed only the computational details that are relevant to the interpretation of the experimental data.

In brief, classical molecular dynamics simulations were conducted using starting structures of iLOV Q₁₀₃X mutant generated by substituting arginine (R⁽⁺⁾), aspartic acid (D⁽⁰⁾), aspartate (D⁽⁻⁾), glutamic acid (E⁽⁰⁾), glutamate (E⁽⁻⁾), glycine (G), alanine (A), and lysine (K⁽⁺⁾) into the crystal structure of iLOV (PDB: 4EES).¹⁰ After minimization and equilibration, each mutant structure was simulated classically in an NPT ensemble for 500 ns. Water dynamics around the N5 atom of the flavin cofactor were analyzed using a custom MDAnalysis-based script. The gmx hbond tool was used to determine the total number of hydrogen bonds formed by each of the N1, O2, N3, O4, and N5 heteroatoms of flavin with the surrounding water and protein environments in the parental iLOV and its mutants in each of the OX and NSQ states.

To simulate the effect of a mutation on the reduction potential of iLOV, a thermodynamic cycle with alchemical transformation of Q₁₀₃ to various mutants was used (**Figure III-1**).^{25,26} The term “alchemical transformation” is used here consistent with its usage in several related free energy protocols where part of the system is gradually mutated

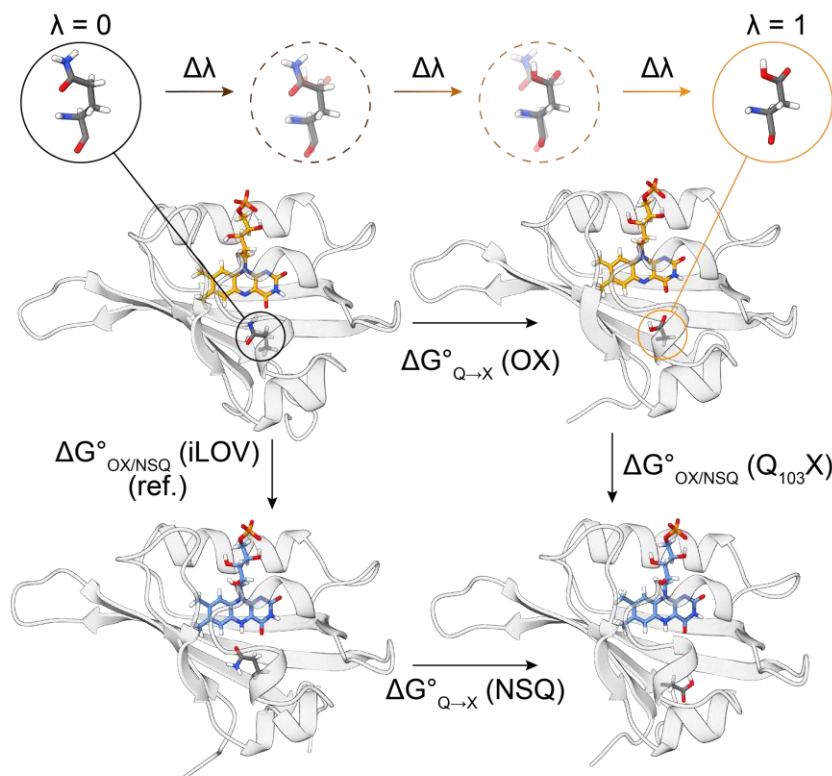


Figure III-1. Thermodynamic cycle used to calculate $\Delta G^{\circ}_{\text{OX/NSQ}}$, and hence E° , for $Q_{103}X$ mutants via alchemical transformations.

in incremental steps (λ).^{27–30} The free energy associated with alchemical mutagenesis of iLOV's Q_{103} residue to the mutant for each of the OX and NSQ states provided two legs of the thermodynamic cycle, while the third leg, corresponding to the free energy of reduction of flavin in the wild-type system, is obtained from the experimental data (*vide infra*, -458 mV or 44.19 kJ/mol). Using those three quantities, the fourth leg of the thermodynamic cycle was computed to provide the free energy of reducing flavin in the mutated iLOV. This free energy is then converted to a reduction potential using **equation III-12**

$$E^{\circ}_{\text{comp}} = -\Delta G_{\text{comp}}/nF \quad (\text{Eq. III-12})$$

Because experimentally observed effects were the largest for the OX/NSQ transition, only these redox states were modelled.

To conduct these calculations, our collaborators used a Bennett Acceptance Ratio (BAR) approach^{31–33} in conjunction with a protocol that builds on the recently automated Average Protein Electrostatic Configuration QM/MM approach for flavoproteins (APEC-F 2.0)^{34,35}. In short, a classical MD simulation is first performed on a structure of wild-type iLOV in both the OX and NSQ states. Next, a QM/MM optimized geometry of the flavin is obtained where the protein and solvent are treated as a superposition of structures obtained from the aforementioned MD simulation (the APEC-F 2.0 method). Finally, this structure is subjected to the BAR protocol in which the QM/MM optimized flavin is kept frozen, while the protein is allowed to gradually evolve via classical MD simulations to one of the Q₁₀₃X mutants in a series of λ steps (the BAR protocol). The alchemical transformation was carried out in a series of 40 discrete λ windows connecting the two physical end points: the parental iLOV protein ($\lambda = 0$) and mutated amino acid ($\lambda = 1$), as shown in **Figure III-1**. A serial equilibration approach was adopted where the last frame of equilibration at a specific λ step is the starting frame for the next one. Specifically, for each λ window, the system (protein + cofactor) was equilibrated for 3 ns in NVT followed by 12 ns production runs, giving a total simulation time of 600 ns for each replica. For each mutant and each of the OX and NSQ states, multiple (≥ 3) replicas were run. A few replicas were also carried out using longer initial equilibration (50 or 100 ns) at the $\lambda=0$ step to serve as an additional reference and reduce uncertainty in the results when needed. To monitor water leakage into the flavin binding site along the BAR perturbation steps, the *gmx rdf* tool was used to determine the radial distribution function (RDF) of water relative to flavin's N5 along each λ trajectory,³⁶ as well as the cumulative number (CN) obtained by integrating the RDF. The CN value represents the average number of particles within a given distance.

RESULTS

During flavin reduction, N5 becomes protonated during the OX→NSQ transition while N1 becomes protonated during the NSQ→NHQ transition, depending on pH and the flavin environment, which modulates the N5 and N1 pK_{as}.^{5,6} We hypothesized that hydrogen bonding interactions at the N5 and N1 sites could confer independent mechanisms of control of the two one-electron couples in flavoproteins via thermodynamic control of proton-coupled electron transfer (PCET). In this work, we focused on N5 using the small flavoprotein, iLOV, as a model system.

The X-ray crystal structure of iLOV reveals a glutamine residue (Q₁₀₃, **Figure I-3**)¹⁰, positioned near both N5 (3.4 Å) and O4 (3.1 Å) of the bound FMN cofactor. To investigate how residues near N5 and O4 of a flavin cofactor affect the flavin reduction potentials, we generated Q₁₀₃X mutations (X = alanine, A; glycine, G; arginine, R; lysine, K; glutamic acid, E; and aspartic acid, D) by site-directed mutagenesis. All purified proteins quantitatively bound FMN at a 1:1 ratio during the reconstitution (Supporting Information **Tables III-S1** and **III-S2** and **Figure III-S1**).

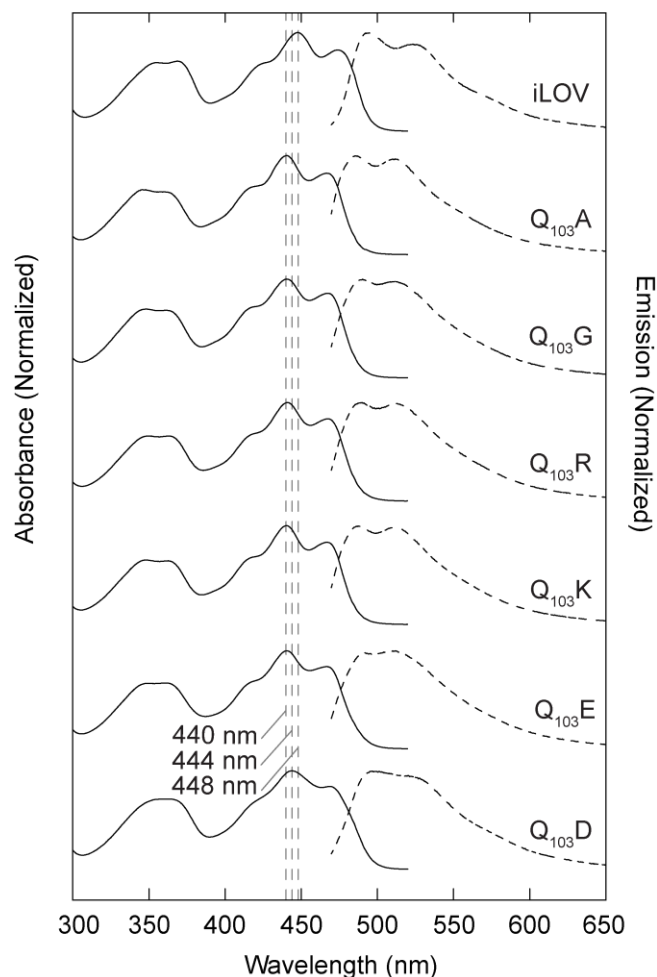


Figure III-2. Effect of $Q_{103}X$ mutations on optical properties of iLOV. Normalized UV-vis absorption and emission spectra ($\lambda_{\text{ex}} = 450 \text{ nm}$) of iLOV and $Q_{103}X$ mutants.

The absorption spectrum of the OX state of the cofactor was modestly perturbed by the $Q_{103}X$ substitutions; the parent iLOV exhibited a λ_{max} of the $S_0 \rightarrow S_1$ transition at 448 nm, whereas the $Q_{103}D$ λ_{max} was blue-shifted to 444 nm, and $Q_{103}E$, $Q_{103}A$, $Q_{103}G$, $Q_{103}K$, and $Q_{103}R$ were further blue-shifted to 440 nm (**Figure III-2**). Emission and excitation spectra, emission λ_{max} , quantum yields (Φ), and associated emission lifetimes (τ) were also modestly changed by the $Q_{103}X$ mutations (Supporting Information **Figure III-S6**, **Figure III-S7**, and **Table III-S4**). For iLOV, the emission λ_{max} is at 492 nm with a Φ of 0.37 ± 0.01 . All of the $Q_{103}X$ mutants had blue-shifted λ_{max} between 2 to 6 nm, generally consistent with their blue-

shifted absorption λ_{max} , with the exception of Q₁₀₃D, which was red-shifted to 498 nm and Q₁₀₃E which was further red-shifted to 510 nm. The Q₁₀₃X mutants exhibited similar Φ , ranging from 0.37 ± 0.01 in Q₁₀₃A to 0.33 ± 0.01 in Q₁₀₃D. The fluorescence emission lifetime for iLOV was 4.37 ± 0.01 ns, while mutations to Q₁₀₃ resulted in a span from 4.01 ± 0.01 for Q₁₀₃A to 3.52 ± 0.01 for Q₁₀₃E. Interestingly, τ did not correlate with measured Φ , a phenomenon that has been observed when placing certain fluorophores in solvents of varying polarity.³⁷ This is thought to result from simultaneous changes to both the radiative and nonradiative relaxation rates, which we expect are sensitive to the N5 environment in iLOV and the associated Q₁₀₃X mutants.³⁸

We next examined how Q₁₀₃ mutation affected the redox behavior of the flavin cofactor, given its proximity to N5, its hydrogen bonding character, and the change in hydrogen bonding character of N5 during redox cycling. Our initial attempts using protein film voltammetry (PFV) and the xanthine/xanthine oxidase (XXO) method were inconclusive. Although iLOV was capable of adsorbing to a pyrolytic graphite edge plane electrode, the electrochemical responses were variable, with unexpected peak current separation and amplitudes, and showed no differences between the parent iLOV and Q₁₀₃X mutants (Supporting Information **Figure III-S8**). The XXO assay was also unsuitable due to slow electron transfer kinetics (Supporting Information **Figure III-S9**). We, therefore, turned to redox titrations, using safranine as a dye redox standard reporter and NaDT as a reductant at pH 7.9 and 8.9 (**Figure III-3**). In this classical approach the ratio of the reduced fraction of dye to reduced fraction of analyte at each solution potential is used to extract the reduction potential of the analyte.^{21–23} Upon addition of the reductant, we observed a loss of the characteristic visible absorbance bands of the OX FMN (448 nm) and the oxidized safranine

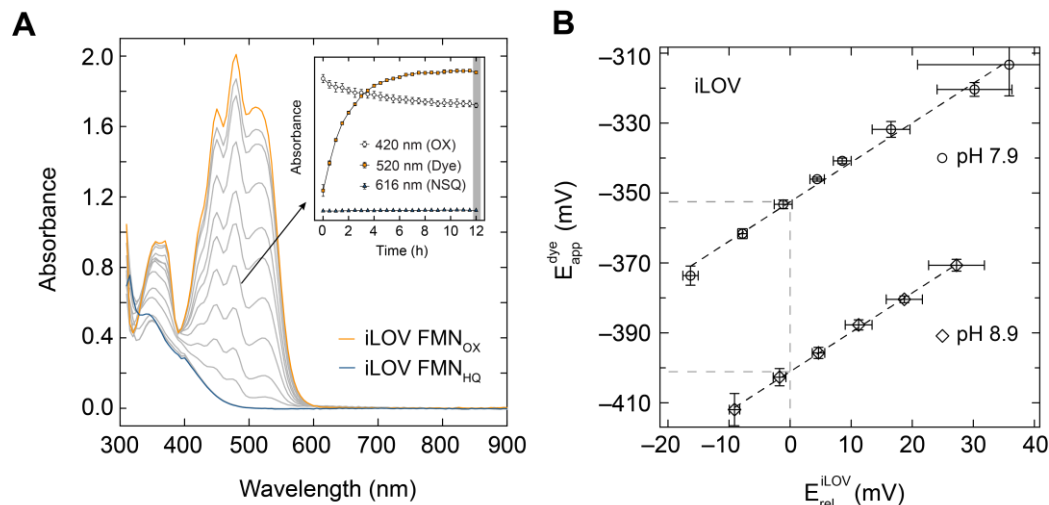


Figure III-3. Spectrophotometric redox titrations of iLOV. **A** Equilibrium iLOV absorbance spectra after adding NaDT reductant from 0 eq (orange) to 3.3 eq. (blue). Inset shows the approach to equilibrium at one point in the titration (arrow) via monitoring the absorption at 420 nm (FMN_{OX}), 520 nm (safranin), and 616 nm (FMN_{NSQ}), and is reproduced in the Supplemental Information **Figure S10** for closer inspection. Safranin is initially reduced faster than iLOV, then reoxidized by reducing iLOV. **B** E_{app} (determined from safranin) vs. E_{rel} (determined from iLOV) plots for pH 7.9 (open circles) and pH 8.9 (open diamonds) and associated linear fits. The gray dashed line shows the E_{app} at which the iLOV FMN has an [OX]/[HQ] ratio of 1 (*i.e.* $E^{\circ}_{OX/NHQ}$).

dye (520 nm, **Figure III-3A**), that disproportionately reduces safranin before equilibrium is reached between safranin and iLOV based on their relative reduction potentials (**Figure III-3A** inset, Supporting Information **Figure III-S10**). After two equivalents of NaDT, iLOV and safranin were completely reduced, and no clear accumulation of an NSQ or ASQ intermediate was observed at the titration midpoint at either pH. This is consistent with significantly crossed potentials and an unstable SQ, as reported previously.³⁹

Assuming an equilibrium dominated by OX and HQ forms of the flavin, the fractional reduction of both iLOV Q₁₀₃X and the safranin reporter dye standard were determined and their natural logarithms were plotted against one another, yielding a linear relationship, where the slope indicates the ratio of the stoichiometric coefficients for the number of electrons transferred between the protein and the indicator dye, the y-intercept reflects the $E^{\circ}_{OX/HQ}$ determined relative to the standard, and the pH dependence reflects the number of

protons involved in the redox process (**Figure III-3B** and **Figure III-S11**). As the apparent solution potential from the dye indicator ($E^{\text{dye}}_{\text{app}}$) decreases, so too does the relative potential of iLOV, ($E^{\text{iLOV}}_{\text{rel}}$), and at the point at which the concentration of the OX and HQ states are equal we determine the $E^{\circ}_{\text{OX/HQ}}$ (**Figure III-3B** dashed lines). The resulting extracted values for $E^{\circ}_{\text{OX/HQ}}$ for iLOV and the $Q_{103}X$ mutants, as well as the reference values for free FMN are summarized in **Table III-1**.

From the redox titrations, the parent iLOV had the lowest $E^{\circ}_{\text{OX/HQ}}$ of -353.9 ± 0.8 mV at pH 7.9 and a pH dependence between pH 7.9 and 8.9 of -47 mV/pH, lower than the theoretical value for a $2 e^{-}/2 H^{+}$ couple of -59 mV/pH, but substantially higher than the expected -29 mV/pH for a $2 e^{-}/1 H^{+}$ couple.⁴⁰ These data are most consistent with iLOV reduction proceeding via concerted OX→NSQ and NSQ→NHQ transitions. The reaction

Table III-1. Reduction potentials, pH dependence, and electron equivalents with the mediator (Slope) of iLOV and $Q_{103}X$ mutants determined from reductive and oxidative (iLOV) titrations. Uncertainties in the last significant digit shown in parentheses

iLOV Variant	$E^{\circ}_{\text{OX/NHQ}}$ (mV)		$\Delta E^{\circ}_{\text{OX/NHQ/pH}}$ (mV)	Slope	
	pH 7.9	pH 8.9		pH 7.9	pH 8.9
iLOV	-353.9 (8)	-401.1 (3)	-47	1.13 (4)	1.13 (4)
iLOV ^a	-351.5 (2)	-392 (2)	-41	1.01 (1)	1.7 (1)
$Q_{103}A$	-337.0 (5)	-393.9 (4)	-57	1.35 (4)	1.11 (3)
$Q_{103}G$	-327.5 (3)	-382.7 (6)	-55	0.93 (3)	0.94 (4)
$Q_{103}R$	-321.0 (5)	-377.9 (1)	-57	1.15 (5)	1.05 (1)
$Q_{103}K$	-318 (1)	-366.6 (3)	-49	1.22 (5)	1.21 (2)
$Q_{103}E$	-327 (2)	-365.6 (4)	-39	1.0 (1)	1.15 (3)
$Q_{103}D$	-323.2 (6)	-365.5 (2)	-42	0.86 (4)	1.21 (1)
FMN	-242 ^b	-272 ^c	-29	n.d. ^d	n.d. ^d

^a Values obtained from oxidative (reverse) titrations.

^b Value taken from ref. 5 at pH 8.00.

^c Value taken from ref. 5 at pH 9.04.

^d n.d. not determined.

was fully reversible; an oxidative titration of reduced iLOV and safranine resulted in an $E^{\circ}_{\text{OX/NHQ}}$ potential only 2.5 mV different, with slightly larger differences at pH 8.9 (9 mV), but significantly more scatter in the data (Supporting Information **Figure III-S12**).

Extrapolation to pH 7.0 yields an $E^{\circ}_{\text{OX/NHQ}}$ of -312 mV, approximately 30 mV lower than that measured for the *Chlamydomonas reinhardtii* LOV1 C₅₇G protein,⁴¹ and 41 mV lower than the homologous *Avena sativa* LOV2.⁴²

The mutants Q₁₀₃A, Q₁₀₃G, and Q₁₀₃R also showed no significant SQ accumulation during the redox titrations. The $E^{\circ}_{\text{OX/NHQ}}$ followed the trend iLOV < Q₁₀₃A < Q₁₀₃G < Q₁₀₃R and an average $\Delta E^{\circ}_{\text{OX/NHQ}}/\text{pH}$ of -56 mV/pH, with the highest $E^{\circ}_{\text{OX/NHQ}}$ shifted +33 mV relative to the parent iLOV. During the titrations of Q₁₀₃K, Q₁₀₃E, and Q₁₀₃D, we did observe some amount of NSQ, based on its characteristic absorbance at 616 nm, with NSQ accumulation followed the trend Q₁₀₃K < Q₁₀₃E < Q₁₀₃D. The NSQ accumulation was not quantitative, suggesting $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ were only moderately separated in these mutants. Because the SQ contribution complicated two-state analysis of $E^{\circ}_{\text{OX/NHQ}}$, we quantified NSQ directly and accounted for it in the calculation of $E^{\circ}_{\text{OX/NHQ}}$. Taking advantage of the unique photosensitivity of the Q₁₀₃D,¹⁹ we quantitatively photoreduced the OX FMN to the NSQ state anaerobically via 450 nm illumination to determine the molar absorptivity at 616 nm (ϵ_{616}) (Supporting Information **Figure III-S2**). This reaction requires no external reductant; presumably the electron is donated by a protein tyrosine or tryptophan. For further validation and to capture any contributions of an ASQ, we also quantitated the total SQ population by EPR, against a 4-hydroxy-TEMPO standard (Supporting Information **Figure III-S13**). The ϵ_{616} determined from these EPR measurements is $12,400 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$, compared to our estimated value of $8,200 \pm 100$

$\text{M}^{-1} \text{cm}^{-1}$ by UV–vis. Due to the challenges of EPR quantitation by double integration, we use the UV–vis-derived ϵ_{616} below. The discrepancy between UV–vis and EPR estimates of ϵ_{616} systematically affects the magnitude of our estimated ΔE by 25 mV, but preserves the relative trend (EPR-based ΔE is reported in the Supporting Information **Table III-S5**). Incorporating NSQ quantitation, the $E^{\circ}_{\text{OX/NHQ}}$ values were determined as -318 mV for Q_{103}K , -327 mV for Q_{103}E , and -323 mV for Q_{103}D at pH 7.9, shifts of $+34$ mV relative to the parental iLOV.

The difference in iLOV Q_{103}X variants' semiquinone accumulation indicates a change in the separation of $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ (ΔE). To determine the NSQ stability for iLOV and Q_{103}X mutants, and hence ΔE , we performed comproportionation reactions, in which we mix equal concentrations OX- and NHQ-prepared iLOV and monitor the accumulation of NSQ via **reaction III-5** by UV–vis and EPR spectroscopy (**Figure III-4** and Supporting Information **Figure SIII-14**). As described in the Methods, the reaction between OX and NHQ is treated as any other oxidation–reduction reaction that is split into two “half-cell” reactions. The final equilibrium concentrations can be used in the Nernst equation to obtain a “full-cell” potential which is equivalent to ΔE (see **equations III-8** and **III-9**). The

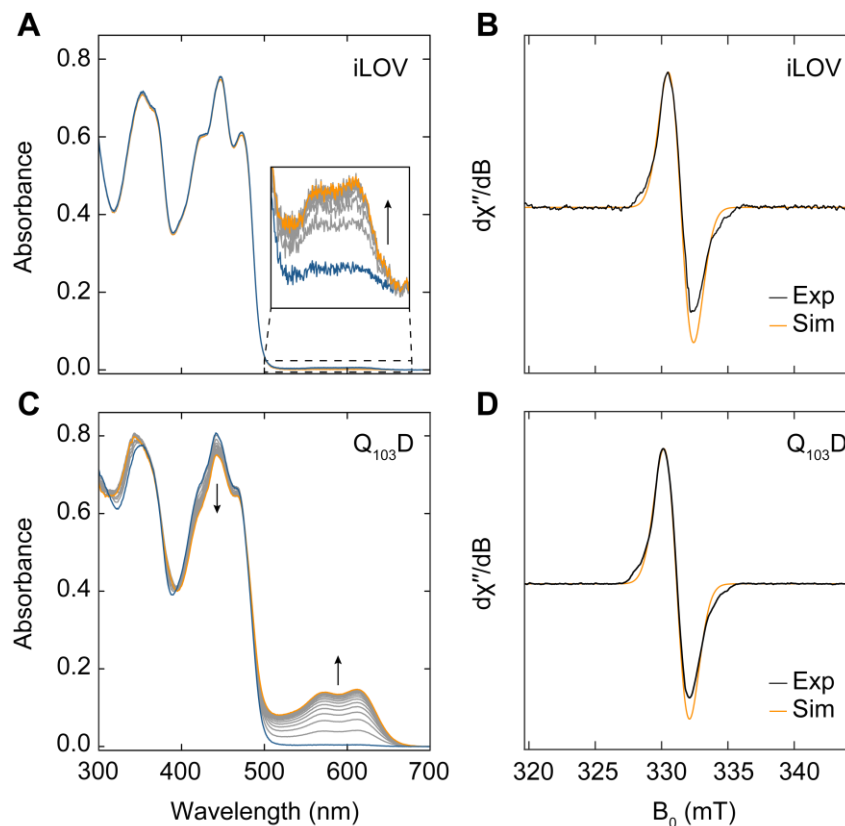


Figure III-4. Comproportionation and characterization of the NSQ. **A** UV-vis time course of iLOV OX and NHQ comproportionation over 24 h. Arrows indicate the time-dependent direction of change in absorbance from beginning (blue) to end (orange) of the reaction. **B** Normalized EPR spectrum (black) and simulation (orange) of the iLOV NSQ. **C** UV-vis time course of $Q_{103}D$ OX and NHQ comproportionation over 24 h. **D** Normalized EPR spectrum (black) and simulation (orange) of the $Q_{103}D$ NSQ.

comproportionation reactions were slow, reaching equilibrium in 10–20 h. Over the course of the reaction, we see a slight decrease in the OX state absorbance at 448 nm for iLOV, with a concomitantly small rise in the characteristic NSQ visible absorption feature at 616 nm (**Figure III-4A**). The EPR spectrum of the iLOV OX/NHQ comproportionation equilibrium mixture showed an isotropic $S = 1/2$ singlet with a g_{iso} of 2.004 and a line width of 1.92 mT (**Figure III-4B**). In general, ASQ radicals have a smaller line width of around 1.5 mT, while NSQ radicals have line widths between 1.8 and 2.0 mT.⁴² Thus, the EPR and UV-vis spectra, and the pH dependence of the redox titrations, support an NSQ assignment. Compared to the $Q_{103}X$ mutants, the parental iLOV formed the least NSQ at equilibrium,

suggesting that $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ are more crossed. Comproportionation reactions of Q₁₀₃D produced the most NSQ (**Figure III-4C**), with identical spectroscopic characteristics of the NSQ by EPR, g_{iso} of 2.004 and a line width of 1.94 mT (**Figure III-4D**). To determine any contribution of ASQ to the comproportionation experiments, we measured the EPR spectrum of a Q₁₀₃D comproportionation reaction at an even higher pH of 9.25, but observed no differences in either the EPR or UV-vis spectra (Supporting Information **Figure III-S15**). These data imply significant differences in ΔE between iLOV and Q₁₀₃D. The EPR spectra and simulation parameters for all comproportionation reactions are reported in the Supporting Information Figure **III-S16** and **Table III-S6**.

With the measurement of iLOV and Q₁₀₃X mutants' $E^{\circ}_{\text{OX/NHQ}}$ in hand, and the relative K_{eq} of comproportionation, we calculated $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ across the series (**Table III-2**). As described in the Methods, these values were calculated by assuming our $E^{\circ}_{\text{OX/NHQ}}$ derived from the redox titrations lay at the exact midpoint of ΔE as derived by the comproportionation experiment. For iLOV, ΔE was -212 mV, corresponding to an $E^{\circ}_{\text{OX/NSQ}}$

Table III-2. ΔE , $E^{\circ}_{\text{OX/NSQ}}$, and $E^{\circ}_{\text{NSQ/NHQ}}$ values for iLOV and Q₁₀₃X mutants from spectrophotometric FMN_{OX} and FMN_{NHQ} comproportionation at pH 7.9. Propagated uncertainties in the last significant digit are reported in parentheses.

iLOV variant	$E^{\circ}_{\text{OX/NHQ}}$ (mV)	ΔE (mV)	$E^{\circ}_{\text{OX/NSQ}}$ (mV)	% of ΔE_{shift}	$E^{\circ}_{\text{NSQ/NHQ}}$ (mV)	% of ΔE_{shift}
iLOV	-354	-212 (4)	-458 (2)	ref.	-246 (2)	ref.
Q ₁₀₃ A	-337	-176 (3)	-427 (2)	86	-251 (2)	14
Q ₁₀₃ G	-328	-112 (1)	-381 (1)	85	-259 (1)	15
Q ₁₀₃ R	-321	-127 (1)	-386 (1)	77	-269 (1)	23
Q ₁₀₃ K	-318	-104 (1)	-372 (1)	79	-269 (1)	21
Q ₁₀₃ E	-327	-89 (4)	-371 (3)	71	-282 (3)	29
Q ₁₀₃ D	-323	-44 (1)	-342 (1)	69	-298 (1)	31
FMN	-242 ^a	-54	-269 ^a	N.A. ^b	-216 ^a	N.A. ^b

^a Values from ref. 5.

^b N.A. not applicable.

of -458 mV and an $E^{\circ}_{\text{NSQ/NHQ}}$ of -246 mV. By comparison, Q₁₀₃D exhibited a ΔE of -44 mV, corresponding to an $E^{\circ}_{\text{OX/NSQ}}$ of -342 mV and an $E^{\circ}_{\text{NSQ/NHQ}}$ of -298 mV. Thus, the Q₁₀₃D mutation diminishes potential crossing relative to iLOV by increasing the $E^{\circ}_{\text{OX/NSQ}}$ by $+116$ mV, while also decreasing the $E^{\circ}_{\text{NSQ/NHQ}}$ by -52 mV. Across the mutants targeting N5, the $E^{\circ}_{\text{OX/NSQ}}$ was increased and the $E^{\circ}_{\text{NSQ/NHQ}}$ was decreased relative to iLOV, resulting in smaller ΔE . The shift in $E^{\circ}_{\text{OX/NSQ}}$ relative to iLOV contributed $\geq 70\%$ to the overall change in ΔE , and the remaining 30% stemming from shifts to $E^{\circ}_{\text{NSQ/NHQ}}$.

To provide insight into the molecular mechanism of flavin redox tuning by mutations proximal to the flavin N5, our collaborators performed classical MD simulations in both the OX and NSQ states. For the parental iLOV in the OX state, multiple replicas of 500 ns trajectories were performed. In most of those trajectories, the glutamine side-chain maintains a hydrogen bonding interaction with the flavin O4, with a representative replica (where hydrogen bonding is maintained throughout most of the trajectory) giving a mean $\text{N}\epsilon\cdots\text{O4}$ distance of 3.2 Å and RMSF of 0.6 Å and mean $\text{N}\epsilon\cdots\text{N5}$ distance of 4.0 Å and RMSF of 0.8 Å (see **Figure III-S17**).

In some replicas of the iLOV OX state, the glutamine side chain loses its hydrogen bonding contact with the flavin O4. This result is somewhat unsurprising in the context of the very hydrophobic environment of the Q₁₀₃ side-chain in the X-ray structure, and the relative flexibility of this residue, as evidenced by the backbone C α RMSD at this site near the C-terminus (**Figure III-17**).¹⁰ We note that in the full *A. thaliana* LOV2 domain of phototropin 2, from which iLOV is derived, Q₁₀₃ is C-terminally flanked by the J α helix, which may stabilize the hydrogen-bonded state to N5/O4 in the OX dark state, and the flipped out conformation resembles that which allosterically controls J α helix unfolding in

the light-activated state.^{43,44} For the remainder of this chapter, we will focus on results from a replica where the hydrogen bonding was maintained throughout the full 500 ns trajectory, consistent with the crystal structure (**Figure I-3**).¹⁰

The reduction of iLOV to the NSQ state appears to result in less conformational flexibility of Q₁₀₃, as indicated by the RMSF analysis in the C-terminal region (**Figure III-S17**). Nevertheless, the conformation of Q₁₀₃ does change in the NSQ state, rotating the side-chain such that its amide carbonyl oxygen, rather than the amide nitrogen, is pointed toward N5. This observation is consistent with what happens in native LOV domains in either the light-activated or reduced NSQ states.^{44,45} The flip comes at the expense of potential hydrogen bonding interactions. The lowest average distance for that of O4•••O Q₁₀₃ carbonyl is 4.3 Å with an RMSF of 0.5 Å and that for N5•••O is 5.6 Å with an RMSF of 0.5 Å (**Figure III-S17**). In place of Q₁₀₃ (strained) hydrogen bonds to the flavin N5/O4, the calculations show that water enters the binding pocket in the NSQ state and mediates the hydrogen bonding interactions between the flavin and Q₁₀₃. Hydrogen bonding between N5 and a water molecule occurs with significantly higher probability (51.8%) in NSQ than the OX state (0.0%). The water occupancy within 4 Å from the flavin N5 in NSQ is significantly higher (94.4%) than the native OX state of iLOV (6.0%).

The $E^{\circ}_{\text{OX/NSQ}}$ was then simulated for the various Q₁₀₃X mutants using relative free energy simulations, benchmarked against the parental iLOV. In previous work, a thermodynamic integration (TI) approach to simulating flavoprotein redox potentials yielded reduction potential errors on the order of <1 kcal/mol (<43 mV) for solvent-exposed residues in flavodoxin.^{46,47} While this approach is promising, the simulations here present added challenges that can contribute additional errors: (1) Q₁₀₃ is directly hydrogen-bonded

to the flavin and those hydrogen bonding interactions are expected to change upon N5 protonation, (2) some mutations involve large changes in the size and charge of the amino acid (e.g., protonated Q₁₀₃R⁽⁺⁾), (3) Q₁₀₃ is buried in a relatively hydrophobic environment, and (4) Q₁₀₃ is near the flexible C-terminus. Such large perturbations at buried but dynamic residues present challenges to the requirement of BAR protocols where each λ step should weakly perturb the system.²⁷ For these reasons, our collaborators opted to use longer MD simulations and 40 λ steps instead of the 8–14 λ steps used for flavodoxin.^{26,30,46}

Their analysis of the mutagenic trajectories and the resultant computed $E^{\circ}_{\text{OX/NSQ}}$ indicated that, even with a relatively long MD period and higher-density λ steps, errors between replicas were >1 kcal/mol (Supporting Information **Table III-S7** and **III-S8**). In the OX state, the free energy associated with introducing Q₁₀₃X mutations yielded standard deviations (σ) between replicas on the order of 0.7–3.7 kcal/mol, with the greatest variations among replicas for the charged mutants Q₁₀₃K⁽⁺⁾ (σ = 3.7 kcal/mol), Q₁₀₃R⁽⁺⁾ (σ = 2.3 kcal/mol), and the deprotonated Q₁₀₃D⁽⁻⁾ (σ = 2.9 kcal/mol). Q₁₀₃G also gave a larger error (σ = 2.1 kcal/mol) due to the large change in size of the amino acid. The Q₁₀₃A, Q₁₀₃E⁽⁰⁾, and Q₁₀₃D⁽⁰⁾ mutations gave more reasonable errors (σ = 0.5 to 0.7 kcal/mol). In comparison to the OX state, σ between replicas of Q₁₀₃X transformations in the NSQ state are generally smaller. For all NSQ mutations, σ ranged from 0.5 to 1.8 kcal/mol, with the exception of Q₁₀₃K⁽⁺⁾, where σ was 3.5 kcal/mol. Nevertheless, since errors for the OX and NSQ states are additive when computing $E^{\circ}_{\text{OX/NSQ}}$ (giving σ of 1.9 to 7.2 kcal/mol, or 82 mV to 312 mV), these uncertainties are too large to reliably capture differences between the iLOV mutants.

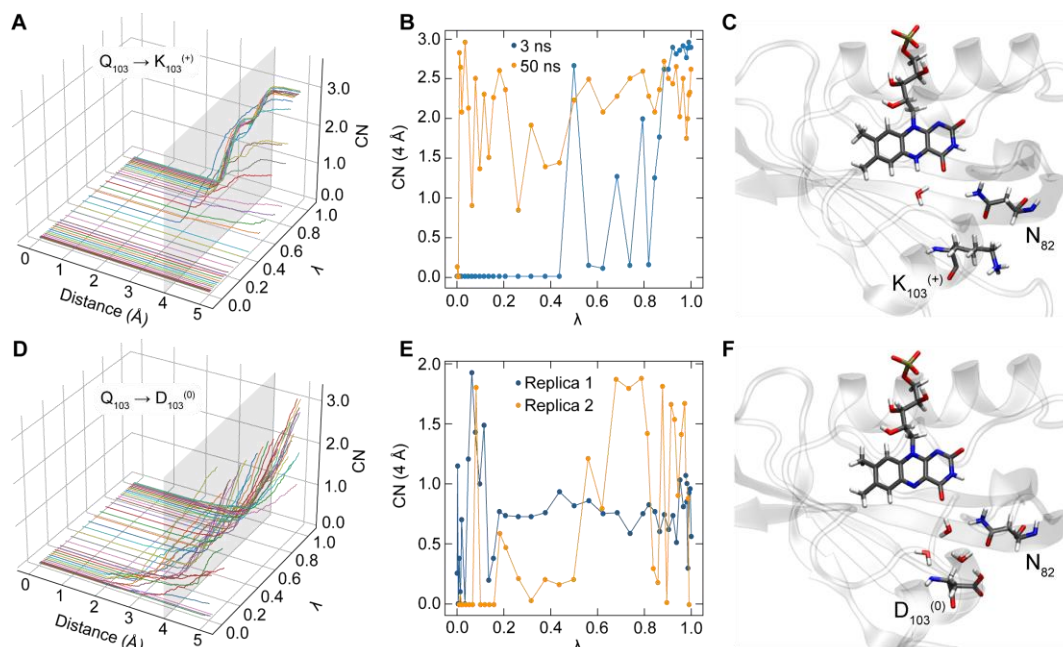


Figure III-5. Water dynamics throughout MD simulations with alchemical transformation **A** Radial distribution of water cumulative number (CN) to N5 in the NSQ state during alchemical Q_{103} ($\lambda = 0$) to $K_{103}^{(+)}$ ($\lambda = 1$) transformation at each λ step with a 3 ns pre-equilibration time. **B** Comparison of CN at 4 Å during the Q_{103} to $K_{103}^{(+)}$ transformation for 3 ns (blue) and 50 ns (orange) pre-equilibration. **C** Representative structure of $Q_{103}K$ with water in the flavin pocket. **D** Radial distribution of water cumulative number (CN) to N5 during alchemical Q_{103} ($\lambda = 0$) to $D_{103}^{(0)}$ ($\lambda = 1$) transformation at each λ step with a 50 ns pre-equilibration time. **E** Comparison of CN at 4 Å during the Q_{103} to $D_{103}^{(0)}$ transformation for two different replicas with 50 ns pre-equilibration. **F** Representative structure of $Q_{103}D$ with water in the flavin pocket.

Based on observations with the parental iLOV, our collaborators suspected that solvent penetration dynamics may significantly contribute to the deviations among replicates. In all OX and NSQ simulations, the native iLOV binding pocket starts out anhydrous, but the gradual introduction of a mutation at Q_{103} causes water to enter the binding pocket near the flavin's N5 region. **Figure III-5** shows the water cumulative number (CN), the number of waters within a variable radius shell centered at N5 at each λ step for one replica of $Q_{103}K^{(+)}$ NSQ state with a pre-equilibration time of 3 ns. Water penetration into the flavin binding site is indicated by an increase in CN at ≤ 4 Å. For $Q_{103}K^{(+)}$ specifically, different behavior was observed between two replicas depending in the pre-equilibration time provided in the

simulation (**Figure III-5B**). In replica 1, which had a short pre-equilibration of only 3 ns at the $\lambda = 0$ step, water does not enter the binding pocket until later in the $Q_{103} \rightarrow K$ transformation. As a result, water entering the binding pocket introduces a large perturbation and, after that point, the trajectories also show larger variations in the CN profile from one λ step to the other. These variations lead to large fluctuations in free energy not consistent with a gradually perturbative regime. By extending the pre-equilibration to 50 ns at the $\lambda = 0$ step this issue is mitigated; all λ trajectories behave more consistently after water enters the flavin binding pocket. A representative structure of the water-penetrated $Q_{103}K^{(+)}$ is shown in **Figure III-5C**. After discarding the 3 ns trajectories for $Q_{103}K^{(+)}$, σ between replicas improved from 3.7 to 2.1 kcal/mol in the OX state, and from 3.5 to 1.0 kcal/mol in the NSQ state of $Q_{103}K^{(+)}$. Similar pre-equilibration time-dependent behavior was observed for $Q_{103}R^{(+)}$ and therefore only the longer pre-equilibration replicates for $Q_{103}R^{(+)}$ are considered further.

The solvent penetration behavior of the OX state of the protonated $Q_{103}D^{(0)}$ was also examined (**Figure III-5D–F**). Again, water consistently entered the binding pocket at some λ steps between 0 $\rightarrow$ 1. Both replicas display variations in the CN over the course of the transformation. In replica 1, water enters the binding pocket at an early λ step and remains inside the pocket until $\lambda = 1$. On the other hand, replica 2 displays intermittent entry of water into the flavin site, which leads to large fluctuations in free energy.

The examples of $Q_{103}K^{(+)}$ and $Q_{103}D^{(0)}$ are indicative of a larger theme, that a major source of error between different replicas of the same system is that not all replicas behave in the same way with regards to water entering the flavin binding pocket. However, the MD behavior can be qualitatively categorized into three empirical bins: (1) water enters the

flavin binding pocket and remains there until the end in both the OX and NSQ state, (2) water initially enters the binding site at some intermediate λ step but is then ultimately expelled from the binding pocket at the end of the BAR protocol (at the final $\lambda=1$ step) in both the OX and NSQ states, and (3) water is expelled from the binding pocket in the OX state at the end of the BAR protocol but stays in the binding pocket in the case of NSQ. Categorizing replicas in this way, comparison of computed $E^{\circ}_{\text{OX/NSQ}}$ from replicas belonging to each of those categories gave more internally consistent results. Specifically, using replicas that exclusively belong to category 3 gave considerably lower σ for almost all mutations. The only exceptions were $\text{Q}_{103}\text{D}^{(0)}$, where category 1 gave slightly lower error than category 3, and $\text{Q}_{103}\text{R}^{(+)}$, where not enough category 3 trajectories were obtained and both categories 1 and 3 are included.

Using the categorized replicas of each Q_{103}X mutant and the thermodynamic cycle shown in **Figure III-1** allowed for the calculation of $E^{\circ}_{\text{OX/NSQ}}$ from 3 to 4 replicas (**Table III-3**). The data from all replicas, including those not considered further herein, are reported in the Supporting Information **Tables III-S7** and **III-S8**. The calculated σ (sum of both OX and NSQ simulations) after binning are all ≤ 2.2 kcal/mol, instead of the 1.9 to 7.2 kcal/mol obtained using all replicas. Overall, the computed potentials are within one σ of the experimental values, the only exception being $\text{Q}_{103}\text{K}^{(+)}$, which is one of the two residues that introduces both a change in the charge state of the amino acid as well as a substantial increase in the side chain length. The general agreement of categories 1 and 3 with the experimental data and their internal consistency suggest that the flavin N5/O4 in the NSQ state interacts with protein-penetrating water in all iLOV mutants, but to varying degrees. Indeed, there is good correlation between the positive potential shift of the $E^{\circ}_{\text{OX/NSQ}}$ couple

Table III-3. A comparison of simulated and experimental redox potentials. The simulated potentials are obtained using a thermodynamic cycle from shifts in redox potentials relative to iLOV by introducing mutations at the Q₁₀₃ site. Standard deviations, reported in parenthesis, are the sum of σ from the OX and NSQ states. H₂O Occupancies reported for the OX state.

Variant	λ Category	E° _{OX/NSQ} (mV)		Difference (mV)	H ₂ O Occupancy (N5, %)	H ₂ O Occupancy (O4, %)
		Computed	Experimental			
iLOV	N.A. ^a	ref. ^b	−458	N.A. ^a	6.0	7.5
Q ₁₀₃ A	3	−433 (31)	−427	−6	1.4	9.3
Q ₁₀₃ G	3	−410 (63)	−381	−29	3.4	36.4
Q ₁₀₃ R	1, 3	−391 (56)	−386	−5	6.2	53.0
Q ₁₀₃ K	3	−444 (42)	−372	−72	6.2	26.9
Q ₁₀₃ E ⁽⁰⁾	3	−397 (94)	−371	−26	1.6	4.7
Q ₁₀₃ D ⁽⁰⁾	1	−364 (59)	−342	−22	32.0	93.7
Q ₁₀₃ D ^(−)	3	−442 (23)	−342	−100	10.8	81.0

^a N.A. not applicable

^b ref. reference value for integrated thermodynamic calculations

and the water occupancy near N5/O4 (**Table III-3**). Furthermore, the protonated Q₁₀₃D⁽⁰⁾ model does substantially better than the corresponding Q₁₀₃D^(−), with replicas yielding an E°_{OX/NSQ} closer to the experiment. These results indicate, in agreement with previous experimental and computational evidence,¹⁹ that the Q₁₀₃D side-chain is protonated. Following the same logic, the Q₁₀₃E side-chain is also expected to be protonated. Overall, the results suggest that the stability and frequency of hydrogen bonding to the NSQ state largely dictate the observed potential tuning of E°_{OX/NSQ}.

Representative structures of the mutated iLOV in the OX state, based on clustering analysis of the classical 500 ns trajectories, are shown in Supporting Information **Figure III-S18**. The same figure also includes a heat map for the RMSF of individual amino acids. In addition to residue 103, two asparagine residues (N₇₂ and N₈₂) as well as one glutamine (Q₄₄) that are involved in a hydrogen-bond network with the flavin and each other are highlighted. In general, this hydrogen bond network is robust in most mutants, with Q₄₄ and

N₇₂ in particular maintaining their conformational states across all mutants and clusters. However, N₈₂, due to its proximity to residue 103, is more strongly affected by the mutations and can have different conformations. This is observed most notably in Q₁₀₃R⁽⁺⁾ which, although is conformationally rigid, displays a different hydrogen bonding pattern around N₈₂ compared to other mutants.

The structures in the Supporting Information **Figure III-S18** indicate that mutations at position 103 can introduce conformational changes distal to the N5 environment, either by allowing water into the binding pocket or by influencing flavin's interactions with other neighboring amino acids. Such subtle (or in the case of N₈₂, not so subtle) changes can propagate through the hydrogen bond network and affect hydrogen bonding at other heteroatom positions on the flavin. Thus, the mutation of Q₁₀₃ and N₈₂ play the major role in forming the hydrogen bonding network near O4 and N5. Any mutation of Q₁₀₃ perturbs the hydrogen bonding network such that either water or Q₁₀₃X mutant has to form hydrogen bonding interactions with N₈₂, otherwise N₈₂ gets closer to the flavin and forms a hydrogen bond with O4.

The heat map in the supplementary **Figure III-S18** also shows that the mutants with the highest flexibility at the C-terminus are Q₁₀₃D⁽⁰⁾ and Q₁₀₃K⁽⁺⁾. Clustering analysis shows two major conformations of Q₁₀₃D⁽⁰⁾. Representative structures from both conformations have a water molecule inside the active site, consistent with earlier results indicating high water occupancy. On the other hand, for Q₁₀₃K⁽⁺⁾, the two largest clusters are significantly different; one cluster has the lysine directly hydrogen bonded with flavin's O4, and the other cluster has the lysine side-chain pointing out away from the flavin and with a water molecule entering the binding pocket. The significant difference between those clusters can

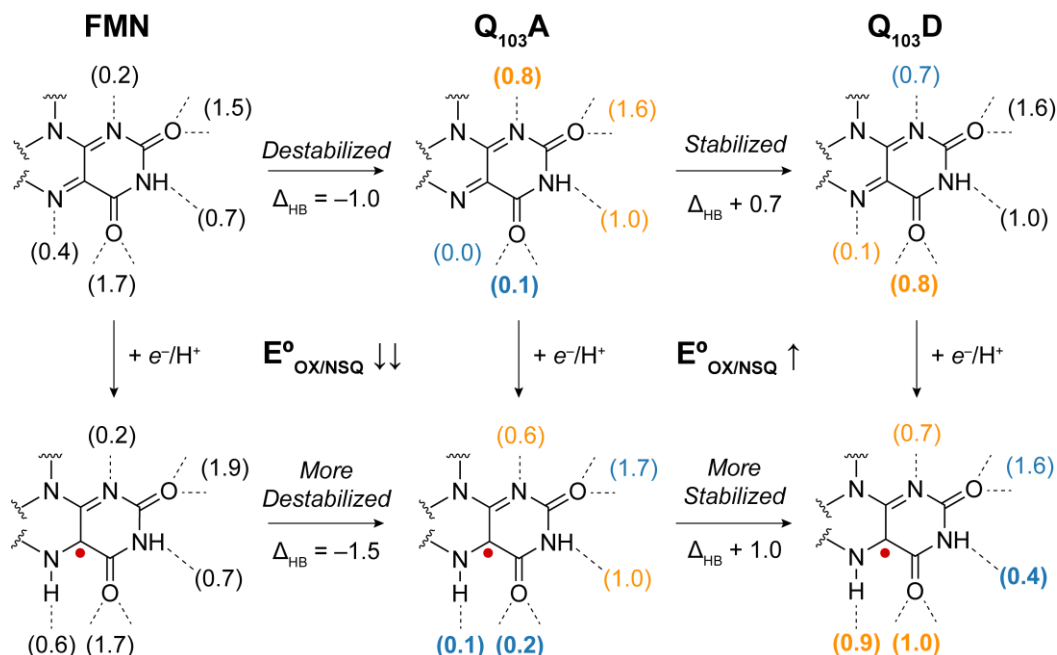
help explain why the redox potentials obtained from free energy simulations had larger standard deviations than for all other mutants.

DISCUSSION

The ability of flavoproteins to tune the reduction potentials of their flavin cofactors to match those of their substrates or electron transfer partners underlies their remarkable catalytic versatility. Although flavin potentials are highly sensitive to the protein environment, the precise mechanisms by which flavoproteins modulate one- and two-electron couples remain unclear. Systematic investigations have been particularly challenging for crossed-potential flavins, where the inherent instability of the NSQ intermediate complicates measurement of individual one-electron potentials.

By targeting Q₁₀₃ in iLOV, which resides near the flavin N5 and O4 atoms, we demonstrated that single site mutations can significantly alter the degree of potential crossing (ΔE), spanning 164 mV across the Q₁₀₃X series, while shifting the overall two-electron potential ($E^{\circ}_{\text{OX/NHQ}}$) by only ~30 mV. The dominant contributor to these changes were positive shifts in the $E^{\circ}_{\text{OX/NSQ}}$ of up to +116 mV. This is somewhat unsurprising, given the proximity of the Q₁₀₃X residue to the flavin N5, which becomes protonated upon reduction in the NSQ state. The trend implies that mutations to Q₁₀₃ destabilize the OX state or stabilize the NSQ state, thereby raising $E^{\circ}_{\text{OX/NSQ}}$. Surprisingly, however, the effect of the Q₁₀₃X mutations did not follow an immediately intuitive trend associated with the side-chain functional groups. Both acidic and basic residues showed similar $E^{\circ}_{\text{OX/NSQ}}$ (e.g., Q₁₀₃K and Q₁₀₃D), and elimination of hydrogen bonding entirely (e.g., Q₁₀₃A and Q₁₀₃G) had only modest effects on $E^{\circ}_{\text{OX/NSQ}}$ relative to the parental iLOV.

Scheme III-1. Proposed role of hydrogen bonding to the isoalloxazine ring affecting the FMN $E^{\circ}_{\text{OX/NSQ}}$ and $E^{\circ}_{\text{NSQ/NHQ}}$ for free FMN, Q₁₀₃A, and Q₁₀₃D. Average hydrogen bonds to the flavin heteroatoms from extended MD trajectories are shown in parentheses. Hydrogen bonding that increases (left to right) is indicated in orange and those that decrease in blue, with bold indicating a change > 0.5. Net change in hydrogen bonding (Δ_{HB}) indicated for each transition.



The $E^{\circ}_{\text{NSQ/NHQ}}$ couple was also affected by Q₁₀₃X mutations, but to a lesser degree, with the most shifted couple in the Q₁₀₃D mutant by -52 mV relative to the parent iLOV. In this, and all other Q₁₀₃X cases, the $E^{\circ}_{\text{NSQ/NHQ}}$ couple becomes more negative, implying a stabilization of the NSQ and/or a destabilization of the NHQ state. Yet again, no clear correlation emerged with side-chain functional groups, underscoring the complexity of interpreting these changes without structural information across all redox states and detailed insights into conformational dynamics.

The QM/MM and MD simulations helped to provide molecular context into the structural and dynamic differences between iLOV mutants, which is summarized in **Scheme III-1**. For the freely solvated FMN analog lumiflavin, hydrogen bonding to the flavin isoalloxazine ring heteroatoms is relatively unrestrained, and all atoms display some degree

of hydrogen bonding in both the OX and NSQ state. These numbers are similar to previous calculations with lumiflavin, with the exception of N1 in the OX state, which formed a hydrogen bond in previous studies,⁴⁸ but the simulations described here with the complete ribityl tail of FMN largely does not. In the X-ray crystallographic structure of iLOV with the FMN cofactor in the OX state, the hydrogen bonding interactions are significantly diminished in the hydrophobic flavin pocket.¹⁰ In particular, no hydrogen bond <3.0 Å is observed for either N1 or N5, destabilizing the cofactor. These interactions are largely reproduced in the extended MD simulations for Q₁₀₃A, with the exception of hydrogen bonding interactions observed for N1, and the loss of the hydrogen bond to O4 via loss of the Q₁₀₃ side-chain. Overall, in the OX state, the isoalloxazine ring loses an average -1.0 (Δ_{HB}) stabilizing hydrogen bond in moving into the iLOV binding pocket. The differences in hydrogen bonding between free and iLOV-bound FMN in the NSQ state are more pronounced. The hydrogen bonding to N5 and O4 is substantially weakened, resulting in a net Δ_{HB} of -1.5 . This greater destabilization of the NSQ partially explains the relatively low $E^{\circ}_{\text{OX/NSQ}}$ couple in iLOV relative to free FMN.

Not all hydrogen bonds will contribute equally to the (de)stabilization of the FMN cofactor in iLOV. We expect the extent of stabilization of either OX or NSQ state through hydrogen bonding is dependent on the ΔpK_a between donor and acceptor and steric constraints imposed by the protein scaffold.⁷ The pK_a of the FMN N5 in the OX state has been computationally estimated to be -10 .⁴⁹ When free in solution, hydrogen bonding between water and N5 is not particularly favored, with a $\Delta pK_a \geq 24$,⁵⁰ which is reflected in the low average hydrogen bond count of 0.4, but there are effectively no steric constraints. Conversely, the pK_a of the FMN NSQ is 8.6 and the associated ΔpK_a for hydrogen bonds

with a water acceptor is 8.6,^{5,19} indicating that hydrogen bonding to or from N5 in aqueous solution preferentially stabilizes the NSQ state over the OX state. We assume the pK_a of N5 in the OX state is not significantly perturbed within the flavin binding pocket (-10), while the pK_a of the NSQ is clearly elevated to >9 , based on our EPR measurements of the Q_{103D} SQ at high pH. As such, when comparing the change in hydrogen bonding between the free and iLOV-bound FMN, the loss of a hydrogen bond acceptor to N5–H in the NSQ state in iLOV is more destabilizing than the loss of a hydrogen bond donor to N5 in the OX state. The opposite argument has been made for the stabilizing effect of the NSQ state in flavodoxins, where a relatively strong hydrogen bond is formed between N5 and a glycine backbone amide carbonyl, increasing hydrogen bonding relative to free FMN.^{51,52}

The FMN C=O4 carbonyl bond is also weakened upon reduction to the NSQ state, making O4 a better hydrogen bond acceptor.^{53–57} Even though hydrogen bonding interactions to O4 are diminished in parental iLOV relative to free FMN in both the OX and NSQ state, we expect the destabilization effect to be greater for the NSQ state. Upon reduction, the aromaticity in the central pyrazine ring loses some degree of delocalization, partially splitting along the N5–N10 axis. We expect that the localization of greater aromaticity to the uracil ring, which contains the heteroatoms O2, O4, N3, and N1, will increase the impact of aromaticity-modulated hydrogen bonding at O4, further destabilizing the NSQ relative to the OX state upon loss of hydrogen bonding.⁵⁸

On comprehensive comparison of the change in total hydrogen bonds (Δ_{HB} , **Scheme III-1**), the NSQ state is destabilized relative to the OX state by an average additional 0.5 hydrogen bonding interactions, notably at N5 and O4, where relatively strong hydrogen bonds should form. The $E^{\circ}_{OX/NSQ}$ of iLOV is shifted -190 mV (18 kJ/mol) in iLOV relative

to free FMN, which is consistent with a net loss of a hydrogen bond to the NSQ state. Additional effects of the protein binding pocket likely contribute to the $E^{\circ}_{\text{OX/NSQ}}$ shift as well, but those that affect both OX and NSQ states similarly will not result in shifts to $E^{\circ}_{\text{OX/NSQ}}$.^{59–61}

Changes in hydrogen bonding character near the flavin N5 and O4 across the $Q_{103}X$ mutations correlate with the $E^{\circ}_{\text{OX/NSQ}}$ shifts. For the $Q_{103}D$ mutation, which raise the $E^{\circ}_{\text{OX/NSQ}}$, hydrogen bonding increases at O4, and to a lesser degree at N5, in the OX state, whereas hydrogen bonding increases significantly for both N5 and O4 in the NSQ state. In both OX and NSQ, the effect of the $Q_{103}D$ mutation is to stabilize the FMN cofactor, but the NSQ is more stabilized by a $\Delta\Delta_{\text{HB}}$ of 0.3. The shifts are relatively modest and due to the increased penetration of water into the flavin binding pocket proximal to N5/O4. Water inside protein cavities is known to behave differently than that of the bulk, including its hydrogen bonding dynamics, pK_a , and corresponding hydrogen bond energetics, and thus may not be directly comparable to solvation of N5 in free FMN.^{57,62,63} Nevertheless, by affecting both OX and NSQ oxidation states, their effect on the overall tuning of the $E^{\circ}_{\text{OX/NSQ}}$ couple (+116 mV, 11 kJ/mol) is more conservative than the net effect of moving FMN from bulk solvent to the iLOV pocket.

For the cationic mutants $Q_{103}R^{(+)}$ and $Q_{103}K^{(+)}$ the side-chain tends to flip out toward the solvent, which creates water penetration pathways in the NSQ state. The greater occupancy of water near N5 over the course of the simulations relative to $Q_{103}G$ correlate with the increase in $E^{\circ}_{\text{OX/NSQ}}$. While electrostatics can tune flavin redox potentials, these effects disproportionately affect proton-uncoupled redox transformations in which an ASQ or AHQ is formed.^{64,65} Our data here are consistent with this, in that, the introduction of an

expectedly charged residue does little to change the reduction potential of a charge-neutral OX→NSQ or NSQ→NHQ transition. Across the series of Q₁₀₃R⁽⁺⁾, Q₁₀₃K⁽⁺⁾, and Q₁₀₃D⁽⁰⁾, the MD simulations indicate water occupancy and hydrogen bonding correlate well with the computed $E^{\circ}_{\text{OX/NSQ}}$, providing a rationale and experimentally testable model for flavin redox tuning via hydrogen bonding interactions to N5 and O4.

For Q₁₀₃D, only the neutral state was found to agree with experiment, but with one methylene carbon fewer than the Q side-chain, no hydrogen bond can be formed with N5. Instead, water overwhelmingly penetrates into the flavin binding pocket, where it can hydrogen bond with N5 and O4, and is additionally stabilized by hydrogen bonding interactions with the carboxylic acid side-chain carbonyl of Q₁₀₃D⁽⁰⁾. This hydrogen bonding interaction likely polarizes the water molecule, making it a better hydrogen bond acceptor within the hydrogen bonding network.

The iLOV scaffold and Q₁₀₃X mutations also change the bound FMN $E^{\circ}_{\text{NSQ/NHQ}}$, relative to the free cofactor. In the parental iLOV, $E^{\circ}_{\text{NSQ/NHQ}}$ is only 30 mV below that of free FMN. The pK_a of the free riboflavin N1 in the NSQ state is 2.3,⁶⁶ and shifts to 6.7 in the NHQ state, corresponding to a ΔpK_a in H₂O•••H–N1 hydrogen bonds of 11.7 and 6.7, respectively. This ΔpK_a difference between the two oxidation states is more modest than for N5 between OX and NSQ states, indicating that hydrogen bonding contributes to stabilization of both NSQ and NHQ states, but more so in the NHQ. The X-ray structure of iLOV (in the OX state) contains a strained hydrogen bond between the amide –NH₂ of Q₄₄ and N1 (N•••N distance of 3.4 Å) and a shorter hydrogen bond with O2 (N•••O distance of 2.9 Å), analogous to the Q₁₀₃ interactions with N5 and O4, whereas our MD simulations show the Q₄₄ amide –NH₂ forms hydrogen bonds to N1 in about 60–80% of frames,

depending on oxidation state. This is in contrast to the situation in water, where only 20% of frames form hydrogen bonds at N1. Assuming Q44 can act as both a hydrogen bond donor and acceptor during the NSQ→NHQ transition, hydrogen bonding capacity to N1 is maintained in both NSQ (acceptor) and NHQ (donor) states. Here, we predict that hydrogen bonding will stabilize both NSQ or NHQ similarly, explaining the small change in $E^{\circ}_{\text{NSQ/NHQ}}$ relative to free FMN. Nevertheless, there are other factors the protein environment likely imposes on the NSQ→NHQ transition that are distinct from those experienced in bulk water, such as the flavin “butterfly” bend in the NHQ state,⁶⁷ which could partially explain the slightly more negative $E^{\circ}_{\text{NSQ/NHQ}}$, but is not addressed in the experiments or simulations presented herein.

Q103E, just like Q103D, is also expected to be protonated based on MD simulations. As shown in the clustering analysis in the Supporting Information **Figure III-S18**, the E103 residue is firmly hydrogen bonded to the flavin O4 atom. The protonated Q103E⁽⁰⁾, therefore, participates in a stabilizing hydrogen bonding network involving both the N82 and flavin. Instead, MD simulations on the deprotonated Q103E⁽⁻⁾ result in a less stable and therefore more dynamic environment near the E103 position (see heat map in the Supporting Information **Figure III-S18**).

It is also useful to compare the $E^{\circ}_{\text{OX/NSQ}}$ potential in native iLOV and Q103E⁽⁰⁾. Both systems have a side chain at position 103 that is at a suitable length to form a direct hydrogen bond to the flavin O4. However, the Q103E⁽⁰⁾ side chain increases $E^{\circ}_{\text{OX/NSQ}}$ relative to Q103 in iLOV. This can be attributed to the difference in the pK_a of the two side chains; E103 is more acidic and is therefore a better hydrogen bond donor than Q103. This difference in hydrogen bonding ability results in a larger stabilizing effect on the OX state in Q103E⁽⁰⁾,

without the need for water penetrating the binding pocket, while in other mutants the $E^{\circ}_{\text{OX/NSQ}}$ trend is related to degree of water solvation.

The trend in $E^{\circ}_{\text{OX/NSQ}}$ across the Q_{103}X mutants was also reflected in the $E^{\circ}_{\text{NSQ/NHQ}}$, but instead decreases the reduction potential from iLOV to Q_{103}D , indicating that the mutations either stabilize the NSQ or destabilize the NHQ. The distal nature of the Q_{103}X mutations relative to N1 makes changes in hydrogen bonding to N1 across the series unlikely. We hesitate to speculate on the molecular origin of this trend, but note that the pK_{a} of N5 in the NHQ state has never been measured to our knowledge, but is likely extremely high (>14), making any N5 hydrogen bonds minimal in the overall NHQ stabilization. In this scenario, systematic increases in stability of the NSQ due to hydrogen bonding to water, as we argue for the tuning of $E^{\circ}_{\text{OX/NSQ}}$, should decrease $E^{\circ}_{\text{NSQ/NHQ}}$, assuming little change in the stability of the NHQ state across the series. This hypothesis will be tested in future studies.

The results from the alchemical free energy calculations performed by our collaborators highlight the difficulties involved with computational prediction of flavin reduction potentials. While several free energy studies have been reported for computing redox potentials or effects of mutations on protein interface (in flavoproteins and other related biological systems), these protocols have often relied on shorter dynamics and fewer λ steps.^{26,47} Such protocols can be suitable for systems like flavodoxin, where flavin is already solvent-exposed. Here, even with 40 λ steps (inclusion of more intermediate states connecting the end points) and longer dynamics (600 ns per replica) than is typically used, there were larger errors in the calculations. This is because free energy protocols are suitable when a change is introduced smoothly and gradually. When flavin is engulfed by a protein environment, the mutational transformation is more easily disrupted by random and

intermittent entering/exiting of water near the flavin. Even with these errors, it is possible to obtain redox potentials in biomolecules with errors on the order of 1–2 kcal/mol, but this requires extensive sampling, multiple replicas to identify outliers, and careful analysis of the trajectories to monitor issues like water access and other indicators of large perturbations occurring along the λ steps. The experiments reported here, which studies the effect of a series of single-point mutations on the reduction potential of iLOV, can serve as a benchmark set for computational methods being developed such as QM/MM MD simulations,^{68,69} polarizable force fields,⁶⁹ and machine learning approaches.^{70,71}

The scientific application of iLOV, as a small engineered fluorescent protein, has been limited to its utility as a genetically encoded alternative to O₂-dependent fluorescent proteins such as GFP. However, its small size, strong binding of FMN, and lack of any other cofactors or subunits make it a perfect model to test theories on reduction potential tuning in crossed flavoproteins. With this model protein, very low concentrations of NSQ can be detected by traditional UV–vis spectroscopy without the convolution of interfering cofactors or substrates. In this work, this has enabled us to estimate the potentials related to one-electron transfer in flavins that preferentially undergo two-electron transfer. Our finding, that hydrogen bonding and solvation preferentially stabilize the NSQ state, lowering the apparent one-electron reduction potential, should be broadly applicable. Interestingly, in the electron bifurcating enzymes, which are estimated to have the lowest E^o_{OX/SQ} couples known, an ASQ is proposed.^{72,73} Further study is necessary to understand how hydrogen bonding and electrostatics will affect OX→ASQ couples.

CONCLUSION

In this chapter, we tuned the reduction potential of a flavin cofactor embedded within the small fluorescent flavoprotein iLOV by changing the identity of a side chain residue nearby the N5 and O4 atoms of the isoalloxazine ring. We showed that the identity of this side chain and the corresponding increase in solvent penetration to the N5 pocket reduced the extent of potential crossing of iLOV, while only modestly affecting the overall two-electron potential of the cofactor. This can help serve as a reference from which to gauge the accuracy of quantum mechanical predictions of flavoprotein reduction potentials and can better inform future engineering of artificial flavoproteins via the thermodynamics of proton-coupled electron transfer.

REFERENCES

1. Tyburski, R.; Liu, T.; Glover, S. D.; Hammarström, L. Proton-Coupled Electron Transfer Guidelines, Fair and Square. *J. Am. Chem. Soc.* **2021**, *143* (2), 560–576. <https://doi.org/10.1021/jacs.0c09106>.
2. Migliore, A.; Polizzi, N. F.; Therien, M. J.; Beratan, D. N. Biochemistry and Theory of Proton-Coupled Electron Transfer. *Chem. Rev.* **2014**, *114* (7), 3381–3465. <https://doi.org/10.1021/cr4006654>.
3. Müller, F. *Chemistry and Biochemistry of Flavoenzymes*; CRC Press: Boca Raton, FL, 1990; Vol. 1.
4. Bashford, D.; Karplus, M. pKa's of Ionizable Groups in Proteins: Atomic Detail from a Continuum Electrostatic Model. *Biochemistry* **1990**, *29* (44), 10219–10225. <https://doi.org/10.1021/bi00496a010>.
5. Draper, R. D.; Ingraham, L. L. A Potentiometric Study of the Flavin Semiquinone Equilibrium. *Arch. Biochem. Biophys.* **1968**, *125* (3), 802–808. [https://doi.org/10.1016/0003-9861\(68\)90517-1](https://doi.org/10.1016/0003-9861(68)90517-1).
6. Mayhew, S. G. The Effects of pH and Semiquinone Formation on the Oxidation–Reduction Potentials of Flavin Mononucleotide. *Eur. J. Biochem.* **1999**, *265* (2), 698–702. <https://doi.org/10.1046/j.1432-1327.1999.00767.x>.
7. Herschlag, D.; Pinney, M. M. Hydrogen Bonds: Simple after All? *Biochemistry* **2018**, *57* (24), 3338–3352. <https://doi.org/10.1021/acs.biochem.8b00217>.
8. Lerner, M. Designer Flavoproteins for the Elucidation of the Fundamental Mechanism of Potential Inversion, University of California, Santa Barbara, Santa Barbara, 2022.
9. Chapman, S.; Faulkner, C.; Kaiserli, E.; Garcia-Mata, C.; Savenkov, E. I.; Roberts, A. G.; Oparka, K. J.; Christie, J. M. The Photoreversible Fluorescent Protein iLOV Outperforms GFP as a Reporter of Plant Virus Infection. *Proc. Natl. Acad. Sci.* **2008**, *105* (50), 20038–20043. <https://doi.org/10.1073/pnas.0807551105>.
10. Christie, J. M.; Hitomi, K.; Arvai, A. S.; Hartfield, K. A.; Mettlen, M.; Pratt, A. J.; Tainer, J. A.; Getzoff, E. D. Structural Tuning of the Fluorescent Protein iLOV for Improved Photostability. *J. Biol. Chem.* **2012**, *287* (26), 22295–22304.
11. Anderson, N. T.; Weyant, K. B.; Mukherjee, A. Characterization of Flavin Binding in Oxygen-Independent Fluorescent Reporters. *AIChE J.* **2020**, *66* (12), 17083. <https://doi.org/10.1002/aic.17083>.
12. Jones, B. J.; Greene, B. L. Singlet Oxygen-Mediated Photochemical Cross-Linking of an Engineered Fluorescent Flavoprotein iLOV. *J. Biol. Chem.* **2024**, *300* (11), 107845. <https://doi.org/10.1016/j.jbc.2024.107845>.

13. Guerrero, F.; Ciragan, A.; Iwai, H. Tandem SUMO Fusion Vectors for Improving Soluble Protein Expression and Purification. *Protein Expr. Purif.* **2015**, *116*, 42–49. <https://doi.org/10.1016/j.pep.2015.08.019>.
14. Cáceres, J. C.; Bailey, C. A.; Yokoyama, K.; Greene, B. L. Selenocysteine Substitutions in Thiyl Radical Enzymes. In *Methods in Enzymology*; Weerapana, E., Ed.; Academic Press, 2022; pp 119–141. <https://doi.org/10.1016/bs.mie.2021.10.014>.
15. Drössler, P.; Holzer, W.; Penzkofer, A.; Hegemann, P. pH Dependence of the Absorption and Emission Behaviour of Riboflavin in Aqueous Solution. *Chem. Phys.* **2002**, *282* (3), 429–439. [https://doi.org/10.1016/S0301-0104\(02\)00731-0](https://doi.org/10.1016/S0301-0104(02)00731-0).
16. Watanabe, T.; Honda, K. Measurement of the Extinction Coefficient of the Methyl Viologen Cation Radical and the Efficiency of Its Formation by Semiconductor Photocatalysis. *J. Phys. Chem.* **1982**, *86* (14), 2617–2619. <https://doi.org/10.1021/j100211a014>.
17. Davari, M. D.; Kopka, B.; Wingen, M.; Bocola, M.; Drepper, T.; Jaeger, K.-E.; Schwaneberg, U.; Krauss, U. Photophysics of the LOV-Based Fluorescent Protein Variant iLOV-Q489K Determined by Simulation and Experiment. *J. Phys. Chem. B* **2016**, *120* (13), 3344–3352. <https://doi.org/10.1021/acs.jpcc.6b01512>.
18. Cáceres, J. C.; Dolmatch, A.; Greene, B. L. The Mechanism of Inhibition of Pyruvate Formate Lyase by Methacrylate. *J. Am. Chem. Soc.* **2023**, *145* (41), 22504–22515. <https://doi.org/10.1021/jacs.3c07256>.
19. Kopka, B.; Magerl, K.; Savitsky, A.; Davari, M. D.; Röllen, K.; Bocola, M.; Dick, B.; Schwaneberg, U.; Jaeger, K.-E.; Krauss, U. Electron Transfer Pathways in a Light, Oxygen, Voltage (LOV) Protein Devoid of the Photoactive Cysteine. *Sci. Rep.* **2017**, *7* (1), 13346. <https://doi.org/10.1038/s41598-017-13420-1>.
20. Andersen, E. M. E.; Wang, H.; Khoo, J. S. H.; Cerda, J. F.; Koder, R. L. Oxidation-Reduction and Photophysical Properties of Isomeric Forms of Safranine. *PloS One* **2022**, *17* (6), e0265105. <https://doi.org/10.1371/journal.pone.0265105>.
21. Minnaert, K. Measurement of the Equilibrium Constant of the Reaction between Cytochrome c and Cytochrome a. *Biochim. Biophys. Acta – Enzymol. Biol. Oxid.* **1965**, *110* (1), 42–56. [https://doi.org/10.1016/s0926-6593\(65\)80093-5](https://doi.org/10.1016/s0926-6593(65)80093-5).
22. Miller, A.-F.; Duan, H. D.; Varner, T. A.; Mohamed Raseek, N. Reduction Midpoint Potentials of Bifurcating Electron Transfer Flavoproteins. In *Methods in Enzymology*; Palfey, B. A., Ed.; Academic Press, 2019; pp 365–398. <https://doi.org/10.1016/bs.mie.2019.03.017>.
23. Massey, V. A Simple Method for the Determination of Redox Potentials. *Flavins Flavoproteins* **1990**.

24. Ma, J. W.; Cunningham, M. F.; McAuley, K. B.; Keoshkerian, B.; Georges, M. K. Nitroxide Partitioning between Styrene and Water. *J. Polym. Sci. Part Polym. Chem.* **2001**, *39* (7), 1081–1089. [https://doi.org/10.1002/1099-0518\(20010401\)39:7%3C1081::AID-POLA1085%3E3.0.CO;2-4](https://doi.org/10.1002/1099-0518(20010401)39:7%3C1081::AID-POLA1085%3E3.0.CO;2-4).
25. Zwanzig, R. W. High-Temperature Equation of State by a Perturbation Method. *Nonpolar Gases J. Chem. Phys.* **1954**, *22* (8), 1420–1426. <https://doi.org/10.1063/1.1740409>.
26. Clark, A. J.; Negron, C.; Hauser, K.; Sun, M.; Wang, L.; Abel, R.; Friesner, R. A. Relative Binding Affinity Prediction of Charge-Changing Sequence Mutations with FEP in Protein–Protein Interfaces. *J. Mol. Biol.* **2019**, *431* (7), 1481–1493.
27. Seeliger, D.; Groot, B. L. Protein Thermostability Calculations Using Alchemical Free Energy Simulations. *Biophys. J.* **2010**, *98* (10), 2309–2316. <https://doi.org/10.1016/j.bpj.2010.01.051>.
28. Gapsys, V.; Michielssens, S.; Seeliger, D.; Groot, B. L. pmx. Automated Protein Structure and Topology Generation for Alchemical Perturbations. *J. Comput. Chem.* **2014**, *36* (5), 348–354. <https://doi.org/10.1002/jcc.23804>.
29. Wade, A. D.; Bhati, A. P.; Wan, S.; Coveney, P. V. Alchemical Free Energy Estimators and Molecular Dynamics Engines: Accuracy, Precision, and Reproducibility. *J. Chem. Theory Comput.* **2022**, *18* (6), 3972–3987. <https://doi.org/10.1021/acs.jctc.2c00114>.
30. Serra, M. A.; Vidossich, P.; Acquistapace, I.; Ganesan, A. K.; Vivo, M. Alchemical Free Energy Calculations to Investigate Protein–Protein Interactions: The Case of the CDC42/PAK1 Complex. *J. Chem. Inf. Model.* **2022**, *62*, 12, 3023–3033. <https://doi.org/10.1021/acs.jcim.2c00348>.
31. Bennett, C. H. Efficient Estimation of Free Energy Differences from Monte Carlo Data. *J. Comput. Phys.* **1976**, *22* (2), 245–268. [https://doi.org/10.1016/0021-9991\(76\)90078-4](https://doi.org/10.1016/0021-9991(76)90078-4).
32. Spoel, D.; Lindahl, E.; Hess, B.; Groenhof, G.; Mark, A. E.; Berendsen, H. J. C. GROMACS: Fast, Flexible, and Free. *J. Comput. Chem.* **2005**, *26* (16), 1701–1718. <https://doi.org/10.1002/jcc.20291>.
33. Shirts, M. R.; Pande, V. S. Comparison of Efficiency and Bias of Free Energies Computed by Exponential Averaging, the Bennett Acceptance Ratio, and Thermodynamic Integration. *J. Chem. Phys.* **2005**, *122* (14), 144107. <https://doi.org/10.1063/1.1873592>.
34. Elhajj, S.; D’Ascenzi, J.; Ajagbe, S. O.; Orozco-Gonzalez, Y.; Olivucci, M.; Gozem, S. An Automated QM/MM Average Protein Electrostatic Configuration Approach for Flavoproteins: APEC-F 2.0. *J. Chem. Phys.* **2025**, *163*, 112501. <https://doi.org/10.1063/5.0287415>.

35. Pedraza-González, L.; Marin, M. d C.; Jorge, A. N.; Ruck, T. D.; Yang, X.; Valentini, A.; Olivucci, M.; Vico, L. Web-ARM: A Web-Based Interface for the Automatic Construction of QM/MM Models of Rhodopsins. *J. Chem. Inf. Model.* **2020**, *60* (3), 1481–1493.
36. Bonfrate, S.; Ferré, N.; Huix-Rotllant, M. Efficient Free Energies from a Simplified Electrostatic Embedding QM/MM Approach Based on Electrostatic Potential Fitted Operators. *J. Chem. Inf. Model.* **2025**, *65* (17), 9196–9207. <https://doi.org/10.1021/acs.jcim.5c01563>.
37. Magde, D.; Wong, R.; Seybold, P. G. Fluorescence Quantum Yields and Their Relation to Lifetimes of Rhodamine 6G and Fluorescein in Nine Solvents: Improved Absolute Standards for Quantum Yields. *Photochem. Photobiol.* **2002**, *75* (4), 327–334. [https://doi.org/10.1562/0031-8655\(2002\)0750327FQYATR2.0.CO2](https://doi.org/10.1562/0031-8655(2002)0750327FQYATR2.0.CO2).
38. Ghosh, P.; Ajagbe, S. O.; Gozem, S. The Photophysical Path to the Triplet State in Light-Oxygen-Voltage (LOV) Domains. *Chem. Eur. J.* **2025**, *31* (22), 202500117. <https://doi.org/10.1002/chem.202500117>.
39. Nöll, G.; Hauska, G.; Hegemann, P.; Lanzl, K.; Nöll, T.; Sanden-Flohe, M.; Dick, B. Redox Properties of LOV Domains: Chemical versus Photochemical Reduction, and Influence on the Photocycle. *ChemBioChem* **2007**, *8* (18), 2256–2264. <https://doi.org/10.1002/cbic.200700304>.
40. Pourbaix, M. *Atlas of Electrochemical Equilibria in Aqueous Solutions*; National Association of Corrosion Engineers: Texas, 1974.
41. Fuentes, A.; Möglich, A. Reduction Midpoint Potential of a Paradigm Light–Oxygen–Voltage Receptor and Its Modulation by Methionine Residues. *RSC Chem. Biol.* *5* (6), 530–543. <https://doi.org/10.1039/d4cb00056k>.
42. Nohr, D.; Weber, S.; Schleicher, E. EPR Spectroscopy on Flavin Radicals in Flavoproteins. In *Methods in Enzymology*; Palfey, B. A., Ed.; Academic Press, 2019; pp 251–275. <https://doi.org/10.1016/bs.mie.2019.03.013>.
43. Nash, A. I.; Ko, W.-H.; Harper, S. M.; Gardner, K. H. A Conserved Glutamine Plays a Central Role in LOV Domain Signal Transmission and Its Duration. *Biochemistry* **2008**, *47* (52), 13842–13849. <https://doi.org/10.1021/bi801430e>.
44. Iuliano, J. N.; Collado, J. T.; Gil, A. A.; Ravindran, P. T.; Lukacs, A.; Shin, S.; Woroniecka, H. A.; Adamczyk, K.; Aramini, J. M.; Edupuganti, U. R.; Hall, C. R.; Greetham, G. M.; Sazanovich, I. V.; Clark, I. P.; Daryaee, T.; Toettcher, J. E.; French, J. B.; Gardner, K. H.; Simmerling, C. L.; Meech, S. R.; Tonge, P. J. Unraveling the Mechanism of a LOV Domain Optogenetic Sensor: A Glutamine Lever Induces Unfolding of the J α Helix. *ACS Chem. Biol.* **2020**, *15* (10), 2752–2765.

45. Ganguly, A.; Thiel, W.; Crane, B. R. Glutamine Amide Flip Elicits Long Distance Allosteric Responses in the LOV Protein Vivid. *J. Am. Chem. Soc.* **2017**, *139* (8), 2972–2980. <https://doi.org/10.1021/jacs.6b10701>.
46. Sattelle, B. M.; Sutcliffe, M. J. Calculating Chemically Accurate Redox Potentials for Engineered Flavoproteins from Classical Molecular Dynamics Free Energy Simulations. *J. Phys. Chem. A* **2008**, *112* (50), 13053–13057. <https://doi.org/10.1021/jp803859j>.
47. Silvestri, G.; Arrigoni, F.; Persico, F.; Bertini, L.; Zampella, G.; Gioia, L.; Vertemara, J. Assessing the Performance of Non-Equilibrium Thermodynamic Integration in Flavodoxin Redox Potential Estimation. *Molecules* **2023**, *28* (16), 6016. <https://doi.org/10.3390/molecules28166016>.
48. Kılıç, M.; Ensing, B. First and Second One-Electron Reduction of Lumiflavin in Water—A First Principles Molecular Dynamics Study. *J. Chem. Theory Comput.* **2013**, *9* (9), 3889–3899. <https://doi.org/10.1021/ct400088g>.
49. Wang, J.; Liu, Y. Systematic Theoretical Study on the pH-Dependent Absorption and Fluorescence Spectra of Flavins. *Molecules* **2023**, *28* (8), 3315. <https://doi.org/10.3390/molecules28083315>.
50. Silverstein, T. P.; Heller, S. T. pKa Values in the Undergraduate Curriculum: What Is the Real pKa of Water? *J. Chem. Educ.* **2017**, *94* (6), 690–695. <https://doi.org/10.1021/acs.jchemed.6b00623>.
51. Ludwig, M. L.; Patridge, K. A.; Metzger, A. L.; Dixon, M. M.; Eren, M.; Feng, Y.; Swenson, R. P. Control of Oxidation–Reduction Potentials in Flavodoxin from *Clostridium Beijerinckii*: The Role of Conformation Changes. *Biochemistry* **1997**, *36* (6), 1259–1280.
52. Chang, F.-C.; Swenson, R. P. The Midpoint Potentials for the Oxidized–Semiquinone Couple for Gly57 Mutants of the *Clostridium Beijerinckii* Flavodoxin Correlate with Changes in the Hydrogen-Bonding Interaction with the Proton on N(5) of the Reduced Flavin Mononucleotide Cofactor As Measured by NMR Chemical Shift Temperature Dependencies. *Biochemistry* **1999**, *38* (22), 7168–7176. <https://doi.org/10.1021/bi982203u>.
53. Zheng, Y.-J.; Ornstein, R. L. A Theoretical Study of the Structures of Flavin in Different Oxidation and Protonation States. *J. Am. Chem. Soc.* **1996**, *118* (39), 9402–9408. <https://doi.org/10.1021/ja9608151>.
54. Sakai, M.; Takahashi, H. One-Electron Photoreduction of Flavin Mononucleotide: Time-Resolved Resonance Raman and Absorption Study. *J. Mol. Struct.* **1996**, *379* (1), 9–18. [https://doi.org/10.1016/0022-2860\(95\)09176-9](https://doi.org/10.1016/0022-2860(95)09176-9).

55. Rieff, B.; Bauer, S.; Mathias, G.; Tavan, P. IR Spectra of Flavins in Solution: DFT/MM Description of Redox Effects. *J. Phys. Chem. B* **2011**, *115* (9), 2117–2123. <https://doi.org/10.1021/jp111334z>.
56. Tegoni, M.; Gervais, M.; Desbois, A. Resonance Raman Study on the Oxidized and Anionic Semiquinone Forms of Flavocytochrome B2 and L-Lactate Monooxygenase. Influence of the Structure and Environment of the Isoalloxazine Ring on the Flavin Function. *Biochemistry* **1997**, *36* (29), 8932–8946. <https://doi.org/10.1021/bi962425x>.
57. Yang, K.-Y.; Swenson, R. P. Modulation of the Redox Properties of the Flavin Cofactor through Hydrogen-Bonding Interactions with the N(5) Atom: Role of α Ser254 in the Electron-Transfer Flavoprotein from the Methylophilic Bacterium W3A1. *Biochemistry* **2007**, *46* (9), 2289–2297.
58. Kakeshpour, T.; Van Wiemeersch, A.; Jackson, J. E. Redox Potential Tuning in Bio-Relevant Heterocycles via (Anti)Aromaticity Modulated H-Bonding (AMHB). *Can. J. Chem.* **2020**, *98* (7), 337–346. <https://doi.org/10.1139/cjc-2019-0410>.
59. Baker, E. N.; Hubbard, R. E. Hydrogen Bonding in Globular Proteins. *Prog. Biophys. Mol. Biol.* **1984**, *44* (2), 97–179. [https://doi.org/10.1016/0079-6107\(84\)90007-5](https://doi.org/10.1016/0079-6107(84)90007-5).
60. Arnold, W. D.; Oldfield, E. The Chemical Nature of Hydrogen Bonding in Proteins via NMR: J-Couplings, Chemical Shifts, and AIM Theory. *J. Am. Chem. Soc.* **2000**, *122* (51), 12835–12841. <https://doi.org/10.1021/ja0025705>.
61. Fleming, P. J.; Rose, G. D. Do All Backbone Polar Groups in Proteins Form Hydrogen Bonds? *Protein Sci.* **2005**, *14* (7), 1911–1917. <https://doi.org/10.1110/ps.051454805>.
62. Baron, R.; Setny, P.; McCammon, J. A. Water in Cavity–Ligand Recognition. *J. Am. Chem. Soc.* **2010**, *132* (34), 12091–12097. <https://doi.org/10.1021/ja1050082>.
63. Cuypers, M. G.; Mason, S. A.; Blakeley, M. P.; Mitchell, E. P.; Haertlein, M.; Forsyth, V. T. Near-Atomic Resolution Neutron Crystallography on Perdeuterated *Pyrococcus Furiosus* Rubredoxin: Implication of Hydronium Ions and Protonation State Equilibria in Redox Changes. *Angew. Chem. Int. Ed.* **2013**, *125* (3), 1056–1059.
64. Zhou, Z.; Swenson, R. P. The Cumulative Electrostatic Effect of Aromatic Stacking Interactions and the Negative Electrostatic Environment of the Flavin Mononucleotide Binding Site Is a Major Determinant of the Reduction Potential for the Flavodoxin from *Desulfovibrio Vulgaris* [Hildenborough. *Biochemistry* **1996**, *35* (50), 15980–15988. <https://doi.org/10.1021/bi962124n>.
65. Zhou, Z.; Swenson, R. P. Electrostatic Effects of Surface Acidic Amino Acid Residues on the Oxidation-Reduction Potentials of the Flavodoxin from

- Desulfovibrio Vulgaris (Hildenborough). *Biochemistry* **1995**, 34 (10), 3183–3192. <https://doi.org/10.1021/bi00010a007>.
66. Land, E. J.; Swallow, A. J. One-Electron Reactions in Biochemical Systems as Studied by Pulse Radiolysis. II. *Riboflavine Biochem.* **1969**, 8 (5), 2117–2125. <https://doi.org/10.1021/bi00833a050>.
 67. Hemmerich, P.; Nagelschneider, G.; Veege, C. Chemistry and Molecular Biology of Flavins and Flavoproteins. *FEBS Lett.* **1970**, 8 (2), 69–83. [https://doi.org/10.1016/0014-5793\(70\)80229-0](https://doi.org/10.1016/0014-5793(70)80229-0).
 68. Kılıç, M.; Ensing, B. Redox Properties of Flavin in BLUF and LOV Photoreceptor Proteins from Hybrid QM/MM Molecular Dynamics Simulation. *J. Phys. Chem. B* **2024**, 128 (13), 3069–3080. <https://doi.org/10.1021/acs.jpcc.3c06245>.
 69. Tazhigulov, R. N.; Gurunathan, P. K.; Kim, Y.; Slipchenko, L. V.; Bravaya, K. B. Polarizable Embedding for Simulating Redox Potentials of Biomolecules. *Phys. Chem. Chem. Phys.* **2019**, 21 (22), 11642–11650. <https://doi.org/10.1039/C9CP01533G>.
 70. Sarangi, R.; Maity, S.; Acharya, A. Machine Learning Approach to Vertical Energy Gap in Redox Processes. *J. Chem. Theory Comput.* **2024**, 20 (15), 6747–6755. <https://doi.org/10.1021/acs.jctc.4c00715>.
 71. Galuzzi, B. G.; Mirarchi, A.; Viganò, E. L.; Gioia, L.; Damiani, C.; Arrigoni, F. Machine Learning for Efficient Prediction of Protein Redox Potential: The Flavoproteins Case. *J. Chem. Inf. Model.* **2022**, 62 (19), 4748–4759. <https://doi.org/10.1021/acs.jcim.2c00858>.
 72. Lubner, C. E.; Jennings, D. P.; Mulder, D. W.; Schut, G. J.; Zadvornyy, O. A.; Hoben, J. P.; Tokmina-Lukaszewska, M.; Berry, L.; Nguyen, D. M.; Lipscomb, G. L.; Bothner, B.; Jones, A. K.; Miller, A.-F.; King, P. W.; Adams, M. W. W.; Peters, J. W. Mechanistic Insights into Energy Conservation by Flavin-Based Electron Bifurcation. *Nat. Chem. Biol.* **2017**, 13 (6), 655–659. <https://doi.org/10.1038/nchembio.2348>.
 73. Wise, C. E.; Ledinina, A. E.; Mulder, D. W.; Chou, K. J.; Peters, J. W.; King, P. W.; Lubner, C. E. An Uncharacteristically Low-Potential Flavin Governs the Energy Landscape of Electron Bifurcation. *Proc. Natl. Acad. Sci.* **2022**, 119 (12), e2117882119. <https://doi.org/10.1073/pnas.2117882119>.

SUPPLEMENTAL INFORMATION

Table III-S1. As-purified and reconstituted FMN loading of iLOV variants. FMN concentrations were determined via UV-vis absorption at 448 nm using an extinction coefficient of $14,800 \text{ M}^{-1} \text{ cm}^{-1}$. For blue-shifted mutants (Q₁₀₃K/E/D/R/A/G) the absorption at 440 nm using an extinction coefficient of $16,100 \text{ M}^{-1} \text{ cm}^{-1}$ was used. Protein concentration was determined via the Pierce 660 assay. Initial FMN:iLOV ratios prior to flavin reconstitution are based on single measurements. Reconstituted FMN:iLOV ratios and uncertainty are based on the average of triplicate measurements and error is reported as the standard deviation.

iLOV Variant	As-purified FMN:iLOV	Reconstituted FMN:iLOV
WT	0.21	1.05 ± 0.02
Q ₁₀₃ A	0.27	1.12 ± 0.02
Q ₁₀₃ G	0.44	1.09 ± 0.01
Q ₁₀₃ R	0.31	0.96 ± 0.02
Q ₁₀₃ K	0.27	0.94 ± 0.02
Q ₁₀₃ E	0.17	0.95 ± 0.01
Q ₁₀₃ D	0.36	1.14 ± 0.02

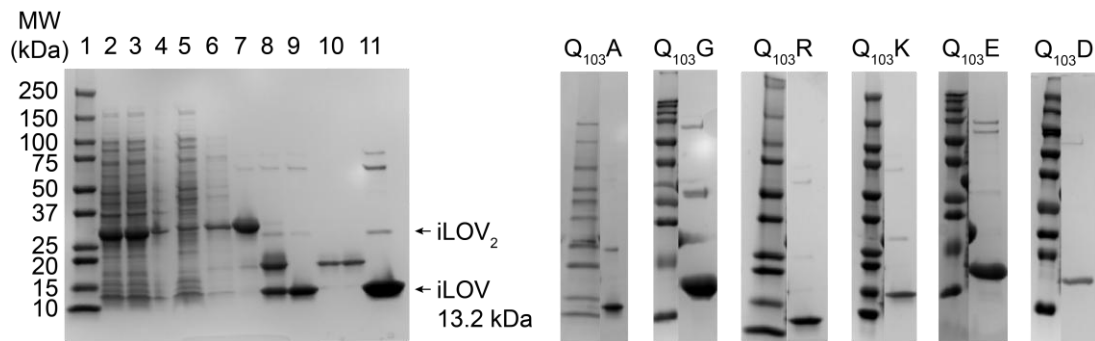


Figure III-S1. Purification (right) and purity (left) SDS-PAGE gels of iLOV and Q₁₀₃X variants. Left: Typical purification gel for iLOV. Lanes as follows: (1) Precision Plus Molecular Weight Standard, (2) total cell lysate, (3) soluble fraction of cell lysate, (4) insoluble pellet of cell debris, (5) Ni-NTA flow through (pre-ULP1 digestion), (6) Ni-NTA wash, (7) Ni-NTA elution ((H)₆-SUMO-iLOV), (8) ULP1 digestion (iLOV and (H)₆-SUMO), (9) digested Ni-NTA flow through and wash (iLOV), (10) ULP1 digestion Ni-NTA elution ((H)₆-SUMO), run in duplicate, and (11) buffer exchanged purified iLOV. Heavier molecular weight bands around 26 kDa, but slightly lower than (H)₆-SUMO-iLOV, present in the purified fraction are small quantities of photo-oxidized and covalently crosslinked iLOV dimers (See Chapter II).

Table III-S2. Site directed mutagenesis (SDM) primer sequences for iLOV variants.

iLOV Variant	Primer Sequences
Q ₁₀₃ A	(forward) 5'-cggtgtgcgctggatggttctg-3' (reverse) 5'-gaaccatccagcgcaacaccgatg-3'
Q ₁₀₃ G	(forward) 5'-cggtgtgggctggatggttctgaccacg-3' (reverse) 5'-cagcccaacaccgatgaaatactgcagctcgcc-3'
Q ₁₀₃ R	(forward) 5'-gcagtatttcacggtgttcggctggatg-3' (reverse) 5'-gaaccatccagccgaacaccgatg-3'
Q ₁₀₃ K	(forward) 5'-gcagtatttcacggtgttaagctggatggttctga-3' (reverse) 5'-ccagcttaacaccgatgaaatactgc-3'
Q ₁₀₃ E	(forward) 5'-ggtgttgagctggatggttctgacc-3' (reverse) 5'-catccagctcaacaccgatgaaatactgc-3'
Q ₁₀₃ D	(forward) 5'-ggtgttgatctggatggttctgaccacg-3' (reverse) 5'-cagaaccatccagatcaacaccgatgaaatactg-3'

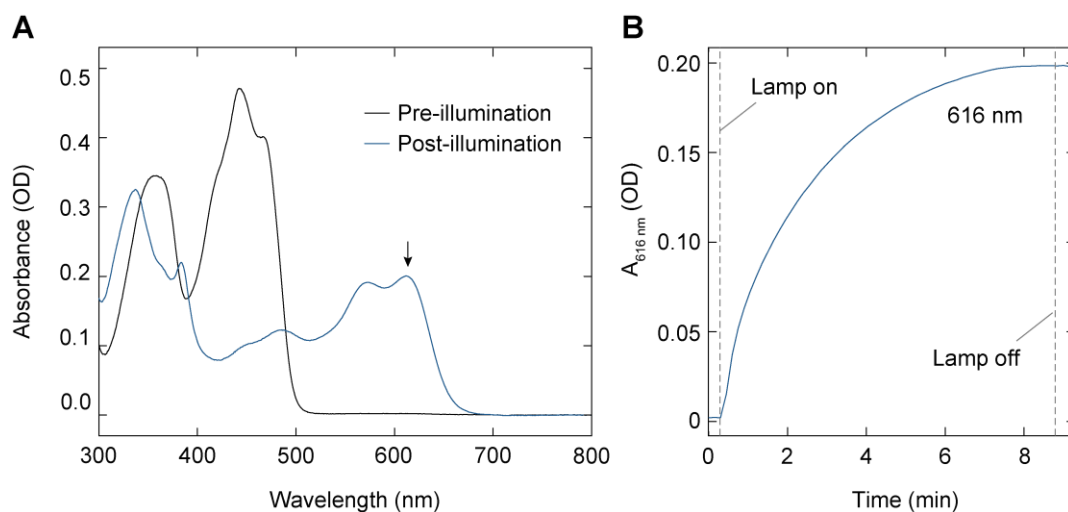


Figure III-S2. Determination of the $Q_{103}D$ NSQ extinction coefficient at 616 nm. **A** UV-vis absorption of $Q_{103}D$ before (black) and after (blue) illumination by a 450 nm LED for 10 mins. **B** Time course of the absorbance at 616 nm during illumination, demonstrating equilibration and complete conversion to the NSQ. The ratio of the absorbance between 616 and 444 nm was multiplied by the extinction coefficient at 444 nm ($16,100 \pm 200 \text{ M}^{-1} \text{ cm}^{-1}$).

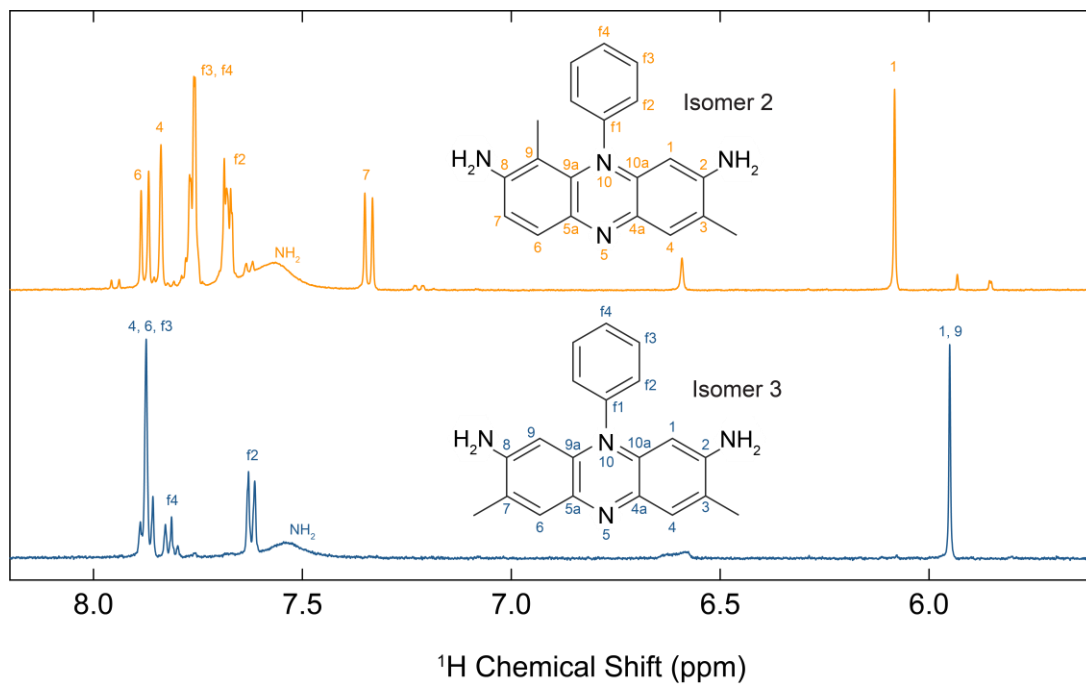


Figure III-S3. ^1H NMR spectra and resonance assignments of safranine isomers in d_6 -DMSO in the aromatic region. Unassigned peaks in the isomer 2 spectrum are thought to be residual contaminants that were not separated during HPLC-MS purification.

Table III-S3. One-dimensional ^1H NMR spectral assignments of safranine isomers.

Isomer 2

^1H -NMR (d_6 -DMSO, 500 MHz): δ 1.34 [3H, s, C(9) CH_3], 2.29 [3H, s, C(3) CH_3], 6.08 [1H, s, $H(1)$], 7.34 [1H, d, $J = 9.2$ Hz, $H(7)$], 7.68 [2H, m, $H(f2)$], 7.77 [3H, m, $H(f3)$ & $H(f4)$], 7.84 [1H, s, $H(4)$], 7.88 [1H, d, $J = 9.2$ Hz, $H(6)$].

Isomer 3

^1H -NMR (d_6 -DMSO, 500 MHz): δ 2.30 [6H, s, C(3/7) CH_3 :($H(17)$)], 5.95 [2H, s, $H(1/9)$], 7.62 [2H, d, $J = 7.5$ Hz, $H(f2)$], 7.81 [1H, t, $J = 7.4$ Hz, $H(f4)$], 7.87 [4H, m, $H(4/6)$ & $H(f3)$].

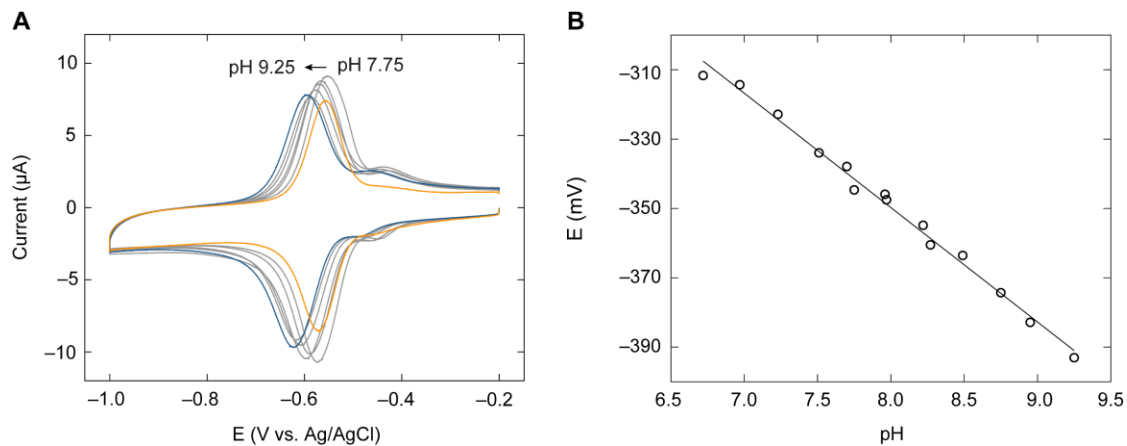


Figure III-S4. Pourbaix analysis of safranine isomer 3. **A** Representative cyclic voltammetry (CV) scans of safranine isomer 3 from pH 7.75 (orange) to 9.25 (blue). **B** Pourbaix diagram (midpoint potential vs. pH) of safranine isomer 3 and associated linear fit. The slope of the fit was -33 mV/pH ($1 \text{ H}^+/2 \text{ e}^-$) with an R^2 of 0.99.

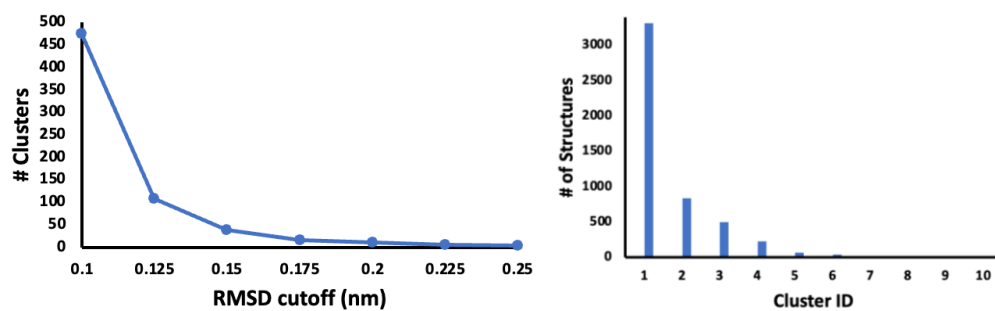


Figure III-S5. Statistical clustering analysis based on RMSD cutoff (left), and number of structures per cluster with an RMSD cutoff of 0.2 nm (right). Ten total clusters were obtained and only 2 had more than 500 MD snapshots out of the total 5001 frames.

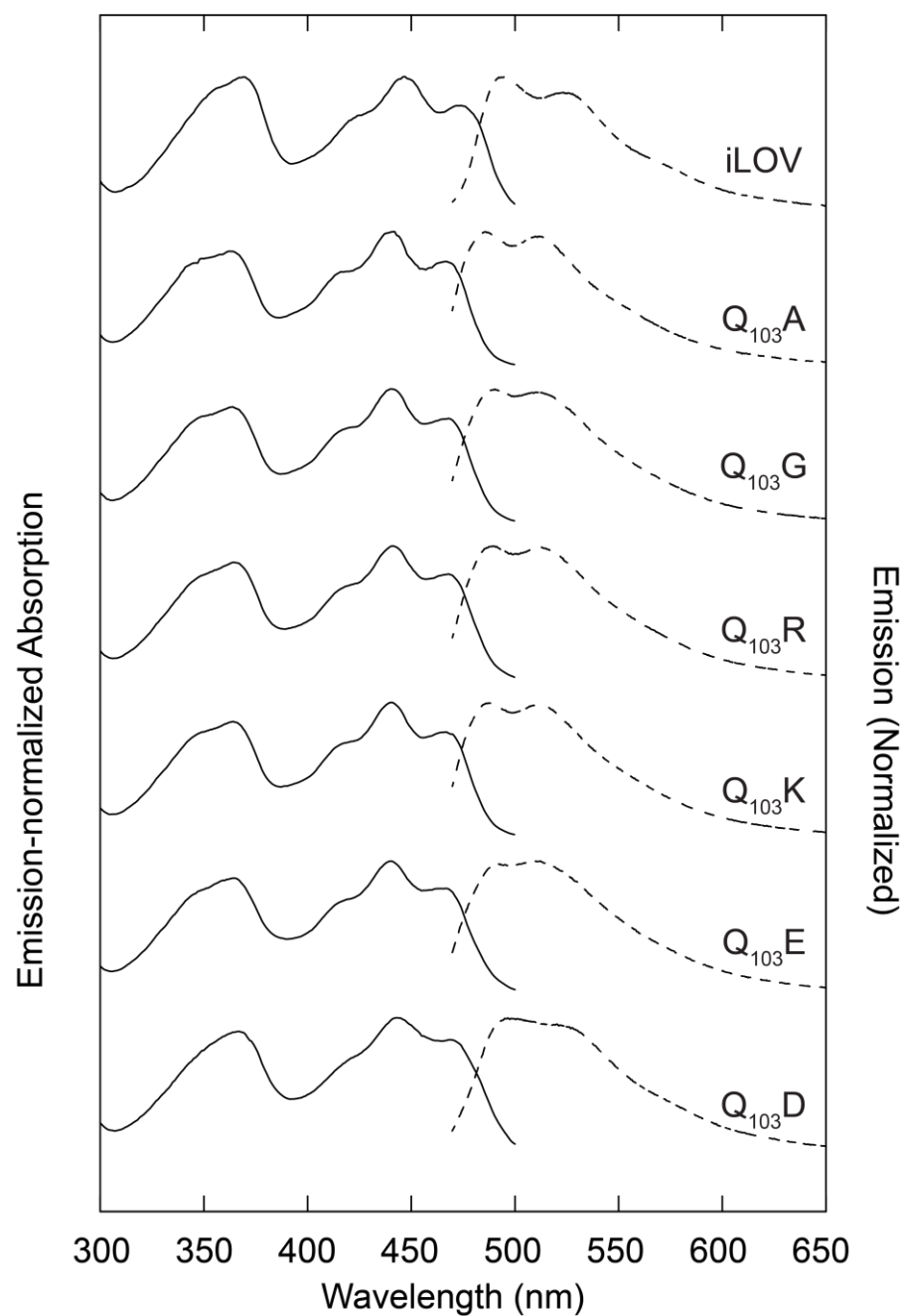


Figure III-S6. Emission-normalized ($\lambda_{em} = 520$ nm) absorption spectra (excitation spectra, solid lines) of iLOV and $Q_{103}X$ mutants. Emission spectra ($\lambda_{ex} = 450$ nm, dashed lines) are the same as those reported in the main text Figure III-2.

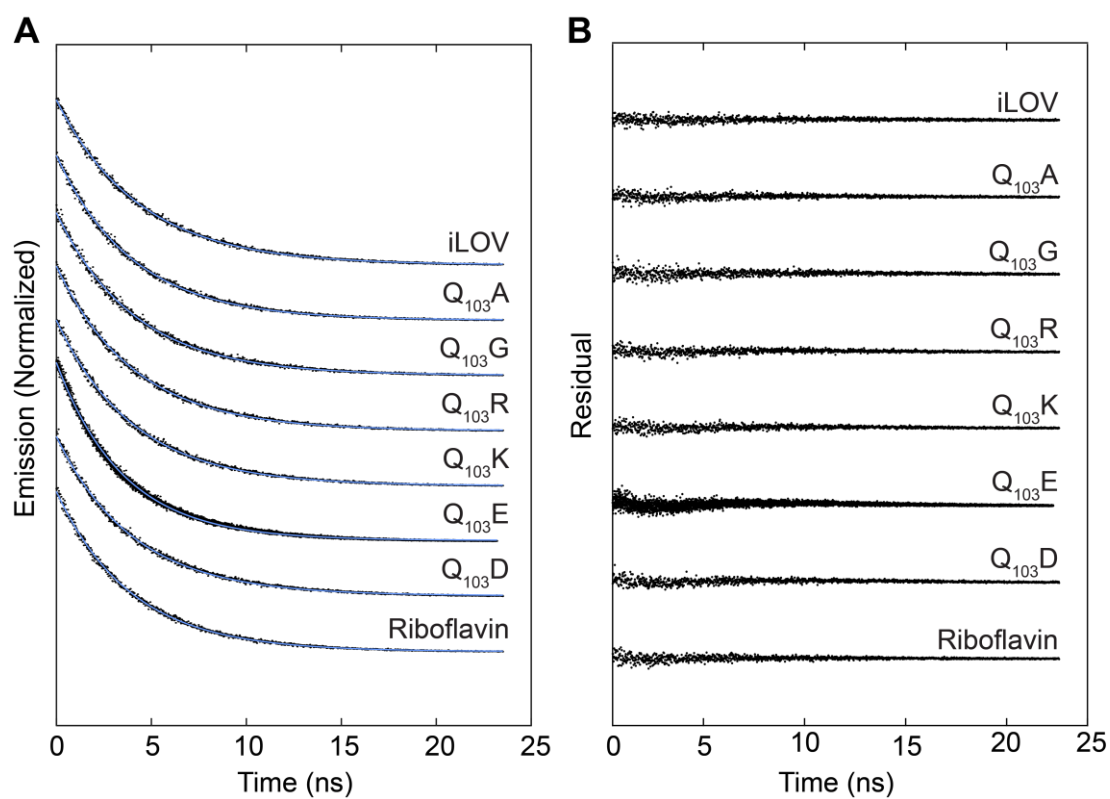


Figure III-S7. Fluorescent lifetimes of iLOV, $Q_{103}X$ mutants, and riboflavin. **A** Emission lifetime traces (dots, $\lambda_{\text{ex}} = 400$ nm) and associated single exponential fits to the experimental data (blue line). **B** Residuals of the experimental data relative to the fit.

Table III-S4. Fluorescence decay lifetimes (τ) and quantum yields (Φ) and emission maxima (λ_{max}) of iLOV and Q₁₀₃X variants.

iLOV Variant	τ (ns)	Φ	λ_{max}
iLOV	4.37 (0.01) ¹	0.37 (0.01) ²	492
Q ₁₀₃ A	4.01 (0.01)	0.37 (0.01)	486
Q ₁₀₃ G	4.09 (0.01)	0.37 (0.01)	490
Q ₁₀₃ R	4.11 (0.008)	0.36 (0.01)	489
Q ₁₀₃ K	4.07 (0.008)	0.35 (0.01)	488
Q ₁₀₃ E	3.516 (0.004)	0.33 (0.02)	510
Q ₁₀₃ D	4.05 (0.009)	0.33 (0.01)	498
Riboflavin	3.923 (0.008)	0.26 ³	534

Numbers in parentheses represent the standard deviation resulting from a fit of a single exponential decay trace.

² Numbers in parentheses represent the error propagation of standard deviation resulting from the integration of triplicate measurements of the iLOV variant emission spectra and the standard deviation resulting from their respective absorbance measurements.

³ Values taken from ref. 9.

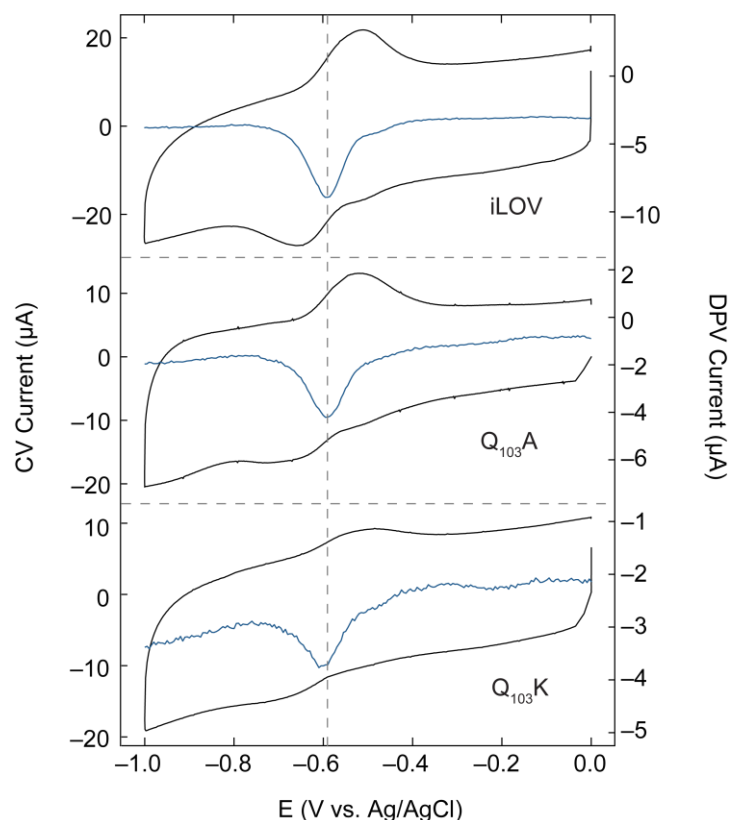


Figure III-S8. Initial attempts to measure $E^{\circ}_{\text{OX/NHQ}}$ for iLOV variants using protein film voltammetry. Cyclic voltammograms (CV, black) and differential pulse voltammograms (DPV, blue) are overlaid. The voltametric setup was identical to that of the safranin measurements. About 5 μL of 1 mM protein was cast onto the surface of a pyrolytic graphite edge-plane working electrode and allowed to dry over a flow of nitrogen. After 1 hour of drying, the electrode was immersed in an electrode solution that was degassed by sparging with Argon gas for 30 minutes prior. A Ag/AgCl (3M NaCl) reference electrode and a platinum wire counter electrode were used. CVs were scanned from 0 to -1.0 V with a scan speed of 1 V/s while DPVs were collected over the same range with a pulse height of 25 mV, a pulse width of 10 ms, a step height of -5 mV and a step time of 100 ms. CVs contained several odd features. The cathodic peak was lower in intensity and broader than the anodic peak, both peak widths were unusually broad at ~ 150 mV, and all traces contained an unusual peak-to-peak separation of around 140 mV (as opposed to the expected value of 0 mV for an adsorbed analyte). No differences were observed between mutants.

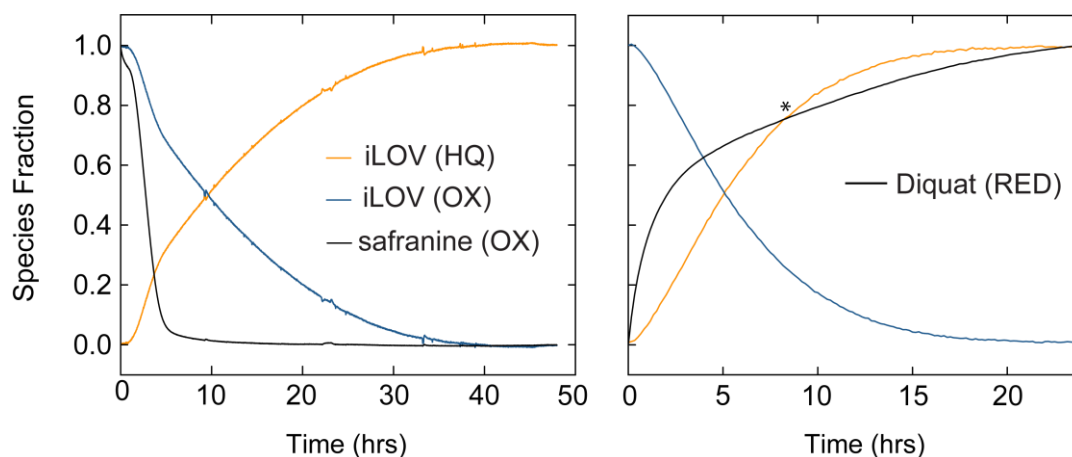


Figure III-S9. Xanthine/xanthine oxidase redox titration assay to measure the $E^{\circ}_{\text{OX/NHQ}}$ couple.

Reactions were performed in a VAC glovebox using the same spectrometer used for comproportionation experiments. For the reaction with safranin, the cuvette contained 169 μM xanthine, 0.3 U/L (10.2 $\mu\text{g/mL}$) xanthine oxidase, 2 μM MV^{2+} as a mediator, 10 μM safranin, and 20 μM iLOV (FMN_{OX}) in 50 mM P_i and 200 mM NaCl at pH 7.9. The fraction of oxidized iLOV was monitored at 420 nm, while the fraction of oxidized safranin was monitored at 520 nm. The fraction of reduced iLOV was assumed to be $1 - \text{FMN}_{\text{OX}}$. The reduction of the standard dye proceeded far faster than the equilibration between the species. For the reaction with diquat as a standard, 130 μM of diquat, 30 μM of protein, 400 μM of xanthine, and 0.23 U/L (7.6 $\mu\text{g/mL}$) of xanthine oxidase was used in the same buffer adjusted to pH 7.9. The concentration of iLOV (OX) was monitored at 475 nm to minimize convolution with diquat, while the concentration of diquat (RED) was monitored at 777 nm. As before, the fraction of iLOV (NHQ) was assumed to equal $1 - \text{FMN}_{\text{OX}}$. In this reaction, the fraction of reduced diquat starts higher than that of iLOV, but at some point in the titration the concentration of reduced iLOV becomes the major species. The reduction of diquat in this assay had an unexpected bi-phasic shape. Together these indicate that the equilibration between the two species was not at equilibrium, and that the oxidation of diquat while initially fast, became retarded as it became consumed by the slower iLOV (OX).

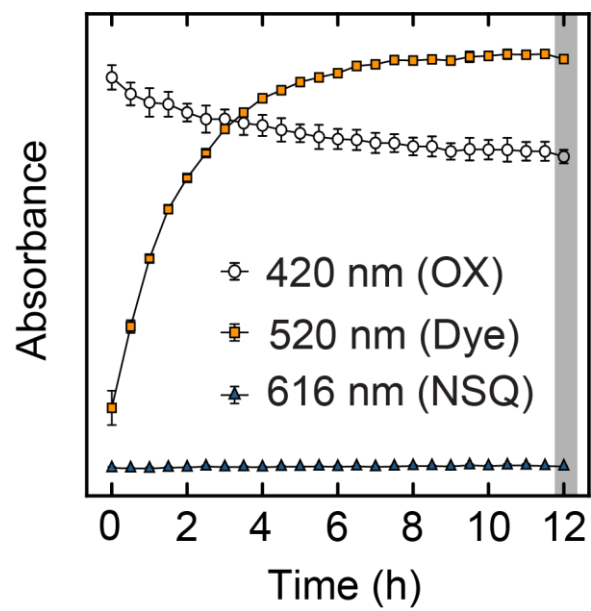


Figure III-S10. Inset from Figure III-3A of the main text reproduced for closer inspection.

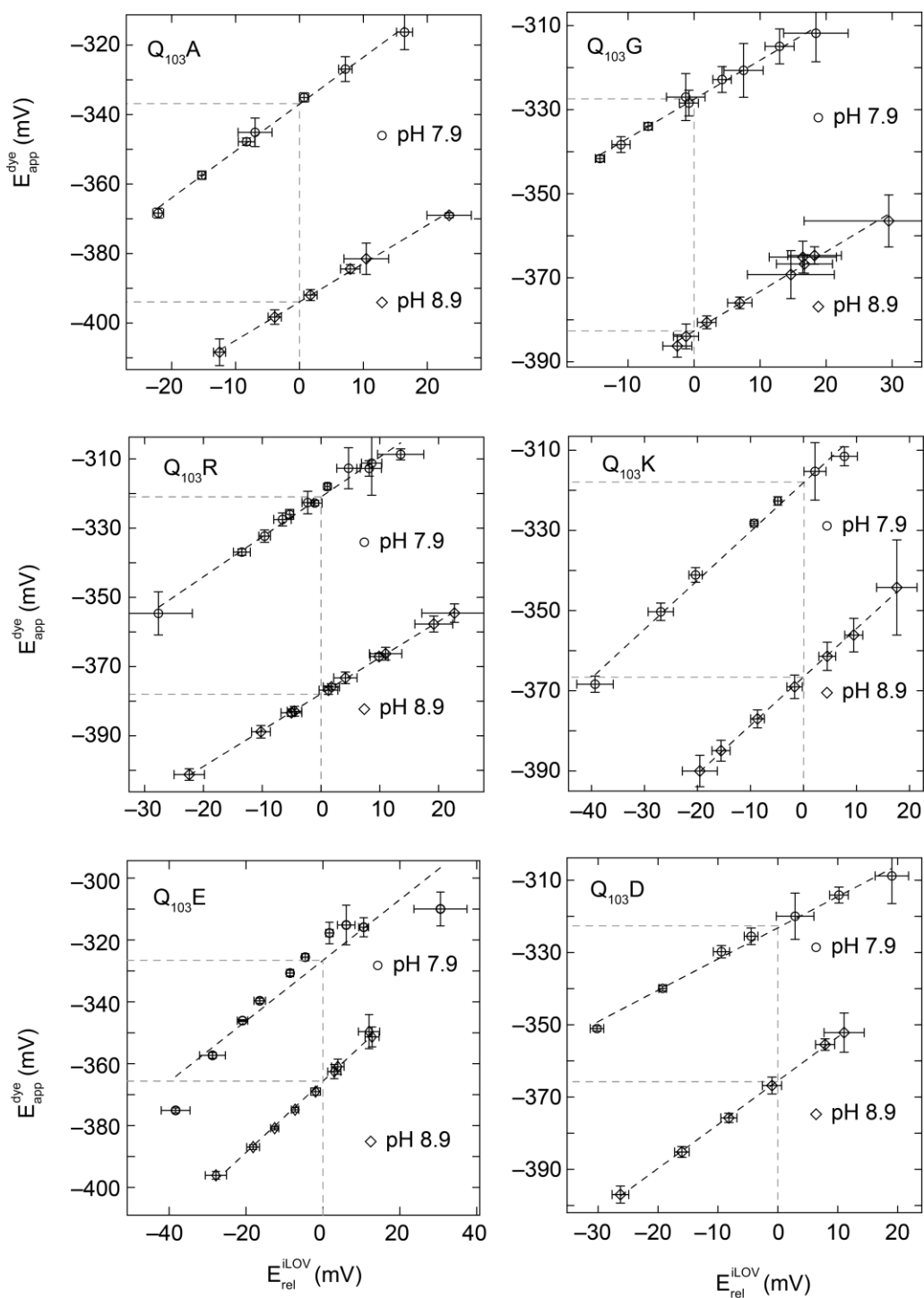


Figure III-S11. Redox titrations of iLOV $Q_{103}X$ mutants. Titrations were conducted at pH 7.9 (circles) and 8.9 (diamonds). The experimental data were fit to a linear function (black dashed lines), and apparent $E^{\circ}_{ox/NHQ}$ potentials are indicated on the y-axis (gray dashed lines). The results are summarized in the main text **Table III-1**. Each data point represents the average of triplicate measurements in separate wells for the same conditions.

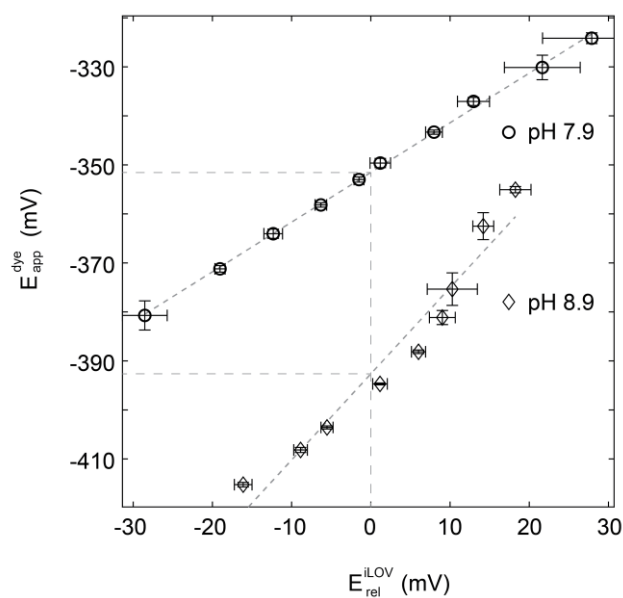


Figure III-S12. Oxidative redox titrations of iLOV with ferricyanide. The results of this titration recreate those of the reductive titration shown in Figure SIII-10 and Main Text Figure III-2. Significantly more scatter is observed in the pH 8.9 data, but the observed reduction potentials remain similar.

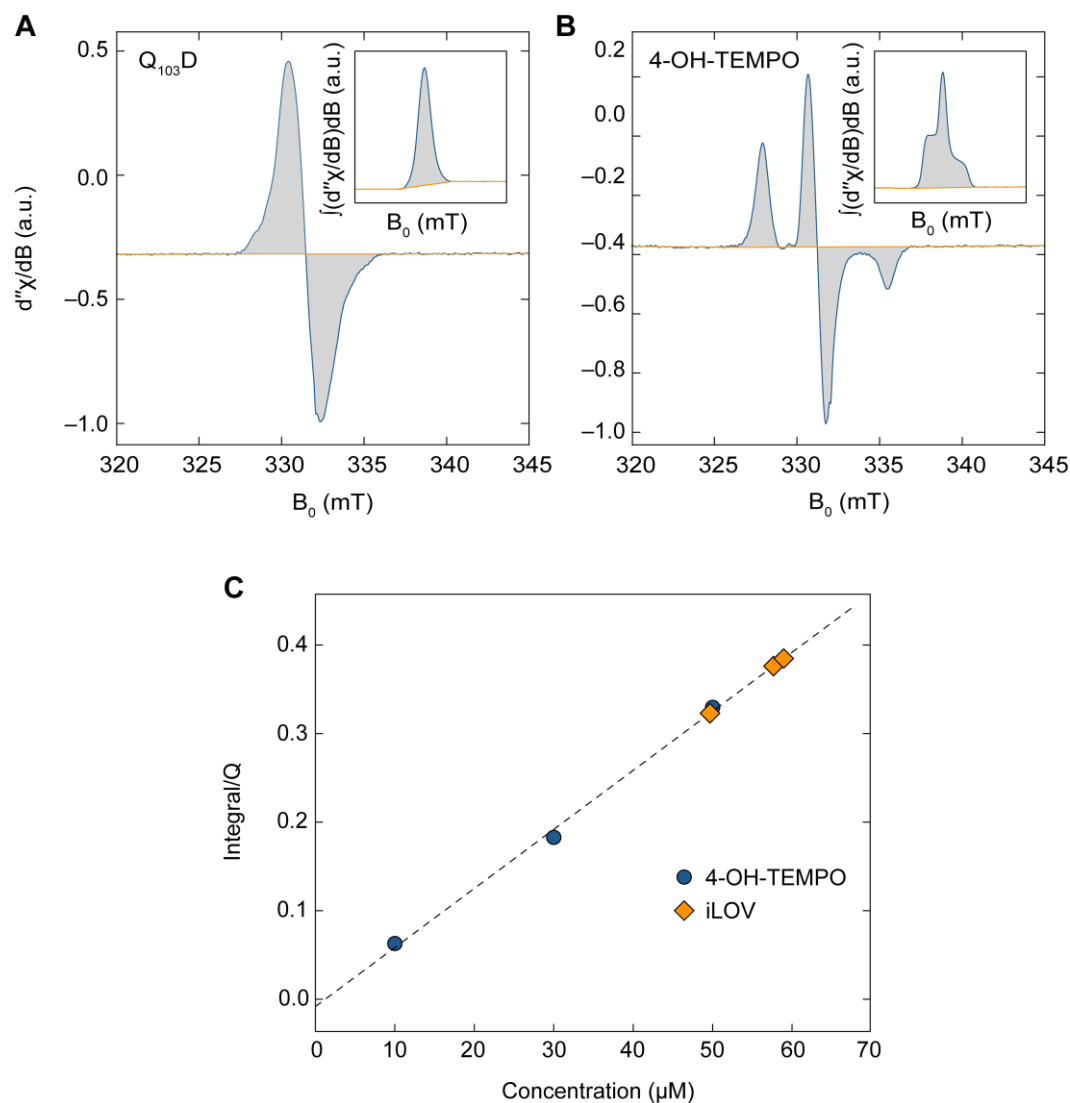


Figure III-S13. Radical quantification by EPR. **A** Representative EPR spectrum of photo-chemically reduced $Q_{103}D$ (gray shaded blue line). A linear baseline (orange) was subtracted from the spectrum, then integrated to yield the integrated spectrum in the inset. A second baseline was subtracted to account for the spectral asymmetry of the original spectrum (orange) and integrated again to yield the apparent total radical content. **B** Representative EPR spectrum of 4-hydroxy-TEMPO, treated identically to $Q_{103}D$. The 4-hydroxy-TEMPO standard concentration was determined by UV-vis to construct the calibration curve in C (blue circles), and compared to $Q_{103}D$ integrations (orange diamonds).

Table III-S5. ΔE values for each mutant using both the UV-Vis estimated extinction coefficient of $8,200 \text{ M}^{-1} \text{ cm}^{-1}$ and using the extinction coefficient estimated using EPR radical counting of $12,400 \text{ M}^{-1} \text{ cm}^{-1}$ for the NSQ radical. Standard deviations are shown in parentheses. While shifts of approximately 25 mV can be observed, the overall trends remain the same.

iLOV Variant	UV-vis ΔE (mV)	EPR ΔE (mV)
WT	-212 (4)	-236 (4)
Q ₁₀₃ A	-176 (3)	-195 (3)
Q ₁₀₃ G	-112 (1)	-133 (1)
Q ₁₀₃ R	-127 (1)	-149 (1)
Q ₁₀₃ K	-104 (1)	-126 (1)
Q ₁₀₃ E	-89 (4)	-112 (4)
Q ₁₀₃ D	-44 (1)	-68 (1)

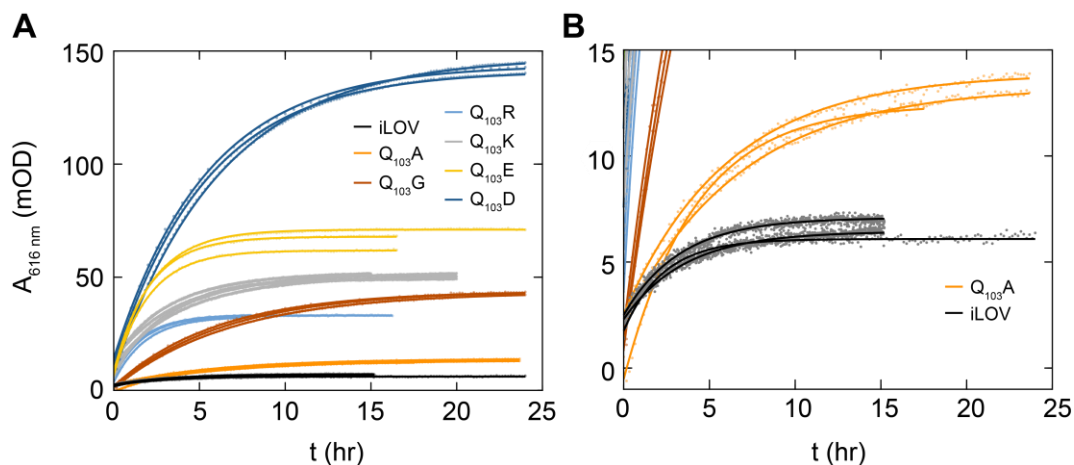


Figure III-S14. UV-vis quantitation of NSQ formation during the time course of comproportionation experiments for iLOV and Q₁₀₃X mutants. **A** Absorbance at 616 nm ($A_{616 \text{ nm}}$, NSQ) data collected for each variant (colored dots) until equilibrium was reached, and single exponential fit (solid colored lines) to the approach to equilibrium. Experiments were performed and fit in triplicate to determine standard deviations. Equilibrium NSQ concentrations were determined from the asymptote of the exponential fit. **B** Expanded view of iLOV and Q₁₀₃A for clarity.

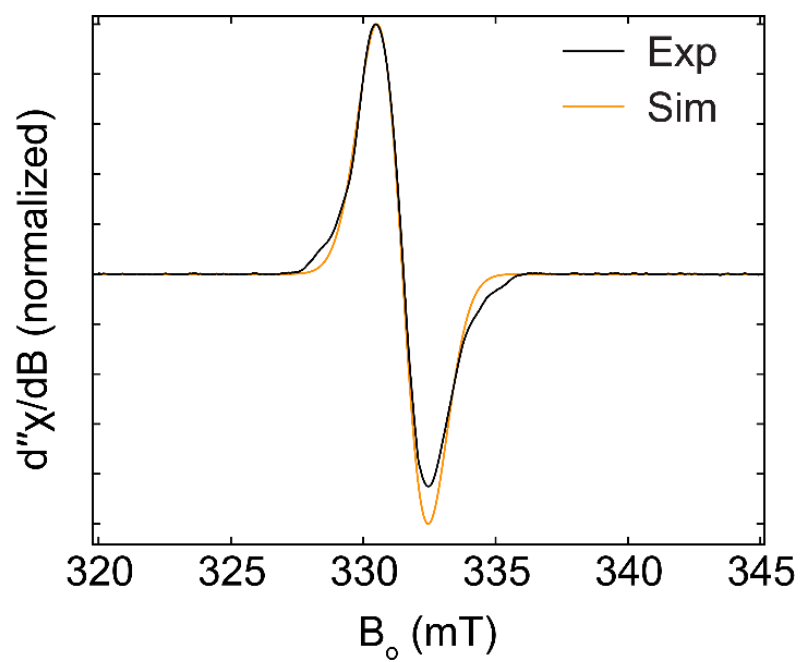


Figure III-S15. EPR spectrum of Q₁₀₃D at pH 9.25. No significant differences in this radical spectrum are observed when compared to Q₁₀₃D at pH 7.9. It is likely that the pK_a of the N5 position in NSQ is >9 .

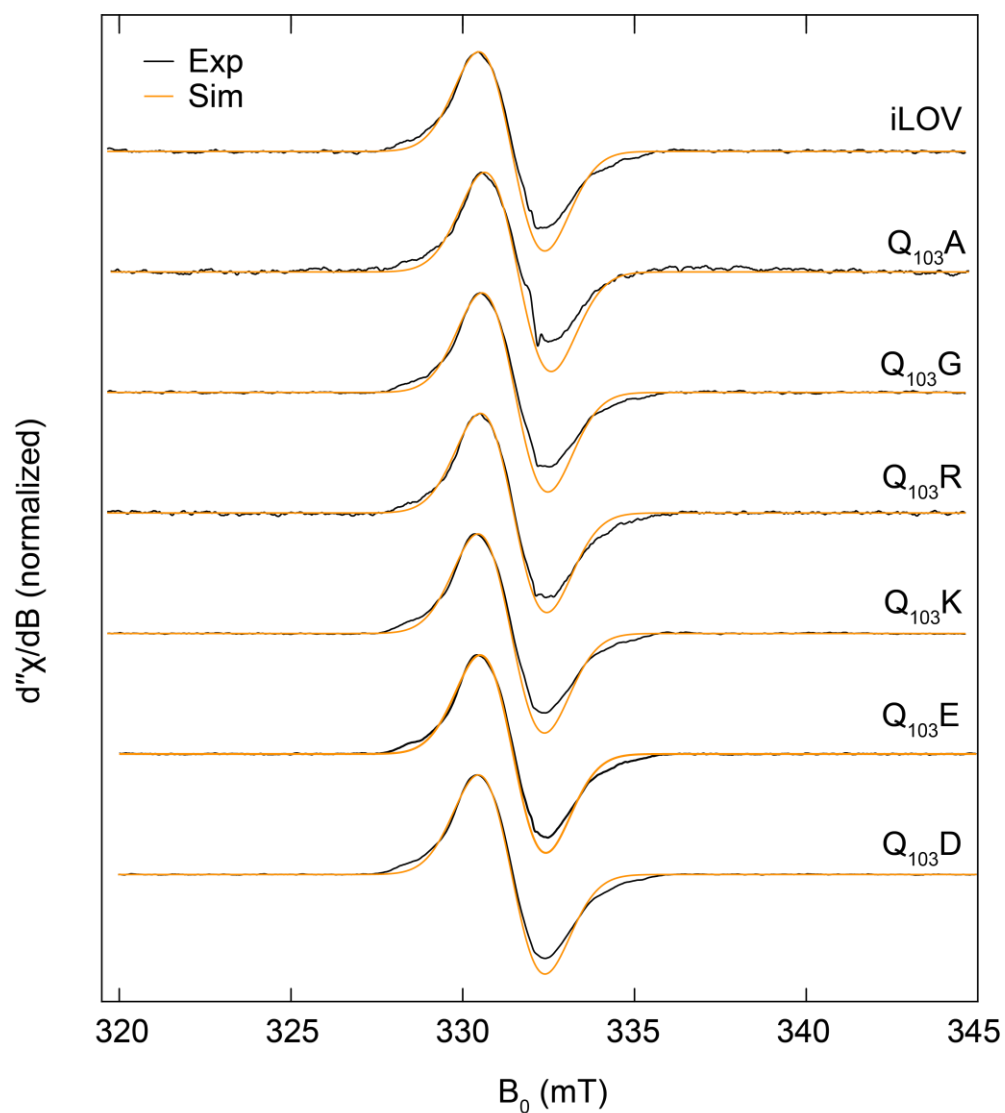


Figure III-S16. Continuous wave X-band EPR spectra of the NSQ radical of each iLOV mutant. Extracted simulation parameters are depicted in Table SIII-6. Each spectrum remained identical.

Table III-S6. EPR simulation parameters for iLOV and Q₁₀₃X NSQ spectra, and associated fit 68% confidence interval.

Variant	g_{iso}	Line width (mT)
iLOV	2.0041 (1)	1.92 (1)
Q ₁₀₃ A	2.0043 (1)	1.94 (2)
Q ₁₀₃ G	2.0042 (1)	1.92 (1)
Q ₁₀₃ R	2.0043 (1)	1.93 (1)
Q ₁₀₃ K	2.0042 (1)	1.94 (1)
Q ₁₀₃ E	2.0045 (1)	1.91 (1)
Q ₁₀₃ D	2.0042 (1)	1.94 (1)
Q ₁₀₃ D (pH 9.5)	2.0043 (1)	1.94 (1)

Table III-S7. Free energies of mutation computed for the oxidized (OX) and neutral semiquinone (NSQ) states of iLOV from different replicas. Indicated in the table are the pre-equilibration time (for $\lambda=0$ step) and the proximity of the water solvent to the N5 atom of flavin, measured as the start of the rise of the radial distribution function (RDF). Free energies highlighted in yellow indicate a “water out” value. Free energies highlighted in red are excluded due to a too-short pre-equilibration for that mutation.

Mutant	State	Rep #	Eq. time (ns)	RDF rise (Å)	ΔG (kcal/mol)
Q₁₀₃D⁽⁰⁾	OX	1	50	1.9	22.9
	OX	2	50	4.6	22.0
	OX	3	3	4.5	22.4
	NSQ	1	3	2.7	21.5
	NSQ	2	3	2.8	20.0
	NSQ	3	50	>5	20.8
Q₁₀₃D⁽⁻⁾	OX	1	3	2.3	-77.2
	OX	2	3	2.7	-74.8
	OX	3	3	1.9	-80.8
	OX	4	50	4	-74.7
	NSQ	1	3	2.5	-75.4
	NSQ	2	3	2.8	-74.7
Q₁₀₃A	OX	1	3	2.2	71.9
	OX	2	3	4.8	72.1
	OX	3	50	4.8	73.2
	NSQ	1	3	>5	69.3
	NSQ	2	3	2.6	71.9
	NSQ	3	50	2.5	72.3
Q₁₀₃G	OX	1	3	1.8	61.1
	OX	2	50	4	62.0
	OX	3	50	2.65	58.0
	NSQ	1	3	2.69	61.1
	NSQ	2	3	2.68	59.4
	NSQ	3	50	2.66	62.2
Q₁₀₃K⁽⁺⁾	OX	1	3	2.8	-19.3
	OX	2	50	2.4	-11.5
	OX	3	50	>5	-15.3
	OX	4	100	2	-11.9
	NSQ	1	3	2.6	-8.2
	NSQ	2	3	2.6	-12.8
	NSQ	3	50	2.68	-16.3
	NSQ	4	100	2.7	-14.9
Q₁₀₃R⁽⁺⁾	OX	1	3	4.1	-170.3
	OX	2	3	1.7	-176.2
	OX	3	50	>5	-174.9
	OX	4	50	1.9	-174.7
	OX	5	100	1.87	-172.6
	NSQ	1	3	2.7	-173.9

	NSQ	2	3	>5	-174.0
	NSQ	3	50	2.8	-175.6
Q₁₀₃E⁽⁰⁾	OX	1	3	2.6	28.5
	OX	2	3	>5	27.5
	OX	3	50	1.9	28.0
	OX	4	50	4.6	29.1
	OX	5	50	>5	28.3
	NSQ	1	3	4.8	28.4
	NSQ	2	3	4.7	29.5
	NSQ	3	50	>5	30.7
	NSQ	4	50	2.6	27.9
	NSQ	5	50	2.6	25.9

Table III-S8: Reduction potentials computed for Q₁₀₃X mutations using different binning for the replicas obtained in Table SIII-7. “All” uses all replicas. For Q₁₀₃K⁽⁺¹⁾ and Q₁₀₃R⁽⁺¹⁾, an extra bin after removing short (3 ns) pre-equilibration trajectories was included. Category 1 includes “water-in” models for both the OX and NSQ state. Category 2 includes “water-out” models for both the OX and NSQ states. Category 3 includes “water-out” for the OX state but “water-in” for the NSQ state. The average reduction potentials, <Q> and <NSQ>, and standard deviations, σ Q and σ NSQ, are reported for different replicas belonging to each bin. The “Error” column reports the deviation from the experimental reference. The average reduction potential and standard deviations are reported for different replicas belonging to each bin, as well as the error relative to the experimental reference. The bin in green displays the data reported in Table III-3.

Q ₁₀₃ D ⁽⁰⁾	<Q> (kcal/mol)	σ Q (kcal/mol)	<NSQ> (kcal/mol)	σ NSQ (kcal/mol)	E _{red} (mV)	Total σ (mV)	Error (mV)
All	22.4	0.5	20.8	0.7	-386	52	-44
Category 1	22.9	N/A	20.7	1.0	-364	45	-22
Category 2	22.2	0.3	20.8	N/A	-399	N/A	-57
Category 3	22.2	0.3	20.7	1.0	-395	59	-53
Exp.					-342		
Q ₁₀₃ D ⁽⁻⁾	<Q> (kcal/mol)	σ Q (kcal/mol)	<NSQ> (kcal/mol)	σ NSQ (kcal/mol)	E _{red} (mV)	Total σ (mV)	Error (mV)
All	-76.8	2.9	-75.0	0.5	-537	147	-195
Category 1	-77.6	3.0	-75.0	0.5	-569	154	-227
Category 2	-74.7	N/A	N/A	N/A	N/A	N/A	N/A
Category 3	-74.7	N/A	-75.0	0.5	-442	23	-100
Exp.					-342		
Q ₁₀₃ A	<Q> (kcal/mol)	σ Q (kcal/mol)	<NSQ> (kcal/mol)	σ NSQ (kcal/mol)	E _{red} (mV)	Total σ (mV)	Error (mV)
All	72.4	0.7	71.2	1.6	-404	101	23
Category 1	71.9	N/A	72.1	0.3	-467	13	-40
Category 2	72.7	0.8	69.3	N/A	-313	N/A	114
Category 3	72.7	0.8	72.1	0.3	-433	46	-6
Exp.					-427		
Q ₁₀₃ G	<Q> (kcal/mol)	σ Q (kcal/mol)	<NSQ> (kcal/mol)	σ NSQ (kcal/mol)	E _{red} (mV)	Total σ (mV)	Error (mV)
All	60.4	2.1	60.9	1.4	-482	155	-101
Category 1	59.5	2.2	60.9	1.4	-518	160	-137
Category 2	62.0	N/A	N/A	N/A	N/A	N/A	N/A
Category 3	62.0	N/A	60.9	1.4	-410	63	-29
Exp.					-381		

$Q_{103}K^{(+)}$	$\langle Q \rangle$ (kcal/mol)	σQ (kcal/mol)	$\langle NSQ \rangle$ (kcal/mol)	σNSQ (kcal/mol)	E_{red} (mV)	Total σ (mV)	Error (mV)
All	-14.5	3.7	-13.0	3.5	-520	312	-76
All, no 3ns	-12.9	2.1	-15.6	1.0	-340	133	32
Category 1	-11.7	0.3	-15.6	1.0	-288	55	84
Category 2	-15.3	N/A	N/A	N/A	N/A	N/A	N/A
Category 3	-15.3	N/A	-15.6	1.0	-444	42	-72
Experiment					-372		

$Q_{103}R^{(+)}$	$\langle Q \rangle$ (kcal/mol)	σQ (kcal/mol)	$\langle NSQ \rangle$ (kcal/mol)	σNSQ (kcal/mol)	E_{red} (mV)	Total σ (mV)	Error (mV)
All	-173.7	2.3	-174.5	1.0	-426	143	0
All (1&3), no 3ns	-174.1	1.3	-175.6	N/A	-391	56	-5
Category 1	-173.6	1.5	-175.6	N/A	-373	66	13
Category 2	-174.9	N/A	-174.0	N/A	-498	N/A	-112
Category 3	-174.9	N/A	-175.6	N/A	-427	N/A	-41
Exp.					-386		

$Q_{103}E^{(0)}$	$\langle Q \rangle$ (kcal/mol)	σQ (kcal/mol)	$\langle NSQ \rangle$ (kcal/mol)	σNSQ (kcal/mol)	E_{red} (mV)	Total σ (mV)	Error (mV)
All	28.3	0.6	28.5	1.8	-467	103	-95
Category 1	28.2	0.4	26.9	1.4	-401	75	-29
Category 2	28.3	0.8	27.9	1.8	-442	113	-70
Category 3	28.3	0.8	26.9	1.4	-397	94	-26
Exp.					-371		

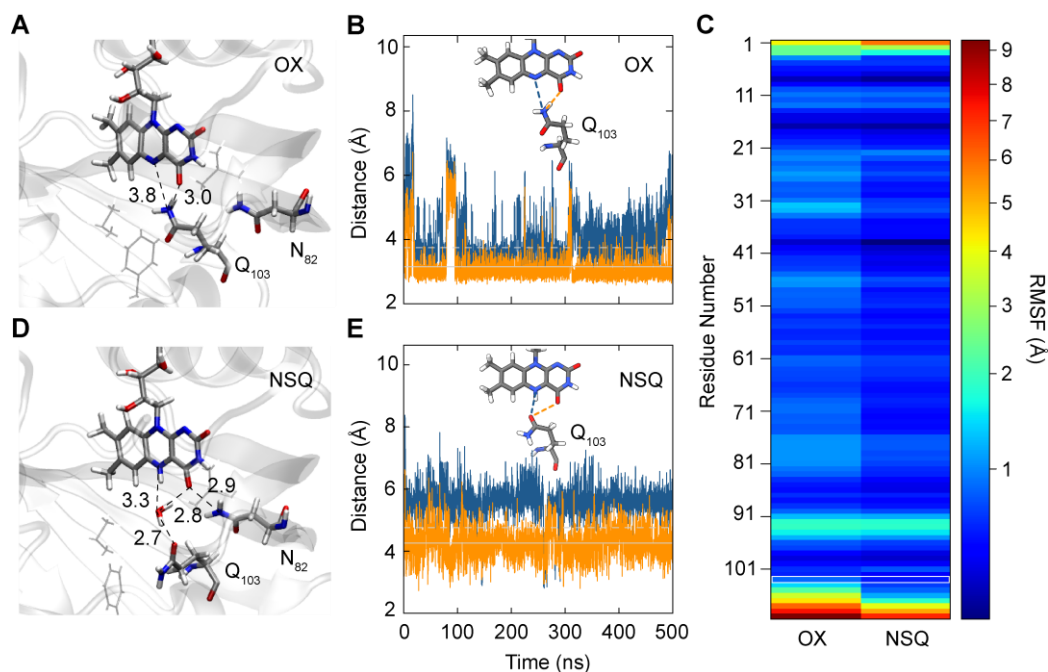


Figure III-S17. MD simulations of iLOV in the OX and NSQ state. **A** Representative structure of iLOV in the OX state from clustering analysis of a 500 ns MD trajectory. **B** Time-dependent distance analysis for Q₁₀₃ amide N to the flavin N5 (blue) and O4 (orange) from a 500 ns MD trajectory of iLOV in the OX state. The solid gray line indicates the mean distance and the dashed line is the mean plus one RMSF. **C** Heat map showing the root-mean-square fluctuation (RMSF) of each residue in the OX and NSQ states of iLOV, with position 103 highlighted in a white box. A power law-transformation to color scaling is applied instead of mapping values linearly with $\gamma = 0.5$ to stretch low values and to enhance the contrast among small RMSF regions. **D** Representative structure of iLOV in the NSQ state from clustering analysis of a 500 ns MD trajectory. **E** Time-dependent distance analysis for Q₁₀₃ amide carbonyl oxygen to the flavin N5 (blue) and O4 (orange) from a 500 ns MD trajectory of iLOV in the NSQ state.

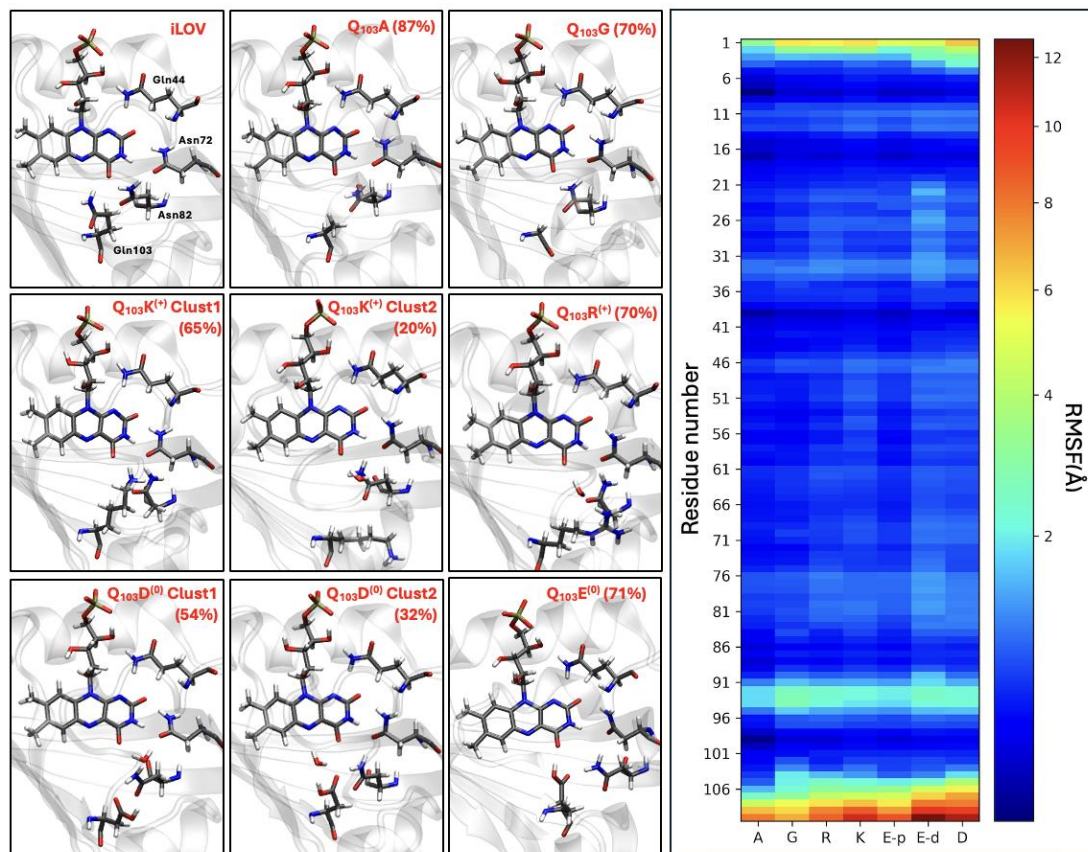


Figure III-S18. Left 9 panels: Structures obtained by clustering analysis for the mutants shown in the OX state. The most populated cluster model is shown in each case, except for Q₁₀₃D⁽⁰⁾ where we instead present the second most populated cluster model (the most populated one has 54% contribution with the aspartic acid pointing away from the flavin, but still has a water inside the binding pocket). The percentages indicate the number of frames belonging to the corresponding cluster model. Right panel: Heat map for the individual residue RMSF for the OX state of different mutants. A power law-transformation to color scaling is applied instead of mapping values linearly with $\gamma = 0.5$ to stretch low values among the various mutants and to enhance the contrast among small RMSF regions. E-p and E-d refer to the protonated and deprotonated forms of E₁₀₃, respectively, while the D refers to the protonated form.

IV. The N1 Site: Modulating iLOV's NSQ/NHQ Redox Couple

Part of the content of this chapter was published in Jones, B. J.; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.

ABSTRACT

This chapter investigates the role that protein side-chain interactions to the N1 atom of a flavin's isoalloxazine ring have on the reduction potentials of the cofactor. Mutation of a glutamine residue near N1 in the model flavoprotein iLOV, results in mutants that have an arrangement of reduction potentials that are more crossed than that of the parental protein. This is demonstrated to arise through the lowering of the potential of the second redox couple (NSQ/NHQ) over that of the first (OX/NSQ). This is consistent with the N1 atom as the site of protonation over the course of proton coupled electron transfer (PCET).

INTRODUCTION

In the last chapter, I demonstrated the ability to increase iLOV's $E_{OX/NSQ}$ reduction potential and achieve a decrease in the extent of potential crossing between the flavins two-one electron couples (ΔE , see **equation III-10**).¹ This was achieved via mutation of glutamine (Q) 103 which lies within hydrogen bonding distance of the N5 atom in the flavin's isoalloxazine ring. This residue was chosen due to its proximity to N5 as the site of protonation during the proton-coupled reduction of the flavin OX to NSQ.² During the proton-coupled reduction of NSQ to NHQ, it is the N1 atom that instead becomes

protonated. Thus, through a similar logic as before, we anticipated that a similar increase in $E_{\text{NSQ/NHQ}}$ would be observed via mutation of glutamine 44, which lies within hydrogen bonding distance of N1 (**Figure I-3**).³ However, unlike the results in the previous chapter, the increase in $E_{\text{NSQ/NHQ}}$ is expected to increase the magnitude of ΔE as the two couples become more thermodynamically separated, resulting in mutants that are more crossed than the wild-type iLOV.

In this Chapter, we demonstrate that mutation of Q44 does indeed result in mutants that are more crossed than iLOV. Through similar methods as before, which included potentiometric titrations, spectrophotometric comproportionation, and electron paramagnetic resonance (EPR) spectroscopy, we show that Q44 variants primarily shift $E^{\circ}_{\text{NSQ/NHQ}}$, while minimally affecting $E^{\circ}_{\text{OX/NSQ}}$, as expected. These hyper-crossed mutants will serve as better analogs for the study of flavin based-bifurcating enzymes which are believed to exhibit more dramatically crossed potentials than typical flavoproteins like wild-type iLOV.⁴⁻⁶ Furthermore, as with the measurements in Chapter III, these measurements will be used in future studies to benchmark the results from computational predictions of iLOV mutant reduction potentials.

MATERIALS AND METHODS

Materials

The iLOV gene within a pQE80L vector backbone was obtained from the Mukherjee lab and cloned into a pSUMO vector as reported previously^{11,12}. The 6× polyhistidine-tagged ubiquitin-like protease 1 (ULP1) was expressed and purified as reported previously.^{13,14} Primers for PCR were ordered from Integrated DNA Technologies (IDT).

DpnI, Phusion HF polymerase, chemically competent DH5- α and BL21 (DE3) *Escherichia coli*, the Qiagen QIAprep Spin Miniprep and Gel Extraction kits, and the NEBuilder HiFi DNA Assembly kit were purchased from New England Biolabs (NEB). IPTG was purchased from Merck. Sephadex G-25 fine resin, LB Miller broth, NaH₂PO₄ (P_i), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), bovine xanthine oxidase (XO), xanthine, 4-hydroxy-(2,2,6,6-tetramethylpiperidin-1-yl)oxyl (4-hydroxy-TEMPO), sodium hydrosulfite (dithionite, NaDT), sodium anthraquinone-2-sulfonate (AQ2S), and Amicon Ultra centrifugal filters were purchased from Millipore Sigma. FMN sodium salt, HisPur nickel nitrilotriacetic acid (Ni-NTA) resin, bicine, phenosafranine, methyl viologen (MV²⁺), 96-well plates, Pierce 660 nm protein assay kit, amber NMR tubes, and Imperial protein stain were obtained from Thermo Fisher Scientific. Bradford reagent was purchased from VWR. Kanamycin sulfate was obtained from Genesee Scientific. Riboflavin was obtained from Acros Organics. Microseal “B” 96-well plate seals were obtained from BioRad. The single-color, cold, mounted, light-emitting diode (LED) with a wavelength of 450 nm and a bandwidth of 18 nm, KG-1, and KG-2 glass filters were obtained from ThorLabs. The pyrolytic graphite edge-plane (PGE) electrode, platinum wire counter electrode, Ag/AgCl (3M) reference electrode, and saturated calomel reference electrode (SCE) were obtained from Bioanalytical Systems (BASi) research products. The SP-200 single channel potentiostat and FC-45 Faraday cage were obtained from BioLogic. Alumina polishing pads and 0.05, 0.3, 1.0 μ m sized alumina were obtained from Buehler. Precision quartz (0.40 mm wall) EPR tubes were obtained from SP Wilmad. Nanopure water was obtained via a Barnstead E-Pure ultrapure water system (>17 M Ω /cm) and was used for all solutions.

Production of iLOV Mutants

Construction of the pSUMO-iLOV plasmid vector, growth and aerobic purification of (H)₆-SUMO-iLOV and mutants, ULP1 digestion, purification of iLOV, and loading and quantitation of FMN were conducted as described in Chapter II.⁷ Associated FMN content before and after reconstitution is provided in **Table IV-S1** and protein purity gels are shown in **Figure IV-S1**.

Site-Directed Mutagenesis of iLOV

Mutagenic primers used for site-directed mutagenesis to generate Q₄₄X (X = A, H, K, and E) iLOV are reported in **Table IV-S2**. Mutagenesis and plasmid preparation were conducted as described in Chapter III.¹

Preparation of Anaerobic iLOV HQ and OX States, Anaerobic UV-vis Measurements, and Measurement of the NSQ Molar Absorptivity

Anaerobic preparation of iLOV HQ and OX, UV-vis measurements, and measurement of iLOV NSQ molar absorptivity were performed as described in Chapter III.¹

Purifying and Characterizing Isomers of Phenosafranine and Anthraquinone-2-Sulfonate (AQ2S).

Initial cyclic voltammograms from commercially obtained phenosafranine were collected as described for safranine in Chapter III, and indicated the presence of multiple isomers with distinct redox properties (**Figure IV-S2**). To ensure reliable redox titrations with a dye indicator of a known reduction potential and extinction coefficient, we purified the isomers of commercially available phenosafranine using a similar protocol as described in Chapter III.¹ Only one major isomer was collected with sufficient purity. CV

measurements of this pure sample indicated enhanced purity of a single phenosafranine isomer (**Figure IV-S3A**). As before, ^1H NMR spectra were collected in d_6 -DMSO on a 500 MHz Bruker Avance NEO spectrometer further confirming the purity of this redox standard⁸ (**Figure IV-S4A**).

Commercially pure (>98% by HPLC) samples of AQ2S only contained a single reversible peak in cyclic voltammograms (**Figure IV-S3C**), indicated sufficient purity by ^1H NMR in d_6 -DMSO (**Figure IV-S4B**), and was not further purified. The reduction potential of pure phenosafranine and AQ2S were determined as a function of pH using a gold working electrode as described for safranine in Chapter III (**Figure IV-S3B and D**).

Measurement of iLOV $E^{\circ}_{\text{OX/HQ}}$

The measurement of equilibrium two-electron $E^{\circ}_{\text{OX/HQ}}$ potential was performed in a 96-well titration assay as described in Chapter III,¹ with minor modifications described here. For the preparation of master mixes, new 3 $\times$ buffers at pH 7.0 and 8.0 were used. The 3 $\times$ concentrations of these buffers are 600 mM NaCl, 60 mM P_i at pH 7.0 (Buffer E) and 600 M NaCl, 60 mM Tris at pH 8.0 (Buffer F). iLOV Q₄₄A and Q₄₄H were measured using phenosafranine as a redox indicator. For consistency wild-type iLOV was also remeasured using phenosafranine. The concentration of phenosafranine used in each master mix remained the same as used for safranine previously. The extinction coefficient of phenosafranine at 527 nm has been reported in methanol to be 48,000 $\text{M}^{-1} \text{cm}^{-1}$.⁹ For the purposes of this protocol which uses initial and final absorbance values to determine fractions reduced, the exact concentrations do not factor into the analysis. Thus, the extinction coefficient in methanol was used to determine the concentration of the stock solution of phenosafranine dissolved in water.

The iLOV mutant Q₄₄K was measured with AQ2S as a standard indicator at a single pH (pH 7.0, using Buffer E). The concentration of a saturated stock solution of oxidized AQ2S dissolved in water was determined by the extinction coefficient at ~ 257 nm of ~ 51,000 M⁻¹ cm⁻¹.¹⁰ The concentration of AQ2S used in the 3 × master mix was 450 μM.

Analysis of Redox Titration Spectra.

The analysis of redox titration data was performed as described previously in Chapter III,^{1,11–13} with some minor modifications. For redox titrations with phenosafranine, the analysis was identical to that of safranine except that the value of E_{app}^{dye} from **Equation III-5** was taken from the new phenosafranine Pourbaix diagram **Figure IV-S3B**. For redox titrations with AQ2S, the reduced species of AQ2S absorbs maximally at around 420 nm¹⁴. Considerable overlap between the absorption spectra of AQ2S and iLOV Q₄₄K exists at 420 nm (the wavelength at which the fraction reduced of iLOV mutants were previously determined). Thus, new wavelengths were chosen. For AQ2S, the ratio of reduced to oxidized species was determined from the increase in absorption at 390 nm, where both iLOV OX and HQ absorb minimally. For iLOV, 480 nm was chosen where AQ2S has negligible contribution. No further spectral deconvolution was performed.

Comproportionation of iLOV HQ and OX, and Quantitative Electron Paramagnetic Resonance (EPR)

To determine ΔE, a similar protocol was used as described previously in Chapter III.¹ However, for the Q₄₄X mutants, the equilibrium concentrations of NSQ were too low to measure accurately with the concentrations of OX and HQ protein used in earlier protocols. For the estimation of ΔE, one replicate was performed with 50 μM HQ and 50 μM OX as

before, while three replicates were performed with 250 μ M HQ and 250 μ M OX. At these concentrations, the absorption below 500 nm saturates the detector, and no features from ASQ can be determined. Furthermore, even minor aggregation, background absorption, or baseline shifts at 616 nm where NSQ is detected can significantly skew the measurement of ΔE . While attempts were made to correct this by subtraction of authentic spectra of OX and HQ, ultimately quantitation via EPR radical counting was chosen to determine the equilibrium concentrations in samples with OX and HQ concentrations greater than 250 μ M. The linewidth of this EPR measurement was used to detect the presence of ASQ. Experimental details of the quantitative EPR measurement are described in Chapter III.^{1,15}

RESULTS

As described in Chapter III, we hypothesized that hydrogen bonding interactions at the N5 and N1 sites of a flavin prosthetic group would provide distinct mechanisms for tuning the two one-electron couples in flavoproteins through thermodynamic control of proton-coupled electron transfer (PCET). In this chapter, we focus on N1 using the small flavoprotein, iLOV, as a model system.

Similar to Chapter III, our approach to this investigation starts with the X-ray crystal structure of iLOV. Here, a glutamine residue (Q₄₄, **Figure 1.2**) is found near both N1 (3.4 Å) and O2 (2.9 Å) atoms of FMN.³ To see how residues near these atoms affect flavin reduction potentials, we constructed Q₄₄X mutations (X = alanine, A; lysine, K; histidine, H; glutamic acid, E) by site directed mutagenesis. Initially purified iLOV mutants bound FMN at levels significantly lower than the wild-type iLOV (<10 % holoprotein), with the exception of Q₄₄K, which exhibited higher loading (~39% holoprotein). After reconstituting these proteins with FMN, all mutants showed an increase in flavin loading to 90%, which is

slightly lower than the quantitative 1:1 binding by wild-type iLOV and Q₁₀₃ mutants, but is sufficient for the experiments described herein (Supplementary **Table IV-S1** and **Figure IV-S1**).

The absorption spectra of Q₄₄X mutants appeared identical to that of wild-type iLOV (**Figure IV-1**). In contrast to the Q₁₀₃ mutants described in Chapter III, which exhibited blueshifts of about 10 nm relative

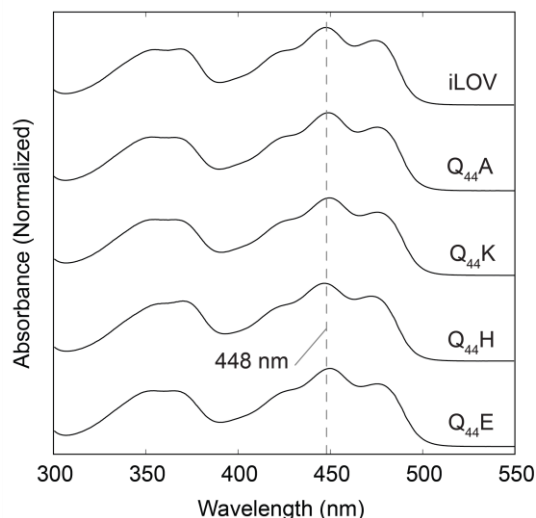


Figure IV-1. Normalized UV-vis absorption spectra of iLOV and Q₄₄X mutants. Each of Q₄₄ mutant exhibited an absorbance spectrum indistinguishable from wild-type iLOV.

to the parent iLOV, the Q₄₄X series of proteins retained the λ_{max} of the S₀ → S₁ transition at 448 nm. Further optical characterization of the emission properties of these proteins will be performed in future studies.

To determine how the Q₄₄ mutations affected the redox behavior of the flavin cofactor, similar methods to those in Chapter III were used. Initial reductive titrations on Q₄₄K and Q₄₄A using safranin as a redox indicator suggested that the 2-electron reduction-midpoint potential of these mutants was more than 50 mV separated from the safranin standard, necessitating the selection of a different redox-active dye. Therefore, we turned to phenosafranin which has a reduction potential roughly 50 mV higher than that of safranin (Supplemental **Figures III-S4B** and **IV-S3B**). For consistency, the reduction potential of the parent iLOV was remeasured using this new indicator and was shown to be approximately 27 mV higher than that measured with safranin (**Figure IV-2**). The use of two different standards should, in principle, reproduce the same result. However, remeasurement of the

standard indicator reduction potentials through cyclic voltammetry (CV), even with different working electrodes (pyrolytic graphite edge-plane versus gold electrode) continued to indicate that the two dyes were about 50 mV separated. It is not immediately clear to us why measurements of the same protein with these two standards are different, but we note that measurement of the reduction potential of the safranine isomer used in Chapter III has been reported to be about 35 mV more positive at the same pH when measured by potentiometric titration in comparison to what we measure via CV.¹⁶ We also note that the reduction potential measured here for the parent iLOV protein using phenosafranine is identical to that which was measured for the *Chlamydomonas reinhardtii* LOV1 C57G protein which used safranine as a redox indicator but utilized the more positive literature value for its reduction potential,¹⁷ and 15 mV lower than the homologous *Avena sativa* LOV2 which also used phenosafranine as a redox standard.¹⁸ Thus for consistency, the measurements performed here are all compared to the reduction potential of parental iLOV measured with phenosafranine rather than the values obtained with safranine reported in Chapter III. Both iLOV Q44H and Q44A were measured with phenosafranine, while the highly positive reduction potential of Q44K was measured with AQ2S at a single pH. Titrations for the mutant Q44E were not obtained due difficulties acquiring sufficient quantities of protein for the measurement. Both Q44K and Q44E will be examined in detail in future studies.

Aside from the systemic 30 mV shift in the 2-electron reduction potential, reductive titrations of proteins using phenosafranine and AQ2S were similar to those with safranine. The ratio of reduced fractions of dye to reduced fraction of analyte are used to determine the difference in reduction potential between the two species.^{11–13} The addition of the NaDT reductant is associated with a decrease in the characteristic absorption features of FMN OX

at 448 nm and oxidized phenosafranine at 527 nm (**Figure IV-2A**). Likewise, reductive titrations with AQ2S as a redox standard show an increase in 390 nm as AQ2S becomes reduced (**Figure IV-2C and D**).¹⁴ Reduction of the small organic molecule proceeds rapidly before the sample can be measured, while reduction of protein occurs slowly over several hours. Thus, as the sample approaches equilibrium electrons slowly migrate from the dye to the protein. This results in an increase in 527 nm for phenosafranine or a decrease in 390 nm for AQ2S as the dye becomes re-oxidized, and a decrease in 448 nm as the protein becomes reduced (**Figure IV-2A and C, inset**). After 2 equivalents of NaDT, the entire sample is fully reduced. For none of the Q_{44X} mutants measured can any spectral features of NSQ or ASQ be observed, suggesting that these mutants are highly crossed.

Reductive titrations of iLOV were analyzed using the same assumptions described in Chapter III and fit to a linearized form of the Nernst Equation.¹ In summary, the fractional reduction of both iLOV Q_{44X} and the reporter dye standard were determined based on the initial (fully oxidized) and final (fully reduced) spectra and their natural logarithms were plotted against one another, producing a linear relationship. The slope indicates the ratio of the stoichiometric coefficients for the number of electrons transferred between the protein and the indicator dye, the y-intercept reflects the $E^{\circ}_{\text{OX/HQ}}$ determined relative to the standard, and the pH-dependence informs on the number of protons involved in the redox process (**Figure IV-2 B, D and Figure IV-S4**). The resulting extracted values for $E^{\circ}_{\text{OX/HQ}}$ for iLOV and the Q_{44X} mutants, as well as the reference values for free FMN are summarized in **Table IV-1**.

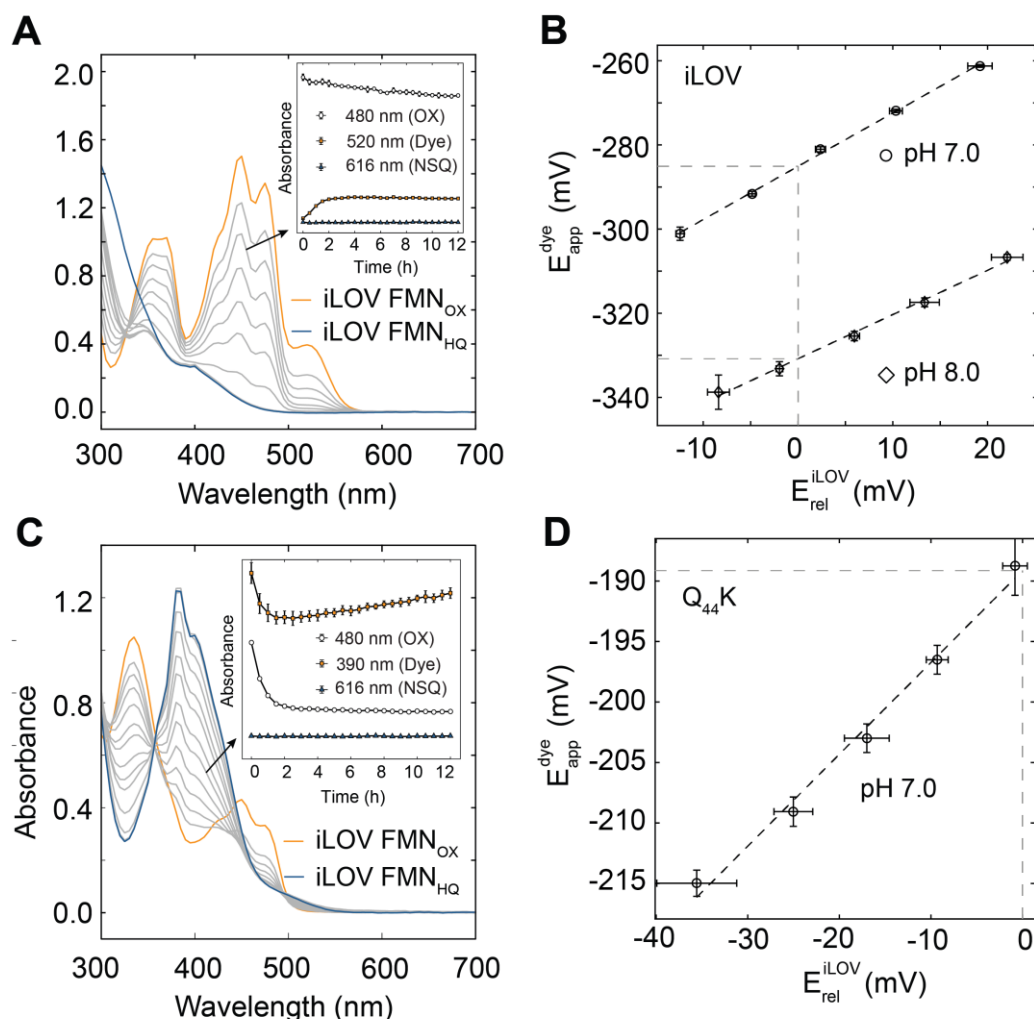


Figure IV-2. Spectrophotometric redox titrations of iLOV and $Q_{44}X$ Mutants. **A** Equilibrium iLOV absorbance spectra with phenosafranine as an indicator after adding NaDT reductant from 0 eq (orange) to 3.3 eq. (blue). Inset shows the approach to equilibrium at one point in the titration (arrow) via monitoring the absorption at 420 nm (FMN_{OX}), 520 nm (phenosafranine), and 616 nm (FMN_{NSQ}). Phenosafranine is initially reduced faster than iLOV, then re-oxidized by reducing iLOV. **B** E_{app} (determined from phenosafranine) vs. E_{rel} (determined from iLOV) plots for pH 7.0 (open circles) and pH 8.0 (open diamonds) and associated linear fits. The gray dashed line shows the E_{app} at which the iLOV FMN has an $[OX]/[HQ]$ ratio of 1 (*i.e.* $E^{\circ}_{OX/NHQ}$). **C** Equilibrium iLOV $Q_{44}K$ absorbance spectra using AQ2S as an indicator. Inset shows the approach to equilibrium at one point in the titration (arrow) via monitoring the absorption at 480 nm (FMN_{OX}), 390 nm (AQ2S), and 616 nm (FMN_{NSQ}). **D** E_{app} (determined from AQ2S) vs. E_{rel} (determined from iLOV $Q_{44}K$) plots for pH 7.0 and associated linear fit.

From these reductive titrations, the parent iLOV had the lowest $E^{\circ}_{OX/HQ}$ of -285.1 ± 0.3 mV at pH 7.0 and a pH dependence between pH 7.0 and 8.0 of -46 mV/pH. Aside from the 27 mV shift when safranine was used a redox standard mentioned above, the pH dependence

Table IV-1. Reduction potentials, pH dependence, and electron equivalents with the indicator (Slope) of iLOV and Q₄₄X mutants determined from reductive titrations. Uncertainties in the last significant digit shown in parentheses

iLOV Variant	Redox Indicator	E° _{OX/NHQ} (mV)		$\Delta E^{\circ}_{\text{OX/NHQ/pH}}$ (mV)	Slope	
		pH 7.0	pH 8.0		pH 7.0	pH 8.0
iLOV	Phenosafranine	−285.1 (3)	−330.8 (5)	−46	1.27 (3)	1.05 (4)
Q ₄₄ A	Phenosafranine	−248.3 (6)	−296.6 (1)	−48	1.28 (4)	1.02 (1)
Q ₄₄ H	Phenosafranine	−246 (2)	−287.6 (4)	−42	1.5 (2)	1.07 (3)
Q ₄₄ K	AQ2S	−189.1 (9)	n.d. ^a	n.d. ^a	0.76 (4)	n.d. ^a
FMN		−242 ^b	−272 ^c	−29	n.d. ^a	n.d. ^a

^a n.d. not determined.

^b Value taken from ref. 1 at pH 8.00.

^c Value taken from ref. 1 at pH 9.04.

remained the same as measured previously¹ which is more consistent with iLOV reduction proceeding via concerted OX→NSQ and NSQ→NHQ transitions.¹⁹ The E°_{OX/NHQ} followed the trend iLOV < Q₄₄A ≈ Q₄₄H < Q₄₄K. The pH dependence of Q₄₄K was not measured, but both Q₄₄A and Q₄₄H exhibited similar $\Delta E^{\circ}_{\text{OX/NHQ/pH}}$ of −48 mV/pH and −42 mV/pH respectively. At pH 7.0, the Q₄₄K mutant exhibited an extraordinarily high E°_{OX/NHQ} shifted +96 mV relative to the parent iLOV.

To investigate whether this shift in 2-electron reduction potential is due to the modulation of E_{OX/NSQ} or E_{NSQ/NHQ}, we set out to measure ΔE as demonstrated in Chapter III. Briefly, this value is obtained by measuring equilibrium concentrations of NSQ in a mixture of equimolar concentrations of iLOV OX and HQ. Initial comproportionation experiments did not show any detectable NSQ or ASQ formation for Q₄₄X mutants under the protein concentrations that were used previously. Therefore, we repeated these experiments using 5× the concentration of protein (250 μ M). This allowed us to detect NSQ concentrations just above the limit of detection, but this approach was not ideal. At these very high

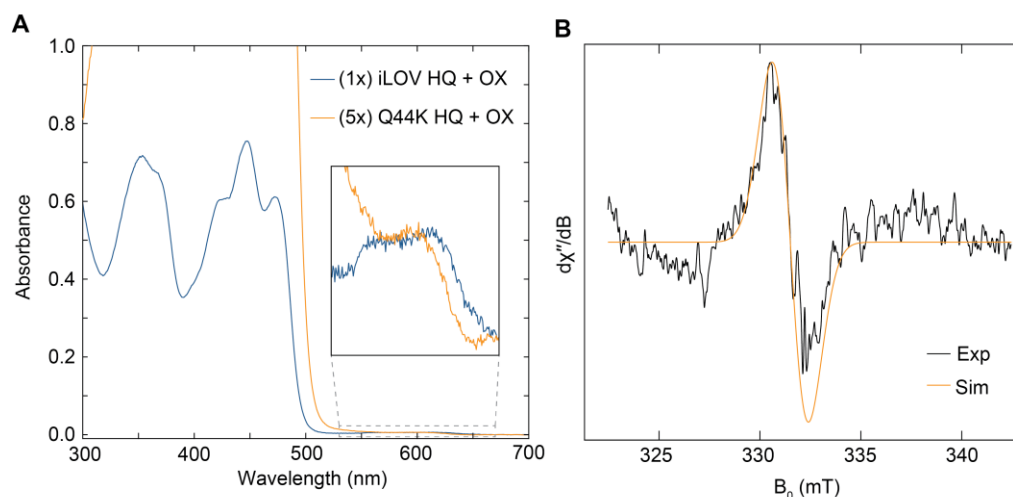


Figure IV-3. Comproportionation and characterization of the NSQ. A. A comparison of absorbance spectra taken at equilibrium of a sample containing a mixture of parental iLOV HQ and OX at 50 μ M each (navy), and a sample containing Q44K HQ and OX at 250 μ M each (orange). The former spectrum is reproduced from Figure 3.4 The latter spectrum was taken prior to attempted deconvolution of OX and HQ spectra used to create Supplemental Figure S4.5. The apparent concentration of NSQ at this concentration as measured by the absorbance at 616 nm is highly skewed by baseline shifts when measured by UV-Vis. **B.** Normalized EPR spectrum (black) and simulation (orange) of the iLOV Q44K NSQ. EPR quantitation was chosen as a preferable, albeit costly, alternative to estimating ΔE .

concentrations of protein, any features of the absorbance spectra below 500 nm saturate the detector, preventing us from identifying any bands unique to potential ASQ. Furthermore, even minor levels of aggregation or background absorbance from the nearby OX and HQ can significantly skew the detected levels of NSQ (**Figure IV-3A**). Nonetheless, these measurements demonstrated two things. First, the kinetics of the approach towards equilibrium for Q44 mutants was significantly faster than that measured for the Q103 series measured in chapter III. Each of the Q44X mutants reached equilibrium levels of NSQ after only 5 minutes from mixing HQ and OX, while the Q103 mutants, including the parental iLOV, took several hours to reach this state (Supplemental **Figures IV-S14** and **IV-S5**). Second, these results suggest that the ΔE of Q44X mutants (especially Q44K) are highly crossed relative to the parental iLOV. The data from these experiments suggests that the $E_{NSQ/NHQ}$ for Q44X mutants is significantly increased relative to $E_{OX/NSQ}$.

To more accurately estimate the value of ΔE , we turned to quantitative X-band EPR at high concentrations of protein (>250 μM each of HQ and OX). Conducting these measurements in triplicate requires a large quantity of protein, but this method is preferable due to the ability to better distinguish ASQ and NSQ radicals and the absence of background interference. As described in Chapter III, the EPR spectrum of the parental iLOV protein comproportionation reaction contains an isotropic $S = 1/2$ singlet with a g_{iso} of 2.004 and a line width of 1.92 mT. Meanwhile, the Q₄₄X mutants exhibited identical g_{iso} values but slightly shorter linewidths of around 1.8 mT. These values lie closer to the expected range for NSQ radicals which typically have line widths between 1.8 and 2.0 mT than the range of ASQ radicals which have line widths around 1.5 mT.²⁰ A tabulated list of simulated EPR parameters is given in Supplementary **Table IV-S4**, while a representative EPR spectrum for each mutant is provided in Supplementary **Figure IV-S6**. For the assignment of the protonation state of the radical species, the UV-Vis results are inconclusive, but the EPR data and the pH dependence indicates that the Q₄₄X mutants are likely to possess an NSQ rather than an ASQ at neutral pH.

The estimates of ΔE produced by UV-vis measurements and those made by quantitative EPR yield different results. By EPR, the absolute magnitude of ΔE followed the trend Q₄₄H < Q₄₄A < Q₄₄K while the UV-Vis data followed the trend Q₄₄A < Q₄₄H < Q₄₄K. The UV-vis data suggests that the Q₄₄H mutant is about 46 mV more crossed than Q₄₄A, while the EPR data instead suggests that the Q₄₄A mutant is 14 mV more crossed. However, in both cases, the Q₄₄K mutant is by far the most crossed with a ΔE of -347 ± 6 mV by UV-vis and -374 ± 9 mV by EPR. We believe that the UV-Vis measurements of ΔE are less reliable due to significant background interference at such high concentrations of total protein and low

Table IV-2. ΔE , $E^{\circ}_{OX/NSQ}$, and $E^{\circ}_{NSQ/NHQ}$ values for iLOV and Q₄₄X mutants from spectrophotometric FMN_{OX} and FMN_{NHQ} comproportionation at pH 7.0. Propagated uncertainties in the last significant digit are reported in parentheses.

iLOV variant	$E^{\circ}_{OX/NHQ}$ (mV)	ΔE_{EPR} (mV)	$E^{\circ}_{OX/NSQ}$ (mV)	% of $E^{\circ}_{OX/NHQ}$ Shift	$E^{\circ}_{NSQ/NHQ}$ (mV)	% of $E^{\circ}_{OX/NHQ}$ Shift
iLOV	-285	-212 (4) ^a	-391 (2)	ref.	-179 (2)	ref.
Q ₄₄ A	-248	-267 ^b	-381	13	-114	87
Q ₄₄ H	-246	-253 (5)	-372 (3)	23	-119 (3)	76
Q ₄₄ K	-189	-374 (9)	-376 (1)	8	-2 (5)	92
FMN	-242 ^b	-54	-269 ^c	N.A. ^d	-216 ^c	N.A. ^d

^a Value from Chapter III or ref 2

^b Value measured with a single replicate

^c Values from ref. 1.

^d Not applicable

concentrations of NSQ, so we will use the ΔE values obtained via EPR for the remainder of the chapter. The ΔE values obtained by UV-vis spectroscopy are listed in **Table IV-S5**

The resultant $E^{\circ}_{OX/NSQ}$ and $E^{\circ}_{NSQ/NHQ}$ across the series is described in **Table IV-2**.

Again, these values are simply calculated assuming that the value for $E^{\circ}_{OX/NHQ}$ is exactly equal to the midpoint between the two one-electron reduction potentials. For none of the mutants does the value of $E^{\circ}_{OX/NSQ}$ change by more than 17 mV. By contrast, the shift in $E^{\circ}_{NSQ/NHQ}$ is far more dramatic, with that of the Q₄₄K mutant exhibiting a stabilization of 177 mV or 17 kJ/mol. For all of the Q₄₄X variants, the shift in $E^{\circ}_{NSQ/NHQ}$ accounts for nearly all of the shift in $E^{\circ}_{OX/NHQ}$ ranging from 76% in Q₄₄H to 92% in Q₄₄K, while the contribution by $E^{\circ}_{OX/NSQ}$ is minor and only decreases the magnitude of ΔE .

DISCUSSION

In previous studies, we demonstrated that mutation to Q₁₀₃ which resides near flavin N5, caused a decrease in the magnitude of potential crossing (ΔE). Since N5 was the site of protonation during the transition from OX to NSQ, this mutation preferentially lowered

$E_{OX/NSQ}$, bringing the two one-electron potentials closer together. With the results of QM/MM molecular dynamics simulations, we determined that this effect was the result of an increase in the number of hydrogen bonding interactions in successive mutants, primarily from solvent water molecules. In this study, we set out to replicate these results by focusing on the alternative flavin atom N1. The N1 atom becomes protonated during the transition from NSQ to NHQ, and we anticipated that a similar, but opposite, effect would be observed here as the ΔE between the two one-electron couples becomes greater. The results described herein confirm this hypothesis. The replacement of Q44, which hydrogen bonds to the N1 position of the flavin, with A, H, and K results in an increase in the absolute value of ΔE . Of these mutants the Q44K is the most crossed by an additional 162 mV or 15.6 kJ/mol. Our results confirm that the increase in potential crossing is primarily caused by the lowering of $E_{NSQ/NHQ}$ relative to $E_{OX/NSQ}$. This implies that the Q44X mutations either destabilize the NSQ redox state or stabilize the NHQ state. Given that the $E_{OX/NSQ}$ is only modestly perturbed, it is likely that any effects on NSQ are similarly observed in OX.

The examination of the redox properties of other mutants including Q44E will be examined in future studies, but currently it is unclear how the acid/base properties of the side chain residue affect the modulation of these potential. It is clear, nonetheless, that the replacement of the native glutamine side-chain with a hydrophobic alanine, has a minor effect on the reduction potentials of the flavin, while the replacement to a polar and likely charged lysine is significant. We believe that this affect similarly stems from the introduction of water molecules to the destabilized flavin binding pocket, resulting in an increased number of hydrogen bonding reactions to the flavin, but computational results from our collaborators have yet to be completed.

In this study, the reduction potential of the Q₄₄K mutant was studied at a single pH, due to limitations in protein availability. While a complete examination of the pH-dependence of this mutant has yet to be performed, it is possible that this mutant possesses an AHQ as opposed to an NSQ. If this is true, then the stabilization of AHQ would likely be an electrostatic effect stemming from the neutralization of negative charge by lysine, as electrostatic stabilization of anionic redox states is well established for other flavoproteins with normally ordered potentials.^{21,22}

While the use of iLOV as a model flavoprotein was not as simple as was initially anticipated, the results from Chapters 3 and 4 demonstrate that the crossed potentials of the FMN prosthetic group can be tuned by mutation of residues nearby N5 and N1 in the flavin binding pocket. Other heteroatoms within the flavin's isoalloxazine ring, such as O2, O4, and N3 are also likely important, as interactions with these sites is predicted to disrupt the electronic distribution around the flavin and modulate the potentials through aromaticity assisted hydrogen bonding.²³ Mutation of N₇₂ and N₈₂, which interacts with these atoms is currently being explored by our group and will be the subject of future studies. The results demonstrated here are likely to be broadly applicable as these principles can be extended to crossed flavoproteins as a group. Furthermore, as a small flavoprotein that can be simulated by molecular dynamics simulations, these results will provide benchmarks for the development of more accurate methods²⁴⁻²⁷ to computationally predict the reduction potentials of flavoproteins in the future.

CONCLUSION

This chapter demonstrated the applicability of iLOV as a model flavoprotein to investigate the effect that interactions near the N1 atom in a flavin have on its reduction

potentials. Mutation of Q₄₄ in the iLOV crystal structure, produces a series of mutants with reduction potential arrangements that are more crossed than that of the parental protein. This is demonstrated to occur by increasing the reduction potential of the NSQ/NHQ couple, further separating the two one-electron reduction potentials. The results of this work will be used in future studies to benchmark the results of computational predictions of iLOV reduction potentials.

REFERENCES

1. Jones, B. J.; Elhajj, S.; Haynes, B.; Wang, S.; O'Connor, I.; Gozem, S.; Greene, B. L. Measurement and Control of Crossed Potentials in a Flavoprotein. *J. Am. Chem. Soc.* **2026**. <https://doi.org/10.1021/jacs.5c16043>.
2. Mayhew, S. G. The Effects of pH and Semiquinone Formation on the Oxidation–Reduction Potentials of Flavin Mononucleotide. *Eur. J. Biochem.* **1999**, *265* (2), 698–702. <https://doi.org/10.1046/j.1432-1327.1999.00767.x>.
3. Christie, J. M.; Hitomi, K.; Arvai, A. S.; Hartfield, K. A.; Mettlen, M.; Pratt, A. J.; Tainer, J. A.; Getzoff, E. D. Structural Tuning of the Fluorescent Protein iLOV for Improved Photostability. *J. Biol. Chem.* **2012**, *287* (26), 22295–22304. <https://doi.org/10.1074/jbc.M111.318881>.
4. Yuly, J. L.; Zhang, P.; Lubner, C. E.; Peters, J. W.; Beratan, D. N. Universal Free-Energy Landscape Produces Efficient and Reversible Electron Bifurcation. *Proc. Natl. Acad. Sci.* **2020** *117* (35), 21045–21051. <https://doi.org/10.1073/pnas.2010815117>.
5. Baymann, F.; Schoepp-Cothenet, B.; Duval, S.; Guiral, M.; Brugna, M.; Baffert, C.; Russell, M. J.; Nitschke, W. On the Natural History of Flavin-Based Electron Bifurcation. *Front. Microbiol.* **2018**, *9*. <https://doi.org/10.3389/fmicb.2018.01357>.
6. L. Yuly, J.; E. Lubner, C.; Zhang, P.; N. Beratan, D.; W. Peters, J. Electron Bifurcation: Progress and Grand Challenges. *Chem. Commun.* **2019**, *55* (79), 11823–11832. <https://doi.org/10.1039/C9CC05611D>.
7. Jones, B. J.; Greene, B. L. Singlet Oxygen-Mediated Photochemical Cross-Linking of an Engineered Fluorescent Flavoprotein iLOV. *J. Biol. Chem.* **2024**, *300* (11), 107845. <https://doi.org/10.1016/j.jbc.2024.107845>.
8. Meth-Cohn, O.; Smith, M. What Did W. H. Perkin Actually Make When He Oxidised Aniline to Obtain Mauveine? *J. Chem. Soc. Perkin 1* **1994**, No. 1, 5. <https://doi.org/10.1039/p19940000005>.
9. Broglia, M. F.; Gómez, M. L.; Bertolotti, S. G.; Montejano, H. A.; Previtali, C. M. Photophysical Properties of Safranin and Phenosafranin: A Comparative Study by Laser Flash Photolysis and Laser Induced Optoacoustic Spectroscopy. *J. Photochem. Photobiol. Chem.* **2005**, *173* (2), 115–120. <https://doi.org/10.1016/j.jphotochem.2005.01.010>.
10. Petrov, M.; Chikin, D.; Abunaeva, L.; Glazkov, A.; Pichugov, R.; Vinyukov, A.; Levina, I.; Motyakin, M.; Mezhev, Y.; Konev, D.; Antipov, A. Mixture of Anthraquinone Sulfo-Derivatives as an Inexpensive Organic Flow Battery Negolyte: Optimization of Battery Cell. *Membranes* **2022**, *12* (10), 912. <https://doi.org/10.3390/membranes12100912>.

11. Miller, A.-F.; Duan, H. D.; Varner, T. A.; Mohamed Raseek, N. Reduction Midpoint Potentials of Bifurcating Electron Transfer Flavoproteins. In *Methods in Enzymology*; Palfey, B. A., Ed.; Academic Press, 2019; pp 365–398. <https://doi.org/10.1016/bs.mie.2019.03.017>.
12. Massey, V. A Simple Method for the Determination of Redox Potentials. *Flavins Flavoproteins* **1990**.
13. Minnaert, K. Measurement of the Equilibrium Constant of the Reaction between Cytochrome c and Cytochrome a. *Biochim. Biophys. Acta – Enzymol. Biol. Oxid.* **1965**, *110* (1), 42–56. [https://doi.org/10.1016/s0926-6593\(65\)80093-5](https://doi.org/10.1016/s0926-6593(65)80093-5).
14. Yang, X. T.; Chai, X. S.; Hou, Q. X.; Zhu, J. Y.; Danielsson, L.-G. Determination of Anthraquinone-2-Sulfonate in Alkaline Pulping Liquor by Spectrophotometry Using a Nafion Membrane Interface. *Anal. Chim. Acta* **2002**, *474* (1), 69–76. [https://doi.org/10.1016/S0003-2670\(02\)01028-0](https://doi.org/10.1016/S0003-2670(02)01028-0).
15. Cáceres, J. C.; Dolmatch, A.; Greene, B. L. The Mechanism of Inhibition of Pyruvate Formate Lyase by Methacrylate. *J. Am. Chem. Soc.* **2023**, *145* (41), 22504–22515. <https://doi.org/10.1021/jacs.3c07256>.
16. Andersen, E. M. E.; Wang, H.; Khoo, J. S. H.; Cerda, J. F.; Koder, R. L. Oxidation-Reduction and Photophysical Properties of Isomeric Forms of Safranin. *PloS One* **2022**, *17* (6), e0265105. <https://doi.org/10.1371/journal.pone.0265105>.
17. Nöll, G.; Hauska, G.; Hegemann, P.; Lanzl, K.; Nöll, T.; von Sanden-Flohe, M.; Dick, B. Redox Properties of LOV Domains: Chemical versus Photochemical Reduction, and Influence on the Photocycle. *ChemBioChem* **2007**, *8* (18), 2256–2264. <https://doi.org/10.1002/cbic.200700304>.
18. Fuentes, A. G. de; Möglich, A. Reduction Midpoint Potential of a Paradigm Light–Oxygen–Voltage Receptor and Its Modulation by Methionine Residues. *RSC Chem. Biol.* **2024**, *5* (6), 530–543. <https://doi.org/10.1039/D4CB00056K>.
19. Pourbaix, M. *Atlas of Electrochemical Equilibria in Aqueous Solutions*; National Association of Corrosion Engineers: Texas, 1974.
20. Nohr, D.; Weber, S.; Schleicher, E. EPR Spectroscopy on Flavin Radicals in Flavoproteins. In *Methods in Enzymology*; Palfey, B. A., Ed.; Academic Press, 2019; pp 251–275. <https://doi.org/10.1016/bs.mie.2019.03.013>.
21. Zhou, Z.; Swenson, R. P. Electrostatic Effects of Surface Acidic Amino Acid Residues on the Oxidation-Reduction Potentials of the Flavodoxin from *Desulfovibrio Vulgaris* (Hildenborough). *Biochemistry* **1995**, *34* (10), 3183–3192. <https://doi.org/10.1021/bi00010a007>.
22. Talfournier, F.; Munro, A. W.; Basran, J.; Sutcliffe, M. J.; Daff, S.; Chapman, S. K.; Scrutton, N. S. α Arg-237 in *Methylophilus Methylophilus* (Sp. W3A1) Electron-

Transferring Flavoprotein Affords ~200-Millivolt Stabilization of the FAD Anionic Semiquinone and a Kinetic Block on Full Reduction to the Dihydroquinone*. *J. Biol. Chem.* **2001**, 276 (23), 20190–20196. <https://doi.org/10.1074/jbc.M010853200>.

23. Kakeshpour, T.; Wiemeersch, A.; Jackson, J. E. Redox Potential Tuning in Bio-Relevant Heterocycles via (Anti)Aromaticity Modulated H-Bonding (AMHB). *Can. J. Chem.* **2020**, 98 (7), 337–346. <https://doi.org/10.1139/cjc-2019-0410>.
24. Kılıç, M.; Ensing, B. Redox Properties of Flavin in BLUF and LOV Photoreceptor Proteins from Hybrid QM/MM Molecular Dynamics Simulation. *J. Phys. Chem. B* **2024**, 128 (13), 3069–3080. <https://doi.org/10.1021/acs.jpcc.3c06245>.
25. Tazhigulov, R. N.; Gurunathan, P. K.; Kim, Y.; Slipchenko, L. V.; Bravaya, K. B. Polarizable Embedding for Simulating Redox Potentials of Biomolecules. *Phys. Chem. Chem. Phys.* **2019**, 21 (22), 11642–11650. <https://doi.org/10.1039/C9CP01533G>.
26. Sarangi, R.; Maity, S.; Acharya, A. Machine Learning Approach to Vertical Energy Gap in Redox Processes. *J. Chem. Theory Comput.* **2024**, 20 (15), 6747–6755. <https://doi.org/10.1021/acs.jctc.4c00715>.
27. Galuzzi, B. G.; Mirarchi, A.; Viganò, E. L.; Gioia, L.; Damiani, C.; Arrigoni, F. Machine Learning for Efficient Prediction of Protein Redox Potential: The Flavoproteins Case. *J. Chem. Inf. Model.* **2022**, 62 (19), 4748–4759. <https://doi.org/10.1021/acs.jcim.2c00858>.

SUPPLEMENTAL INFORMATION

Table IV-S1. As-purified and reconstituted FMN loading of iLOV Q₄₄X variants. FMN concentrations were determined via UV-vis absorption at 448 nm using an extinction coefficient of 14,800 M⁻¹ cm⁻¹. Protein concentration was determined via the Pierce 660 assay. Initial FMN:iLOV ratios prior to flavin reconstitution are based on single measurements. Reconstituted FMN:iLOV ratios and uncertainty are based on the average of triplicate measurements and error is reported as the standard deviation.

iLOV Variant	As-purified FMN:iLOV	Reconstituted FMN:iLOV
WT	0.21	1.05 ± 0.02
Q ₄₄ A	0.10	0.90 ± 0.02
Q ₄₄ H	0.08	0.90 ± 0.02
Q ₄₄ K	0.39	0.84 ± 0.02
Q ₄₄ E	0.06	0.88 ± 0.02

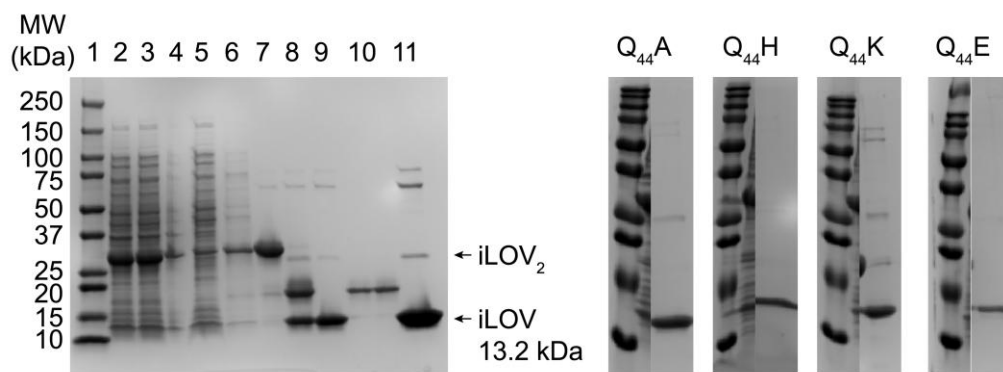


Figure IV-S1. Purification (right) and purity (left) SDS-PAGE gels of iLOV and Q₄₄X variants. Left: Typical purification gel for iLOV. Lanes as follows: (1) Precision Plus Molecular Weight Standard, (2) total cell lysate, (3) soluble fraction of cell lysate, (4) insoluble pellet of cell debris, (5) Ni-NTA flow through (pre-ULP1 digestion), (6) Ni-NTA wash, (7) Ni-NTA elution ((H)₆-SUMO-iLOV), (8) ULP1 digestion (iLOV and (H)₆-SUMO), (9) digested Ni-NTA flow through and wash (iLOV), (10) ULP1 digestion Ni-NTA elution ((H)₆-SUMO), run in duplicate, and (11) buffer exchanged purified iLOV. Heavier molecular weight bands around 26 kDa, but slightly lower than (H)₆-SUMO-iLOV, present in the purified fraction are small quantities of photo-oxidized and covalently crosslinked iLOV dimers (See Chapter II).

Table IV-S2. Site directed mutagenesis (SDM) primer sequences for iLOV variants.

iLOV Variant	Primer Sequences
Q ₄₄ A	(forward) 5'-gttttctggcgggcccggagacc-3' (reverse) 5'-gcccgccagaaaacgagcattgcg-3'
Q ₁₀₃ H	(forward) 5'-gttttctgcacggcccggagacc-3' (reverse) 5'-gccgtgcagaaaacgagcattgcg-3'
Q ₁₀₃ K	(forward) 5'-gttttctgaaggcccggagacc-3' (reverse) 5'-gcccttcagaaaacgagcattgcg-3'
Q ₁₀₃ E	(forward) 5'-gttttctggaggcccggagacc-3' (reverse) 5'-gccctccagaaaacgagcattgcg-3'

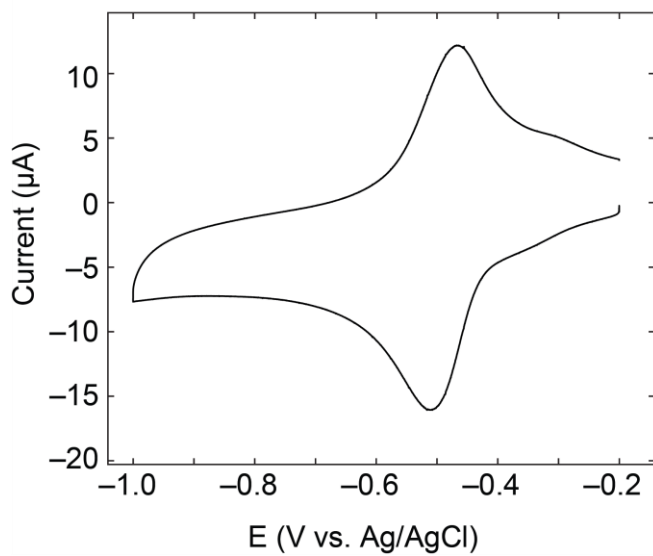


Figure IV-S2. A cyclic voltammogram of commercially obtained phenosafranine. Aberrant shoulders at high potentials suggest the presence of impurities which will affect the results from reductive titrations.

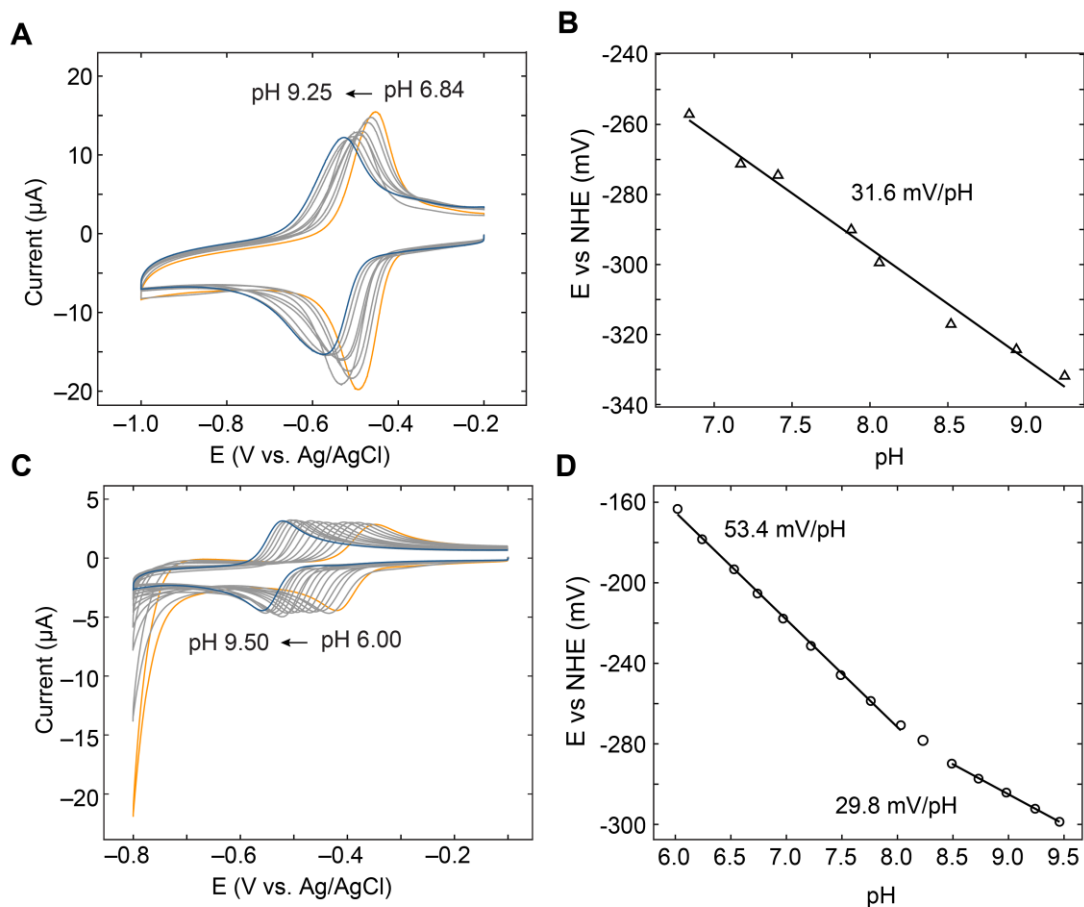


Figure IV-S3. Cyclic voltammograms and resultant Pourbaix diagrams from redox indicators phenosafranine and AQ2S. **A** Representative cyclic voltammetry (CV) scans of pure phenosafranine from pH 6.84 (orange) to 9.25 (blue). **B** Pourbaix diagram (midpoint potential vs. pH) of pure phenosafranine and associated linear fit. The slope of the fit was -32 mV/pH ($1 \text{ H}^+/2 e^-$) with an R^2 of 0.99. **C** Representative cyclic voltammetry (CV) scans of pure AQ2S from pH 6.00 (orange) to 9.50 (blue). **D** Pourbaix diagram (midpoint potential vs. pH) of pure AQ2S. This dye has a pK_a near pH 8.3 and thus contains two regimes of pH dependence. The associated linear fits for both ranges (pH 6.0-8.0 and pH 8.5-9.5) are displayed. Our experiments were performed at pH 7.0 and 8.0, so the fit between 6.0 and 8.0 was used for experiments. The slope of this fit was -53 mV/pH ($1 \text{ H}^+/2 e^-$) with an R^2 of 0.99.

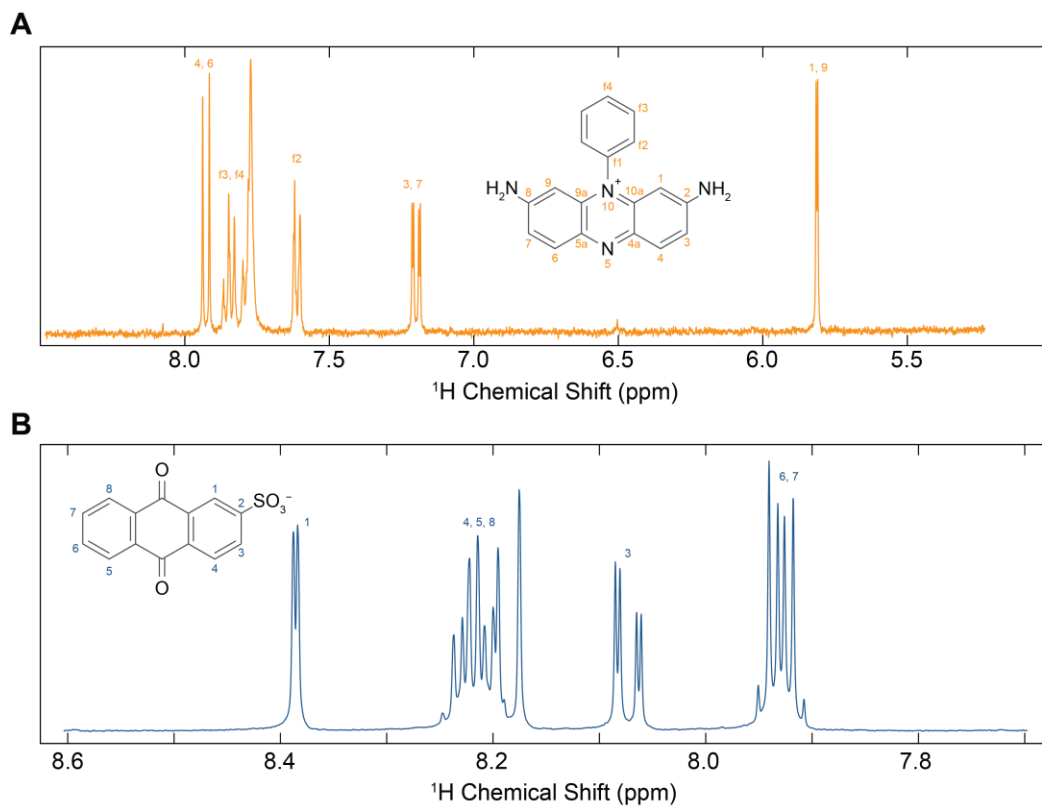


Table IV-S3. One-dimensional ^1H NMR spectral assignments of phenosafranine and AQ2S

Phenosafranine

^1H NMR (400 MHz, d_6 -DMSO) δ 7.92 [d, $J = 9.2$ Hz, 2H, $H(4/6)$], 7.88 – 7.75 [m, 3H, $H(f3/f4)$], 7.61 [d, $J = 7.3$ Hz, 2H, $H(f2)$], 7.20 [dd, $J = 9.2, 2.3$ Hz, 2H, $H(3/7)$], 5.81 [d, $J = 2.2$ Hz, 2H, $H(1/9)$].

Anthraquinone-2-Sulfonate (AQ2S)

^1H NMR (400 MHz, d_6 -DMSO) δ 8.39 [d, $J = 1.5$ Hz, 1H, $H(1)$], 8.27 – 8.15 [m, 3H, $H(4/5/8)$], 8.07 [dd, $J = 8.0, 1.8$ Hz, 1H, $H(3)$], 7.93 [dd, $J = 5.8, 3.4$ Hz, 2H, $H(6/7)$].

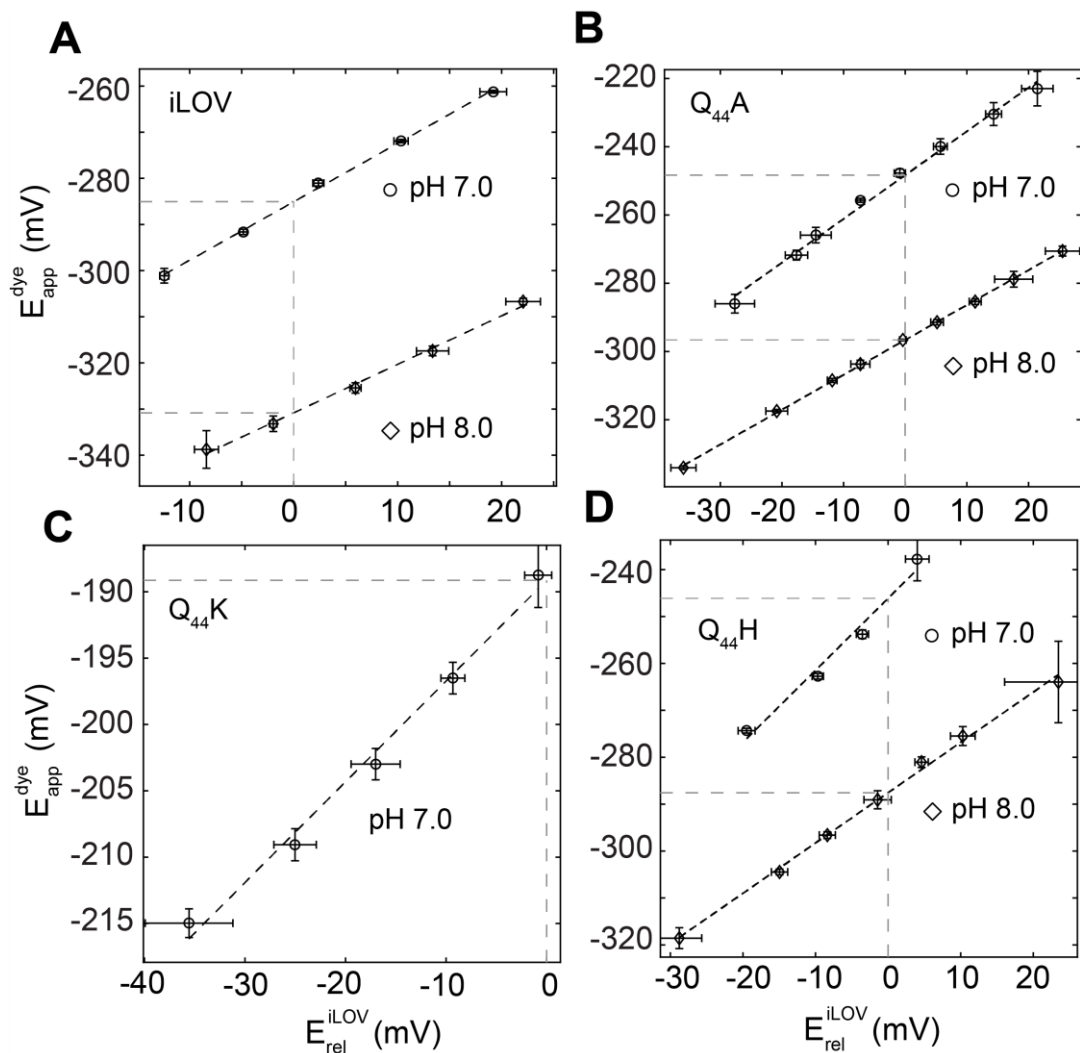


Figure IV-S5. Redox titrations of iLOV Q₄₄X mutants. Titrations were conducted at pH 7.0 (circles) and 8.0 (diamonds). The experimental data were fit to a linear function (black dashed lines), and apparent $E_{OX/NHQ}^{\circ}$ potentials are indicated on the y-axis (gray dashed lines). The results are summarized in the main text **Table 4.1**. Each data point represents the average of triplicate measurements in separate wells for the same conditions.

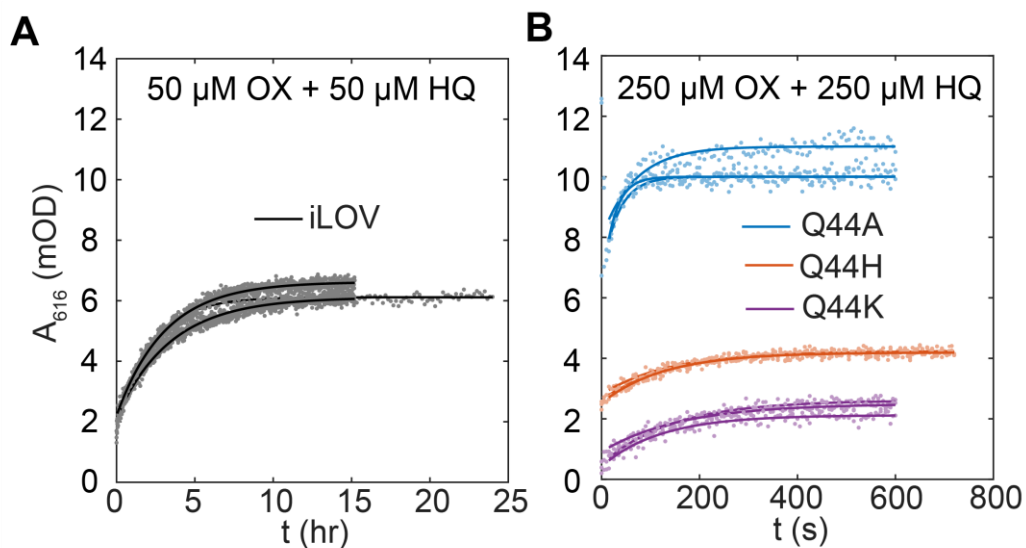


Figure IV-S6. UV-Vis based comproportionation experiments detecting the concentration of NSQ via the absorbance at 616 nm. **A.** Data reproduced from Figure S3.14 depicting the comproportionation of parental iLOV using 50 μM OX and 50 μM HQ. Equilibrium is approached after several hours. **B.** Data from the comproportionation of $Q_{44}X$ mutants (with the exception of $Q_{44}E$ which was not obtained in sufficient quantities to perform this experiment) using 250 μM OX and 250 μM HQ. Equilibrium was reached after a couple minutes. Considerable interference with the background persisted, even after subtracting authentic OX and HQ spectra from the resultant spectra.

Table IV-S4. EPR simulation parameters for iLOV and Q₄₄X NSQ spectra, and associated fit 68% confidence interval.

Variant	g_{iso}	Line width (mT)
iLOV	2.0041 (1) ^a	1.92 (1) ^a
Q ₄₄ A	2.0044 (1)	1.71 (2)
Q ₁₀₃ H	2.0048 (1)	1.84 (1)
Q ₁₀₃ K	2.0052 (1)	1.8 (1)

^a value taken from chapter III or reference 2.

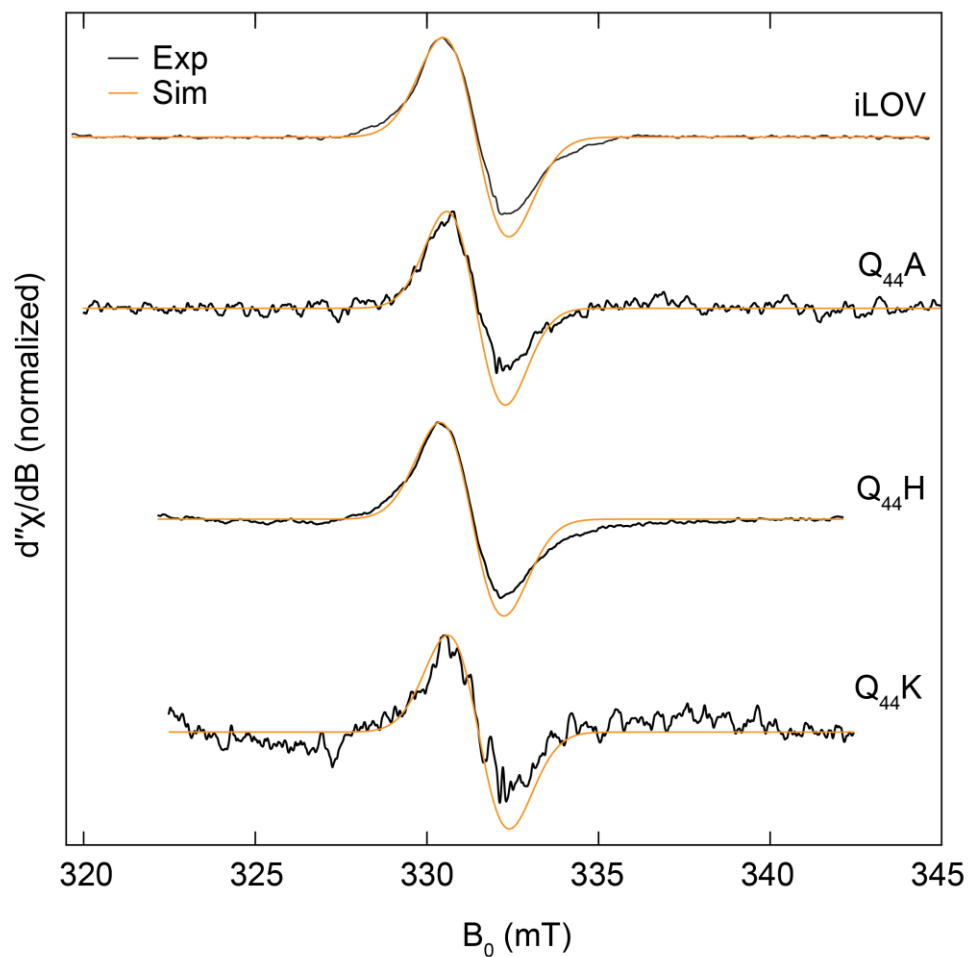


Figure IV-S7. Continuous wave X-band EPR spectra of the NSQ radical of each iLOV $Q_{44}X$ mutant. Extracted simulation parameters are depicted in Table IV-S4. The parent iLOV spectrum is reproduced from Figure S3.16. $Q_{44}A$ was measured with 220 μM each of OX and HQ. $Q_{44}H$ was measured with 700 μM each of OX and HQ. $Q_{44}K$ was measured with 300 μM each of OX and HQ.

Table IV-S5. ΔE values for each mutant determined via comproportionation experiments monitored by UV-Vis.

iLOV Variant	UV-Vis ΔE (mV)	EPR ΔE (mV)
WT	−212 (4) ^a	n.d.
Q ₄₄ A	−272 (3)	−267 ^b
Q ₄₄ H	−317.7 (3)	−253 (5)
Q ₄₄ K	−347 (6)	−374 (9)

^a Reproduced from Chapter III.

^b Measured from a single replicate.