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Measurement and Control of Crossed Potentials in a Flavoprotein

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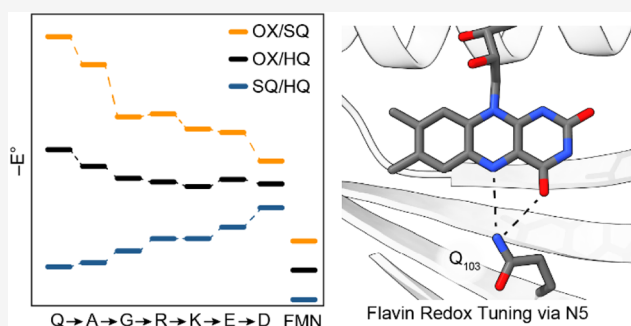


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ABSTRACT: Flavoproteins constitute 1–3% of prokaryotic and eukaryotic proteins, functioning as electron transfer agents, catalysts, and sensing or regulatory modules. Their versatility as redox-active proteins stems from the tunability of the flavin cofactor's one- and two-electron reduction potentials via interactions with the protein scaffold. Although several mechanisms have been proposed to explain how the flavin-binding pocket modulates redox thermodynamics, a holistic model enabling interpretation and prediction remains to be established. In this study, we investigate how the flavin N5 environment influences the redox properties of the flavin mononucleotide cofactor in the “improved” light-oxygen-voltage (iLOV) sensing protein using site-directed mutagenesis, redox titrations, and hybrid quantum mechanical molecular mechanical (QM/MM) methods combined with classical alchemical free energy simulations. Mutating the residue Q_{103} , 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 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

Flavins play crucial roles in biology as redox-active enzyme cofactors and electron transfer agents.^{1–3} The versatility of the flavin cofactor arises from its ability to mediate both one- and two-electron transfer reactions.^{4,5} The oxidized (OX) cofactor can be reduced by one electron to generate a radical semiquinone (SQ) state or accept two electrons to form the fully reduced hydroquinone (HQ) state (Figure 1). These redox transformations can be proton-coupled, resulting in either neutral (N) or anionic (A) forms of the SQ and HQ states. The reduction potentials of these couples ($E^{\circ}_{OX/SQ}$ and $E^{\circ}_{SQ/HQ}$) are highly sensitive to the local protein environment, allowing enzymes to tune flavin redox chemistry for optimal thermodynamic efficiency and kinetic performance.⁶

In solution at neutral pH, free flavin mononucleotide (FMN) exhibits “crossed” potentials: the reduction potential of the OX/NSQ couple ($E^{\circ}_{OX/NSQ} = -238$ mV vs NHE) is more negative than the subsequent NSQ/AHQ couple ($E^{\circ}_{NSQ/AHQ} = -172$ mV, Figure 2A).⁷ As a result, reduction occurs as concerted two-electron process, and the NSQ is unstable. Flavoproteins can stabilize or destabilize an SQ intermediate to achieve a wide range of redox behaviors. For example, consider flavin-based electron bifurcating enzymes, including the NADPH-dependent

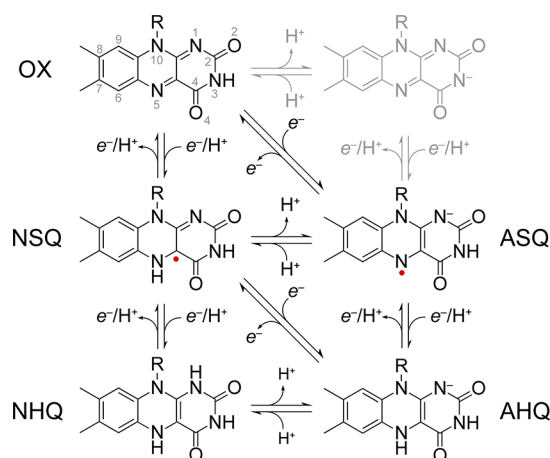


Figure 1. Redox and protonation states of the flavin isoalloxazine ring.

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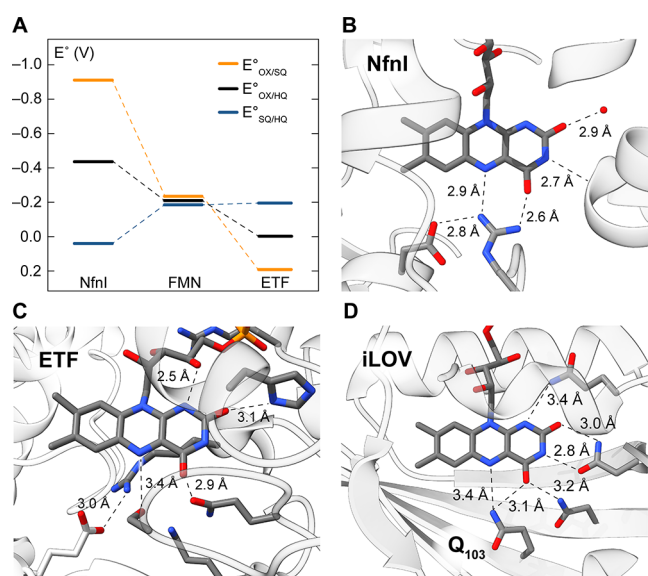


Figure 2. Flavin redox chemistry and tuning in flavoproteins. (A) Reduction potentials for the three redox couples of *Pyrococcus furiosus* (Pf) NfnI, free FMN, and *Methylophilus methylotrophus* (Mm) ETF, demonstrating the span of modulation the protein environment exerts on flavin redox chemistry. (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. (D) Hydrogen bonding interactions of iLOV FMN cofactor (PDB ID: 4EES) with the protein environment.

ferredoxin: NAD⁺ oxidoreductases (Nfn), heterodisulfide reductases (Hdr), and bifurcating electron transfer flavoproteins (Etf), which couple the oxidation of a moderate two-electron donor to the one-electron reduction of both an exergonic and endergonic acceptor.^{8,9} In these enzymes, electron bifurcation occurs at a flavin adenine dinucleotide (FAD) cofactor with significantly crossed one-electron potentials and an unstable (A)SQ intermediate, a feature proposed to be critical for electron bifurcation.^{10,11} In the *Pyrococcus furiosus* NfnI, which catalyzes the NADPH-dependent reduction of both NAD⁺ (exergonic) and ferredoxin (endergonic), the potential splitting ($\Delta E = E^{\circ}_{\text{OX/SQ}} - E^{\circ}_{\text{SQ/HQ}}$) between the $E^{\circ}_{\text{OX/ASQ}}$ and $E^{\circ}_{\text{ASQ/AHQ}}$ couples is estimated to be −951 mV.^{12,13} By contrast, the *Methylophilus methylotrophus* nonbifurcating electron transfer flavoprotein (ETF), which facilitates electron transfer from trimethylamine dehydrogenase to the electron transport chain,¹⁴ exhibits normally ordered potentials; the $E^{\circ}_{\text{OX/ASQ}}$ midpoint potential is +196 mV and a much lower −197 mV for the $E^{\circ}_{\text{ASQ/AHQ}}$, and a corresponding ΔE of +393 mV. The higher potential OX/ASQ couple is well matched to the electron donor, an iron–sulfur cluster of trimethylamine dehydrogenase with a reduction potential of +79 mV, thus enabling facile one-electron transfer, and the $E^{\circ}_{\text{ASQ/AHQ}}$ couple is metabolically irrelevant.¹⁵

Comparison of the redox behavior of *P. furiosus* NfnI and *M. methylotrophus* ETF reveals that the flavin environment can tune the two one-electron potentials by more than 1 V (>100 kJ/mol), ranging from strongly crossed to normally ordered. Yet a simple inspection of their respective flavin protein environments does not immediately reveal the origin(s) of these differences

(Figure 2B,C). Hence, how the flavin environment achieves this control remains poorly understood. It is likely that the combined effects of sterics,^{16,17} hydrogen bonding,^{18,19} aromaticity,^{18,20,21} and Coulombic interactions^{22–24} contribute to this tuning, and are known to modulate flavin redox properties in flavoproteins. Yet, there are relatively few definitive experimental or theoretical studies isolating scaffold contributions to flavin redox tuning, particularly in flavoproteins with crossed one-electron potentials. Such structure–function relationships have been experimentally challenging to demonstrate due to the weak binding of flavin cofactors, variations that can be seen by post-translational modifications or substrate/product/effector binding, and the difficulty of measuring the effects on crossed potential flavins, where the SQ intermediate is unstable.

Here, we leverage the “improved” light-oxygen-voltage sensing (iLOV) protein 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 2D).^{25,26} iLOV is a fluorescent protein derived from the LOV2 domain of *Arabidopsis thaliana* phototropin 2 via truncation of the protein and mutation of the photoreactive cysteine residue involved in light-dependent conformational changes, to alanine.^{25,26} The simplicity, structural stability, and depth of flavin–protein interactions in this flavoprotein make iLOV an ideal model to test theories of reduction potential tuning of the prosthetic group in flavoproteins more broadly. 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.²⁶ 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 . Hybrid quantum mechanical molecular mechanical (QM/MM) and classical alchemical free energy simulations reproduce these experimental trends 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.

EXPERIMENTAL DETAILS

Materials

The iLOV gene within a pQE80L vector backbone was obtained from the Mukherjee lab and cloned into a pSUMO vector as reported previously.^{27,28} The 6× polyhistidine-tagged ubiquitin-like protease 1 (ULP1) was expressed and purified as reported previously.^{29,30} 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, safranine O, methyl viologen (MV^{2+}), 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 μm 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.

Methods

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 previously.²⁸ Total protein yields and associated FMN content is provided in [Supporting Information Table S1](#) and protein purity gels are shown in [Supporting Information Figure S1](#).

Site-Directed Mutagenesis of iLOV

Mutagenic primers used for site-directed mutagenesis to generate Q₁₀₃X (X = G, A, R, K, E and D) iLOV are reported in [Supporting Information Table 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.²⁸

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 [eq 1](#).

$$\Phi_S = \Phi_R \times \frac{I_S}{I_R} \times \frac{1 - 10^{-A_R}}{1 - 10^{-A_S}} \quad (1)$$

Here, Φ_S is the quantum yield of the sample unknown (iLOV and Q₁₀₃X mutants), Φ_R is the quantum yield of some reference,

(in this case riboflavin with a reported quantum yield of 0.26),³¹ 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 Q₁₀₃X 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 ([eq 2](#))

$$I(t) = I_0 \times e^{-t/\tau} + C \quad (2)$$

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

Preparation of anaerobic iLOV samples in both OX and HQ flavin redox states is described in detail in the [Supporting Information Supplemental Methods](#). Briefly, to prepare anaerobic samples of iLOV or Q₁₀₃X mutants in the flavin HQ state, a degassed sample of protein in the flavin OX state was mixed with NaDT and MV^{2+} at 100-fold and 10-fold molar excess over iLOV respectively in an anaerobic atmosphere. The sample was then desalted into anaerobic buffer on a G25 desalting column before use. Samples of OX were also desalted on the same column for consistency.

Anaerobic UV–vis Measurements

Anaerobic UV–vis measurements were carried out as previously described,³² with minor modifications described in the [Supporting Information Supplementary Methods](#).

Measurement of the NSQ Molar Absorptivity

The photochemical production of the Q₁₀₃D NSQ and measurement of its molar absorptivity at 616 nm is described in the [Supporting Information Supplementary Methods](#) and the spectroscopic results are shown in the [Supporting Information Figure S2](#).

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

To measure the equilibrium two-electron $E^{\circ}_{\text{OX/HQ}}$ potential, we developed a method, based on previous work,^{33–35} 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 O₂). The purification of the safranin reference dye isomers, equilibrium redox titration of iLOV and Q₁₀₃X mutants, and analysis of the titration data is described in the [Supporting Information Supplementary Methods](#), and the characterization of purified safranin are shown in the [Supporting Information Figure S3](#), [Table S3](#), and [Figure S4](#).

Comproportionation of iLOV HQ and OX

To measure ΔE , pure samples of iLOV or Q₁₀₃X mutants were mixed in equimolar concentrations inside a VAC Atmospheres glovebox (<0.2 ppm) and monitored spectroscopically over several hours for the formation of NSQ, which has a characteristic absorption band at 616 nm. Equilibrium concentrations of each species were extracted from the data using the extinction coefficient of NSQ, measured as described earlier, and used to estimate ΔE via the Nernst equation. A full description of the experimental details and the subsequent analysis for this measurement is provided in the [Supporting Information Supplementary Methods](#).

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 N₂-cooled isopentane (<−130 °C) cold well within the glovebox. The sample was then removed from the glovebox and placed into a storage dewar in liquid N₂ 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.³² 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 M^{−1} cm^{−1}.³⁶

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

We conducted molecular dynamics (MD) simulations of iLOV and Q₁₀₃X mutants in the OX and NSQ states. Details about the force field parameters and solvated protein model setup are included in the [Supporting Information Supplementary Methods](#). Na⁺ ions were added to neutralize the system. Additional Na⁺ and Cl[−] ions were added to mimic the experimental salt concentration (0.2 M). MD simulations were performed using GROMACS 2024.4 software package along with amber99sb force field.³⁷ The system was minimized and equilibrated following steps described in the [Supporting Information Supplementary Methods](#), followed by MD simulations in the NPT ensemble for 500 ns for both OX and NSQ states for subsequent analysis. A similar procedure was followed to simulate 500 ns trajectories for each of iLOV's Q₁₀₃ mutations—arginine (R⁽⁺⁾), aspartic acid (D⁽⁰⁾), aspartate (D^(−)), glutamic acid (E⁽⁰⁾), glutamate (E^(−)), glycine (G), alanine (A), and lysine (K⁽⁺⁾). The mutations were introduced to the model using SCWRL4.0.³⁸

Using the 500 ns MD simulations described above, we further conducted statistical clustering analysis following the approach described in the [Supporting Information Supplementary Methods](#). Root mean square fluctuation (RMSF) values were computed for the C α atoms of the wild-type protein in both the oxidized (OX) and neutral semiquinone (NSQ) states as well as for selected mutants using MDAnalysis.³⁹ The results were visualized as a residue-by-state heatmap.

Water dynamics around the N5 atom of the flavin cofactor were analyzed using a custom MDAnalysis-based script. The protein backbone was aligned to remove global translation and rotation. For each frame, the closest water oxygen distances to N5 and O4 were identified, and the following two metrics were recorded: hydrogen-bond formation (distance ≤ 3.5 Å, angle $\geq 150^\circ$) and whether the water occupied a defined pocket (within 4 Å of N5 or O4; this parameter indicates presence of water nearby but one that is not necessarily in the right orientation to form a hydrogen bond).

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 3](#)).^{40,41} 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 in incremental steps.^{42–45} The free energy associated with alchemical mutagenesis of iLOV's Q₁₀₃ 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 [eq 3](#)

$$E^{\circ}_{\text{comp}} = -\Delta G_{\text{comp}}/nF \quad (3)$$

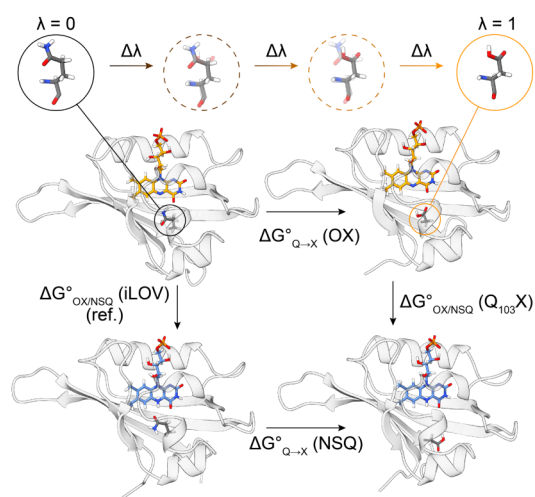


Figure 3. Thermodynamic cycle used to calculate $\Delta G^{\circ}_{\text{OX/NSQ}}$ and hence E° , for $Q_{103}X$ mutants via alchemical transformations.

The Bennett Acceptance Ratio (BAR) approach was used to compute the free energy of the alchemical transformation steps.^{46–48} For this purpose, we adopted a protocol that builds on the recently automated Average Protein Electrostatic Configuration QM/MM approach for flavoproteins (APEC-F 2.0).⁴⁹ A similar idea, albeit with several modifications in the details of the protocol, has been used to simulate spectroscopic properties,^{50–53} as well as relative free energies and isomerization barriers for retinal binding proteins and molecular switches in solution.^{54–56} Briefly, with APEC-F 2.0, QM/MM models of iLOV in its oxidized (OX) and neutral semiquinone (NSQ) states were constructed starting from the X-ray crystallographic structure (PDB ID 4EES).²⁶ We focus on the OX/NSQ simulations because these are the ones displaying the strongest variations with mutations experimentally. Using APEC-F 2.0, the charges and geometry of the flavin optimized quantum mechanically were computed at the B3LYP/ANO-L-VDZP level of theory in a superposition of 100 solvated protein configurations extracted from molecular dynamics (MD). As done for the 500 ns MD simulations, the system was neutralized using a 0.2 M salt concentration. Details of the QM/MM model construction and methodology were reported recently.⁴⁹ Software employed in the construction of the APEC-F 2.0 models include GROMACS, OpenMolcas, Tinker, and Dowser.^{47,57–59}

The APEC-F 2.0 QM/MM structure provides a starting point for further free energy simulations computed with BAR as implemented in the GROMACS 2024.4 *gmx bar* tool. Since the redox site on flavin is localized on the rigid isoalloxazine moiety, the cofactor was kept frozen in its QM/MM-optimized geometry throughout the entire BAR protocol, while relaxing the protein, TIP3P water molecules, and solution ions during the MD. Keeping the frozen chromophore approximation adopted in APEC, the initial zeroth λ window is the only one starting from 0 K with a 300 ps heating to 298 K, whereas all the following begin from the last configuration sampled in the previous 3 ns equilibration. The protocol was run in the NVT ensemble using the equilibrated volume that was originally determined at the level of APEC using NPT dynamics. Additional details of those NVT simulations are reported in the [Supporting Information Supplemental Methods](#). The mutations were introduced using the pmx library adopting the

amber99sbmut force field,⁶⁰ which includes dummy atoms in a dual topology fashion. 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 3](#). We adopted a serial equilibration approach where the last frame of equilibration at a specific λ step is the starting frame for the next one. Specifically, for each λ window, we equilibrated the system (protein + cofactor) 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, we ran multiple (≥ 3) replicas. 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.

Redox potential shifts were computed in this way for mutating Q_{103} in iLOV. For mutations involving the introduction of a charge ($Q_{103}D^{(-)}$, $Q_{103}E^{(-)}$, $Q_{103}K^{(+)}$, and $Q_{103}R^{(+)}$), we adopt the coalchemical ion approach where an ion is coperturbed into existence along with the mutant at each λ to keep the net charge of the system constant.⁶¹ Specifically, for $Q_{103}D^{(-)}$, a growing negative charge, arising in the simulation as the glutamine is annihilated and aspartate forms, was neutralized with the gradual morphing of a water molecule into a sodium ion. Similarly, for $Q_{103}K^{(+)}$ and $Q_{103}R^{(+)}$, the introduced positive charge was neutralized by morphing a water into a chloride ion.

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,⁶² 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_a s.^{7,63} 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 (Q_{103} , [Figure 2D](#)),²⁶ 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 $Q_{103}X$ 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 S1 and S2 and Figure S1](#)).

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 4](#)). 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](#)

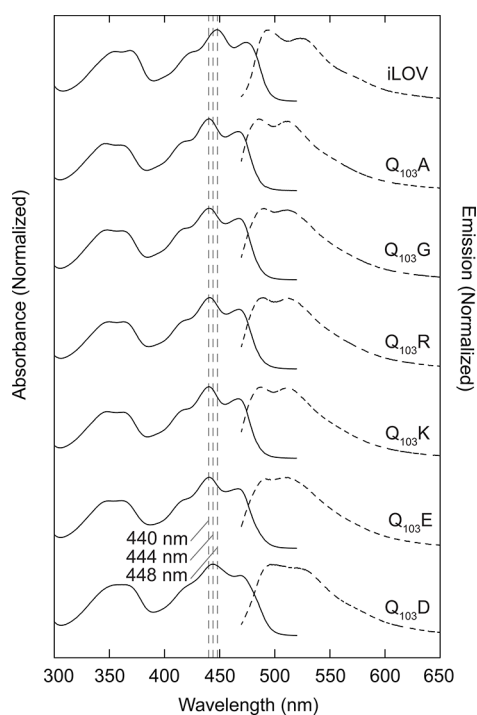


Figure 4. Effect of $Q_{103}X$ mutations on optical properties of iLOV. Normalized UV–vis absorption and emission spectra ($\lambda_{\text{ex}} = 450$ nm) of iLOV and $Q_{103}X$ mutants.

Information Figure S6, Figure S7, and Table 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 $Q_{103}D$, which was red-shifted to 498 nm and $Q_{103}E$ which was further red-shifted to 510 nm. The $Q_{103}X$ mutants exhibited similar Φ , ranging from 0.37 ± 0.01 in $Q_{103}A$ to 0.33 ± 0.01 in $Q_{103}D$. The fluorescence emission lifetime for iLOV was 4.37 ± 0.01 ns, while mutations to Q_{103} resulted in a span from 4.01 ± 0.01 for $Q_{103}A$ to 3.52 ± 0.01 for $Q_{103}E$. Interestingly, τ did not correlate with measured Φ , a phenomenon that has been observed when placing certain fluorophores in solvents of varying polarity.⁶⁴ This is thought to result from simultaneous changes to both the radiative and nonradiative relaxation rates, which we expect are sensitive to the NS environment in iLOV and the associated $Q_{103}X$ mutants.⁶⁵

We next examined how Q_{103} mutation affected the redox behavior of the flavin cofactor, given its proximity to NS, its hydrogen bonding character, and the change in hydrogen bonding character of NS 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 $Q_{103}X$ mutants (Supporting Information Figure S8). The XXO assay was also unsuitable due to slow electron transfer kinetics (Supporting Information Figure S9). We, therefore, turned to redox titrations, using safranin as a dye redox standard reporter and NaDT as a reductant at pH 7.9 and 8.9 (Figure 5). In this classical approach the ratio of the reduced fraction of dye to reduced fraction of analyte at each solution

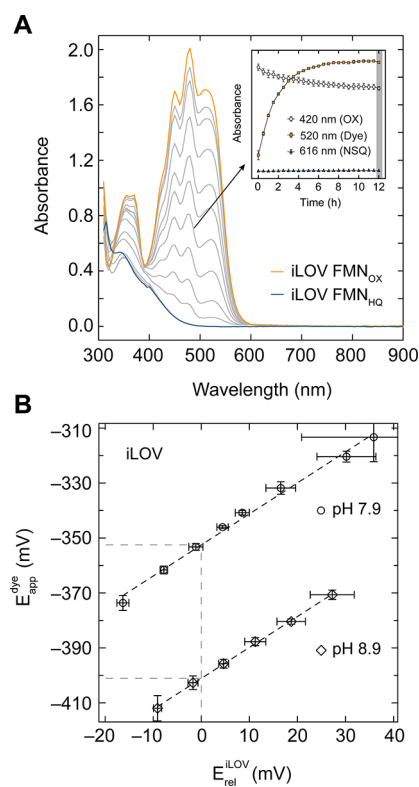


Figure 5. Spectrophotometric redox titrations of iLOV. (A) Equilibrium iLOV absorbance spectra after adding NaDT reductant from 0 eq (orange) to 3.3 eq (blue). Inset shows the approach to equilibrium at one point in the titration (arrow) via monitoring the absorption at 420 nm (FMN_{OX}), 520 nm (safranin), and 616 nm (FMN_{NSQ}), and is reproduced in the Supporting 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., $E^{\circ}_{\text{OX}/\text{NHQ}}$).

potential is used to extract the reduction potential of the analyte.^{33–35} Upon addition of the reductant, we observed a loss of the characteristic visible absorbance bands of the OX FMN (448 nm) and the oxidized safranin dye (520 nm, Figure 5A), that disproportionately reduces safranin before equilibrium is reached between safranin and iLOV based on their relative reduction potentials (Figure 5A inset, Supporting Information Figure 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.⁶⁶

Assuming an equilibrium dominated by OX and HQ forms of the flavin, the fractional reduction of both iLOV $Q_{103}X$ 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 $E^{\circ}_{\text{OX}/\text{HQ}}$ determined relative to the standard, and the pH dependence reflects the number of protons involved in the redox process (Figure 5B, Supporting Information Supplemental Methods, and Figure S11). As the apparent solution potential from the dye indicator ($E_{\text{app}}^{\text{dye}}$) decreases, so too does the

Table 1. Reduction Potentials, pH Dependence, and Electron Equivalents with the Mediator (Slope) of iLOV and Q₁₀₃X Mutants Determined from Reductive and Oxidative (iLOV) Redox Titrations^a

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 ^b	−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 ^c	−272 ^d	−29	n.d. ^e	n.d. ^e

^aUncertainties in the last significant digit shown in parentheses. ^bValues obtained from oxidative (reverse) titrations. ^cValue taken from ref 7 at pH 8.00. ^dValue taken from ref 7 at pH 9.04. ^en.d. not determined.

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 5B dashed lines). The resulting extracted values for $E^{\circ}_{\text{OX/HQ}}$ for iLOV and the Q₁₀₃X mutants, as well as the reference values for free FMN are summarized in Table 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.⁶⁷ These data are most consistent with iLOV reduction proceeding via concerted OX→NSQ and NSQ→NHQ transitions. The reaction was fully reversible; an oxidative titration of reduced iLOV and safranin 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 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 C₃₇G protein,⁶⁸ and 41 mV lower than the homologous *Avena sativa* LOV2.⁶⁹

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 $\Delta E^{\circ}_{\text{OX/NHQ/pH}}$ 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,⁷⁰ 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 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 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 S5). Incorporating NSQ quantitation, the $E^{\circ}_{\text{OX/NHQ}}$ values were determined as -318 mV for Q₁₀₃K, -327 mV for Q₁₀₃E, and -323 mV for Q₁₀₃D at pH 7.9, shifts of +34 mV relative to the parental iLOV.

The difference in iLOV Q₁₀₃X 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 Q₁₀₃X 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 1 by UV–vis and EPR spectroscopy (Figure 6 and Supporting Information Figure S14). 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 eqs S6–S9). The 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 6A). 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 6B). 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.⁶⁹ Thus, the EPR and UV–vis spectra, and the pH dependence of the redox titrations, support an NSQ assignment. Compared to the Q₁₀₃X 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 6C), with identical spectroscopic characteristics of the NSQ by EPR, g_{iso} of 2.004 and a line width of 1.94 mT (Figure 6D). 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 S15). These data imply significant differences in ΔE between iLOV and Q₁₀₃D. The EPR spectra and

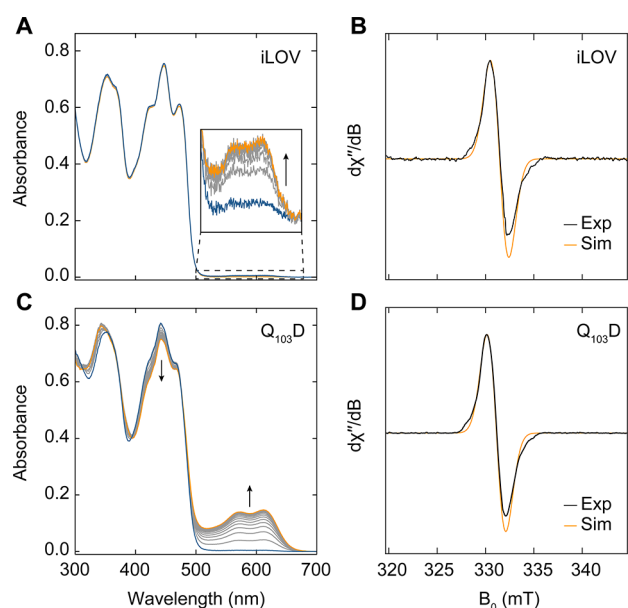


Figure 6. Comproportionation and characterization of the NSQ. (A) UV-vis time course of iLOV OX and NHQ comproportionation over 24 h. Arrows indicate the time-dependent direction of change in absorption from beginning (blue) to end (orange) of the reaction. (B) Normalized EPR spectrum (black) and simulation (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.

simulation parameters for all comproportionation reactions are reported in the Supporting Information Figure S16 and Table S6.

With the measurement of iLOV and $Q_{103}X$ mutants' $E^{\circ}_{OX/NHQ}$ in hand, and the relative K_{eq} of comproportionation, we calculated $E^{\circ}_{OX/NSQ}$ and $E^{\circ}_{NSQ/NHQ}$ across the series (Table 2). As described in the Methods, these values were calculated by assuming our $E^{\circ}_{OX/NHQ}$ 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 $E^{\circ}_{OX/NSQ}$ of -458 mV and an $E^{\circ}_{NSQ/NHQ}$ of -246 mV. By comparison, $Q_{103}D$ exhibited a ΔE of -44 mV, corresponding to an $E^{\circ}_{OX/NSQ}$ of -342 mV and an $E^{\circ}_{NSQ/NHQ}$ of -298 mV. Thus, the $Q_{103}D$ mutation diminishes potential crossing relative to iLOV by increasing the $E^{\circ}_{OX/NSQ}$ by $+116$ mV, while also decreasing the $E^{\circ}_{NSQ/NHQ}$ by -52 mV. Across the mutants targeting N5, the $E^{\circ}_{OX/NSQ}$ was increased and the $E^{\circ}_{NSQ/NHQ}$ was decreased relative to iLOV, resulting in smaller ΔE . The shift in $E^{\circ}_{OX/NSQ}$ relative to iLOV contributed $\geq 70\%$ to the overall

change in ΔE , and the remaining 30% stemming from shifts to $E^{\circ}_{NSQ/NHQ}$.

To provide insight into the molecular mechanism of flavin redox tuning by mutations proximal to the flavin N5, we performed classical MD simulations in both the OX and NSQ states. For the parental iLOV in the OX state, we ran multiple replicas of 500 ns trajectories. 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 $N\epsilon\cdots O4$ distance of 3.2 Å and RMSF of 0.6 Å and mean $N\epsilon\cdots N5$ distance of 4.0 Å and RMSF of 0.8 Å (see Figure 7A,B).

In some replicas of the iLOV OX state, we found that 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 Q_{103} side-chain in the X-ray structure, and the relative flexibility of this residue, as evidenced by the backbone $C\alpha$ RMSD at this site near the C-terminus (Figure 7C).²⁶ We note that in the full *A. thaliana* LOV2 domain of phototropin 2, from which iLOV is derived, Q_{103} is C-terminally flanked by the $J\alpha$ 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 $J\alpha$ helix unfolding in the light-activated state.^{71,72} We focus our analysis throughout the remainder of the manuscript on a replica where the hydrogen bonding is maintained throughout the full 500 ns trajectory, consistent with the crystal structure (Figure 2D).²⁶

The reduction of iLOV to the NSQ state appears to result in less conformational flexibility of Q_{103} , as indicated by the RMSF analysis in the C-terminal region (Figure 7C). Nevertheless, the conformation of Q_{103} 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 (Figure 7D). This observation is consistent with what happens in native LOV domains in either the light-activated or reduced NSQ states.^{72,73} The flip comes at the expense of potential hydrogen bonding interactions. The lowest average distance for that of $O4\cdots O$ Q_{103} carbonyl is 4.3 Å with an RMSF of 0.5 Å and that for $N5\cdots O$ is 5.6 Å with an RMSF of 0.5 Å (Figure 7E). In place of Q_{103} (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 Q_{103} . 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

Table 2. ΔE , $E^{\circ}_{OX/NSQ}$ and $E^{\circ}_{NSQ/NHQ}$ Values for iLOV and $Q_{103}X$ Mutants from Spectrophotometric FMN_{OX} and FMN_{NHQ} Comproportionation at pH 7.9^a

iLOV Variant	$E^{\circ}_{OX/NHQ}$ (mV)	ΔE (mV)	$E^{\circ}_{OX/NSQ}$ (mV)	% of ΔE_{shift}	$E^{\circ}_{NSQ/NHQ}$ (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 ^b	-54	-269 ^b	N.A. ^c	-216 ^b	N.A. ^c

^aPropagated uncertainties in the last significant digit are reported in parentheses. ^bValues from ref 7. ^cN.A. not applicable.

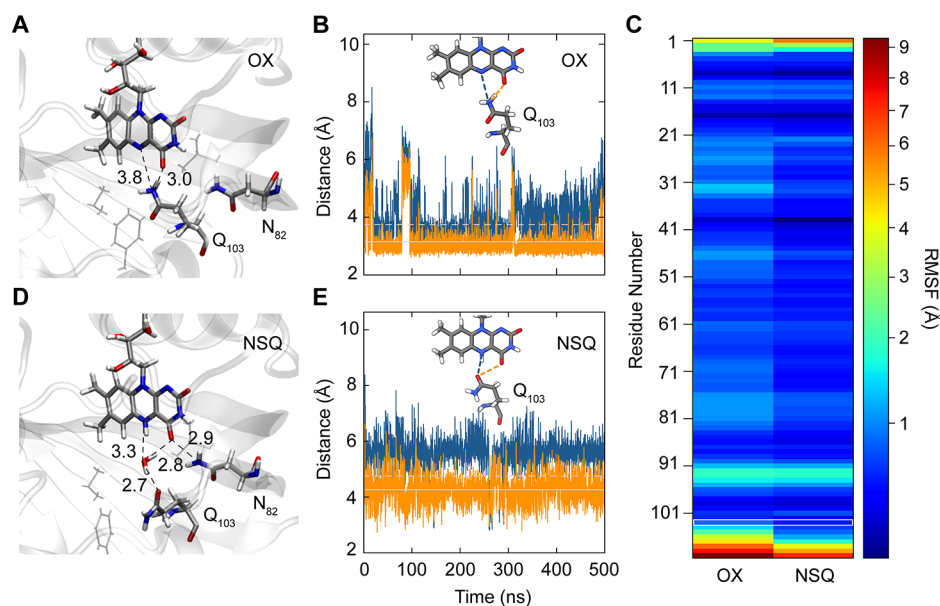


Figure 7. MD simulations of iLOV in the OX and NSQ state. (A) Representative structure of iLOV in the OX state from clustering analysis of a 500 ns MD trajectory. (B) Time-dependent distance analysis for Q_{103} amide N to the flavin N5 (blue) and O4 (orange) from a 500 ns MD trajectory of iLOV in the 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 Q_{103} amide carbonyl oxygen to the flavin N5 (blue) and O4 (orange) from a 500 ns MD trajectory of iLOV in the NSQ state.

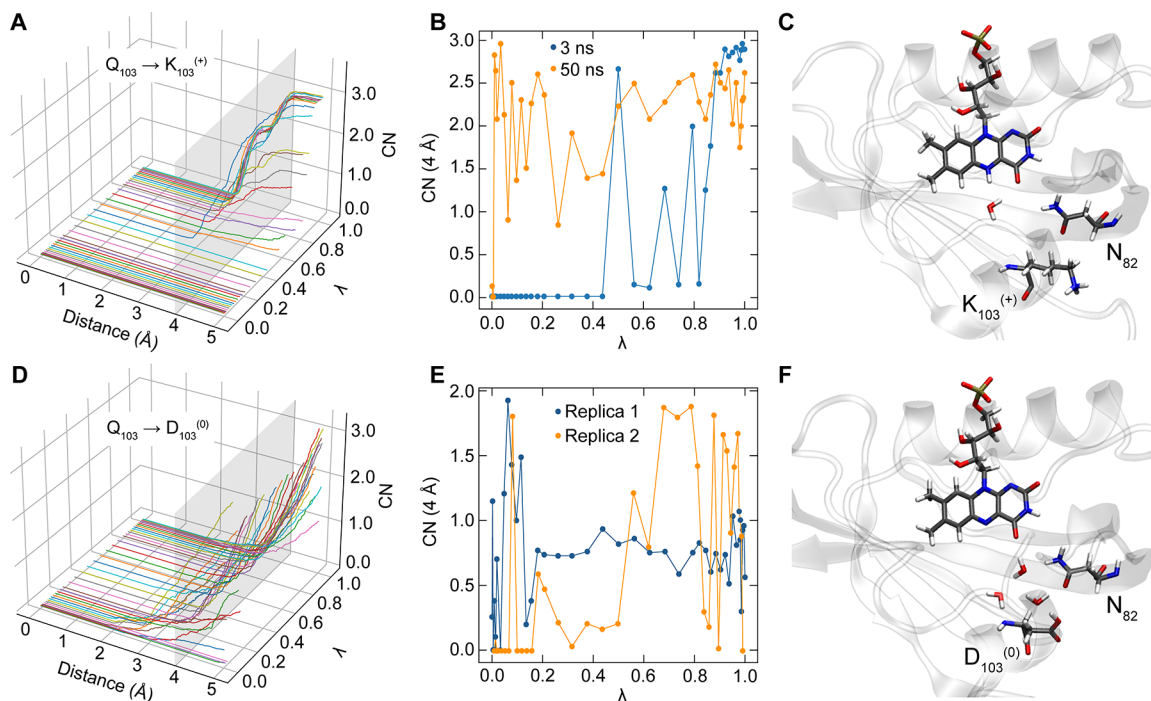


Figure 8. (A) Radial distribution of water cumulative number (CN) to N5 in the NSQ state during alchemical Q_{103} ($\lambda = 0$) to $K_{103}^{(+)}$ ($\lambda = 1$) transformation at each λ step with a 3 ns pre-equilibration time. (B) Comparison of CN at 4 Å during the Q_{103} to $K_{103}^{(+)}$ transformation 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.

occupancy within 4 Å from the flavin N5 in NSQ is significantly higher (94.4%) than the native OX state of iLOV (6.0%).

We next simulated the $E^{\circ}_{OX/NSQ}$ for the various $Q_{103}X$ mutants using relative free energy simulations, benchmarked against the parental iLOV. In previous work, a thermodynamic

Table 3. A Comparison of Simulated and Experimental Redox Potentials^a

Variant	λ Category	$E^{\circ}_{\text{OX/NSQ}}$ (mV)		Difference (mV)	H_2O Occupancy (NS, %)	H_2O Occupancy (O4, %)
		Computed	Experimental			
iLOV	N.A. ^b	ref ^c	−458	N.A. ^b	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

^aThe 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 parentheses, are the sum of σ from the OX and NSQ states. H_2O Occupancies reported for the OX state. ^bN.A. not applicable. ^cReference value for integrated thermodynamic calculations.

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.^{74,75} 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 NS 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.⁴² For these reasons, we opted to use longer MD simulations and 40 λ steps instead of the 8–14 λ steps used for flavodoxin.^{41,45,74}

Our analysis of the mutagenic trajectories and the resultant computed $E^{\circ}_{\text{OX/NSQ}}$ indicate that, even with a relatively long MD period and higher-density λ steps, errors between replicas were >1 kcal/mol (Supporting Information Table S7 and 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.

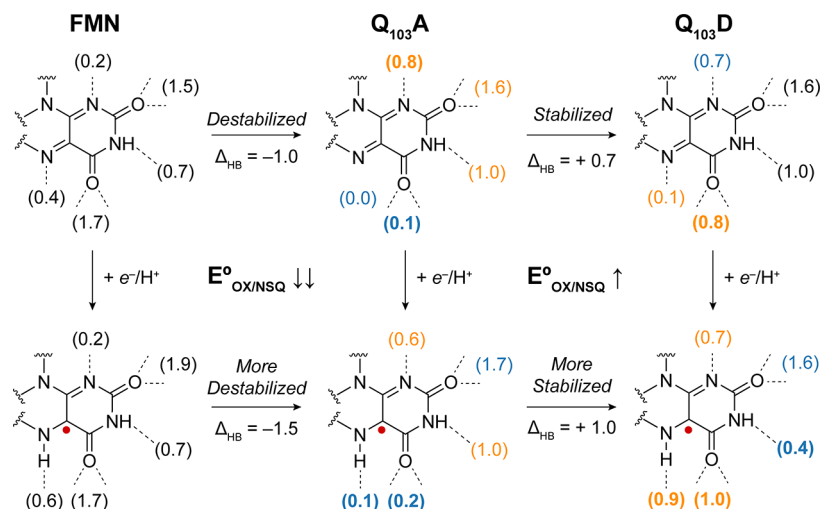
Based on our observation with the parental iLOV, we suspected that solvent penetration dynamics may significantly contribute to the deviations among replicates. In all OX and NSQ simulations, the native iLOV binding pocket starts out anhydrous, but the gradual introduction of a mutation at Q₁₀₃ causes water to enter the binding pocket near the flavin's NS region. Figure 8A shows the water cumulative number (CN), the number of waters within a variable radius shell centered at NS at each λ step for one replica of Q₁₀₃K⁽⁺⁾ 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 Q₁₀₃K⁽⁺⁾ specifically, we find different behavior between two replicas depending in the pre-equilibration time provided in the simulation (Figure 8B). In replica 1, which had a short pre-equilibration of only 3 ns at the λ = 0 step, water does not enter the binding pocket until later in the Q₁₀₃→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 λ = 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 Q₁₀₃K⁽⁺⁾ is shown in Figure 8C. After discarding the 3 ns trajectories for Q₁₀₃K⁽⁺⁾, 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 Q₁₀₃K⁽⁺⁾. We observed similar pre-equilibration time-dependent behavior for Q₁₀₃R⁽⁺⁾ and consider only the longer pre-equilibration replicates further here for Q₁₀₃R⁽⁺⁾ as well.

We also examined the solvent penetration behavior of the OX state of the protonated Q₁₀₃D⁽⁰⁾ (Figure 8D–F). Again, we consistently see water entering the binding pocket at some λ steps between 0→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 λ = 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 Q₁₀₃K⁽⁺⁾ and Q₁₀₃D⁽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, we can qualitatively categorize the MD behavior 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 λ =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, we notice that the comparison of computed $E^{\circ}_{\text{OX/NSQ}}$ from replicas belonging to each of those categories gives more internally consistent results. Specifically, using replicas that exclusively belong to category 3 gave

Scheme 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_{103}A , and Q_{103}D ⁱ



ⁱAverage 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.

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 3 allowed us to calculate $E^{\circ}_{\text{OX/NSQ}}$ from 3 to 4 replicas (Table 3). The data from all replicas, including those not considered further herein, are reported in the Supporting Information Tables S7 and 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 and the water occupancy near N5/O4 (Table 3). Furthermore, the protonated $\text{Q}_{103}\text{D}^{(0)}$ model does substantially better than the corresponding $\text{Q}_{103}\text{D}^{(-)}$, with replicas yielding an $E^{\circ}_{\text{OX/NSQ}}$ closer to the experiment. These results indicate, in agreement with previous experimental and computational evidence,⁷⁰ that the Q_{103}D side-chain is protonated. Following the same logic, the Q_{103}E 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 $E^{\circ}_{\text{OX/NSQ}}$.

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 S17. The same figure also includes a heat map for the RMSF of individual amino acids. In addition to residue 103, we highlight two asparagine residues (N_{72} and N_{82}) as well as one glutamine (Q_{44}) that are involved in a hydrogen-bond network with the

flavin and each other. In general, this hydrogen bond network is robust in most mutants, with Q_{44} and N_{72} in particular maintaining their conformational states across all mutants and clusters. However, N_{82} , 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 $\text{Q}_{103}\text{R}^{(+)}$ which, although is conformationally rigid, displays a different hydrogen bonding pattern around N_{82} compared to other mutants.

The structures in the Supporting Information Figure S17 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 N_{82} , 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 Q_{103} and N_{82} play the major role in forming the hydrogen bonding network near O4 and N5. Any mutation of Q_{103} perturbs the hydrogen bonding network such that either water or Q_{103}X mutant has to form hydrogen bonding interactions with N_{82} , otherwise N_{82} gets closer to the flavin and forms a hydrogen bond with O4.

The heat map in the Supporting Information Figure S17 also shows that the mutants with the highest flexibility at the C-terminus are $\text{Q}_{103}\text{D}^{(0)}$ and $\text{Q}_{103}\text{K}^{(+)}$. Clustering analysis shows two major conformations of $\text{Q}_{103}\text{D}^{(0)}$. Representative structures from both conformations have a water molecule inside the active site, consistent with our earlier results indicating high water occupancy. On the other hand, for $\text{Q}_{103}\text{K}^{(+)}$, 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.

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.

Our QM/MM and MD simulations help provide molecular context into the structural and dynamic differences between iLOV mutants, which is summarized in Scheme 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. Our 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,⁷⁶ but in our simulations 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.²⁶ In particular, no hydrogen bond <3.0 Å is observed for either N1 or N5, destabilizing the cofactor. These interactions are largely reproduced in our 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.⁷⁷ The pK_a of the FMN N5 in the OX state has been computationally estimated to be -10 .⁷⁸ When free in solution, hydrogen bonding between water and N5 is not particularly favored, with a $\Delta pK_a \geq 24$,⁷⁹ 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,^{7,70} 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 Q₁₀₃D 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.^{80,81}

The FMN C=O4 carbonyl bond is also weakened upon reduction to the NSQ state, making O4 a better hydrogen bond acceptor.^{82–86} 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.¹⁸

On comprehensive comparison of the change in total hydrogen bonds (Δ_{HB} , Scheme 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 $E^{\circ}_{\text{OX/NSQ}}$ 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 $E^{\circ}_{\text{OX/NSQ}}$.^{87–89}

Changes in hydrogen bonding character near the flavin N5 and O4 across the Q₁₀₃X mutations correlate with the $E^{\circ}_{\text{OX/NSQ}}$ shifts. For the Q₁₀₃D 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 Q₁₀₃D mutation is to stabilize the FMN cofactor, but the NSQ is more stabilized by a Δ_{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.^{86,90,91} Nevertheless, by affecting both OX and NSQ oxidation states, their effect on the overall tuning of the $E^{\circ}_{OX/NSQ}$ 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 $Q_{103}K^{(+)}$ we observe that 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 $E^{\circ}_{OX/NSQ}$. While electrostatics can tune flavin redox potentials, these effects disproportionately affect proton-uncoupled redox transformations in which an ASQ or AHQ is formed.^{22,92} 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 $OX \rightarrow NSQ$ or $NSQ \rightarrow NHQ$ transition. Across the series of $Q_{103}R^{(+)}$, $Q_{103}K^{(+)}$, and $Q_{103}D^{(0)}$, the MD simulations indicate water occupancy and hydrogen bonding correlate well with the computed $E^{\circ}_{OX/NSQ}$, providing a rationale and experimentally testable model for flavin redox tuning via hydrogen bonding interactions to N5 and O4.

For $Q_{103}D$, 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 $Q_{103}D^{(0)}$. 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 $Q_{103}X$ mutations also change the bound FMN $E^{\circ}_{NSQ/NHQ}$ relative to the free cofactor. In the parental iLOV, $E^{\circ}_{NSQ/NHQ}$ is only 30 mV below that of free FMN. The pK_a of the free riboflavin N1 in the NSQ state is 2.3,⁹³ and shifts to 6.7 in the NHQ state, corresponding to a ΔpK_a in $H_2O \cdots H-N1$ 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 $-NH_2$ of Q_{44} and N1 ($N \cdots N$ distance of 3.4 Å) and a shorter hydrogen bond with O2 ($N \cdots O$ distance of 2.9 Å), analogous to the Q_{103} interactions with N5 and O4, whereas our MD simulations show the Q_{44} amide $-NH_2$ 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 Q_{44} can act as both a hydrogen bond donor and acceptor during the $NSQ \rightarrow NHQ$ 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 $E^{\circ}_{NSQ/NHQ}$ relative to free FMN. Nevertheless, there are other factors the protein environment likely imposes on the $NSQ \rightarrow NHQ$ transition that are distinct from those experienced in bulk water, such as the flavin “butterfly” bend in the NHQ state,¹ which could partially explain the slightly more negative

$E^{\circ}_{NSQ/NHQ}$ but is not addressed in the experiments or simulations presented herein.

$Q_{103}E$, just like $Q_{103}D$, is also expected to be protonated based on our MD simulations. As shown in the clustering analysis in the Supporting Information Figure S17, the E_{103} residue is firmly hydrogen bonded to the flavin O4 atom. The protonated $Q_{103}E^{(0)}$, therefore, participates in a stabilizing hydrogen bonding network involving both the N_{82} and flavin. Instead, MD simulations on the deprotonated $Q_{103}E^{(-)}$ result in a less stable and therefore more dynamic environment near the E_{103} position (see heat map in the Supporting Information Figure S17).

It is also useful to compare the $E^{\circ}_{OX/NSQ}$ potential in native iLOV and $Q_{103}E^{(0)}$. 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 $Q_{103}E^{(0)}$ side chain increases $E^{\circ}_{OX/NSQ}$ relative to Q_{103} in iLOV. This can be attributed to the difference in the pK_a of the two side chains; E_{103} is more acidic and is therefore a better hydrogen bond donor than Q_{103} . This difference in hydrogen bonding ability results in a larger stabilizing effect on the OX state in $Q_{103}E^{(0)}$, without the need for water penetrating the binding pocket, while in other mutants the $E^{\circ}_{OX/NSQ}$ trend is related to degree of water solvation.

The trend in $E^{\circ}_{OX/NSQ}$ across the $Q_{103}X$ mutants was also reflected in the $E^{\circ}_{NSQ/NHQ}$ 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 $E^{\circ}_{OX/NSQ}$, should decrease $E^{\circ}_{NSQ/NHQ}$ assuming little change in the stability of the NHQ state across the series. This hypothesis will be tested in future studies.

It is worthwhile to discuss the methodological approach to the alchemical free energy calculations more thoroughly, as these methods may be useful in other redox calculations in complex and dynamic redox proteins and enzymes. First, we notice that the computed reduction potentials reported for all mutants in Table 3 are systematically more negative than the experimentally measured potentials. One possible explanation for such a systematic error is the different force fields for the OX and NSQ flavin states, even though the charges and geometries were obtained from QM/MM calculations. Another source of error may be introduced by the difference in solvation of those two states, since in most simulations (e.g., belonging to category 3) the OX of the final mutated protein at $\lambda = 1$ is dry while for NSQ it is solvated. As more information is collected on flavin redox tuning, these types of experiments can serve as an experimental benchmark from which to compare computational predictions of the reduction potentials of flavoproteins. 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.^{8,41,75} 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, we find 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. Often, computing redox potentials in solution can be done with reasonably high accuracy using implicit solvation,⁹⁴ but here we instead highlight both the challenges, as well as the capability to compute redox potential in a complex and heterogeneous environment such as iLOV. 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,^{95,96} polarizable force fields,¹⁴ and machine learning approaches.^{97,98}

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 about potential tuning of 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 $E^\circ_{\text{OX/SQ}}$ couples known, an ASQ is proposed.^{12,13} Further study is necessary to understand how hydrogen bonding and electrostatics will affect OX→ASQ couples.

CONCLUSION

In this study, 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.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.5c16043>.

Supplemental Methods: initial and postreconstitution FMN/protein content, protein purification and purity

gels, iLOV Q₁₀₃X mutagenic primers, photoreduction and ϵ 616 determination for the Q₁₀₃D NSQ, statistical clustering analysis of MD trajectory, 1H NMR spectra and assignment of safranin isomers, cyclic voltammograms and Pourbaix diagram of safranin isomer 3, excitation spectra of iLOV and Q₁₀₃X mutants, emission lifetimes for iLOV and Q₁₀₃X mutants, emission lifetime fit constants and quantum yields, protein film voltammetry of iLOV and Q₁₀₃X mutants, xanthine/xanthine oxidase redox titrations of iLOV and Q₁₀₃X mutants, equilibrium redox titrations of all Q₁₀₃X mutants, X-band EPR quantitation of Q₁₀₃D NSQ, comproportionation approach to equilibrium for iLOV and Q₁₀₃X mutants, X-band EPR spectra of iLOV and Q₁₀₃X mutant NSQs, X-band EPR spectrum of Q₁₀₃D NSQ at pH 9.25, EPR simulation parameters for iLOV and Q₁₀₃X mutant NSQs, and comparison of ΔE estimates using UV–vis vs EPR NSQ quantitation, free energies of mutation for OX and NSQ states of iLOV and Q₁₀₃X, computed reduction potentials for Q₁₀₃X mutations, and statistical clustering analysis and representative structures of Q₁₀₃X (PDF)

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Notes

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