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Integrated Structural Studies for Elucidating Carotenoid-Protein Interactions

Corie Y. Ralston and Cheryl A. Kerfeld

Abstract

Carotenoids are ancient pigment molecules that, when associated with proteins, have a tremendous range of functional properties. Unlike most protein prosthetic groups, there are no recognizable primary structure motifs that predict carotenoid binding, hence the structural details of their amino acid interactions in proteins must be worked out empirically. Here we describe our recent efforts to combine complementary biophysical methods to elucidate the precise details of protein-carotenoid interactions in the Orange Carotenoid Protein and its evolutionary antecedents, the Helical Carotenoid Proteins (HCPs), CTD-like carotenoid proteins (CCPs).

Keywords

Carotenoid · Chromophore · CTD-like carotenoid proteins (CCP) · Helical carotenoid protein (HCP) · Hydrogen-deuterium exchange (HDX) · Orange carotenoid protein (OCP) · Photoprotection · Protein crystallography · Small angle X-ray scattering (SAXS) · X-ray footprinting mass spectrometry (XFMS)

1 Carotenoids and Carotenoproteins

Carotenoids are a diverse family of isoprenoid-based pigment molecules with a variety of functions in biological systems (Frank and Cogdell 1996; Cogdell et al. 2000; Frank and Brudvig 2004; Krinsky and Johnson 2005). They play critical roles across the tree of life, including light-harvesting and photoprotection in photosynthesis, coloration (e.g. in birds, lobsters, fruits), the formation of retinal in the eye, and anti-oxidant activity that is of significant interest for the development of therapeutics. Carotenoids play a key role in protecting cells by scavenging radicals and quenching $^1O_2$ (Telfer et al. 1994). $^1O_2$ is a strong oxidizer and a potent initiator of radical oxidation; it reacts with all major macromolecules, leading to a complex cascade of deleterious chemical, biological and physiological reactions. Given that a single carotenoid

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molecule can quench many $^1O_2$ molecules (Gust et al. 1993), carotenoids confer a powerful protective function in biological systems.

A major limitation in the development of carotenoid-based systems for broad and diverse uses such as therapeutics or artificial photosynthesis is their lability and the dearth of understanding of how carotenoid function is influenced by its environment. Carotenoids are photosensitive, highly unsaturated hydrophobic molecules that need to be protected from oxidation and degradation (Kispert and Polyakov 2010). Their limited water solubility is typically an impediment to their study and applications (Polyakov and Kispert 2015; Tan et al. 2014; Tachaprutinun et al. 2009; Offord et al. 2002) because of the requirement for liposome-based experiments or use of toxic organic solvents (Polyakov and Kispert 2015).

Carotenoids are thought to have evolved to stabilize lipid membranes (Vershinin 1999). The eventual association of carotenoids with proteins greatly expanded their range of properties and functions. The conjugated polyene chain of the carotenoid molecule is mainly responsible for its chemical reactivity toward oxidizing agents and free radicals. For carotenoids in solution, $^1O_2$ quenching reactivity increases with the number of conjugated double bonds and is attenuated by structural features, such as the presence of end rings, functional groups, and nonlinear configurations (Hirayama et al. 1994). Surrounding the carotenoid with protein changes its optical and quenching properties (Diversé-Pierluissi and Rodgers 1983; Bilski et al. 2000). In photosynthetic systems, the protein environment precisely tunes the spectral properties of carotenoids to enable them to serve as accessory light harvesting pigments or as excitation energy quenchers. Protein structural features also strongly affect the energy of the T_1 energy level that serves as the quencher in the physical mechanism. Furthermore, the solvent accessibility of amino acids and of the carotenoid impacts the reaction with ROS because the quenching reactions involve collisionally-mediated excitation energy transfer mechanisms.

How carotenoids become bound to proteins is also poorly understood. The association is non-covalent, and there are no identifiable sequence motifs that predict binding, making the elucidation of carotenoid-protein interactions a necessary step in the characterization of any carotenoprotein. Here we describe our efforts to combine various biophysical methods to understand the structure and function of carotenoproteins. Such integrated approaches are underway for crustacyanin, a water soluble protein for coloration of the lobster carapace; however those studies aim at elucidating the higher order assembly of carotenoproteins (Rhys et al. 2011). In contrast, we discuss our recent results in combining protein crystallography, X-ray footprinting mass spectrometry, Hydrogen-Deuterium Exchange and Small Angle X-ray scattering to focus on the structural basis of carotenoid-protein interaction in the Orange Carotenoid Protein and its evolutionary antecedents, the Helical Carotenoid Proteins (HCPs) and the CTD-like Carotenoproteins (CCPs) (Fig. 1). Together, the OCP, CCP and HCPs constitute an ideal model system for comparative studies of the evolution of carotenoid binding and function, and to elucidate how protein-pigment interactions tune the spectral and functional properties of carotenoproteins. This article focuses on our efforts to integrate primarily X-ray based methods for the study of a protein with a non-covalently associated prosthetic group.

2 Hybrid Structural Methods in the Study of the OCP

Studies on naturally occurring carotenoproteins, typically from photosynthetic pigment-protein complexes, have revealed the exquisite level of spectral and functional tuning conferred by the protein as it engages with the chromophore, placing it in specific environments and configurations. However, in photosynthetic systems carotenoids are primarily bound to membrane proteins and typically, as part of photosystems, contain multiple additional pigments (chlorophylls) which makes it difficult to disentangle the specific

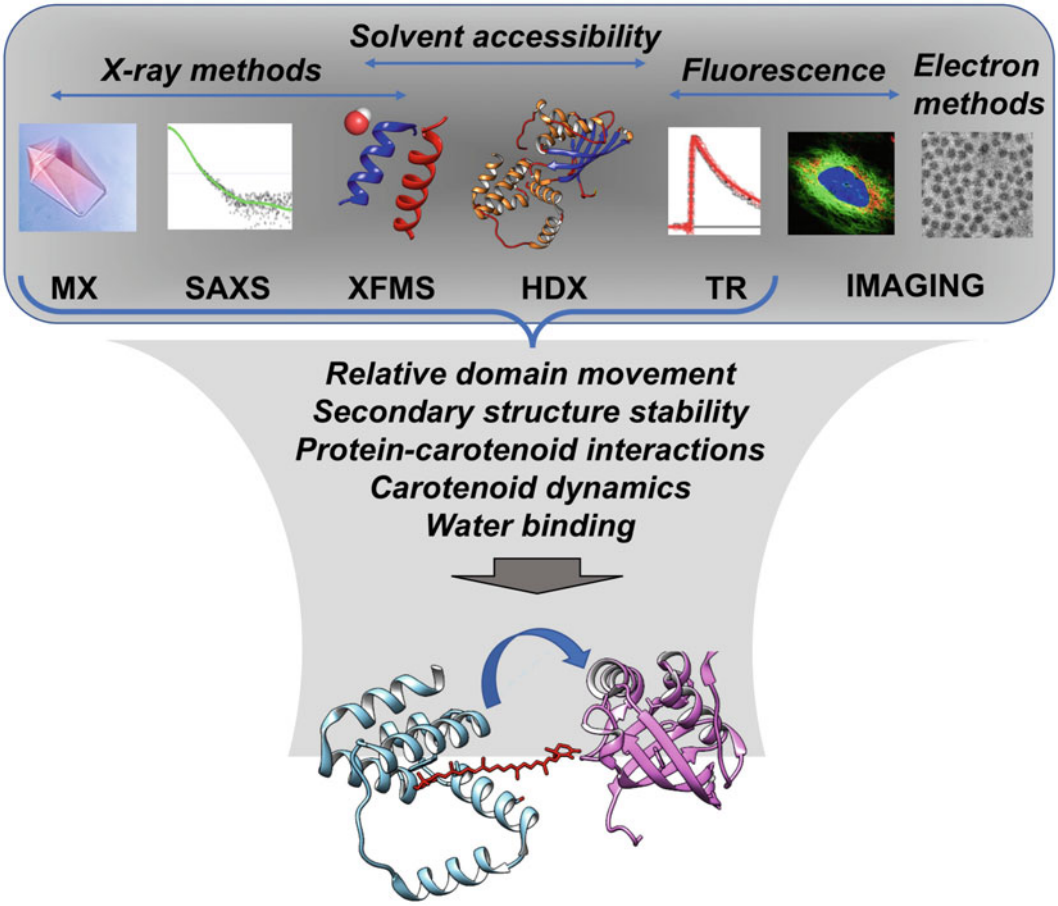

Fig. 1 The integration of various structural, spectroscopic and imaging methods is increasingly necessary to develop a comprehensive understanding of biological systems. In order to contribute to the understanding of the carotenoid-protein environment and the effect of subtle changes in that environment on both optical properties and global conformation and dynamics, we have combined data from crystallography (MX), small angle scattering (SAXS), X-ray footprinting mass spectrometry (XFMS), hydrogen deuterium exchange (HDX), and time-resolved fluorescence (TR) studies were combined

details of protein-carotenoid interaction and their contribution to the overall spectral and functional properties.

One model system, derived from a photoprotective mechanism of Cyanobacteria, offers an ideal model system for probing the carotenoid-protein interactions. The OCP is a 34 kDa water-soluble protein that binds a single carotenoid molecule (Kerfeld et al. 2003), typically echinenone or canthaxanthin. The pronounced influence of the protein on the spectral properties of the carotenoid is striking; canthaxanthin, for example, has an absorption maximum at 475 nm in methanol, when bound to the OCP the absorption maximum shifts to 495 nm (Fig. 2). The spectral shift is induced by the conformation imposed on the carotenoid by the protein, as well as the specific environment created by the amino acids that line the carotenoid binding pocket (Fig. 3).

The OCP is composed of two domains, an all alpha-helical N-terminal domain (NTD), and a mixed alpha beta C-terminal domain. The carotenoid spans the two domains, and is almost fully

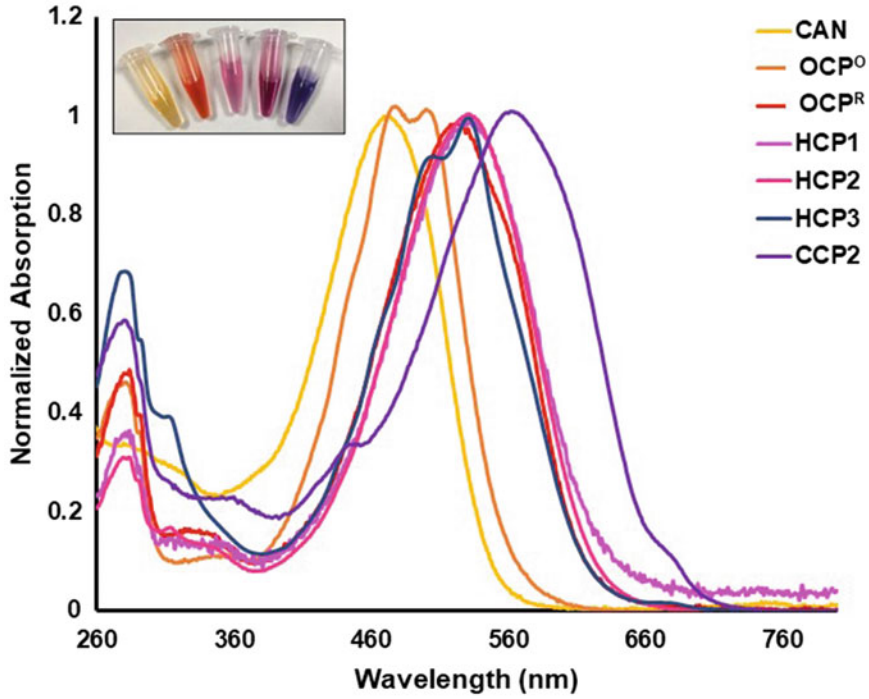

Fig. 2 UV–Vis absorbance spectra of canthaxanthin in methanol, OCP in the orange (resting) and red (active) forms, HCPs and CCP2. Inset: Solutions of canthaxanthin methanol and in OCP, HCP1, HCP3 and CCP2

enveloped (3.4% solvent exposed) by protein (Kerfeld et al. 2003) (Fig. 3). In this configuration, the OCP appears orange and is inactive in photoprotection. However, when it is illuminated by intense blue-green light, corresponding to growth conditions in which available light energy is in excess of what can be used for photochemistry, the OCP visibly turns red and becomes active for photoprotection; in this form it binds the light harvesting antenna and dissipates captured light energy as heat.

A mechanistic understanding of the process of light absorption by the OCP, and how carotenoid light absorption and motion ultimately leads to the spectral conversion of the orange to the red form of the OCP, has been probed by our groups through a combination of complementary structural characterization methods. As described here, macromolecular crystallography, small angle X-ray scattering (SAXS), hydrogen deuterium exchange (HDX), and in particular XFMS were essential to discovering and characterizing carotenoid movement and subsequent protein conformational changes induced upon light absorption by the OCP.

The crystal structure of the OCP was determined before its function was known (Kerfeld et al. 2003); it had been isolated in the course of purification of c-type cytochromes from cyanobacterial cells harvested from the environment (David Krogmann personal communication). In the course of the purification, a derivative of the OCP was also isolated; this corresponded to a proteolytic fragment of the OCP and was red-colored, and so named the Red Carotenoid Protein (RCP) (Diversé-Pierluissi and Krogmann 1988). Placing this observation in the context of the structure created a puzzle, as modeling the RCP structure on that of the OCP would place about half of the carotenoid in solvent (see Figure 5 in (Kerfeld 2004)). Given the hydrophobic nature of the carotenoid, this was implausible, but it was clearly more than an artifact, when it was shown that such proteolytic

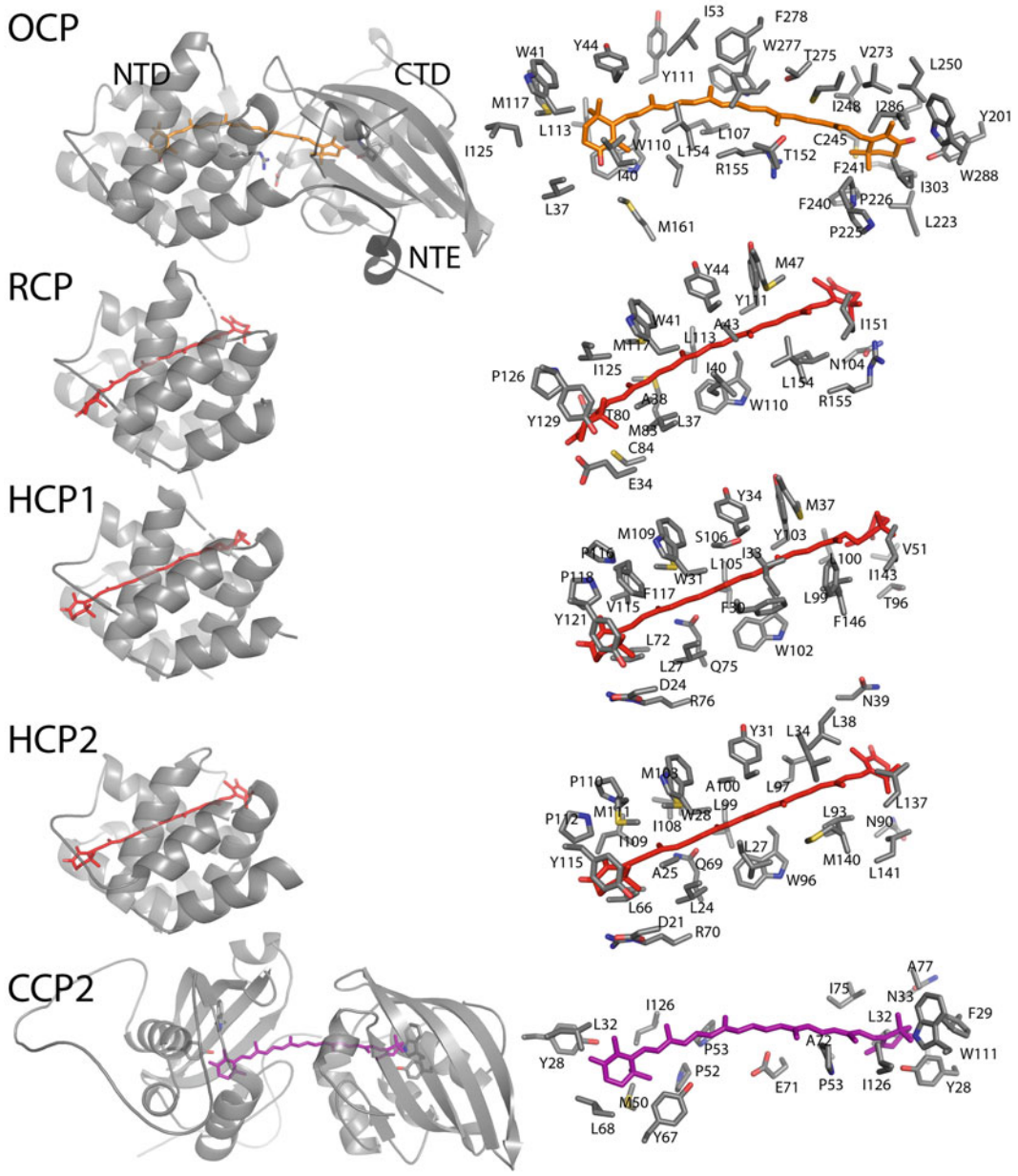

Fig. 3 Overview of the structures of the OCP-related family of carotenoproteins. Left:. Crystal structures of the monomers; only CCP2 is an obligate homodimer. Note in the OCP how the carotenoid (orange) spans the two structural domains (NTD and CTD). The HCPs are structural homologs of the NTD and the CCP2 is a structural homolog of the CTD. NTE: N-terminal extension. Residues hydrogen bonding to the carotenoid in the OCP and CCP2 are shown in sticks. Right:. Close-up view of protein-carotenoid interactions. Sidechains within 4 Å of the carotenoid (red or orange) are shown in sticks. The orientation is the same as on the left. The following PDB IDs (www.rcsb.org) were used for the models: 4XB5 for OCP, 4XB4 for RCP, 5FCX for HCP1, 6MCJ for HCP2 and the small angle scattering biological data bank ID SASDHD for the CCP2 holo-dimer (www.sasbdb.org)

fragments were active in photoprotection (Leverenz et al. 2014). This led to our proposal that the OCP was structurally and functionally modular, composed of two domains, a sensor C-terminal domain (CTD), and an effector N-terminal domain (NTD). Yet the mystery of how the carotenoid was bound exclusively by the NTD remained. This was solved through the use of a combination of structural methods.

The crystal structure of the RCP (Fig. 2) revealed that the carotenoid was almost fully enveloped by the NTD (Leverenz et al. 2015). HDX, SAXS and particularly XFMS (Fig. 1) were required then to show that the carotenoid translocation was triggered by conditions that elicit photoprotection. These solution state structural methods, along with crystal structures, allowed a full description of the protein-carotenoid interactions that change in response to photoactivation. First, the XFMS data revealed a large increase in solvent accessibility at the NTD-CTD interface upon photoactivation of the OCP (Gupta et al. 2015). The largest observed change was at an arginine on the NTD, which forms a salt bridge with a glutamate on the CTD, and increases were also observed at several conserved residues which help stabilize the carotenoid in the interdomain interface. These changes were consistent with SAXS pairwise distribution plots pre- and post- illumination with blue light, indicating a global change from a compact globular shape to an elongated form (Gupta et al. 2015). HDX was also performed as part of the study, and backbone accessibility in the two states was consistent with the retention of secondary structure during photoactivation. Together, this hybrid approach provided compelling evidence that domain separation of the NTD and CTD is one of the structural rearrangements induced by light illumination. Further structural changes upon photoactivation within the carotenoid binding pocket were also determined using XFMS. In the OCP, the carotenoid is hydrogen bonded to two absolutely conserved tyrosine and tryptophan residues in the hydrophobic carotenoid binding pocket of the CTD. These residues were observed to become solvent accessible upon photoactivation, indicating that the hydrogen bonding to the pigment had been disrupted. In addition, solvent accessibility decreased at key residues within the carotenoid binding pocket in the NTD. Together, these reciprocal changes – the increase in solvent accessibility in the CTD carotenoid binding pocket and the decrease in accessibility in the NTD pocket – provided strong evidence that the carotenoid shifts into the NTD during photoactivation, initially spanning both domains, but becoming buried completely in the NTD upon photoactivation. Further time-resolved XFMS studies (Gupta et al. 2019), as well as time-resolved visible and UV spectroscopy (Konold et al. 2019), corroborated these results and showed an order of events during photoactivation Upon absorption of light, carotenoid movement first destabilizes the protein-water network at the major interdomain interface, which leads to full domain dissociation, and is then followed by final structural arrangement of a small N-terminal helix. During relaxation to the inactivated state, the reverse order is observed, though with the addition of a possible intermediate incorrectly folded state, serving to slow the change to fully inactive state in the absence of the Fluorescence Recovery Protein (FRP) (Sutter et al. 2013), which is a non-pigmented soluble protein that in cyanobacteria catalyzes the fast conversion back to the inactivated state. Further structural analysis of the OCP-FRP interactions were undertaken using XFMS to identify the site of interaction between the two proteins (Gupta et al. 2019). Data was consistent with the FRP binding at the CTD interface and bridging both the CTD and NTD, bringing the two domains into proximity and reducing the time for proper re-association of the domains. Previous mutational and fluorescence studies have also yielded structural details that point to the FRP acting as a scaffold for OCP domain association (Sluchanko et al. 2017a; Sluchanko et al. 2017b; Moldenhauer et al. 2018). The FRP may help to align the domains such that the carotenoid can move into the CTD and form hydrogen bonds in the hydrophobic pocket, though further studies remain to be done to completely understand the mechanism by which FRP accelerates back-conversion to the inactivated state.

The temperature dependence of the photoactivation and the relaxation processes of the OCP has been shown to be complex, with some reports indicating a temperature independence of some components of the forward process, and other reports indicating temperature dependence of both processes (Maksimov et al. 2015; Wilson et al. 2008). It is possible that the dense water network in the carotenoid cavity serves to mitigate local conformational fluctuations near the carotenoid due to thermal fluctuations by a mechanism related to that used by thermophilic proteins (Chakraborty et al. 2015; Sterpone et al. 2010; Melchionna et al. 2006), and thus under certain conditions enables the mechanism of photoprotection to be driven by the intensity of light as the single controlling factor. Recently, a new family of OCPs was discovered in cyanobacteria (Bao et al. 2017; Kuznetsova et al. 2020). Dubbed OCP2, these proteins do not require the FRP for accelerated back-conversion to the inactivated state. The proposed evolutionary progression in this family of proteins, from the HCPs and CCPs (Lechno-Yossef et al. 2017) (next section) to the various families of OCP, highlights the increasing control of photoprotection that organisms can evolve through residue changes that affect water networks, protein-protein interactions, and protein-carotenoid interactions.

3 Hybrid Structural Methods in the Study of the HCPs and CCP

The initial crystal structure characterization of the OCP coincided with the advent of the genomic sequencing era. Already at that time (2003), it was evident from the few available cyanobacterial genome sequences that the NTD and CTD of the OCP were encoded as single proteins in some cyanobacterial genomes (Kerfeld et al. 2003). It is now apparent that these proteins are widespread across the phylum Cyanobacteria and that both are single domain, globular water soluble carotenoproteins. The NTD homologs are referred to as Helical Carotenoproteins (HCPs) because of the all alpha-helical structure of the NTD. Based on the primary structure, the HCPs can be subdivided into at least nine evolutionarily distinct clades, each predicted to bind carotenoid (Melnicki et al. 2016). To-date, the crystal structure of HCP1 and HCP2 have been determined, confirming the prediction that the HCPs bind carotenoids in a manner similar to the binding of the carotenoid in RCP (Dominguez-Martin et al. 2019; Khan et al. 2020). We expect that representatives from all nine HCP clades will have a distinctive carotenoid-protein environment that underlies differences in optical properties and function. We also speculate that the functions of the HCPs are only fully evident when they are in complex with other proteins; identifying and structurally characterizing these complexes is an emerging frontier of carotenoprotein research.

Even more enigmatic are the functions of the CTD homologs which are referred to as **C**-terminal domain-like **C**arotenoid **P**roteins (CCPs). Whereas cyanobacterial genomes typically contain multiple HCP paralogs, the CCP is present in only one copy. In 2017 it was shown that the CCPs bind carotenoids (Lechno-Yossef et al. 2017; Moldenhauer et al. 2017) and are visibly intensely blue in color (absorption maximum 558 nm, Fig. 2). Very recently the structure of a CCP (CCP2, which is distinguished from CCP1 by a conserved Cys residue) was determined using SAXS-MALS, providing the first insight into how the carotenoid is bound (Dominguez-Martin et al. 2020). In contrast to the HCPs in which the carotenoid is enveloped by an HCP monomer, CCPs are dimers or higher order oligomers, with the carotenoid spanning the homodimer interface (Fig. 3); accordingly, in contrast to the structure of the OCP and of the HCPs, the two halves of the symmetrical canthaxanthin molecule are in identical protein environments. Analytical SEC and SAXS models indicated that the protein exists as both a dimer and a tetramer in solution, with the dimer the dominant species, and the tetramer forming at higher protein concentrations. The arrangement of monomers relative to each other in the higher oligomeric states was dependent on carotenoid binding. The holo-dimer form was elongated

relative to the apo-form, with a slight separation between the monomers. In the apo-dimer a short C-terminal helix appears to be docked between the two monomers, and this helix is displaced in the holo-dimer form, leaving access to the carotenoid pocket. Further analysis using XFMS was consistent with the carotenoid spanning both monomers symmetrically in the holo-dimer form; comparison of the apo- and holo- forms of CCP for both dimer and tetramer forms revealed the residues directly protected by the presence of carotenoid and lining the binding pocket (Dominguez-Martin et al. 2020). Two of these residues, a tyrosine and tryptophan (Fig. 3), are absolutely conserved in both the CCP and the OCP, and are essential for photoactivation (Kerfeld et al. 2003; Leverenz et al. 2015). In the OCP, carotenoid movement necessarily disrupts hydrogen bonding to these residues within the binding pocket, because the carotenoid moves completely out of the CTD and into the NTD during photoactivation (Leverenz et al. 2015). For CCP binding canthaxanthin, these residues provide hydrogen bonding to the keto ring of the carotenoid, and this hydrogen bonding stabilizes the dimer form of CCP by pinning each end of the carotenoid in each monomer of the dimer. The stabilization of the pigment within the binding pocket also affects the visible light absorption spectrum of the pigment. The extreme red shift in absorption observed in the binding of canthaxanthin to CCP, for instance, might be due at least partially to the hydrogen bonding of the pigment at both ends to these conserved residues, which binds the pigment in an s-trans conformation as it spans the dimer.

In contrast to the dimer form of CCP, the holo-tetramer form appears to exist in an antiparallel arrangement of holo-dimers which interact via an interface which also exists in the apo-tetramer form. The pattern of protection due to carotenoid binding as determined via XFMS was the same in holo- and apo-tetramer forms, indicating that the carotenoid spans the monomers in a specific configuration independent of higher order oligomeric states. Interestingly, in the SAXS models the monomers are oriented such that the single conserved cysteine is in position to form a disulfide bond at the interface, consistent with denaturing gel electrophoresis. Reducing the disulfide bond had no effect on the absorption spectra of the carotenoid, indicating that disulfide bond formation did not alter the carotenoid environment.

The spectral properties of canthaxanthin binding in the CCP dimer are thus explained through these hybrid structural studies, and highlight the importance of understanding the protein-carotenoid environment. Work is currently ongoing to unravel the biological significance of the dimer form, and to understand the biological role of this carotenoprotein.

4 Conclusions and Prospects

Development of water-soluble carotenoproteins with predictable binding selectivity for carotenoids, the potential for tuning spectral and quenching properties via amino acid substitutions, and control over their delivery could provide a broadly useful tool for exploiting the natural functions of carotenoids for human nutrition and therapeutics. Likewise, developing artificial photosynthesis relies on precise structural knowledge of the positioning and orientation of pigment molecules for energy transfer. Our studies suggest that the OCP, with its two distinct spectral forms, evolved from the fusion of two discrete water soluble carotenoproteins, the CCP and HCP, that are proposed to have functioned as a heterodimer (Lechno-Yossef et al. 2017). OCP, HCP and CCP illustrate the profound influence of the protein environment on the diversity of optical and functional properties of carotenoproteins (Fig. 2). Applying a suite of biophysical methods has been essential to understanding the structural basis of this diversity, since subtle structural changes in the protein-carotenoid environment can lead to large changes in light absorption or global protein conformation or dynamics.

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