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Origins of the Intermediate Spectral Form in M100 Mutants of Photoactive Yellow Protein

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Abstract

Numerous single site mutants of photoactive yellow protein (PYP) from *H. halophila* and as well as PYP homologs from other species exhibit a shoulder on the short wavelength side of the absorbance maximum in their dark-adapted states. The structural basis for the occurrence of this shoulder, called the “intermediate spectral form,” has only been investigated in detail for the Y42F mutation. Here we explore the structural basis for occurrence of the intermediate spectral form in a M121E derivative of a circularly permuted *H. halophila* PYP (M121E-cPYP). The M121 site in M121E-cPYP corresponds to the M100 site in wild-type *H. halophila* PYP. High resolution NMR measurements with a salt-tolerant cryoprobe enabled identification of those residues directly affected by increasing concentrations of ammonium chloride, a salt that greatly enhances the fraction of the intermediate spectra form. Residues in the surface loop containing the M121E (M100E) mutation were found to be affected by ammonium chloride as well as a discrete set of residues that link this surface loop to the buried hydroxyl group of the chromophore via a hydrogen bond network. Localized changes in the conformational dynamics of a surface loop can thereby produce structural rearrangements near the buried hydroxyl group chromophore while leaving the large majority of residues in the protein unaffected.

Abstract

Introduction

Photoactive yellow protein (PYP) is a small, water-soluble, blue light sensory protein found in a range of bacterial species (1, 2). Numerous biophysical studies have made PYP a prototype for understanding signaling via conformational changes in the widespread PAS

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SUPPORTING INFORMATION

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superfamily (3-5). As well, PYP can serve a switching module in the design of optogenetic tools (6-9).

The active site of the PYP contains the chromophore *p*-coumaric acid (*p*-hydroxycinnamic acid) in a thioester linkage with Cys69. In the dark-adapted state, high resolution X-ray structures, neutron diffraction studies, Raman, FTIR and NMR studies have shown that the chromophore is ionized and forms H-bonds to two nearby residues, Glu46 and Tyr42, in their the neutral forms at pH 7 (10). The pKa of the chromophore in the denatured protein is 9.0 ± 0.5 (11) and Glu side-chains typically have pKas of 4.3 ± 0.5 (12). Thus, the pKas of the *p*-coumaryl and Glu46 groups are significantly perturbed in the folded dark state of PYP. The ionized form of the chromophore in dark-adapted PYP absorbs maximally near 446 nm, accounting for the yellow color of the protein. Upon absorption of blue light by dark-adapted PYP, a photocycle ensues that produces the *cis* isomer of the chromophore which becomes solvent exposed and protonated. This species absorbs maximally near 350 nm. The isomerization of the chromophore results in substantial changes in protein conformational dynamics (4, 13).

First noticed with a Y42F mutant of PYP, mutations or sequence changes to PYP can sometimes lead to the occurrence of a dark-adapted species with an absorbance between that of *cis*-protonated (350 nm) or *trans*-deprotonated (446 nm) protein (14-16). This species has been termed the “intermediate spectral form”. It has now been observed in a variety of PYP mutants (17-21) as well as PYP homologues from other species (22-25). The position of the absorbance band (~380 nm) suggests a protonated chromophore since denaturation of PYP mutants or wild-type PYP, which causes solvent exposure and protonation of the *trans* chromophore, leads to absorbance near this wavelength (17). Resonance Raman studies by El-Mashtoly *et al*, also indicate that the intermediate spectral form seen with Y42F, E46Q and Y42A mutants of PYP is due to a protonated *p*-coumaric acid chromophore (26).

For the Y42F mutant, the intermediate spectral form appears to arise from a very localized H-bonding rearrangement. Heyn and colleagues studied the photophysical behavior of the Y42F mutant in detail using a variety of techniques (10). They showed that the ionized and neutral forms of the *trans* chromophore coexist in the folded state of dark-adapted PYP, can interconvert easily via an excited state proton transfer reaction, and share the same photocycle. Heyn and colleagues attributed the appearance of a fraction of protonated *trans* chromophore to a strengthening of the hydrogen bond between Glu46 and the *p*-coumaryl moiety, caused by the removal of the Tyr42 H-bond.

It is unclear however, to what extent such localized changes in H-bonding are the reason for other occurrences of the intermediate spectral form. Some mutations that produce an intermediate spectral form are far away from the hydroxyl group of the chromophore. Indeed, a protonated *trans p*-coumaric acid chromophore could arise from any conformational change that altered the specific environment of the wild-type protein since the default state of the chromophore would be the protonated state at neutral pH. Indeed as noted above, the intermediate spectral form is observed when most PYPs are denatured (*e.g.* by adding guanidinium HCl)(17).

Mutations at residue M100 in PYP have been studied in detail since these lead to marked slowing of the photocycle (19-21, 27). Mutations at this site also lead to production of the intermediate spectral form, to varying extents depending on the nature of the mutation (19-21). During the course of protein engineering of optogenetic tools, we prepared an M121E mutant of a circular permutant of PYP (M121E-cPYP) (28). Circularly permuted PYP (cPYP) has an essentially the same dark-state structured as wild-type PYP and undergoes a very similar photocycle (28). M121 in this construct corresponds to M100 in wild-type PYP. We observed that the M121E mutation in cPYP produced a significant fraction of the intermediate spectral form, just as the corresponding M100E mutation does in wild-type PYP. NMR measurements of the protein backbone and CD measurements indicated that the mutation did not cause unfolding or large structural rearrangements (28). The M121E (M100E) site is a significant distance from the hydroxyl group of the chromophore, so we were interested in understanding how it was influencing the protonation state there. In particular, if mutations were causing significant long range changes in side chain packing, dynamics, or in tertiary contacts, it would be important to identify which residues are involved since this may be relevant for engineering coupling to target proteins for optogenetic applications.

Materials and Methods

Protein expression and purification

Expression and purification of M121E-cPYP and cPYP was adapted from the work of Devanathan *et al.* (29) as described previously (28).

UV/Vis spectra and photoisomerization

UV-Vis spectra were obtained using a PE Lambda 35 or 25 spectrophotometer or using a diode array UV-Vis spectrophotometer (Ocean Optics Inc., USB4000), in each case coupled to a temperature controlled cuvette holder (Quantum Northwest, Inc.). Protein concentrations were determined using an extinction coefficient at λ_{max} (~446 nm) of $29 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ M121E-cPYP. Irradiation of the protein sample was carried out by using a Luxeon III Star LED Royal Blue (455 nm) Lambertian operating at approximately 700 mA (~50 mW/cm²)

NMR

Labeled protein samples (¹⁵N-M121E-cPYP, ¹⁵N-cPYP) were prepared as described previously (28). NMR experiments were recorded at CSICOMP (Dept. of Chemistry, University of Toronto). Salt titrations were performed on an Agilent DD2 700 MHz spectrometer equipped with a salt-tolerant HFCN cold probe. All pulse sequences were used as obtained from the Varian 'Biopack' sequence library. HSQC spectra were acquired with 40 increments spanning 1520 Hz in the ¹⁵N dimension. All studies were performed in Tris-acetate EDTA buffer and 100 mM NaCl (pH 7.5) having 0.25, 0.5, 0.75, 1.0, 1.15, 1.35, 1.5 or 2 M (NH₄)₂SO₄ or NH₄Cl at 20°C. Samples were fully dark adapted before HSQC spectra were acquired. Spectra were processed using the NMRPipe (30) processing suite. Typically, FID signals were zero filled to double the original data size and apodized using a squared-cosine window function prior to Fourier transformation. In the indirect

dimension(s), linear prediction was used to double the original data size prior to zero-filling and apodization as above. Samples were referenced in the proton dimension using 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an external standard in solutions prepared in exactly the same manner as the protein containing solutions (*e.g.* 0-2 M ammonium chloride added to tris-acetate EDTA100 mM NaCl) (31). Referencing in the ^{15}N dimension was performed using the ratio of ^{15}N and ^1H gyromagnetic ratios (0.101329118). After referencing, a large subset of peaks appeared essentially unaffected by salt. To correct for small inaccuracies in the referencing process, one of these peaks (Leu 54) was taken to be invariant with salt and the corresponding peak in all spectra were overlaid using the reference-shift spectrum option of NMRViewJ. Assignment of backbone resonances were taken from (28).

Protein Models

The three dimensional structure of wild-type PYP obtained by high resolution X-ray crystallography (1NWZ) was used to create models and examine H-bonding. The Accelrys DSVisualizer suite was employed. Standard protocols for mutating the Met100 site to Glu were employed followed by simulated annealing. H-bonds were visualized using Pymol (Schrödinger Inc.).

Results and Discussion

An examination of the dark state UV-Vis spectrum of M121E-cPYP in Tris-acetate buffer at pH 7.0 (Fig. 2a) compared to cPYP or wild-type PYP (Fig. 2a) reveals the presence of the intermediate spectral form as a shoulder near ~380 nm in addition to the main absorbance band at 446 nm. As noted above, this shoulder has been observed previously in M100E, M100A, M100L mutants of wild-type PYP (19-21). The M121E-cPYP protein undergoes a functional photocycle, *i.e.* the changes typical of trans-cis isomerization and the production of the cis-pronated state are observed upon blue light irradiation (Fig. 2b) (27).

The presence of the intermediate spectral form is not sensitive to the pH of the medium between pH 5 and pH 9 (28) indicating the protonation/deprotonation of the chromophore must not involve proton transfer to solvent but proton transfer reactions within the protein itself. The same is true for the well-studied intermediate spectral form in the Y42F mutant (10).

The ratio of protonated to anionic chromophore is affected by temperature (28) and also by altering the ionic environment (15-18). The chaotropic salt ammonium chloride significantly stabilizes the protonated form of the chromophore; at 2 M NH_4Cl , the protonated form predominates (fig. 2c). In contrast, the kosmotropic (order-making) salt ammonium sulfate stabilizes the anionic form slightly (Fig. 2d). Under all salt conditions, the protein appears capable of undergoing a blue-light triggered photocycle (Fig. 2b,c,d).

The ability to alter the fraction of the intermediate spectral form by changing the salt type and concentration, the recent development of salt-tolerant cryoprobes for NMR, together with the availability of backbone NMR assignments for the M121E-cPYP protein provided an opportunity to probe the residue specific changes underlying occurrence of this species.

We therefore recorded NH-HSQC spectra of M121E-cPYP as a function of salt concentration for both ammonium chloride and ammonium sulfate. Variation in salt concentration is expected to influence chemical shifts by altering the bulk magnetic susceptibility of the solvent, the screening of electrostatic interactions, the van der Waals forces between the solute and solvent, and the extent of hydrogen bond formation between solvent and solute molecules (32, 33). These effects may alter protein dynamics, rates of solvent exchange, pK_as of ionizable groups, and may cause protein conformational changes. However, we expected that signals that changed with ammonium chloride selectively, and not with ammonium sulfate, could be related to changes underlying the production of the intermediate spectral form.

Figure 3 shows spectra of dark-adapted M121E-cPYP at increasing concentrations of either ammonium chloride (left) or ammonium sulfate (right). With both salts, the large majority of the resonances are unaffected by salt, when referenced to DSS (see Methods section). Several peaks are affected by both salts as might be expected, for instance, from a solvent exposed residue whose ionization or H-bonding was affected by ionic strength. For each peak that was affected by ammonium chloride, we also checked whether it was affected in the non-mutated cPYP (*i.e.* M121-cPYP) (see Supplementary Information), for which the UV-Vis spectrum is unaffected by ammonium chloride. A specific subset of resonances behaves in an obviously different manner in the ammonium chloride spectra of M121E-cPYP compared to the ammonium sulfate spectra of M121E-cPYP (Fig. 3). These resonances also behave differently in the M121E-cPYP versus the M121-cPYP spectra (Fig. S1). This subset of resonances is boxed in Figure 3 and close-up views are provided in Figure 4. Corresponding residue numbers in wild-type PYP are shown in brackets.

Figure 5 shows a model of M121E-cPYP with those NH chemical shifts that are affected selectively by ammonium chloride indicated as cyan-colored spheres. Affected residues fall into two groups: (i) residues in the loop containing the M121E mutation site and (ii) residues near the ionizable end of the chromophore (E67(46), Y63(42), G50(29)) (wild-type numbering in brackets). These groups are linked by interactions between T71(50), R73(52) and E121(100) as described below.

Ammonium chloride appears to selectively affect the conformational dynamics of the loop containing the M121E (M100E) mutation. Whereas in ammonium sulfate, the E121(100) peak (Fig. 4, panel (a)) and the Q120(99) peak (not shown) are of reduced intensity suggesting enhanced solvent exchange, other residues in the loop (117(96), 119(98), 122(101) (residue 118(97) could not be assigned) are virtually unaffected. In contrast, these peaks undergo large shifts with increasing ammonium chloride concentrations (Fig. 4 panels (b,c,d)). The corresponding residues occur at different chemical shifts in the unmutated M121(100)-cPYP spectra, as expected, but are not affected by changes in ammonium chloride concentration.

Altered conformational dynamics of the M121E (M100E) loop also affect residue C90(69), the residue that makes a thioester linkage to the chromophore (Fig. 4 panel (a)). Movement of C90(69) may then be transmitted to movement at the hydroxyl end of the chromophore, directly affecting the hydrogen bonds between that group and residues E67(46) and Y63(42)

(Fig. 4, panels (e,b). Movement of the loop containing the M121E (M100E) mutation can also be transmitted to the residues interacting with the chromophore hydroxyl group through a network of hydrogen bonds involving residues R73(52) and T71(50). The high resolution X-ray structure of wild-type photoactive yellow protein enables one to trace these H-bond networks in detail (34). A model for how the H-bonds may be affected by the M121E (M100E) mutation was created using the wild-type structure as a starting point, creating the point mutant, and carrying out a standard simulated annealing protocol implemented in Accelrys software using the CHARMM forcefield. Models showing H-bond networks that link the surface loop to the chromophore hydroxyl group in wild-type and M121E (M100E) are shown in Figure 6. The residue Thr71(50) appears to play a central role in this network, as it does in the production of the intermediate spectral form in the Y42F mutant (10).

Specifically, as noted above (Fig. 1), H-bonds connect the hydroxyl group of the chromophore with the side chains of Tyr63(42) and Glu67(46). The Tyr63(42) side chain hydroxyl group is also H-bonded to the side chain of Thr71(50), as is the backbone carbonyl oxygen of Glu67(46). The backbone carbonyl oxygen of Glu67(46) also H-bonds to the backbone NH of Thr71(50) (which is markedly affected by ammonium chloride, Fig. 4, panel (g)). The backbone carbonyl oxygen of Thr71(50) makes an H-bond to the backbone NH of Arg73(52) (which is also markedly affected, Fig. 4, panel (c)) as well as to the side chain guanidinium group of Arg73(52). Whereas in the wild-type protein the side chain of Arg73(52) cannot H-bond to Met121(100), and instead interacts with the backbone carbonyl oxygen atom of Tyr98, when Met121(100) is mutated to Glu121(100), the Arg73(52) side chain can make two new H-bonds to this Glu side chain. This new H-bond pattern could alter the strength and persistence of H-bonding in the local network, and thereby alter the protonation state of the chromophore. While the present data do not provide direct data on the protonation state of Arg73(52), they imply the residue exists in solution in its protonated form rather than its neutral form as has been observed in neutron diffraction studies of wild-type PYP (35). We note that Arg73(52) has been proposed previously to play important roles in PYP including as a key mediator of chromophore electronic transitions (36), a mediator of chromophore isomerization (37), and as a gateway for conformational change in signaling (38). Repositioning on Arg73(52) may also alter water access to the chromophore (37). A bound water molecule is seen H-bonded to Arg52 in wild-type PYP (34).

In summary, these data indicate that a specific subset of resonances can transmit altered conformational dynamics of a surface loop to key residues surrounding the hydroxyl group of the chromophore while leaving most regions of the protein unaffected. It is likely that other mutations at the M121(100) site would lead to production of the intermediate spectral form through a similar subset of residues although the details (*e.g.* interactions between the E121(100) and R73(52) side chains) will be different. It is interesting that the changes in conformational dynamics created by the M121E (M100E) mutation, although apparently more extensive than those caused by the Y42F mutation, do not alter the specific environment of the chromophore hydroxyl group enough prevent the protein undergoing a functional photocycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Key residues and hydrogen bonds at the active site of photoactive yellow protein.

Figure 2.

(a) UV-Vis spectrum of cPYP (solid line) and M121E-cPYP (dashed line) in the dark-adapted state. (pH 7.5 Tris-acetate EDTA buffer, 100 mM NaCl) (b) M121E-cPYP (pH 7.5 Tris-acetate EDTA buffer, 100 mM NaCl) in the dark-adapted state (solid line), immediately following irradiation with blue light (dashed line) and then at 2 min intervals during recovery in the dark (gray lines). (c) As in (b) but with the addition of 2 M ammonium chloride. (d) As in (b) but with the addition of 2 M ammonium sulfate.

Figure 3. (Left) ^{15}NH HSQC spectra of M121E-cPYP (pH 7.5 Tris-acetate EDTA buffer, 100 mM NaCl) in the dark-adapted state (black), with increasing concentrations of ammonium chloride (0, 0.25, 0.5, 0.75, 1.0, 1.15, 1.35, 1.5 or 2 M). (Right) with ammonium sulfate instead of ammonium chloride. Resonances that are uniquely affected by ammonium chloride case are boxed and labeled (with wild-type numbering in brackets).

Figure 4.

Close-up views of those resonances that are boxed in Fig. 3. (Left) with increasing concentrations of ammonium chloride from 0 to 2 M. (Middle) with increasing concentrations of ammonium sulfate. (Right) the corresponding region in M121-cPYP with increasing concentrations of ammonium chloride. Residue numbering in wild-type PYP is shown in brackets for those residues that are affected by salt.

Figure 5.

Model of the M121E-cPYP protein showing NH groups that are selectively affected by ammonium chloride as cyan spheres. The chromophore is shown in yellow sticks. The model is based on the structure of wild-type PYP (1NWZ) (28). The mutated side chain (E121) is shown in stick format as well as R73(52), with which it interacts.

Figure 6.

Models of the hydrogen bond networks in (a) wild-type PYP and (b) M121E (M100E) mutants. H-bonds (black dotted lines) connect the chromophore hydroxyl group to T71(50) and from there to R73(52). The side chain of R73(52) makes new H-bonds when M121(100) is mutated to E121(100). The new H-bonds affect H-bond strength and detailed positioning of T71(50), and thereby of Y63(42) and E67(46). Only one rotamer of R73(52) is shown here, but simulations suggest other rotamers are possible.