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A Single CH/π Weak Hydrogen Bond Governs Stability and the Photocycle of the Photoactive Yellow Protein

Miki Harigai, Mikio Kataoka, and Yasushi Imamoto*

Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma Nara 630-0192, Japan

Received March 28, 2006; E-mail: imamoto@ms.naist.jp

Loss of the single CH/π weak hydrogen bond causes substantial alteration of the stability and photocycle of the photoactive yellow protein (PYP), which is a clear example that a CH/π weak hydrogen bond has a specific role in the proteins. Focusing on the phenyl ring of Phe6 adjacent to the alkyl chain of Lys123, characterization of the mutants for these amino acid residues demonstrated that lacking a π-electron at position 6 or an alkyl chain at position 123 substantially decreases stability and slows the photocycle.

PYP is a blue light sensor (absorption maximum, 446 nm) of a purple photosynthetic bacterium, *Halorhodospira halophila*.[1] As its high-resolution tertiary structure is available,[2,3] PYP has been studied in detail based on its three-dimensional structure. PYP is a structural prototype for the PAS domain superfamily that contains many proteins that function as various sensors in a wide range of organisms from humans to bacteria.[4] A thioester-linked *p*-coumaric acid chromophore of PYP is photoisomerized from trans to cis form. Subsequently, several intermediates are formed, then revert to the dark state over a 100 ms time scale.[5] The putative active intermediate (signaling state) in the photocycle is a long-lived near-UV intermediate (PYP_M, it is also called I_2 or pB) that has a partially denatured structure as the result of substantial protein conformational change.[6,7]

We have previously reported that PYP with the N-terminal six amino acid residues (Met-Glu-His-Val-Ala-Phe) truncated (T6) has a 100 times slower photocycle than that of intact PYP.[8] This is not accounted for by the overall structural disorder as this truncation does not markedly alter the structure of other parts of the protein.[6,9] X-ray crystallography demonstrated that the only strong hydrogen bond involving an N-terminal amino acid residue is present between the peptide nitrogen of Glu2 and the peptide oxygen of Gly25.[2] However, truncation of the N-terminal three residues showed no marked effect on the decay of PYP_M (data not shown). Thus, candidates for the key residue(s) of the photocycle are Val4, Ala5, and Phe6. Val4 and Ala5 are located on the surface of PYP; however, Phe6 projects inward and its phenyl ring is adjacent to the alkyl chain of Lys123 (Figure 1), suggesting there is interaction between these residues, although interaction between the side chains of Phe and Lys is likely to be weak.

To evaluate the interaction between Phe6 and Lys123, site-directed mutants were prepared and characterized. Positively charged Lys123 was replaced by small-sized Ala (K123A), negatively charged Glu (K123E), or hydrophobic Leu (K123L). Aromatic Phe6 was replaced by small-sized Ala (F6A), negatively charged and ionic-bondable Asp (F6D), imidazole-containing His (F6H), hydrophobic Leu (F6L), or aromatic Tyr (F6Y).

No pigment was yielded from the K123A apoprotein, indicating that this small side chain does not serve as a partner for Phe6. The loss of volume at position 123 and possible penetration of the water would substantially alter the chromophore binding pocket. Other mutants were reconstituted into yellow proteins with absorption maxima at 445−447 nm (see Supporting Information), strongly suggesting that their structures are comparable to wild-type. These mutants were characterized using flash photolysis, Fourier transform infrared (FTIR) spectroscopy, and thermal denaturation.

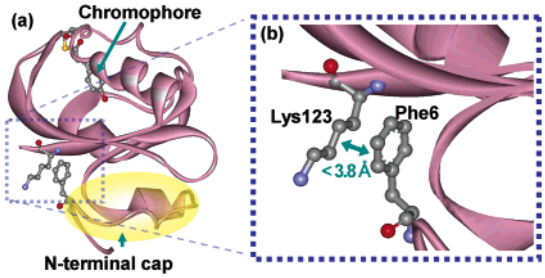

Figure 1. Spatial relationship between Phe6 and Lys123. (a) Overall structure of wild-type PYP (WT) in the dark (1nwz).[10] (b) Expanded view. Distance between the alkyl carbon of Lys123 and the phenyl carbon of Phe6 is indicated.

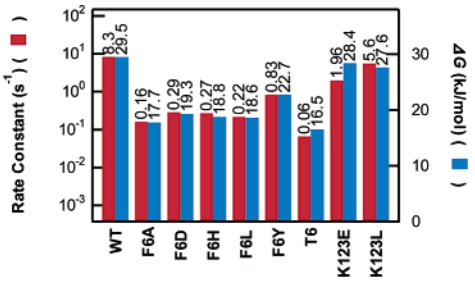

Figure 2. The photocycle rate constant and thermal stability. The decay rate constants for the M-intermediates and ΔG for the dark states were measured in 10 mM TAPS buffer at pH 8.0. The values at 20 °C are presented. No pigment was yielded from the K123A apoprotein.

Transient spectroscopy after flash excitation demonstrated that the decay rate constant for the M-intermediate formed from F6A ($F6A_M$) was 0.16 s^{-1}. The effect of substitution of Ala for Phe6 was comparable to that of the six amino acid truncation (T6), showing that loss of the phenyl ring of Phe6 is the main factor in the stabilization of $T6_M$ (Figure 2). For other Phe6 mutants, decay rate constant of M-intermediates was also significantly smaller than that of wild-type PYP_M (WT_M), indicating that Phe is optimal for position 6. However, the rate constant of $F6Y_M$ was the closest to WT_M. Absorption maxima of M-intermediates of Phe6 mutants except $F6Y_M$ were 1−3 nm red-shifted from WT_M (Supporting Information). $K123E_M$ and $K123L_M$ showed absorption maxima and rate constants similar to those of WT_M, despite the electrostatic properties at position 123.

Light-induced structural changes were studied using FTIR spectroscopy (Figure 3). Difference FTIR spectra in amide I and II regions, assigned to vibrational modes of peptide bonds, indicate conformational change of the protein. Although the site-specific information has not been available, the protein structural change is evaluated by comparison of the spectral shape. While the difference

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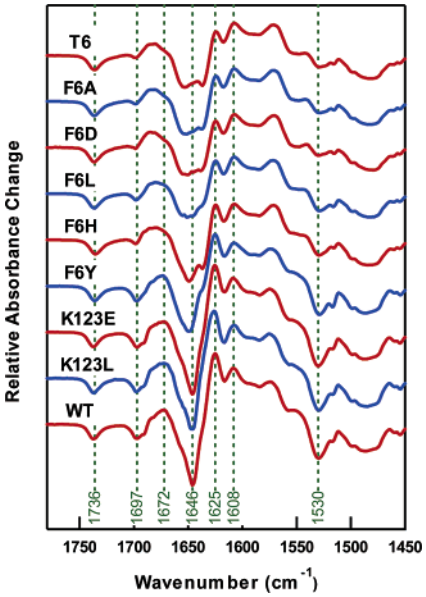

Figure 3. Light-induced difference FTIR spectra measured at pH 8.0 [M-intermediates (positive) *minus* dark state (negative) spectra].

in UV−visible spectra of M-intermediates among mutants was small, clear difference was observed in FTIR spectroscopy. The difference FTIR spectrum for WT_M/WT is characterized by a single intense negative band at 1646 cm^{-1}, a positive 1625 cm^{-1} band greater than the 1608 cm^{-1} band, and clear negative bands at 1697 and 1530 cm^{-1} (Figure 3). In this respect, difference FTIR spectra for F6Y, K123E, and K123L agree with WT, while the corresponding bands for T6, F6A, F6D, and F6L have reduced intensity. F6H has intermediate properties between WT and T6.

Stability of these mutants in the dark state was then studied using thermal denaturation experiments (Figure 2). Thermal bleaching was monitored by absorbance at 456 nm (the isosbestic point of thermal broadening for WT spectra from 20 to 90 °C). Free energy of bleaching (ΔG) at 20 °C was estimated by extrapolating the plot of $\ln[B]/[N] = -\Delta G/RT$ versus $1/T$ to 20 °C, where R is the gas constant, T is temperature, and [B] and [N] are the fractions of bleached and native (yellow) states at T, respectively (data not shown). Rank order of stability evaluated by ΔG was WT > K123E > K123L > F6Y > other mutants.

The present results demonstrate that the best substitute for Phe6 is Tyr. Altered properties of F6L suggest that the requirement for position 6 is not hydrophobicity. As van der Waals volume of Phe (135 $Å^3$) is between Tyr (141 $Å^3$) and Leu (124 $Å^3$) and the bulkiness of Leu is greater than Phe or Tyr,[11] the effect of mutation is not simply explained by the size of side chain. Asp6 in F6D would form a salt bridge with Lys123, but this strong bond is not appropriate for PYP. Glu and Leu can similarly substitute for Lys123, but Ala does not, showing that the presence of an alkyl chain rather than the electrostatic properties of the end of the side chain is essential for position 123. Thus the interaction between π-electrons at position 6 and the alkyl chain at position 123 governs the stability and photocycle of PYP.

Since the energy of a CH/π hydrogen bond is very weak (2−8 kJ/mol),[12] this bond seems to be a less important interaction than the conventional OH/O or NH/O hydrogen bond. However, recent progress in theoretical and experimental studies has shown the importance of weak hydrogen bonds in proteins.[13] The difference in ΔG between F6L and WT is 11 kJ/mol, greater than the energy for a single CH/π hydrogen bond. Thus other interaction would be simultaneously disrupted when the CH/π hydrogen bond between positions 6 and 123 is lost. While F6Y possesses a π-electron system, ΔG for F6Y is 7 kJ/mol smaller than WT, which would be caused by the possible interaction between phenolic oxygen of Tyr6 and the β-sheet.

Although this study clearly demonstrates that a single CH/π weak hydrogen bond can be an essential intramolecular interaction for PYP, the distance between $C_{\delta 2}$ of Phe6 and C_ϵ of Lys123 in the PYP crystal (PDB 1nwz)[10] is 3.78 Å (Figure 1), greater than the reported value for the CH/π hydrogen bond (∼3.5 Å). However, 26 NMR structures deposited in the PDB (3phy)[3] suggest that the spatial relationship between Phe6 and Lys123 fluctuates, and the distance between the phenyl carbon of Phe6 and the alkyl carbon of Lys123 is possibly very close (∼3 Å). Importantly, the weak hydrogen bond between positions 6 and 123 cannot be substituted by a tight salt bridge (F6D) or hydrophobic collapse (F6L). Therefore, a weak but flexible attraction would be required for the reversible conformational change observed in a photocycle. The weak hydrogen bond would have a specific role that cannot be performed by any other interaction.

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Supporting Information Available: Absorption spectra and transient difference spectra of mutants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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