Sensitive Circular Dichroism Marker for the Chromophore Environment of Photoactive Yellow Protein: Assignment of the 307 and 318 nm Bands to the $n \rightarrow \pi^*$ Transition of the Carbonyl

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The absorption and CD spectra of wild-type PYP, apo-PYP, and the mutants, E46Q and M100A, were measured between 250 and 550 nm. At neutral pH, the two very weak absorption bands of wild-type PYP at 307 and 318 nm ($\epsilon_{max} = 600 \pm 100$ M^{-1} cm^{-1} at 318 nm) are associated with quite strong positive CD bands ($\Delta\epsilon_{max} \approx 6.8$ M^{-1} cm^{-1}). Both sets of bands are absent in the apoprotein. On the basis of this evidence, we assign these optical signals to the $n \rightarrow \pi^*$ transition of the oxygen of the carbonyl group of the 4-hydroxycinnamic acid chromophore, which is expected to be electric dipole forbidden but magnetic dipole allowed. The progression of narrow bands at 307 and 318 nm with a shoulder in the CD around 329 nm is due to vibrational fine structure with a frequency of about 1050 ± 50 cm^{-1}. This is the carbonyl stretch frequency in the electronically excited state and is well-known from the vibrational structure in the CD spectra of carbonyl compounds. The positive sign of the CD in the near UV is in accordance with the octant rule and the high-resolution X-ray structure, if we assume that the NH group of cysteine 69 to which the carbonyl is hydrogen bonded is the principle perturbant. Similar absorption and CD spectra were observed in the range of 300−340 nm for the mutants E46Q and M100A at neutral pH. Protonation of the trans chromophore by lowering the pH in the dark (without photoisomerization) broadens the 307 and 318 nm CD bands in the mutant E46Q but does not significantly affect their positions or alter their sign. For the long-lived I_2 photointermediate of the mutant M100A with protonated cis chromophore, we observed that the sign of the rotational strength in the 310−320 nm range is negative (i.e., opposite to that in the dark state with trans chromophore). This suggests that the light-induced isomerization of the chromophore, which leads to breaking of the hydrogen bond with the backbone amide of C69, brings the carbonyl into a new protein environment with different asymmetry than in the unbleached protein. The observed change in sign is mainly due to this effect, but a change in chromophore twist may also contribute. Thus, the 318 nm CD signal is a sensitive marker for the environment of the chromophore carbonyl, which samples various environments and configurations during the photocycle.

Introduction

Photoactive yellow protein is a bacterial photoreceptor from *Halorhodospira halophila* that is the prototype of the large class of PAS-domain signal proteins (for reviews, see refs 1 and 2). The initial event of light absorption by the 4-hydroxy cinnamic acid chromophore is followed by rapid isomerization around the $C_7{=}C_8$ double bond.[3] The chromophore is covalently linked to the protein backbone via a thioester linkage with cysteine 69. In the dark, the chromophore is deprotonated at neutral pH and anchored in the binding pocket by three hydrogen bonds.[4] The hydroxy group of the chromophore is hydrogen bonded to tyrosine 42 and glutamate 46.[4] Following light-induced isomerization, the chromophore passes through a number of intermediates before returning to the initial dark state. The longest-lived of these intermediates, I_2, is believed to be the signaling state[1,2] and has a protonated chromophore.[1,2] Protonation of the chromophore occurs either intramolecularly from E46[5] or from the aqueous medium.[6−8] At least in the mutant E46Q, chromophore protonation from the aqueous medium has been established.[8] The third hydrogen bond in the dark state is between the chromophore carbonyl and the backbone NH group of cysteine 69. It has been shown that it is this carbonyl that flips over upon isomerization.[9] In this way, the hydrogen bonds with Y42 and E46 are initially preserved, whereas the hydrogen bond with the protein backbone is broken. Isomerization thus brings the chromophore carbonyl into a different environment in the early intermediate I_1 as well as in the late intermediate I_2 in which the chromophore is exposed to the protein surface. In this paper, we assign the optical transitions in absorption and CD of PYP at 306 and 318 nm to the $n \rightarrow \pi^*$ transition of the chromophore carbonyl. It is well-known that the sign and magnitude of carbonyl CD are very sensitive to the position of perturbing groups in its vicinity.[10−15] Thus, we identified a useful localized marker band that can be used to monitor the carbonyl environment during the photocycle.

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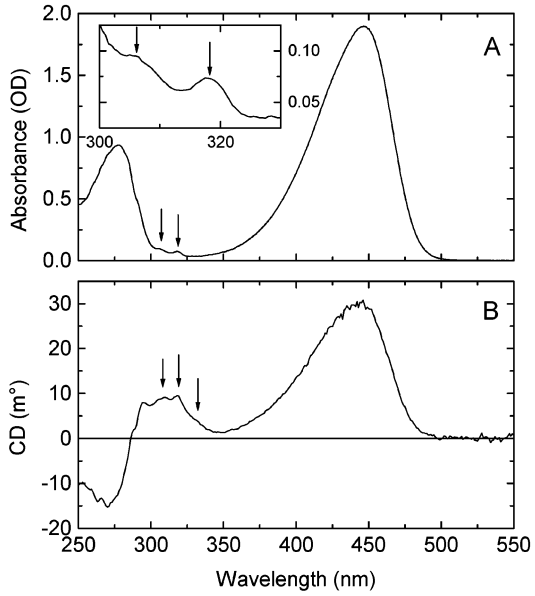

Figure 1. Absorption (A) and CD (B) spectra of wild-type PYP from 250 to 550 nm. The weak absorption bands at 318 and 307 nm are displayed on an expanded scale in the inset of (A) and marked by arrows. In panel B, arrows point to transitions at 307, 318, and 329 nm. Conditions: pH 6.9, 50 mM Tris, 50 mM KCl, room temperature.

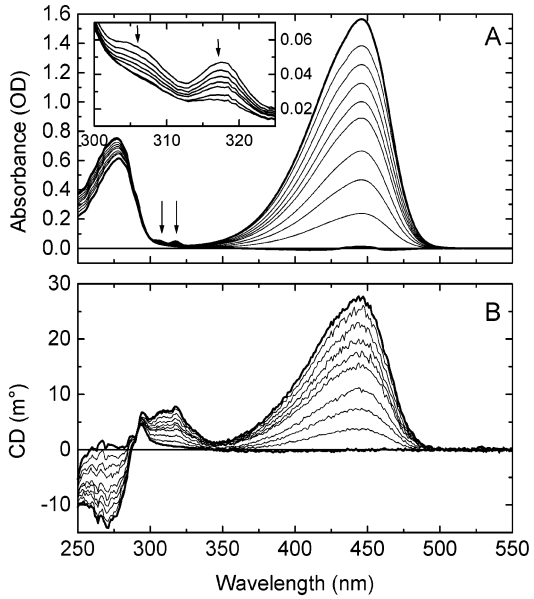

Figure 2. Time course of chromophore removal monitored by absorption (A) and CD (B). The bleaching reaction was initiated at time $t = 0$ by adding 100 mM hydroxylamine. With decreasing amplitude the spectra were taken 12, 32, 52, 72, 92, 137, 192, and 297 min after the start of the reaction (thin lines). The spectra of the initial unbleached state and the completely bleached state (thick lines) were determined by extrapolation. Inset of A: bleaching of the 307 and 318 nm bands. Conditions: 10 mM Tris, pH 7.4, 200 mM NaCl, 1 mM DTT, 20 °C.

Materials and Methods

Wild-type PYP and the mutants E46Q and M100A were prepared as described.[16,17] Apo-PYP was prepared according to refs 18 and 19. Absorption spectra were measured with a Shimadzu UV-260 or UV-2102 PC spectrophotometer. CD spectra were collected with a Jasco 500A instrument modified and updated with a homemade data acquisition system.[20] All spectra were taken at room temperature. The spectral bandwidth in the CD measurements was 1 or 2 nm.

Results

The absorption and CD spectra of wild-type PYP above 250 nm are shown in parts A and B of Figure 1, respectively. The strong bands around 446 nm in absorbance and CD are due to the lowest $\pi \rightarrow \pi^*$ transition of the PYP chromophore. The absorption and CD between 250 and 300 nm is principally due to the aromatic amino acid side chains of PYP (W119 and five tyrosines) with a possible contribution from higher energy transitions of the chromophore. Notable are the two very weak absorption bands around 307 and 318 nm (inset of Figure 1A), which have disproportionally strong positive counterparts in the CD spectrum. It is reasonable to assume that the two absorption bands with λ_{max} values of 307 and 318 nm are associated with the corresponding CD bands centered at 306 and 317 nm. Since these bands are absent in the absorption and CD spectra of the apoprotein (Figure 2A,B), they are due to the chromophore. The time course of the chromophore removal by hydroxylamine is shown in the absorption and CD spectra of Figure 2. The inset of Figure 2A shows on an expanded scale that the absorbance at 307 and 318 nm decreases in parallel with the decrease of the chromophore band at 446 nm. We note that there is also a significant drop in the absorbance at 280 nm, which may be due to the chromophore, to the aromatic amino acids, or both. The corresponding time course of the CD spectra (Figure 2B) indicates that in apo PYP the visible and near UV CD vanishes except for the peak at 295 nm, which is reduced to approximately half its amplitude. Presumably, the positive CD band at 295 nm is due to W119. The fact that its amplitude differs between apo and holo PYP in the 295 nm region may be due to a loss of chromophore contribution or to a change in the tryptophan conformation. Using the extinction coefficient of 45 500 M^{-1} cm^{-1} at the maximum of the 446 nm band,[1] we estimate extinction coefficients of 600 ± 100 and 250 ± 50 M^{-1} cm^{-1} for the 318 and 307 nm bands, respectively. Typically, very weak absorption in the range of 280−320 nm, in association with very strong CD, is the signature of the $n \rightarrow \pi^*$ transition of a carbonyl oxygen.[11−13] For an isolated planar carbonyl group, the $n \rightarrow \pi^*$ transition of the lone pair electron on O is electric dipole forbidden, with the small observed absorbance due to vibronic coupling.[21] We note that the CD spectrum has also a clear shoulder at around 329 nm. This suggests that we are dealing here with vibrational fine structure in absorption and CD with a spacing of about 10.5 nm. This splitting corresponds to a vibrational frequency of $\bar{\nu} \approx 1050$ cm^{-1}.

In the $n \rightarrow \pi^*$ transition, the lone pair p-orbital is rotated by 90° to the π^* state. This large charge circulation leads to a very large magnetic transition dipole moment $\vec{m}_{mag}$ parallel to the C═O bond direction. Since the rotational strength R is given by $R = Im(\vec{\mu}_{el} \cdot \vec{m}_{mag})$, the large rotational strength observed requires in addition to a large $\vec{m}_{mag}$ a sufficiently large electric transition dipole moment $\vec{\mu}_{el}$ in the same direction. Together, these two transition dipole moments produce a helical charge motion. Various mechanisms have been proposed for the generation of $\vec{\mu}_{el}$, such as symmetry breaking or an admixture of a small contribution from the π-state.[11−14] The CD of the $n \rightarrow \pi^*$ transitions of carbonyls is well-understood,[11−13] and the sign of the rotational strength is very sensitive to the local environment and the positions of the perturbing groups. Sector rules (octant rule) for the sign of carbonyl CD have been successfully used to answer structural questions in organic chemistry.[10,13,15] The structure of the dark state of PYP is known with a resolution of 0.82 Å and shows that the thioester carbonyl

is hydrogen-bonded to the backbone amino group of cysteine 69.[4] The direction of the hydrogen bond is clearly not coaxial with the carbonyl bond and is also out of the C═O plane. On the basis of its proximity to the carbonyl oxygen, it is reasonable to assume that the NH group is the strongest perturbant. Using the known coordinates of the NH group with respect to the C═O group (RCSB Protein Data Bank 1NWZ), we calculated the sign of the carbonyl CD according to the octant rule. In agreement with the experiment, we found that the sign is positive. It is reasonable to expect that the sign and magnitude of the CD of the PYP chromophore carbonyl differ in its various photocycle intermediates as the carbonyl position changes.

To test this hypothesis, we altered the carbonyl environment using photoinduced isomerization. From FTIR[9] and high-resolution X-ray diffraction,[22−24] it is known that the light-induced isomerization around the C_7═C_8 double bond is associated with a 180° flip of the carbonyl leaving the hydrogen bonds of the phenolic O^- with E46 and Y42 initially (up to and including the I_1 intermediate) intact. Thus, the hydrogen bond of the carbonyl with the backbone amide of C69 is broken, and the carbonyl is in a different environment in I_1[22] as well as in the longest lived signaling state I_2.[23] To carry out a CD measurement on the I_2 intermediate, we used the mutant M100A in which this intermediate has a lifetime of approximately 30 min at pH 6.15 and room temperature.[17] The absorption and CD spectra of M100A in the dark and immediately after bleaching are shown in Figure 3A,B. We note that the absorption spectrum of M100A in the dark has a significant shoulder on the blue side of the main 446 nm band, which is absent in wild-type (Figure 1A). A similar shoulder was observed for the mutant M100L and was shown to be due to a species with protonated trans chromophore (350 nm) which is in a temperature-dependent equilibrium with the major species with deprotonated chromophore.[25] In the corresponding CD spectrum (Figure 3B), we observe in the same wavelength range (330−370 nm) a positive contribution that is also absent in wild-type and is evidently associated with the species absorbing at 350 nm. This interpretation of the additional CD in the 330−370 nm region (with respect to wild-type) is supported by our results with the E46Q mutant (Figure 4) and is discussed in the next paragraph. In the region 250−330 nm, the absorption and CD spectra of M100A in the dark are quite similar to those of wild-type. The small absorption bands around 307 and 318 nm are associated with corresponding positive CD bands. Following illumination, the absorption at 450 nm is very small with a corresponding increase in the 350 nm absorbance due to the formation of the I_2 intermediate. In addition, there is a clear decrease in absorbance around 280 nm, suggesting a chromophore contribution in this wavelength range. The CD spectrum taken immediately after bleaching shows a strong negative CD band around 318 nm. Thermal relaxation or photoreversal with UV light result in the return to the dark spectrum with a positive CD band centered at 318 nm. The sign reversal of the CD in the 318 nm band is a direct manifestation of the change in environment of the chromophore carbonyl induced by the isomerization. This light-induced change in CD between the dark and I_2 state was reversible.

In the I_2 intermediate, the chromophore is not only isomerized but also protonated; thus, the observed change could be due to protonation. As a control experiment, we analyzed PYP in which the chromophore was protonated but not isomerized. For these purposes, we used the mutant E46Q. The structure of this mutant was recently determined.[26] The chromophore is still hydrogen-bonded to Q46, Y42, and the amide group of C69 but is

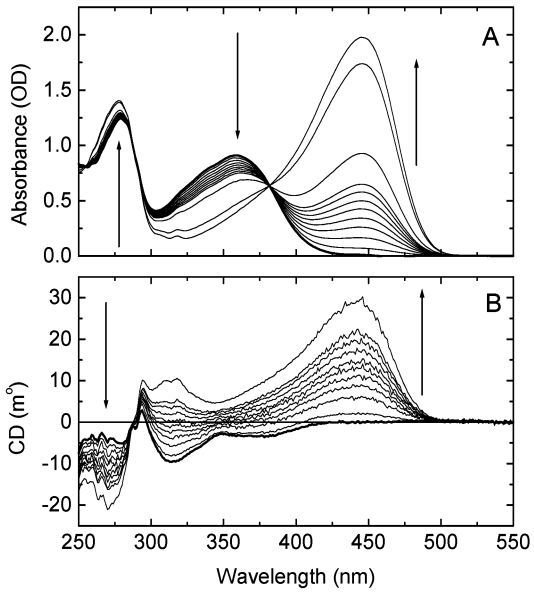

Figure 3. Absorption (A) and CD (B) spectra of the mutant M100A from 250 to 550 nm in the dark and after illumination. Conditions: pH 6.1, 18 mM phosphate buffer, 1.2 mM Tris, 40 mM KCl. The cuvette was illuminated for 2 min with a Schott KL1500 lamp with BG 37 and GG 435 filters. The arrows indicate the progression of the spectral changes in the recovery of the dark state. (A) Twelve spectra in the recovery sequence after illumination at room temperature. Completely bleached state (spectrum with zero absorbance at 450 nm) obtained by extrapolation from the time sequence. Spectra with increasing absorbance at 450 nm: 1, 2, 3, 4, 5, 6, 7, 8, 13, and 48 min after the bleach. Initial dark spectrum: spectrum with highest absorbance at 450 nm. (B) Eleven CD spectra in the recovery sequence after illumination at 10 °C. Completely bleached state (spectrum with zero CD at 450 nm) obtained by extrapolation from time sequence. Spectra with increasing CD at 450 nm: 5, 15, 25, 35, 45, 55, 70, 90, 110 min after the bleach. Spectrum with highest CD at 450 nm: initial dark spectrum.

protonated in the dark, without isomerization, at pH values below the pK_a of 4.8.[8,27] Figure 4A,B shows the absorption and CD spectra above and below this pK_a. Also shown are the spectra for the completely bleached form P_{bl} with protonated trans chromophore, obtained by extrapolation. The data of Figure 4 show that the pH changes in absorbance and CD were reversible. At pH 7.45 (deprotonated chromophore), the absorption spectrum has two weak peaks at 309 and 320 nm, as in wild-type but redshifted by approximately 2 nm. The CD spectrum shows the corresponding positive bands and is quite similar to that of the wild-type dark state. This suggests that in the dark state of E46Q with deprotonated chromophore, it is anchored in its binding pocket as in wild-type. This is in accordance with the results from X-ray diffraction.[26] From the absorption spectrum, it is clear that, at the lowest pH value of 4.6, the protein is mainly in the bleached state absorbing around 350 nm with protonated chromophore. The CD spectra in Figure 4B show that with decreasing pH the well-resolved peaks between 295 and 320 nm are progressively broadened but with sign remaining positive. At the same time, a positive CD band develops at 350 nm, which is apparently due to the absorbance band at 350 nm from the protonated chromophore. If we assume that upon protonation, without isomerization, the chromophore remains anchored in the binding pocket as in the deprotonated state, these observations can be readily explained. The hydrogen bond of the carbonyl remains intact, and its environment is unaltered. Thus, the sign of the CD in the 295−320 nm range is unaltered. Since the environment of the rest of the chromophore is also unchanged, the positive CD from the 462 nm

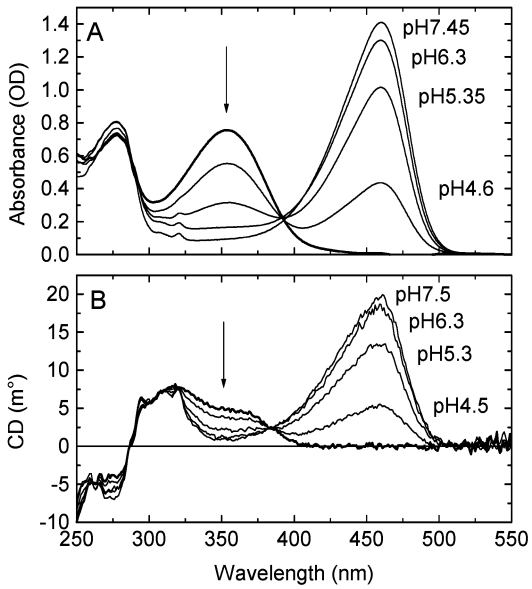

Figure 4. Absorption (A) and CD (B) spectra of the mutant E46Q from 250 to 550 nm at various pH values. The pH was varied in the sequence: 7.45, 5.35, 4.60, and 6.30 in panel A and 7.5, 5.3, 4.5, and 6.3 in panel B, to ascertain reversibility. The pK_a is at 4.8. The spectra with zero absorbance or CD at 460 nm were obtained by extrapolation and refer to the completely bleached low pH form P_{bl}.

band is transferred to the 350 nm band. The cause of the broadening of the vibrational structure in the CD spectra near 320 nm is unclear but does explain why these bands can no longer be discerned in the absorption spectra at low pH where they are superimposed on the steep flank of the 350 nm band (Figure 4A).

Discussion

The 318 nm absorption and CD bands of wild-type PYP have been observed previously,[28,29] but their origin has not yet been explained. Here, we assign them to the $n \rightarrow \pi^*$ transition of the thioester carbonyl of the chromophore. That these transitions are due to the chromophore and not to the protein follows from the fact that the absorbance and CD of the apoprotein is zero above 300 nm (Figure 2). Moreover, absorbance of tryptophan or tyrosine residues at 318 nm with such a low extinction coefficient is without precedent. The combination of very low dipole strength with high rotational strength ($\Delta\epsilon/\epsilon \approx 10^{-2}$) is exactly what is expected for a carbonyl $n \rightarrow \pi^*$ transition. The λ_{max} values of 308 and 318 nm are also in the expected range for a carbonyl $n \rightarrow \pi^*$ transition in a moderately hydrophobic environment (see, e.g., ref 30).

We interpreted the lines at 307, 318, and 329 nm in CD and absorbance as vibrational fine structure with $\bar{\nu} \approx 1050$ cm^{-1}. This frequency is well-known for the vibrational structure in the CD spectra of numerous carbonyl compounds[15] and is the carbonyl stretch frequency in the electronically excited state.[15,31] The observed frequency further supports our assignment of these bands to the carbonyl group.

The near UV spectroscopic transitions described here serve as a valuable local marker for the chromophore geometry and environment. It is, for example, well-known that the λ_{max} value of this type of transition is sensitive to the environment and exhibits a blue shift in polar and hydrogen bonding solvents.[30] The sign of the rotational strength depends moreover on the positions of neighboring perturbing groups (octant rule[10,12,13,15]) and thus provides another sensitive marker for the carbonyl environment.

The carbonyl of the chromophore plays an important role in the mechanism of PYP and changes its position upon isomerization and chromophore rotation. During the early events of the photocycle, up to and including the I_1 intermediate, the hydrogen bonds of the chromophore phenolic O^- with E46 and Y42 are preserved and the thioester part of the chromophore, including the carbonyl, flips in the isomerization.[9,23] In this process, the hydrogen bond of the thioester carbonyl with the protein backbone is broken, and the carbonyl enters a different environment. In the latter part of the photocycle, additional structural changes occur, leading to further changes in the carbonyl environment. On the basis of the octant rule, we may expect to observe corresponding changes in the magnitude and sign of the carbonyl CD. By environment, we mean the rest of the chromophore and thioester linkage, as well as nearby perturbing groups in the protein (−NH,..).

As an example of the potential application of the near UV CD as a probe, we showed here that the sign of the rotational strength in the I_2 intermediate of the photocycle in the mutant M100A is inverted with respect to the dark state. In I_2, the chromophore is not only cis but also protonated. To resolve the issue, we showed with the mutant E46Q that protonation in the dark, without isomerization, leaves the sign and amplitude of the 318 nm CD band unaltered. Thus, in this experiment, the chromophore remained in its binding pocket and did not change its geometry.

It is well-established[5,7] that there are at least two forms of the photocycle intermediate I_2 with protonated chromophore: an early form I_2 with a rise time of about 200 μs and a late form I_2' in which in addition a major conformational change has occurred (rise time about 2 ms). It is unclear at present whether the CD spectrum of Figure 3B of the bleached form of M100A refers to I_2, I_2', or an I_2/I_2' equilibrium. It is conceivable that time-resolved CD spectroscopy may be able to distinguish between these two states, provided the carbonyl environments differ. In time-resolved optical rotatory dispersion measurements (ORD), three components with lifetimes of 10 μs, 1.5 ms, and 515 ms were detected for the chromophore structural changes, and these (in part) may reflect the change in carbonyl environment.[32] Whereas the ORD signal depends on all CD transitions, the CD kinetics have the advantage that it reports on the change of one particular localized transition.

In a recent time-resolved resonance Raman study, evidence was presented that the carbonyl stretching frequency of the chromophore (in the electronic groundstate) shifted from 1633 cm^{-1} in the dark state to 1666 cm^{-1} in I_1.[33] This large upshift of 33 cm^{-1} was interpreted as the breaking of the hydrogen bond of the carbonyl with the backbone amide of cysteine 69 due to the isomerization. Between I_1 and I_2, a small downshift of 6 cm^{-1} was observed, followed by another downshift of 9 cm^{-1} in the transition between I_2 and I_2'. The authors concluded that in I_2' (1651 cm^{-1}) the chromophore reformed an H-bond, possibly with the amide of cysteine 69, although the C═O stretching frequency was still 18 cm^{-1} above its value in the dark state.

The first structural data on the I_2 intermediate were derived from experiments in which a photostationary state, presumably containing a mixture of I_2 and I_2', was created.[23] In this structure, the chromophore carbonyl is hydrogen bonded to the amide of cysteine 69, but in a different geometry as in the dark state and of course with the C_7═C_8 bond cis. In recent time-resolved diffraction experiments, two distinct I_2 species could be resolved by SVD analysis.[34] In the early state (possibly similar to the I_2 intermediate in solution), there is no H-bond between the

carbonyl and the backbone, whereas in the later state (possibly I_2') such an H-bond is likely. Since the global protein conformational change that occurs in solution has not been observed so far in the crystallographic studies, further work is required before the later intermediates from the diffraction work may be identified with the corresponding photocycle intermediates in solution.

In connection with our CD results, we point out that there is a further difference in the carbonyl environment between P and I_2: the isomerization around the C_7═C_8 bond and the amount of twist around this bond. This difference in chromophore geometry may also contribute to the change in the CD signal. The resonance Raman results suggested, for example, from the intensity of the HOOP modes, that in I_2' the chromophore is largely planar but is more twisted in I_2.[33] To make further progress, a characterization of the photostationary state of M100A and time-resolved CD measurements are clearly required.

We note that we also observed significant differences in the CD in the 350 nm band between states with cis or trans protonated chromophores. This band is due to the lowest $\pi \rightarrow \pi^*$ transition of the protonated chromophore. For the cis chromophore in the I_2 state of M100A, the CD in the 350 nm band is close to zero (Figure 3B). For the trans chromophore in the low pH bleached dark state of E46Q, the CD in the 350 nm band is large and positive as is the 460 nm band of the deprotonated dark chromophore (Figure 4B). We interpret these observations as follows. In the case where the CD 350 nm band is positive (E46Q), the protonated trans chromophore retains the same structure and stays in the same environment as the deprotonated trans chromophore. Therefore, the rotational strength is of the same sign and scales with $|\vec{\mu}_{el}|$. In the case where the 350 nm band is largely absent (M100A), the protonated cis chromophore has left the asymmetric binding pocket exposing its hydroxyl group to the aqueous medium. The fact that the CD is near zero suggests that, in I_2, the planar chromophore is in a symmetric environment.

Previous workers have observed CD in the 300−330 nm range for wild-type[28,29] and the mutant M100L[25] without establishing its origin. For example, using wild-type PYP, CD measurements were performed in the bleached I_2 state by lowering the pH to 4.[29] In agreement with our results for M100A, these data show that the 318 nm band reverses in sign in I_2. In the absence of an assignment of the 318 nm CD band, no conclusion could be drawn from this observation. In connection with our present assignment, it means that the carbonyl environment in I_2 is similar in the mutant M100A as in wild-type.

Now that the nature of the 307 and 318 nm optical transitions have been clarified, we may anticipate further applications, in particular, with time-resolved CD.

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References and Notes

(1) Cusanovich, M. A.; Meyer, T. E. *Biochemistry* **2003**, *42*, 4759.
(2) Hellingwerf, K. J.; Hendriks, J.; Gensch, T. *J. Phys. Chem. A* **2003**, *107*, 1082.
(3) Groot, M. L.; van Wilderen, L. J. G. W.; Larsen, D. S.; van der Horst, M. A.; van Stokkum, I. H. M.; Hellingwerf, K. J.; van Grondelle, R. *Biochemistry* **2003**, *42*, 10054.
(4) Getzoff, E. D.; Gutwin, K. N.; Genick, U. K. *Nat. Struct. Biol.* **2003**, *10*, 663.
(5) Xie, A.; Kelemen, L.; Hendriks, J.; White, B. J.; Hellingwerf, K. J.; Hoff, W. D. *Biochemistry* **2001**, *40*, 1510.
(6) Meyer, T. E.; Cusanovich, M. A.; Tollin, G. *Arch. Biochem. Biophys.* **1993**, *306*, 515.
(7) Borucki, B.; Devanathan, S.; Otto, H.; Cusanovich, M. A.; Tollin, G.; Heyn, M. P. *Biochemistry* **2002**, *41*, 10026.
(8) Borucki, B.; Otto, H.; Joshi, C. P.; Gasperi, C.; Cusanovich, M. A.; Devanathan, S.; Tollin, G.; Heyn, M. P. *Biochemistry* **2003**, *42*, 8780.
(9) Xie, A.; Hoff, W. D.; Kroon, A. R.; Hellingwerf, K. J. *Biochemistry* **1996**, *35*, 14671.
(10) Moffitt, W.; Woodward, R. B.; Moscowitz, A.; Klyne, W.; Djerassi, C. *J. Am. Chem. Soc.* **1961**, *83*, 4013.
(11) Woody, R. W. Theory of circular dichroism of proteins. In *Circular dichroism and conformational analysis of biomolecules*; Fasman, G. D., Ed.; Plenum Press: New York, 1996; p 25.
(12) Schellman, J. A. *Acc. Chem. Res.* **1968**, *1*, 144.
(13) Rodger, A.; Norden, B. *Circular dichroism and linear dichroism*; Oxford University Press: Oxford, 1997; pp 90 and 123.
(14) Höhn, E. G.; Weigang, O. E. *J. Chem. Phys.* **1968**, *48*, 1127.
(15) Lightner, D. A.; Gurst, J. E. *Organic Conformational Analysis and Stereochemistry from Circular Dichroism Spectroscopy*; Wiley-VCH: New York, 2000.
(16) Genick, U. K.; Devanathan, S.; Meyer, T. E.; Canestrelli, I. L.; Williams, E.; Cusanovich, M. A.; Tollin, G.; Getzoff, E. D. *Biochemistry* **1997**, *36*, 8.
(17) Devanathan, S.; Genick, U. K.; Canestrelli, I. L.; Meyer, T. E.; Cusanovich, M. A.; Getzoff, E. D.; Tollin, G. *Biochemistry* **1998**, *37*, 11563.
(18) Imamoto, Y.; Ito, T.; Kataoka, M.; Tokunaga, F. *FEBS Lett.* **1995**, *374*, 157.
(19) Devanathan, S.; Genick, U. K.; Getzoff, E. D.; Meyer, T. E.; Cusanovich, M. A.; Tollin, G. *Arch. Biochem. Biophys.* **1997**, *340*, 83.
(20) Borucki, B.; Otto, H.; Rottwinkel, G.; Hughes, J.; Heyn, M. P.; Lamparter, T. *Biochemistry* **2003**, *42*, 13684.
(21) Atkins, P. W. *Molecular Quantum Mechanism*, 3rd ed.; Oxford University Press: Oxford, 1997; p 369.
(22) Genick, U. K.; Soltis, S. M.; Kuhn, P.; Canestrelli, I. L.; Getzoff, E. D. *Nature* **1998**, *392*, 206.
(23) Genick, U. K.; Borgstahl, G. E. O.; Ng, K.; Ren, Z.; Pradervand, C.; Burke, P. M., Šrajer, V.; Teng, T.-Y., Schildkamp, W.; McRee, D. E.; Moffat, K.; Getzoff, E. D. *Science* **1997**, *275*, 1471.
(24) Ren, Z.; Perman, B.; Šrajer, V.; Teng, T.-Y.; Pradervand, C.; Bourgeois, D.; Schotte, F.; Ursby, T.; Kort, R.; Wulff, M.; Moffat, K. *Biochemistry* **2001**, *40*, 13788.
(25) Sasaki, J.; Kumaucki, M.; Hamada, N.; Oka, T.; Tokunaga, F. *Biochemistry* **2002**, *41*, 1915.
(26) Anderson, S.; Crosson, S.; Moffat, K. *Acta Crystallogr.* **2004**, *D60*, 1008.
(27) Meyer, T. E.; Devanathan, S.; Woo, T.; Getzoff, E. D.; Tollin, G.; Cusanovich, M. A. *Biochemistry* **2003**, *42*, 3319.
(28) Meyer, T. E.; Yakali, E.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1987**, *26*, 418.
(29) Lee, B.-C.; Croonquist, P. A.; Sosnick, T. R.; Hoff, W. D. *J. Biol. Chem.* **2001**, *276*, 20821.
(30) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry II*; W. H. Freeman: San Francisco, 1980; p 387.
(31) Weigang, O. E. *J. Chem. Phys.* **1965**, *43*, 3609.
(32) Chen, E.; Gensch, T.; Gross, A. B.; Hendriks, J.; Hellingwerf, K. J.; Kliger, D. S. *Biochemistry* **2003**, *42*, 2062.
(33) Pan, D.; Philip, A.; Hoff, W. D.; Mathies, R. A. *Biophys. J.* **2004**, *86*, 2374.
(34) Schmidt, M.; Pahl, R.; Šrajer, V.; Anderson, S.; Ren, Z.; Ihee, H.; Rajagopal, S.; Moffat, K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4799.