Lack of Negative Charge in the E46Q Mutant of Photoactive Yellow Protein Prevents Partial Unfolding of the Blue-Shifted Intermediate†

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Received May 23, 2003; Revised Manuscript Received October 13, 2003

ABSTRACT: The long-lived light-induced intermediate (pB) of the E46Q mutant (glutamic acid is replaced by glutamine at position 46) of photoactive yellow protein (PYP) has been investigated by NMR spectroscopy. The ground state of this mutant is very similar to that of wild-type PYP (WT), whereas the pB state, formed upon illumination, appears to be much more structured in E46Q than in WT. The differences are most striking in the N-terminal domain of the protein. In WT, the side-chain carboxylic group of E46 is known to donate its proton to the chromophore upon illumination. The absence of the carboxylic group near the chromophore in the E46Q mutant prohibits the formation of a negative charge at this position upon formation of pB. This prevents the partial unfolding of the mutant, as evidenced from NMR chemical shift comparison and proton/deuterium (H/D) exchange studies.

Photoactive yellow protein (PYP)[1] is a small (14 kDa) water-soluble blue light photoreceptor found in *Halorhodospira halophila* (*1*). The wavelength dependence of negative phototaxis of the bacterium is similar to the absorption spectrum of PYP, and therefore it is believed that PYP plays a role as the primary photoreceptor in this process. As a model for other photoactive proteins, PYP and its light cycle have been the subject of many studies. PYP belongs to the PAS family and has a covalently linked chromophore, *p*-coumaric acid, which undergoes a trans to cis isomerization upon illumination (*2*, *3*). During the light cycle various intermediates are formed, of which the long-lived blue-shifted intermediate (also known as pB, I_2, or PYP_M) is thought to be the signaling state. The pB state has been characterized by many techniques and it was shown that its structure in solution differs significantly from its structure in the crystal environment (*4*, *5*). The pB state in crystals is well-described by a single structure (*5*), whereas both thermodynamic and NMR studies in solution indicate that the pB state shows structural and dynamic disorder (*4*, *6*). Xie et al. (*7*) showed by FTIR that in WT the protonation of the chromophore occurs prior to the large global conformational change, a phenomenon that was not observed for E46Q. The authors suggested that this partial unfolding in solution is caused by the buried negative charge on E46 in the pB state, which is formed when this residue donates its proton to the chromophore (*7*, *8*). FTIR measurements also revealed that E46 in the pB state of WT in the crystalline state remains protonated (*7*). The chromophore must then be protonated by other routes, which might require only little structural changes in the protein, as is observed in the crystal structure of pB (*5*). In contrast, Cusanovich and Meyer (*9*) recently proposed a hydrophobic collapse model as the signaling mechanism for PAS domains, thereby reducing the role for E46.

To test the idea of the negative charge on E46 being the cause of partial unfolding in pB, we have studied the mutant E46Q in various NMR experiments performed both with and without illumination. We have chosen this PYP mutant in order to specifically study the effect of the absence of a negative charge in the hydrophobic core of the protein upon formation of pB, as the replacement of the glutamic acid at position 46 by a glutamine prohibits the transfer of a proton from this residue to the chromophore. As a consequence, the chromophore is protonated via another route in this mutant, e.g., protonation via Y42 or via surrounding water molecules, and requires little structural changes.

The intensity and chemical shift differences in the HSQC spectra of the ground state and the long-lived intermediate of E46Q are compared with those of WT to elucidate the differences between these two states. Furthermore, proton/deuterium (H/D) exchange experiments with and without illumination were performed on E46Q to see whether there is a change in stability in parts of the protein compared to WT. These studies show that the ground state is hardly affected by the mutation, while the pB state differs substan-

† Financial support of NWO (Dutch Foundation for Scientific Research) is gratefully acknowledged.

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[1] Abbreviations: FID, free induction decay; FTIR, Fourier transform infrared spectroscopy; H/D, proton/deuterium; HSQC, heteronuclear single-quantum coherence; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; PAS, Per, Arnt, Sim; pB, long-lived intermediate of the photoactive yellow protein photocycle; pBdark, low-pH form of photoactive yellow protein in the ground state; pG, ground state of photoactive yellow protein; PYP, photoactive yellow protein; PF, protection factor; SCOTCH, spin coherence transfer in chemical reactions; 3D, three-dimensional; TOCSY, total correlation spectroscopy; WT, wild type.

10.1021/bi034877x CCC: $25.00
Published on Web 11/20/2003

tially from the pB state of WT, mainly in the N-terminal part of the protein.

MATERIALS AND METHODS

Uniformly ^{15}N-labeled wild-type PYP (WT) and E46Q were expressed and purified as described previously (*10*, *11*). All samples contained 50 mM phosphate buffer and a protease inhibitor cocktail (Complete, Roche Applied Science). Samples were at pH 6.5 for E46Q data. Data for WT were recorded at pH 5.8. As the pK_a for the protonation of the chromophore is higher in E46Q than in WT (*12*), this slightly higher pH (6.5) was chosen instead of pH 5.8 normally used for experiments on WT (*4*, *13*, *14*) to avoid formation of pB_{dark} (*15*). All spectra were acquired on a Bruker Avance 500 MHz spectrometer, except for 3D ^{15}N−NOESY−HSQC and 3D ^{15}N−TOCSY−HSQC spectra, which were recorded on Bruker Avance 750 MHz. NMRPipe (*16*) was used for processing the spectra, and data were analyzed by use of NMRview (*17*). An argon laser (Stabilite 2017, Spectra-Physics) with mechanical shutter connected to a glass fiber light guide was used to illuminate the sample in the probe of the spectrometer (*13*). To reach maximal conversion during illumination, the experiments with E46Q were carried out at 283 K, and at 311 K for WT. Assignment of the HSQC peaks of E46Q-pG was initially done by direct comparison with WT and was confirmed by the use of both ^{15}N−NOESY−HSQC and ^{15}N−TOCSY−HSQC spectra. E46Q-pB was assigned by the SCOTCH experiment (*13*). H/D exchange experiments with and without illumination were performed on E46Q, similar to what was previously done for WT (*14*). A series of HSQC spectra was recorded to follow the exchange without illumination. These data were compared with HSQC spectra interspersed with 5, 20, and 80 min illumination periods. The illumination period consisted of 0.5 s laser bursts, repeated every 5 s to prevent too much heating (i.e., less than 1 K). This resulted in a total illumination time of 10.5 min. The whole experiment including the illumination period was performed in 3.8 h. The pH of the samples after dissolution in D_2O was 6.1 (uncorrected meter output). Data collection was started 15 min after the protein was dissolved. The decrease in intensity of a particular signal as a function of the exchange time was converted in protection factors by using the method of Bai et al. (*18*, *19*).

To observe changes in exchange rates for amides in H/D exchange experiments without illumination (a so-called dark experiment) and with illumination (light experiment), the following has to be kept in mind: Only residues that exchange slowly enough in the dark experiment can be used to monitor any change in the light experiment. Furthermore, due to the limited time of illumination, only residues that show a significant decrease in intensity during this time will give rise to observable changes in exchange rate. Residues for which the exchange rate is only slightly altered upon illumination will thus not show a difference in such an experiment.

The data for the light experiment were then fitted by use of

$$Y = A_1 \exp[-t_D/\tau_D - t_L/\tau_L] \quad (1)$$

where A_1 is the intercept, t_D the time the protein spent in the dark, t_L the time it was illuminated, τ_D the time constant in the dark, and τ_L the time constant during illumination. A_1 and τ_D were determined from the data from the dark experiment. The values for these parameters were kept constant during the fitting of the data of the light experiment. As the conversion to and recovery of pB is rapid at this pH, the period spent in the pB state was taken to be equal to the illumination time without further correction. In this way τ_L is a measure for the time constant in the pB state.

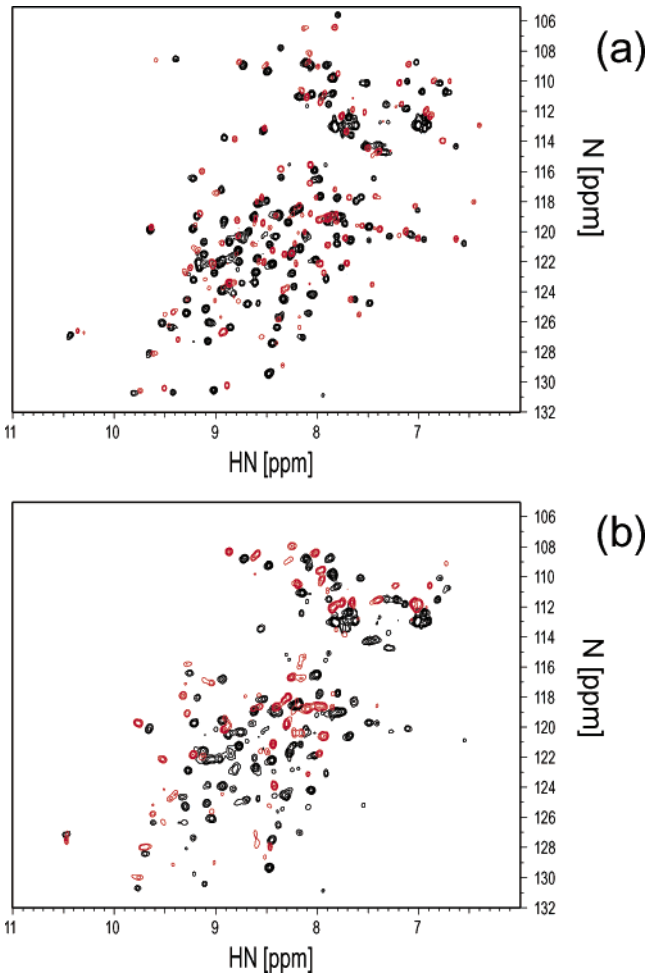

FIGURE 1: Overlay of HSQC spectra for the pG states (a) and pB states (b) of E46Q and WT PYP. Spectra of E46Q are shown in black and those of WT are shown in red. The chemical shift differences in panel a are mainly due to the different experimental conditions needed to achieve maximum conversion: the spectra of E46Q were recorded at pH 6.5 and 283 K, and those of WT, at pH 5.8 and 311 K.

RESULTS AND DISCUSSION

Comparison of the Ground State of WT and E46Q. Figure 1a shows the HSQC spectra of the pG states of WT and E46Q. The spectra are very similar although small shifts are visible that are mainly due to the different experimental conditions. The high similarity of the spectra made the backbone assignment of E46Q straightforward. The ^{1}H and ^{15}N assignments thus obtained were confirmed by the use of 3D ^{15}N−NOESY−HSQC and 3D ^{15}N−TOCSY−HSQC spectra. The ^{1}Hα chemical shift indices (CSI) are closely similar for the pG states of WT and E46Q and correspond to the secondary structure elements found in the WT structure (see Supporting Information). These data indicate that the

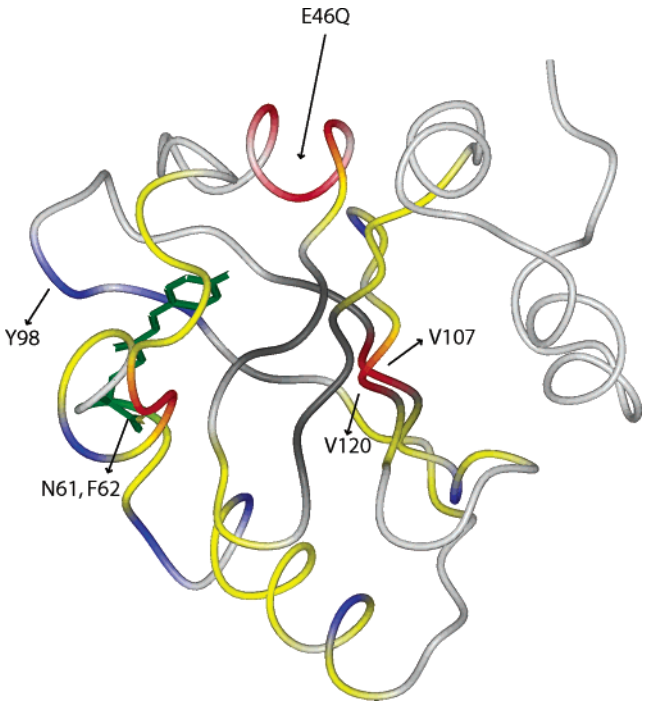

FIGURE 2: Differences in protection factor (PF) of the pG states of E46Q and WT mapped on the X-ray structure of WT PYP (PDB entry 2phy). The chromophore is shown in dark green. Residues with largest differences in PF are labeled. Color coding: (red) $PF_{WT} - PF_{E46Q} > 0.5$; (yellow) $-0.5 < PF_{WT} - PF_{E46Q} < 0.5$; (blue) $PF_{WT} - PF_{E46Q} < -0.5$; (gray) ambiguous data, prolines and rates too fast to determine PF; (dark gray) exchange rate in E46Q too slow to determine PF.

structure of the pG state of the mutant protein strongly resembles that of WT PYP.

Protection factors obtained from H/D experiments could be determined for 67 out of the total of 125 residues of E46Q. A few amides exchanged extremely slowly in E46Q and no exact value of the time constant of exchange could be derived from these data. These residues are all positioned in the core of the β-sheet and are colored dark gray in Figure 2. These residues showed a very high protection factor in WT as well (*14*). Figure 2 highlights the difference in the protection factors of the pG states of E46Q and WT PYP mapped on the 3D structure of the WT protein. Most residues showed a change in protection factor of less than 0.5 (color-coded yellow in Figure 2). In both proteins the N-terminal part shows hardly any protection. For the regions where the protection could be measured, only the region near the mutated site as well as the strand comprising residues N61 and F62, and V107 and V120 show slightly less protection in E46Q compared to WT (indicated in red in Figure 2). Only a few amide protons are more protected in E46Q (color-coded blue in Figure 2). Of these, Y98 shows the highest protection compared to WT (difference with WT is −1.08), most likely resulting from local rearrangements near the chromophore loop. The small differences found in the H/D exchange behavior are in agreement with the strong similarity in the structures of the pG states of E46Q and WT PYP judged from the chemical shift comparison discussed above.

Comparison of the Long-Lived Intermediates of WT and E46Q. The photocycle of E46Q is similar to that of WT. The recovery time from pB to pG also lies in the subsecond time scale but is more pH-dependent than for WT (*20*). More than 90% conversion was achieved by illuminating the sample with a power of 1.5 W during 300 ms of illumination prior to and during the recording of the FID. HSQC spectra of pB E46Q resemble the spectra of pG E46Q and a similar number of peaks is visible in both spectra. This is in strong contrast to the situation for WT, where only half the number of peaks visible in the pG HSQC spectrum are detectable in the spectrum of the pB state (*4*). Figure 1b shows an overlay of the pB spectra of E46Q (color-coded black) and WT (red). This clearly shows the much larger number of peaks

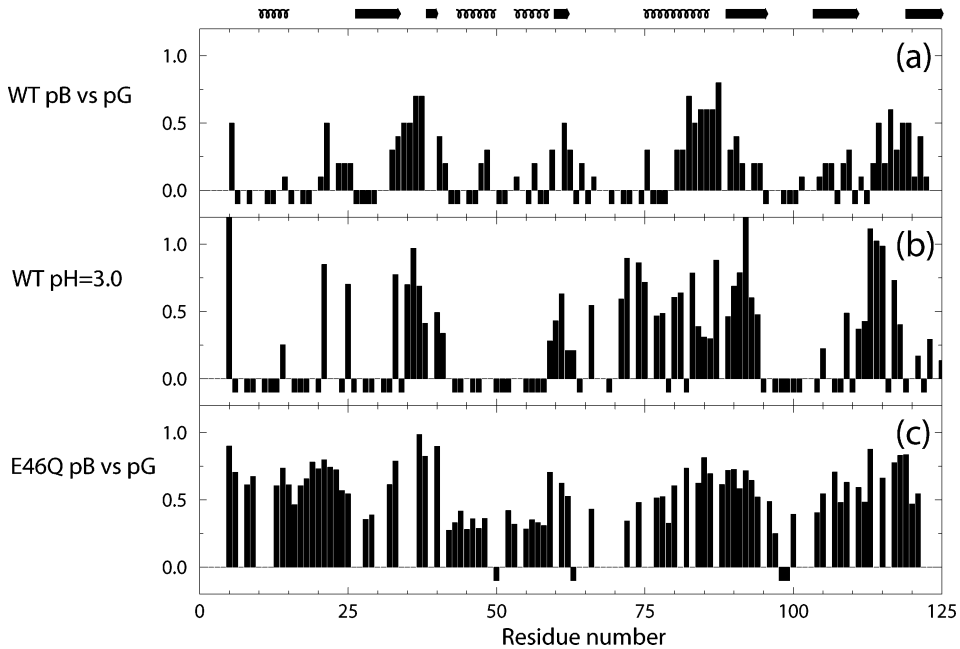

FIGURE 3: Comparison of the intensities of HSQC peaks of (a) pB versus pG of WT, (b) pH 3.0 versus pH 5.8 of WT, and (c) pB versus pG of E46Q. A small negative bar indicates that the peak becomes too weak to detect. A missing bar indicates that the peak cannot be represented due to ambiguity or spectral overlap. The secondary structure of WT PYP in solution, as determined by Dux et al. (*10*), is shown above the figure.

observable in the spectrum of E46Q compared to WT (116 backbone peaks versus 67).

When the intensities of the HSQC peaks in pG and pB for both WT and E46Q are compared (Figure 3a,c), the most striking differences are observed in the N-terminal part of the protein. In WT, the N-terminal residues are no longer visible in pB, as is also the case for WT upon lowering pH (Figure 3b) (*14*), whereas in E46Q these peaks retain high intensity. This shows that in E46Q the N-terminal part exhibits smaller conformational changes than is the case for WT. The decrease of signals in WT and E46Q cannot be attributed to increased correlation time or aggregation, as these phenomena would affect all the signals in the spectrum. The other regions that show dramatic decrease of intensity in WT, i.e., Y42−I58, C69−K78, and T95−M100, also show a decrease in intensity in E46Q but to a much lesser extent. Apparently the pB state of both WT and E46Q shows exchange behavior in these regions. Besides the N-terminal part, the structural changes upon formation of pB are therefore similar for WT and E46Q but less pronounced in E46Q. When the chemical shift differences between the HSQC peaks of pG and pB are visualized on the X-ray structure for both WT (Figure 4a) and E46Q (Figure 4b), the same trend can be seen. The main differences in E46Q are located in the chromophore region and the mutation site, whereas in WT the differences affect larger parts of the protein.

In our previous studies, a decrease of protection against H/D exchange of WT PYP due to exposure of light was observed for 14 out of 51 residues for which exchange could be measured (*14*). We have performed the same type of H/D exchange experiments on E46Q. In Figure 5 the time constants for H/D exchange with and without illumination are shown for WT (upper panel) and E46Q (lower panel), where the first 25 residues are omitted as they exchanged within the dead time of experiment. Time constants obtained for E46Q and WT without illumination are similar (full-height boxes in Figure 5), with small differences arising from different experimental conditions. The general trend is the same and comparison of the protection factors (where differences in temperature and pH are compensated for) showed that there are only subtle differences (Figure 2). The residues in E46Q for which no time constant could be determined due to extremely slow exchange (color-coded dark gray in Figure 2) are marked with an open circle in the lower panel of Figure 5.

Of the 54 slowly exchanging residues in E46Q, 13 showed a significantly faster exchange behavior during illumination (Figure 5, lower panel, solid boxes). For these residues the hydrogen-bonding network is altered upon formation of pB. For the other 41 residues no difference in exchange behavior in the pB state could be observed, which means that these residues exchanged also slowly during illumination. In WT the same number of residues showed observable differences in exchange behavior in pB compared to pG (Figure 5, upper panel, solid boxes) (*14*).

Comparing the exchange behavior in pB, for E46Q and WT, it is seen that mainly at the beginning or ends of secondary structure elements a decrease in exchange rate between pG and pB is observed (e.g., F28 and G29 at the beginning of the first strand and N43 at the second helix). In WT the deprotection seems to be slightly larger, resulting in more perturbed residues at the termini of secondary structure elements. For example, in both E46Q and WT the beginning of the first strand, comprising residues 27−34, shows a clear decrease in exchange rates upon formation of pB, and for WT also a decrease for A30 is observed. The

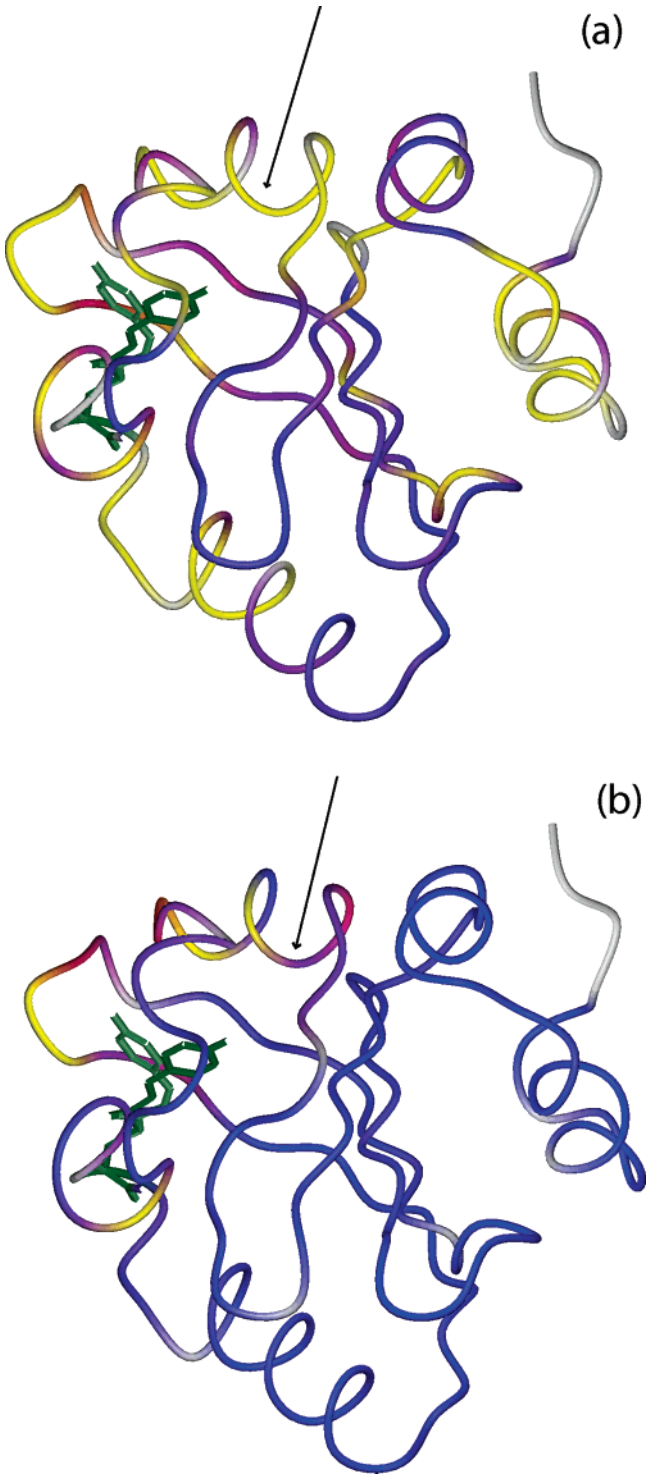

FIGURE 4: Chemical shift differences between pG and pB superimposed on the structure of WT PYP (PDB entry 2pyp) for WT (a) and for E46Q (b). Colors range from blue via red to yellow with increasing chemical shift difference. Peaks that were no longer visible in pB are also colored yellow. Peaks that could not be detected due to ambiguity or spectral overlap are colored gray. The chromophore is shown in dark green in the pG state and in light green in the pB state in the crystal environment. An arrow indicates the mutation.

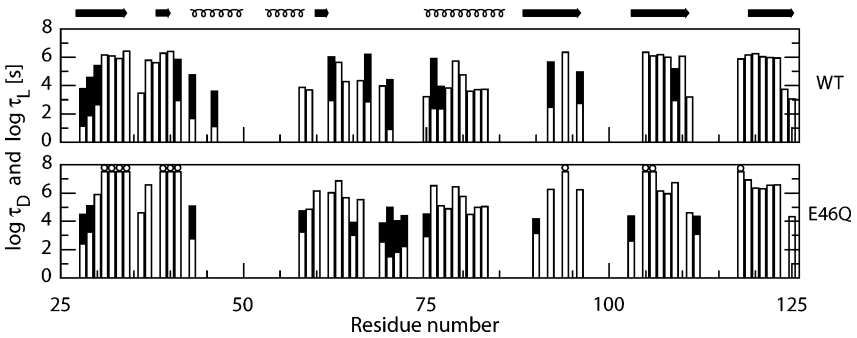

FIGURE 5: Logarithmic time constants (in seconds) per residue for exchange experiments without illumination (τ_D) and with illumination (τ_L). Data for WT are shown in the upper panel, and for E46Q, in the lower panel. Detectable changes in time constants upon illumination are color-coded black, i.e., the time constant with illumination (τ_L) is represented by the height of the open box for these residues. For the other residues no change was observed. Open circles indicate residues of E46Q that exchanged too slowly to determine the time constant. The secondary structure of PYP in solution, as determined by Dux et al. (*10*), is shown above the figure.

same is true for the helix comprising residues 75−86. In E46Q only F75 shows a decrease in exchange rate, but in WT both Y76 and G77 show a decrease. The strand comprising residues 88−96 exhibits in WT both for F92 and F96 a decrease in exchange rate. For E46Q this is only the case for T90. All these observations are in agreement with the less dramatic intensity changes in E46Q discussed above.

The chromophore loop (C69−S72) in E46Q shows a decrease in exchange rates between pG and pB. Unfortunately, the time constants in WT for this region were too low to determine a difference with pB. It should be mentioned that the information obtained from these H/D exchange experiments is limited, as small changes in exchange behavior of very slowly exchanging residues and very fast exchanging residues (like those in the N-terminal part of PYP) are not observable.

Concluding Remarks. In this paper we have shown that the replacement of glutamate at position 46 by glutamine causes hardly any changes in the structure of pG compared to WT. In the long-lived light-induced intermediate pB, however, the large conformational changes observed in the WT protein are largely absent in the mutant. We are now able to pinpoint these conformational changes in WT in a residue-specific manner: the chemical shift and intensity changes between pG and pB indicate that mainly the N-terminal part is responsible for the unfolding behavior upon formation of pB in WT.

Although residue 46 in E46Q can no longer donate a proton to the chromophore, and therefore no negative charge can be formed in the interior of the protein on this residue, the differences in H/D exchange behavior in pG and pB between E46Q and WT still show much resemblance. This confirms that the major differences must be in the regions that are not observable with this method, i.e., in the N-terminal region. Our findings are in agreement with studies on truncated forms of PYP where the N-terminal part is lacking (*21*, *22*). These studies revealed that the transient functional unfolding of PYP in the pB state has essentially been abolished in the N-terminally truncated variants.

Additionally, Xie et al. (*7*) concluded that the large-amplitude "protein quake" in the WT protein is due to the negative charge located on E46, after donation of the proton to the chromophore. This is in contrast to the proposed hydrophobic collapse model of Cusanovich and Meyer (*9*). However, the results described in this paper indicate that E46 plays a key role in the unfolding of the N-terminal part upon formation of pB. For both WT and E46Q the exchange rate for G29 is enhanced upon formation of pB, which means that also in the mutated form this region is altered, but the changes are less pronounced in E46Q as the amide signals for the N-terminal part remain visible in the HSQC spectrum of pB. This favors the "protein quake" model over a hydrophobic collapse model, although the latter cannot be excluded on the basis of our data.

A conclusive understanding of the underlying mechanism that triggers the protein to partly unfold upon illumination awaits a high-resolution structure in solution. The reduced exchange broadening in the spectrum of E46Q compared to WT makes this mutant an attractive candidate to characterize the pB state by NMR spectroscopy.

ACKNOWLEDGMENT

We thank Michiel Hilbers for help with the laser setup.

SUPPORTING INFORMATION AVAILABLE

Figure with the ^{1}Hα chemical shift indices (CSI) for the pG states of WT and E46Q. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI034877X