Site-Specific Mutations Provide New Insights into the Origin of pH Effects and Alternative Spectral Forms in the Photoactive Yellow Protein from *Halorhodospira halophila*†

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ABSTRACT: Acid/base titrations of wild-type PYP and mutants, either in buffer or in the presence of chaotropes such as thiocyanate, establish the presence of four spectral forms including the following: a neutral form (446−476 nm), an acidic form (350−355 nm), an alkaline form (430−440 nm), and an intermediate wavelength form (355−400 nm). The acidic species is formed by protonation of the oxyanion of the para-hydroxy-cinnamyl cysteine chromophore as a secondary result of acid denaturation (with pK_a values of 2.8−5.4) and often results in precipitation of the protein, and in the case of wild-type PYP, eventual hydrolysis of the chromophore thioester bond at pH values below 2. Thus, the large and complex structural changes associated with the acidic species make it a poor model for the long-lived photocycle intermediate, I_2, which undergoes more moderate structural changes. Mutations at E46, which is hydrogen-bonded to the chromophore, have only two spectral forms accessible to them, the neutral and the acidic forms. Thus, an intact E46 carboxyl group is essential for observation of either intermediate or alkaline wavelength forms. The alkaline form is likely to be due to ionization of E46 in the folded protein. We postulate that the intermediate wavelength form is due to a conformational change that allows solvent access to E46 and formation of a hydrogen-bond from a water molecule to the carboxylic acid group, thus weakening its interaction with the chromophore. Increasing solvent access to the intermediate spectral form with denaturant concentration results in a continuously blue-shifted wavelength maximum.

PYP was discovered in 1985 (*1*) as a water-soluble protein in the halophilic purple phototrophic bacterium, *Ectothiorhodospira* (now *Halorhodospira*) *halophila*. Its photoactivity was first characterized in 1987 (*2*). It has an intense yellow color because of an absorption peak located at 446 nm (ϵ = 45 mM^{-1} cm^{-1}) (*1*−*3*). Following a laser flash at 450 nm, the protein forms a bleached intermediate on the millisecond time scale and recovers within a second (*2*). As a result of recent picosecond and femtosecond kinetic studies (*4*, *5*), we now know that there are at least four room-temperature intermediates in the PYP solution photocycle. Following the initial discovery, five other species of PYP have been found in purple bacteria, including *Chromatium* (now *Halochromatium*) *salexigens* (*6*), *Rhodospirillum* (now *Rhodothallasium*) *salexigens* (*6*), *Rhodobacter sphaeroides* (*7*), *Rhodobacter capsulatus* (*8*), and *Rhodospirillum centenum* (now *Rhodocista centenaria*) (*8*). The latter protein exists as a naturally occurring chimera of PYP with a phytochrome and is the only PYP for which the physiological function is unequivocally known, namely the regulation of a chalcone synthase homologue by light (*8*). The three-dimensional structure of PYP has been determined as have those of some photocycle intermediates (*9*−*12*). Mutagenesis has shown that amino acid residues near the chromophore binding site, E46, Y42, and M100 have mechanistically important roles in color tuning and photocycle kinetics and that T50 and R52 play somewhat lesser roles in modulating these properties (*13*−*17*). E46 and Y42 are directly hydrogen-bonded to the chromophore phenolate oxygen, T50 is hydrogen-bonded to Y42, R52 must swing out of the way of the chromophore during the photocycle bleach/recovery process, and M100 facilitates cis−trans isomerization of the chromophore double bond during dark recovery (*9*, *10*, *15*).

Solution pH has a pronounced effect on the kinetics of the PYP photocycle (*13*). The rate constant for photobleaching increases in the low pH range with a pK_a of approximately 5.7. Dark recovery shows a bell-shaped dependence on pH with maximum activity at pH 8 and pK_a values of 6.4 and 9.4. The pK_a values at 5.7−6.4 are currently believed to represent ionization of the E46 carboxylic acid and the pK_a at 9.4 to deprotonation of the chromophore phenolic oxygen (*18*) when these functional groups are exposed to solvent in the photobleached form. When both E46 and chromophore are either protonated or ionized, recovery is much slower than at pH 8, presumably where only E46 is ionized.

Mutant Y42F appears to be unique in that it has a 391 nm shoulder on the 458 nm peak in the ground state spectrum (*14*) that is relatively insensitive to ambient light or pH but

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is strongly affected by kosmotropes and chaotropes (*17*). Kosmotropes, such as ammonium sulfate, stabilize the protein and diminish the 391 nm shoulder while increasing the 458 nm absorbance. Chaotropes, such as thiocyanate and ammonium chloride, have the opposite effect in that the shoulder is enhanced at the expense of the 458 nm peak and becomes dominant at the highest concentrations. It was concluded that the hydrogen bond from E46 to the chromophore phenolate differs in the two spectral forms of Y42F, possibly through the formation of an additional hydrogen bond from solvent water in the intermediate wavelength form (*17*).

There remains some controversy about the nature of the pK_a of 2.8 for protonation of the chromophore in dark-adapted protein (i.e., whether it is the pK_a of the chromophore in folded protein or represents acid denaturation or unfolding of the protein resulting in exposure of the chromophore to solvent and an increase in its pK_a). NMR spectroscopy (*19*) shows that some structure remains in the acid-denatured protein at pH 1.7, that the photobleached protein in solution is more unfolded than in the crystalline form, and that the photobleached form has similarities to the acid-denatured protein (*19*, *20*). It is not possible to completely characterize the fully protonated form because the protein both precipitates, and the chromophore thioester is slowly hydrolyzed below pH 2. The chromophore also hydrolyzes at high pH (>11), and the nature of the alkaline form is also controversial because of the presence of mixed species in solution (*18*). We have now further studied the effects of chaotropes, pH, and temperature on PYP mutants and explored the possibility that mutant Y42F may not be unique in its spectral properties.

MATERIALS AND METHODS

Recombinant *H. halophila* PYP and mutants were prepared in *Escherichia coli* as described previously (*17*). Acid/base titrations were performed on a Cary 300 UV−vis spectrophotometer in a 5 mM solution in each of the following buffers: potassium phosphate, sodium acetate, BIS-TRIS,[1] HEPES, TAPS, CHES, and CAPS. The thiocyanate experiments were performed in the same buffer mixture, which was either adjusted to pH 7 or varied to determine pK_a. Protein stability was evaluated by measurement of the midpoint in guanidine concentration for loss of ellipticity at 222 nm in the circular dichroism spectrum as previously described (*17*). Denaturation was carried out in 20 mM Tris-Cl, 40 mM NaCl, pH 7.5, plus varying concentrations of guanidine. Circular dichroism spectra were measured with an Aviv Instruments modified Cary model 60 (Lakewood, NJ). Thermal titrations of the visible and near-UV absorbance in 20 mM MOPS, pH 7, was monitored with the Cary 300 spectrophotometer. Temperature was changed at 5° intervals, equilibrated for 4 min after reaching temperature (in approximately 1 min) before spectra were recorded. Calorimetry was performed with a differential scanning microcalorimeter (model VP-DSC) from Microcal Corp, Northampton MA. The samples were scanned at 1°/min up to 100°.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane sulfonic acid (pK_a 7.5); CAPS, 3-(cyclohexylamino)-1-propane sulfonic acid (pK_a 10.4); CHES, 2-(*N*-cyclohexylamino)-ethane sulfonic acid (pK_a 9.3); BIS-TRIS, bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl)-methane (pK_a 6.5); TAPS, tris-(hydroxymethyl)-methylamino-1-propane sulfonic acid (pK_a 8.4); MOPS, 3-(*N*-morpholino)-propane sulfonic acid (pK_a 7.2). WT, wild-type protein; Y42F, mutation of tyrosine 42 to phenylalanine; Y42F/T50V, double mutation including threonine 50 to valine. The same notation is used for other mutations.

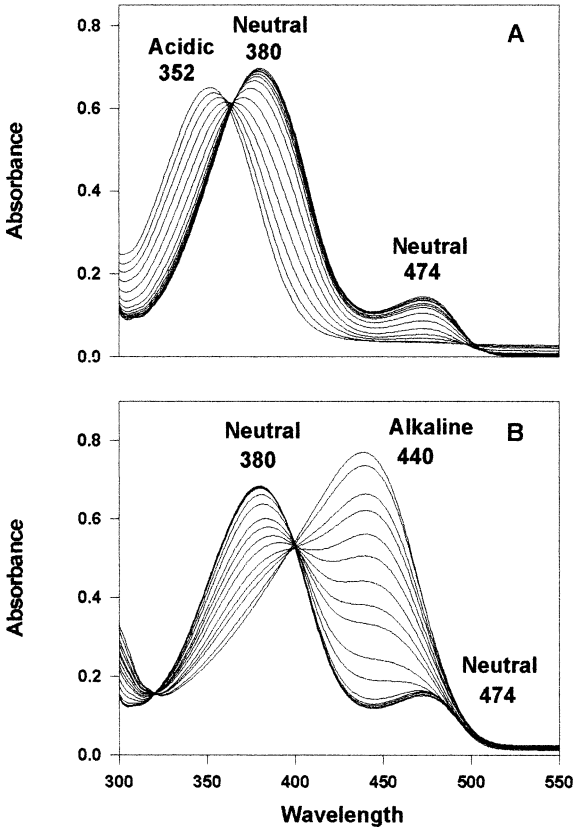

FIGURE 1: Acid/base titrations of double mutant Y42F/T50V performed in 5 mM buffer (see Materials and Methods) using 1 M HCl or 1 M NaOH to adjust pH. (A) Acidic titration from approximately pH 7 to pH 3.3 (note slight turbidity for the last four spectra near the endpoint). (B) Basic titration from pH 7 to pH 11.7, above which there was significant chromophore hydrolysis (note loss of isosbestic point for the last two spectra).

RESULTS AND DISCUSSION

Low pH: Acid Denaturation or pK_a of the Chromophore? It has been reported that the structural changes that occur at low pH are similar to those occurring upon photoexcitation (*19*, *20*), but we believe that they are fundamentally different. To clarify the pH effects and spectral properties of PYP, we first performed acid/base titrations on wild type (WT) and a series of PYP mutants. An example is shown in Figure 1 for mutant Y42F/T50V. The pK_a values obtained are shown in Table 1. In most cases, the acidic titrations were not simple but required more than one pK_a or nonintegral slopes (i.e., more than one proton) for best fit, as shown in Figure 2. The higher of the two pK_a values (pK_{2acid} in Table 1) generally had a smaller amplitude and will be discussed below. We believe that the lower of the two pK_a values (pK_{1acid} in Table 1) generally represents partial protein denaturation. The nonintegral slope (*n* value) was larger for WT than for T50V, while other mutants with higher pK_a values have slopes of 1. It should be noted that the pK_a values for WT and T50V are below the range observed for aspartic acid and glutamic acid residues exposed to solvent at the protein suface (nominally 3.9 and 4.3 for the free amino acids). Thus, the spectral change because of protonation of the chromophore phenolate anion may be modulated by

Table 1: Wavelength Maxima, Results of Acid/Base Titrations, and Thermal Transitions of PYP Mutants

	neut. (λ_{max})[a]	pK_1 acid[b]	pK_2 acid[c]	alk. (λ_{max})	pK_{alk}	guan. (C_m)[d]	KSCN (λ_{max})[e]	cal (T_m)[f]	vis (T_m)[g]
WT	446	2.8(1.8)	NA	442	>11.5	2.73	446(363,4M)	87	82
Y42A	450(374, 5.5)	5.2(1)	NA	440	11.0				
Y42W	455(364, 3.8)	5.4(1)	7.4(0.43)	440	9.8				
Y42F	458(391, 0.62)	4.4(1)	6.4(0.25)	433	10.2	2.29	383(458, 0.4 M)	86	65(28)
Y42F/T50V	474(380, 4.2)	4.6(1)	6.1(0.36)	440	11.1			86	56(38)
Y42F/E46Q	476	7.4(1)	8.5(0.49)	NA	NA		476 (1M)		
E46Q	460	4.8(1)	NA	NA	NA	2.65	460 (4M)	87	73
E46A	469	7.9(1)	NA	NA	NA		469 (1M)		
E46D (16)	444	8.6(1)	NA	NA	NA				
T50V	457	3.5(1.4)	NA	436	>11.5	2.46	457(370, 2 M)	87	69
R52A	449	3.9(1)	5.1(0.38)	438	10.6	2.37	449(380, 1 M)		57
M100A	446	3.8(1)	5.8(0.18)	432		2.40	442(373, 1 M)		68

[a] Additional peaks and relative amplitudes at pH 7 are indicated in parentheses. [b] The *n* value or steepness of the titraton curve is indicated in parentheses. [c] The relative amplitude of the second acidic pK_a is indicated in parentheses. [d] C_m is the midpoint concentration of guanidine at which half the 222 nm ellipticity in the circular dichroism spectrum is lost. [e] Spectral shoulders and concentration of thiocyanate needed to elicit a change are shown in parentheses. [f] The calorimetric melting temperature was simple in all cases. [g] The visible/UV absorbance vs temperature was fit with a two-state model except in the case of Y42F and Y42F/T50V, where the long-wavelength peak was converted to the intermediate form at the lower melting temperature (in parentheses), and the intermediate form melted at the higher temperature.

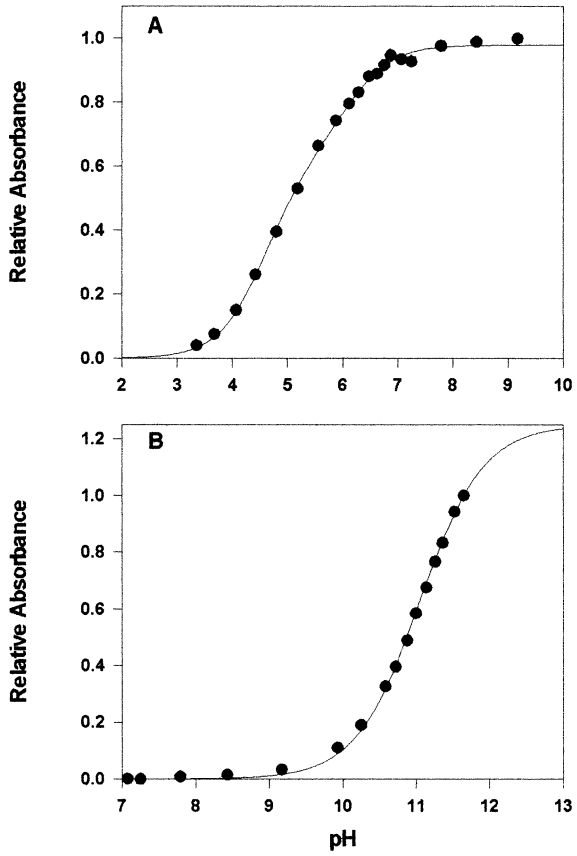

FIGURE 2: Plot of the titration data for Y42F/T50V in Figure 1. (A) Acidic titration analyzed at 474 nm (the solid line is the theoretical curve generated using a nonlinear fit to the Henderson−Hasselbach equation in Sigmaplot with $n = 1$ and two pK_a values, 4.6 (64% amplitude) and 6.1. (B) Basic titration analyzed at 440 nm (the solid line is the theoretical curve generated using a nonlinear fit to a single pK_a of 11.1 ($n = 1$) and a floating endpoint that was 20% larger than the last data point).

protonation of one or more surface residues within this range, and the nonintegral *n* value is consistent with denaturation below pH 3.8. In any case, most PYP proteins precipitated below the lowest acidic pK_a, both in WT where the pK_a is very low, or in those mutants with pK_a values closer to neutrality, which suggests at least partial denaturation between pH 3.8 and 5.4. Thus, the acidic pK_a represents denaturation that is coupled to partial unfolding and aggregation of the protein, except for E46 mutants as discussed below. In all cases, precipitation was virtually completely reversible upon neutralization (i.e., raising the pH to well above the pK_a). Although it may be possible that the acid-denatured form of PYP has some structural similarity to the photobleached protein for mutants with pK_a values above those for solvent exposed Asp and Glu, it would not apply to WT protein. However, the degree of unfolding that results in aggregation and precipitation at a pH below the pK_a would still be significantly different from that which occurs during photobleaching where there is no apparent aggregation on the second time scale. If they were similar, then the short lifetime of I_2 in WT and most mutant proteins might preclude observation of aggregation. However, mutant M100A, which has a long-lived photobleached intermediate that recovers on the hour time scale, does not aggregate (become turbid) in the bleached state at pH 5−10 (*15*). Mutant M100A reversibly loses only about 12% of its ellipticity at 222 nm upon photobleaching at pH 7, which is less than implied from NMR measurements on photobleached WT protein at what should be a relatively safe pH 5.75 (*21*). Our results, which indicate a modest structural change for I_2, suggest that the acidic form is a poor model for photobleached protein because of the complications of denaturation at low pH.

Further evidence for acid denaturation at the lower of the two pK_a values is provided by an apparent inverse correlation between the acidic pK_a and the midpoint for guanidine denaturation (as measured by loss of ellipticity at 222 nm in the circular dichroism spectrum at neutral pH) for all mutants studied except E46Q (Figure 3). If the loss of ellipticity represents a conformational change, then so does the acidic pK_a. As will be shown below, the acidic pK_a also correlates with the midpoint for thermal denaturation. Thus, the answer to the question of whether the acidic pK_a is the pK_a of the chromophore in the folded protein or is due to denaturation is clearly indicated to be the latter in WT and all PYP mutants but those at E46 (i.e., protonation of chromophore results from the effects of acid denaturation and exposure to solvent and does not initiate unfolding).

Mutant E46Q is nearly as stable as WT protein to guanidine denaturation but has a pK_a that is higher than for

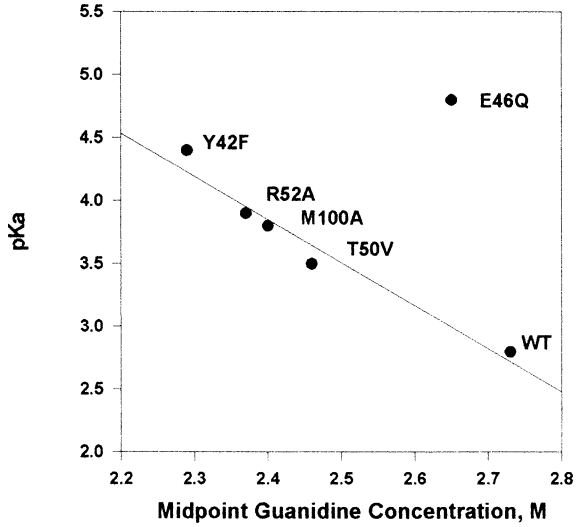

FIGURE 3: Inverse correlation between the midpoint molarity for guanidine denaturation at pH 7.5 and the pK_a for acid denaturation. Note that E46Q does not group with the others.

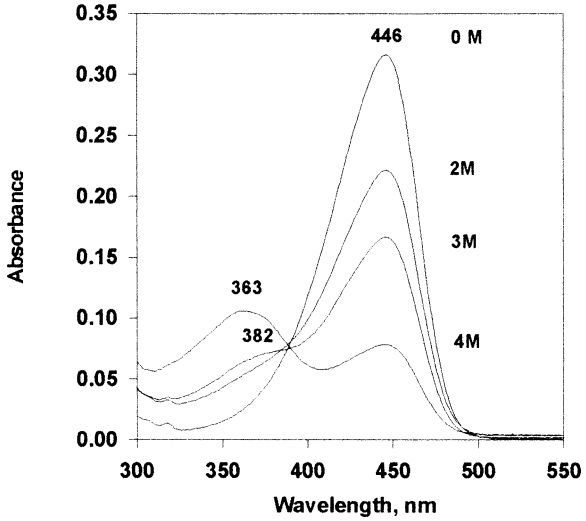

FIGURE 4: Effect of potassium thiocyanate on the absorption spectrum of WT PYP at pH 7. Note formation of the intermediate form prior to conversion to the completely bleached form absorbing at about 350 nm.

mutations at other positions for which both pK_a and guanidine midpoints of unfolding were determined (see Figure 3). The pH effects with mutant E46Q, and possibly with other E46 mutants with high pK_a values (E46A and E46D), are thus more likely to be due to the pK_a of the chromophore in the folded protein than for WT and for mutations at other positions. In support of this conclusion, we found no change in 222 nm ellipticity or protein secondary structure between pH 6 and 11 for mutant E46A, which is above and below the pK_a of 7.9 where acid denaturation is not an issue. That is, protonation of the chromophore does not initiate unfolding. This experiment could not be carried out for WT and for mutants at other positions because of turbidity at pH values below the pK_a.

What Is the Nature of the Alkaline Form of PYP? The spectral shift of the alkaline form of WT protein is not very pronounced, and hydrolysis of the chromophore thioester bond begins to occur above pH 11 before the conversion is complete. It is reasonable to suggest that hydrolysis occurs as a result of exposure of the chromophore to solvent following deprotonation of surface lysines and alkaline denaturation or unfolding triggered by electrostatic repulsion of negative charges on the protein surface. It was previously reported that the alkaline form of WT protein absorbs light at 410 nm and has a pK_a > 11.7 (*22*). However, where the titration is still isosbestic, we observe that the wavelength maximum is nearer 442 nm; thus, the 410 nm form previously reported may be a mixture of 442 and 340 nm (completely denatured and free chromophore) forms. The alkaline titration of PYP species such as the double mutant, Y42F/T50V, is much more dramatic because the peak at 474 nm is small (relative amplitude 0.24), and alkaline conversion to a 440 nm form is nearly complete before significant hydrolysis occurs, as shown in Figure 1. We did not observe any significant change in 222 nm ellipticity for this mutant at pH 10 (data not shown), which indicates that protein denaturation has not yet commenced. Wild-type PYP and all of the mutants except those at position E46 have an alkaline form that absorbs at shorter wavelength than the neutral form, as shown in Table 1. The precise wavelength maxima and pK_a values are uncertain because none of the titrations were complete before denaturation and hydrolysis commenced. Because the alkaline form is present in all but the E46 mutants, it is a likely consequence of ionization of the E46 carboxylic acid in the folded protein. This pK_a value of about 10−12 for E46 is similar to the pK_a of 11.7 based upon electrostatic calculations for the crystalline protein (*18*), in which the interior protein dielectric constant was assumed to be 2. Nevertheless, E46 may have some dynamic access to solvent in both the native and the photobleached forms consistent with the results of NMR analysis of WT protein (*19*).

Is the 391 nm Shoulder in Mutant Y42F Unique? Prior to this study, Y42F was the only mutant that had been reported to have a pronounced shoulder (at 390 nm) on the 458 nm peak at pH 7 (*14*), which we designate as the intermediate spectral form. To address the question as to whether the spectral properties of Y42F are unique, we constructed additional mutants and investigated the effects of chaotropes as recently reported (*17*). In the new single mutants, Y42W and Y42A, and in the double mutant, Y42F/T50V, the intermediate spectral form is not only present but is dominant at pH 7 as shown in Figure 1. However, the formation of an intermediate spectral form is not unique to mutations at position Y42 as would appear from the earlier studies (*14*, *17*). It is possible that the large concentration of the intermediate spectral form in Y42F is related to the fact that it is the least stable of the mutants studied to date (see the results of guanidine denaturation in Table 1) and is missing a hydroxyl, which may create a space that can be occupied by water. That protein stability can have an effect on formation of the intermediate form is shown by the impact of chaotropes on the absorption spectrum of WT PYP as shown in Figure 4. Thus, the addition of potassium thiocyanate to WT and all mutants (except those at E46) results in destabilization and formation of an intermediate species, which is in equilibrium with native and unfolded protein. Even in Y42F, the shoulder shifts to shorter wavelengths and becomes dominant in the presence of thiocyanate (or ammonium chloride (*17*)). In all cases, the actual wavelength maximum of the intermediate form is dependent upon the concentration of the chaotrope or other denaturant. For WT

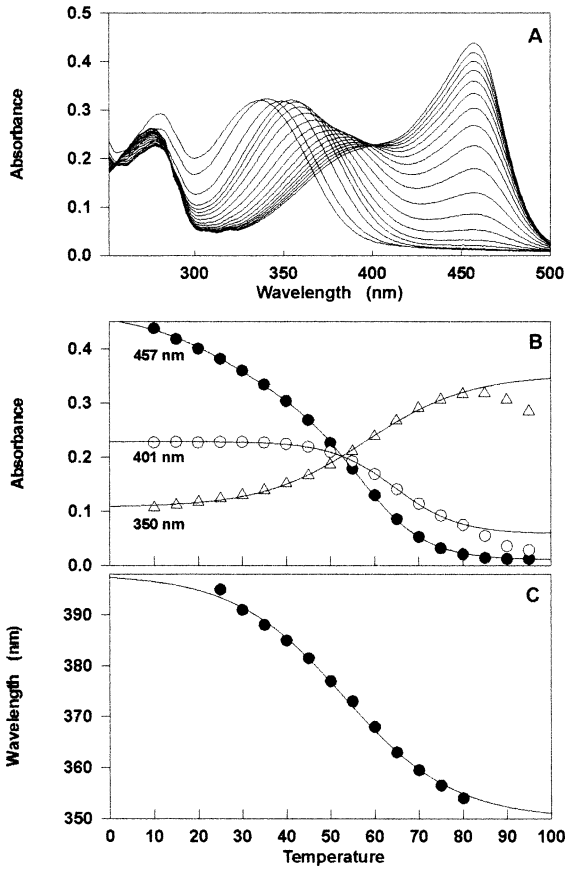

FIGURE 5: Thermal denaturation of mutant Y42F in 20 mM MOPS buffer, pH 7.0. (A) The temperature was ramped in 5 °C intervals from 10 to 95 °C. The 457 nm form of the protein is dominant at 10 °C and is converted to the intermediate form up to 40 °C with an isosbestic point at 401 nm. Both are converted to the 355 nm form up to 80 °C, and the protein completely denatures above 80 °C. (B) The absorbance vs temperature data at 457 nm were fit with a three-state model with T_m 28 and 57 °C. At 401 and 350 nm, the data were fit with a two-state model with T_m of 65 and 58 °C, respectively. (C) The wavelength maximum of the intermediate form vs temperature was fit with a two-state model with T_m 52 °C.

protein, the intermediate form absorbs at approximately 382 nm in 3 M KSCN, at 363 nm in 4 M KSCN, and presumably shifts to 350−355 nm upon further denaturation. The fact that acid denaturation of PYP mutants most often occurs in two closely spaced steps (see Table 1, pK_1 acid and pK_2 acid) may be accounted for in part by partial conversion to the intermediate form prior to complete denaturation. During guanidine (*17*) and temperature denaturation of Y42F, the 458 nm form is largely converted to the intermediate form before it is converted to the 355 nm form and ultimately to the 340 nm (completely denatured) form as shown in Figure 5. Thus, both the construction of additional mutants and the application of chaotropes demonstrate that the spectral properties of Y42F are not unique and that the intermediate form involves a conformational state accessible to WT and most mutants (under denaturing conditions) except those at E46 (see below). Therefore, the existence of the intermediate spectral form is dependent upon the presence of the E46 carboxylic acid group. The molar ellipticity at 222 nm of mutants Y42F and Y42F/T50V is virtually identical to WT, indicating that the conformational change, resulting in the intermediate form, does not involve significant changes in secondary structure.

Thermal Denaturation and the Intermediate Spectral Form. The calorimetric melting point for WT PYP and all mutants that have been measured is 86−87 °C (see Table 1). However, the 446 nm absorption peak of WT shifts to 355 nm at a midpoint of 82 °C. Provided the temperature does not exceed 80 °C, the titrations are fully reversible. Above 80 °C, the spectrum shifts to 340 nm, and reversibility is time dependent for WT and all mutants studied, which we believe is due to hydrolysis of the chromophore in completely denatured protein. However, all of the mutants have midpoints for spectral shifts that are considerably less than the 82 °C for WT and range from 56 to 73 °C as shown in Table 1. These temperatures, reflecting the conversion to a mixture of intermediate form and partially unfolded protein, may be correlated with the pK_a values for acid denaturation and the midpoints for guanidine denaturation. Thus, our results with several denaturants reflect localized rather than overall protein stability, the latter of which is indicated by the calorimetric melting temperature for complete unfolding. In the case of mutant Y42F, the native 457 nm form is partially converted to the intermediate spectral form with a melting temperature of 28 °C before both forms are converted to the 355 nm form at a melting temperature of 65 °C and finally to the fully denatured 340 nm form at 86 °C as shown in Figure 5A,B. The wavelength maximum for the intermediate form is not unique but continually shifts from about 395 to 350 nm at temperatures of 25−80 °C with a midpoint of 52 °C, Figure 5C, which is near the average of the melting temperatures of the 457 nm form and the intermediate form, which appeared to be 57−58 °C when monitored at 457 and 350 nm. We interpret this as a continuous change in the internal dielectric constant in the vicinity of the chromophore of the intermediate form and thus is a measure of the strength of the E46−chromophore hydrogen bond in the folded protein.

E46: A Critical Amino Acid Residue in PYP. Up to this point, we have focused our attention on mutations other than those at E46. This residue is special for two reasons; it has a buried hydrophilic side chain that is protonated at neutral pH, and it is hydrogen-bonded to the ionized chromophore phenolate. Mutations at E46, including E46Q (*13*), E46A (*16*), and E46Q/Y42F, are exceptional in several ways. First, the acidic pK_a for conversion of E46Q to a 355 nm form is simple and does not inversely correlate with protein stability (measured by guanidine denaturation), as described above and shown in Figure 3. The second exceptional characteristic of E46 mutants is that they do not have an intermediate spectral form in the presence of thiocyanate or upon thermal denaturation (data not shown). Third, none of the E46 mutant proteins have an alkaline spectral form distinguishable from the neutral form at high pH, as shown in Figure 6 for mutant E46Q/Y42F. Thus, the alkaline pK_a, where it can be measured in mutations other than those at E46, is likely to represent the pK_a of E46 in the folded protein when it is hydrogen-bonded to the ionized chromophore as noted above. With both E46 and the chromophore ionized inside the WT protein (above the alkaline pK_a), it is unlikely that they would still interact except via water-mediated hydrogen bonds.

The acidic pK_a for E46Q (4.8) is higher than for any of the previously characterized mutants or for WT protein, despite the fact that E46Q is one of the more stable mutants to both guanidine and thermal denaturation as described

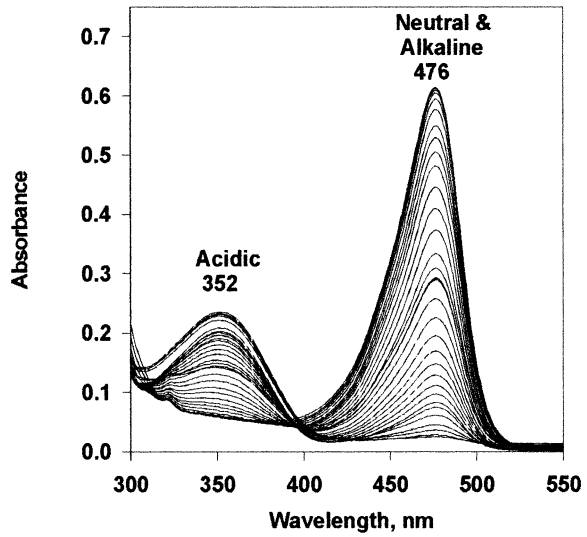

FIGURE 6: Acid/base titration of mutant Y42F/E46Q over the pH range of 5.9−11.1. The data could be fit with two pK_a values of 7.4 and 8.5 of approximately equal amplitude (not shown). Note the slight turbidity at the low pH end of the titration.

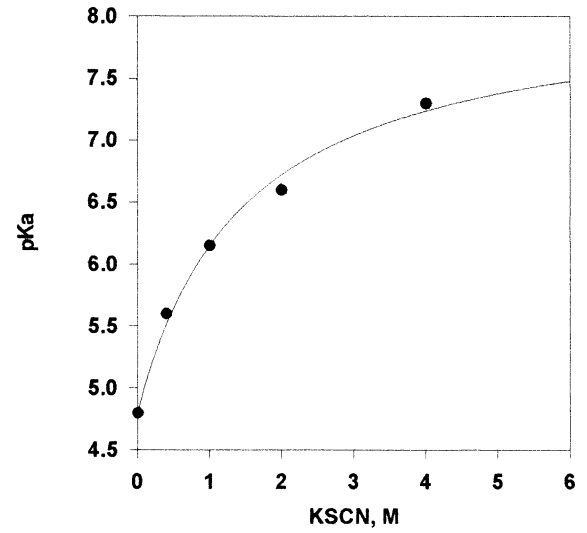

FIGURE 7: Shift in the acidic pK_a of mutant E46Q in the presence of potassium thiocyanate. The solid line is a hyperbolic fit to the data with the limiting value of the pK_a being 8.1.

above and shown in Table 1. Furthermore, addition of thiocyanate to E46Q does not result in an intermediate wavelength form, whereas the pK_a for conversion to a 352 nm form increases to a limiting value of approximately 8.1 at the highest thiocyanate concentration, as shown in Figure 7. If the pK_a of 4.8 for native E46Q represents the pK_a of the chromophore in the folded protein, then the higher pK_a values in thiocyanate solutions indicate increased solvent exposure of the chromophore, which has a pK_a of approximately 9 in water. This can also be interpreted as a continuous change in the internal dielectric constant with denaturant concentration. The fact that the limiting value of the E46Q pK_a in thiocyanate (8.1) does not quite reach the pK_a of the chromophore in water (*23*) indicates that the chromophore is not completely exposed to solvent even at the highest thiocyanate concentrations. The much higher pK_a values for mutants E46A and E46D suggest lower protein stability and increased solvent exposure of the chromophore.

CONCLUSIONS

On the basis of the studies described above, it appears that the E46 carboxylic acid group is necessary for occurrence of both the intermediate and the alkaline spectral forms. Previously, it was proposed that the intermediate spectral form results from increased solvent exposure of the chromophore or to an additional hydrogen bond to either E46 or the chromophore (*17*). Because we have now found that the presence of the intermediate form is dependent upon the presence of the E46 carboxylic acid group, it is likely that E46 is directly involved in its formation. Since there is no unique wavelength for the intermediate spectral form, its position may represent differences in the length or geometry of the E46−chromophore hydrogen bond and thus its strength. It may also represent varying degrees of solvent exposure and thus a changing dielectric constant in the vicinity of the chromophore. The shift of this peak toward the 355 nm spectral form, which represents fully protonated chromophore, may indicate that E46 is less electronegative in the intermediate spectral form, perhaps as a result of increased exposure to solvent and/or to acceptance of a hydrogen bond as previously suggested (*17*). The double mutant, Y42F/T50V, has an intermediate spectral form, which eliminates either residue as a possible H-bond donor to E46 in that species. Remaining possibilities are a peptide amide or solvent water. Because there are no peptide amides in the crystal structure sufficiently near E46 to form a hydrogen bond, water is considered to be the more likely hydrogen-bond donor to E46. The cavity created by loss of the phenolic oxygen in mutants Y42F and Y42F/T50V is an obvious location for the hydrogen-bonding water molecule. The equilibrium for the conformational change, which allows access to water, also shifts with denaturant concentration, thus allowing a continuous increase in dielectric constant at the chromophore, which is proportional to the concentration of the intermediate form, thus causing a blue-shift in the absorption spectrum.

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