Mimic of Photocycle by a Protein Folding Reaction in Photoactive Yellow Protein*

The light receptor photoactive yellow protein (PYP) displays rhodopsin-like photochemistry based on the trans to cis photoisomerization of its p-coumaric acid chromophore. Here, we report that protein refolding from the acid-denatured state of PYP mimics the last photocycle transition in PYP. This implies a direct link between transient protein unfolding and photosensory signal transduction. We utilize this link to study general issues in protein folding. Chromophore trans to cis photoisomerization in the acid-denatured state strongly decelerates refolding, and converts the pH dependence of the barrier for refolding from linear to nonlinear. We propose transition state movement to explain this phenomenon. The cis chromophore significantly stabilizes the acid-denatured state, but acidification of PYP results in the accumulation of the acid-denatured state containing a trans chromophore. This provides a clear example of kinetic control in a protein unfolding reaction. These results demonstrate the power of PYP as a light-triggered model system to study protein folding.

Signal transduction in biology starts with the activation of receptor proteins. In the case of photochemical signal transduction, signals are generated by photoreceptors containing light-absorbing chromophores like retinal. Photoactive yellow protein (PYP) is a water-soluble blue-light receptor in *E. coli* and related eubacteria (1, 2). *E. halophila* displays negative phototaxis because of its life time and its resemblance to the S373 intermediate of the archaebacterial sensory rhodopsin I (1, 13, 14). A number of results indicate that a large structural change occurs during the formation of the pB state, resulting in the partial unfolding of PYP (12, 15–21).

To explore the relationship between protein folding in PYP and signaling state formation during the photocycle, we have studied the kinetics of protein folding in PYP. Recently, we demonstrated that refolding of PYP from the fully denatured state containing a cis chromophore involves the pB photocycle intermediate as an on-pathway folding intermediate (19). Thus, the folding pathway in PYP can be studied both by conventional stopped-flow techniques and by photoexcitation. Here we extend this novel approach for studying protein folding to the transitions between native PYP and its acid-denatured state pBtrans (also called pBdark) in rapid mixing pH jump experiments.

**EXPERIMENTAL PROCEDURES**

**Absorbance and Fluorescence Spectroscopy**—PYP was overexpressed in *Escherichia coli* and purified as described previously (9). PYP was used at a concentration of 5 μM for absorbance measurements and 2 μM for fluorescence spectroscopy. Steady state equilibrium measurements of the PYP absorbance spectrum at different pH values and temperatures were performed using a Cary 300 UV-visible spectrophotometer (Varian) equipped with a Cary 1 × 1 Peltier element. The kinetics of the last photocycle step were recorded at 446 and 340 nm at different pH values (in the same buffer as the stopped-flow experiments; see below) after 20 s illumination with broadband blue actinic light using the Cary 300 UV-visible spectrophotometer (Varian) or a Hewlett-Packard 8453 diode array spectrophotometer. Intrinsic aromatic and chromophore fluorescence from PYP was measured using a PerkinElmer Life Sciences LS50B spectrometer both in steady state experiments at pH 2.0 and in kinetic experiments on the photocycle at pH 3.9. Fluorescence excitation was set at 280 nm for intrinsic aromatic fluorescence, 350 nm for chromophore fluorescence from the acid-denatured state, and 440 nm for fluorescence from the pBtrans state. The fluorescence emission spectrum of the pH photocycle intermediate at pH 3.9 was reconstructed from each kinetic trace at different wavelengths by fitting the data as a monoeponential decay, and extrapolation to t = 0, i.e. immediately after the actinic illumination.

**Stopped-flow Absorbance Spectroscopy**—Stopped-flow absorbance spectroscopy for pH jump experiments on PYP unfolding and refolding was performed using an SX-18MV stopped-flow spectrophotometer (Applied Photophysics) with a volumetric ratio of 1:1. For unfolding transitions to low pH, yielding pBtrans, PYP in 10 mM potassium phosphate (pH 7.3) and 50 mM KCl was mixed rapidly with a buffer solution of 50 mM citrate, 50 mM KCl, and different concentrations of HCl or KOH. For refolding transitions, PYP was first denatured at pH 2.4 or 2.0 in 10 mM potassium phosphate, 25 mM citrate, and 10 or 20 mM HCl, and subsequently refolded by rapid mixing with solutions containing different concentrations of KOH, to yield final pH values from pH 3.2 to 7.6. To investigate the effect of the pC isomerization state on the unfolding process, acid-denatured PYP at pH 2.0 in 10 mM potassium phosphate, 25 mM citrate, and 20 mM HCl was exposed to UV-A light from a Cuda 1-150 light source for 4 min to photoisomerize the pC from tran to cis, yielding pH2trans. This PYP solution was mixed with solutions containing different concentrations of KOH. Stopped-flow absorbance traces at 445 and 340 nm were recorded at least four times, averaged, and analyzed using the SX-18MV software. The refolding jump experiments from pH2trans to a final pH value of 7.6 were also performed at different tem-
peratures to allow the thermodynamic analysis of this folding transition.

Data Analysis—The equilibrium absorbance data on the acid denaturation of PYP were described as a two-state transition using Equation 1, where the equilibrium constant $K_{\text{on}}$ is defined by $10^{(\Delta G^\circ /RT)}$; $\text{Abs}$ is the observed absorbance at 446 or 340 nm; and $a_0$, $b_0$, and $a$, $b$ are the parameters for the sloping base lines to describe the pH dependence of native and acid-denatured PYP, $pK$ and $n$ are the pH at the transition midpoint and the number of protons taken up in the unfolding transition, respectively.

$$\text{Abs} = [(a_0 + b_0pH) + (a + b_pH)10^{(\Delta G^\circ /RT)}] (1 + e^{(\Delta G^\circ /RT)})$$

(Eq. 1)

The rate constants for the unfolding and refolding reactions observed by stopped flow absorbance spectroscopy were analyzed as follows. First, the data were fit as a monoexponential process. The resulting rate of the protein refolding rate ($k_u$) and unfolding rate ($k_o$) ($k_u = k_o + k_t$), with the ratio of $k_o$ to $k_u$ determined by the equilibrium constant $K_{\text{on}}$, $K_{\text{on}} = k_o/k_u$. Combining these two equations, $k_t$ and $k_o$ can be obtained from $K_{\text{on}}$ and $k_u$. The resulting equations link the equilibrium and kinetic data, providing a quantitative test of the two-state nature of the transition.

$$k_t = k_u/(1 + K_{\text{on}})$$

(Eq. 2)

$$k_o = K_{\text{on}}k_u/(1 + K_{\text{on}})$$

(Eq. 3)

The pH dependence of the kinetics of protein unfolding and refolding is caused by the uptake or release of protons upon reaching the transition state. In general, the pH dependence of the refolding kinetics can be described by the following equation, where a change in pH of $\Delta pH$ causes a change in the folding rate $d\text{log}k_u/dpH$ (22, 23).

$$d\text{log}k_u/dpH = -(1/2.3RT\Delta G^\circ/\Delta pH) = -\Delta\text{Q}_D$$

(Eq. 4)

$R$ is the gas constant, $T$ is the absolute temperature, $\Delta G^\circ$ is the activation free energy for reaching the transition state from the denatured state, and $\Delta\text{Q}_D$ is the number of moles of protons involved upon reaching the transition state.

Two different phenomenological descriptions of the pH dependence of $d\text{log}k_u/dpH$ were used: (i) a linear dependence, in which the $\Delta\text{Q}_D$ does not depend on pH (Equation 5); and (ii) a phenomenological description, in which deviations from linearity are described by an additional quadratic term (Equation 6).

$$\log k_t = y_0 + a[pH]$$

(Eq. 5)

$$\log k_o = y_0 + a[pH] + b[pH]^2$$

(Eq. 6)

The temperature dependence of the rate constants $k$ for folding and photocycle transitions was described using activation changes in enthalpy, entropy, and heat capacity $\Delta H^\circ$, $\Delta S^\circ$, and $\Delta C^\circ_v$ at an arbitrarily chosen reference temperature of 298 K (Equation 7).

$\ln K^\circ = S_{\text{GND}}RT - \Delta S_{\text{GND}}RT - (\Delta C^\circ_v/RT)(1 - 298K/RT)$

(Eq. 7)

$T$ is the temperature in degrees Kelvin, $h$ is Planck’s constant, and $k$ is Boltzmann’s constant.

Equilibrium thermal denaturation curves for $pB^\text{trans}$ and $pB^\text{cis}$ at pH 2.0 were analyzed using Equation 8 as an equilibrium between the native and the unfolded state and two sloping base lines. The temperature dependence for the free energy for unfolding $\Delta G_J(T)$ was described as Equation 9, where $T_n$ is the temperature at the transition midpoint.

$$\Delta G_J(T) = \Delta H(T_n)(1 - T/T_n) - \Delta C_v(T_n - T) + T \ln(T/T_n)$$

(Eq. 9)

RESULTS AND DISCUSSION

Effect of the Chromophore Isomerization State on Refolding Kinetics—At low pH the initial $pG^\text{trans}$ state of PYP is converted to the blue-shifted, partially unfolded $pB^\text{trans}$ state with an apparent $pK_a$ of 2.8 (24, 25) (Fig. 1C). This acid denaturation process involves the protonation of the pCA chromophore, resulting in the blue-shift of the absorbance spectrum of PYP from 446 to 350 nm. We studied the kinetics of the transitions between $pG^\text{trans}$ and $pB^\text{trans}$ as determined in pH jump experiments using stopped-flow absorbance spectroscopy. Because of the large difference in absorbance maximum, these transitions can be sensitively monitored at both 350 and 445 nm. At 25 °C the transitions between $pG^\text{trans}$ and $pB^\text{trans}$ were beyond the time resolution (2 ms) of the rapid mixing device (Fig. 1A) in the pH range 2.0 to 7.5. By performing the pH jump experiments at 5 °C, the kinetics were slowed down sufficiently to allow the accurate determination of the rate constant for the transitions between $pG^\text{trans}$ and $pB^\text{trans}$ in a wide pH range (Fig. 1B, filled circles). At all pH values the signals could be described as a monoeponential transition. The logarithm of the refolding rate ($k_t$) and unfolding rate ($k_o$) show a linear pH dependence, with slopes of 0.30 and 0.85, respectively. This indicates that the value of the relevant changes in charge $\Delta Q_D$
and \( \Delta Q_{\text{deg}} \) (see Equation 4) are independent of pH in the range 1.7 to 7.5. The rates of unfolding and refolding were found to be identical at pH 2.8, which is the midpoint transition in equilibrium pH titrations (Fig. 1C). Equilibrium titrations at 5 °C were essentially identical to those performed at 25 °C (25), with a \( pK_a \) of 2.8 and an \( n \) value of 1.15 (see Equation 1). Thus, the sum of the slopes of the pH dependence observed for the unfolding and refolding transitions is equal to number of protons taken up during the transition as found in the equilibrium data. The monoexponential behavior and good correspondence between equilibrium and kinetic data (using Equations 2 and 3) indicate that the transition between pG\text{trans} to pB\text{trans} can be described as a two-state process.

To directly study the effect of the isomerization state of the pCA chromophore on the kinetics of refolding from the acid-denatured state to the native pG\text{trans} state, pB\text{trans} was illuminated with UV-A light at pH 2.0 to generate pB\text{cis}. The formation of pB\text{cis} could be monitored by a small but characteristic red-shift in the absorbance spectrum of the acid-denatured state (data not shown), as the absorbance maximum of pB\text{trans} is at 350 nm, and that of pB\text{cis} at 355 nm (25). Both species are fully stable at low pH. The formation of pB\text{cis} had a strong effect on the protein refolding kinetics; strongly biexponential behavior was observed. The very fast phase of \(~50\%\) could be attributed to refolding from pB\text{trans}. The remaining 50% of the signal displayed dramatically decelerated folding kinetics, caused by refolding from pB\text{cis} (Fig. 1A). Apparently, the UV-A illumination resulted in the formation of a stable mixture of equally populated pB\text{trans} and pB\text{cis} states. The pH dependence of the kinetics for refolding from pB\text{cis} to pG\text{trans} was determined, revealing that isomerization of pCA to its cis isomer decelerated the kinetics of refolding by \( 3–5 \) orders of magnitude (Fig. 1B, open circles at 25 °C and open squares at 5 °C). The deceleration of refolding is attributed to the energy barrier caused by the cis to trans isomerization of the pCA that needs to occur for refolding from pB\text{cis}. For free pCA the energy barrier for thermal isomerization has been estimated to be 125 kJ/mol (see Ref. 18). This experiment demonstrates how the energy landscape for protein refolding can be experimentally modified by the introduction of a specific chemical process into the energy landscape for folding.

The pH dependence of the logarithm of refolding rates for pB\text{cis} significantly deviates from linear behavior both at 25 and 5 °C (Fig. 1B). This reveals that, for pB\text{cis}, the value of \( \Delta Q_{\text{deg}} \) is pH-dependent, in contrast to the situation found for pB\text{trans}. The change in \( \Delta Q_{\text{deg}} \) for pB\text{cis} refolding as a function of pH was estimated by analysis of the data using a phenomenological quadratic equation (Equation 6). This analysis revealed that the number of protons \( \Delta Q_{\text{deg}} \) released by pB\text{cis} upon reaching the transition state decreases from \(-1\) to \(-0\) in the pH range from 3.2 to 7.5. The interpretation of these observations is discussed below.

The deceleration of PYP refolding by isomerization of the pCA to the cis conformation is analogous to the effect of isomerization of the imide bond of Pro residues (26). Because such Pro isomerization events occur with a significant energy barrier (85 kJ/mol), they can be rate-limiting for protein folding, as we found for pCA isomerization during PYP folding. However, the pCA isomerization effect exhibits two attractive features not found for Pro isomerization; (i) the extent of pCA isomerization in unfolded PYP is under direct experimental control (photoisomerization), and (ii) pCA isomerization is a central part of the functional cycle of PYP.

**Mimic of the Last Photocycle Transition by pH Jump Refolding**—We directly tested the relationship between the protein refolding reaction from pB\text{cis} to pG\text{trans}, as observed by stopped-flow absorbance spectroscopy, and the pB to pG\text{trans} photocycle transition, as observed after photoexcitation of PYP. To this end, the kinetics of the last PYP photocycle transition were measured by time-resolved absorbance spectroscopy as a function of pH. These measurements showed that the photocycle kinetics at different temperatures at pH 7.5 were reported previously (15) (filled circles). We attribute the small difference between the two curves to minor differences in experimental condition, such as buffer composition.

**Comparison of the Structure of Kinetic and Equilibrium pB Species**—To further investigate the structure of pB\text{trans}, pB\text{cis}, pB, and pG\text{trans}, we studied and compared the aromatic fluorescence signals from these four species. Excitation of pG\text{trans} at 280 nm (pH 7.0) elicits a fluorescence emission peak at 330 nm (Fig. 3A, spectrum 1), consistent with the 5 Tyr residues and single Trp side chain in PYP. In the pB\text{trans} state at pH 2.0, the amplitude of this peak is reduced by \(-39\%\) and the emission maximum is shifted from 330 to 350 nm (Fig. 3A, spectrum 2). In addition to this aromatic fluorescence band, the spectra of pG\text{trans} and pB\text{trans} both display a second emission band of lower intensity, at 495 and 435 nm, respectively. These bands are caused by fluorescence emission from the pCA chromophore. The pCA in the pG\text{trans} state is known to have an emission maximum at 495 nm (27) (Fig. 3C, spectrum 13). To confirm that the emission band at 435 nm is caused by pCA, the
emission spectrum of this species was measured upon excitation of the chromophore at 350 nm, and was indeed found to be at 435 nm (Fig. 3C, spectrum 9). This reveals fluorescence resonance energy transfer (FRET) from the aromatic amino acids to the pCA chromophore. The FRET pCA emission band in pbtrans is a factor of 7.5 stronger than that in pgtrans, presumably because of the increased spectral overlap between the emission from the aromatic amino acids and the absorbance of the pCA chromophore in pbtrans.

The fluorescence emission spectra of pbcis were obtained by performing fluorescence spectroscopy on a mixture containing 50% pbtrans and 50% pbcis, obtained as described above (Fig. 3, A (spectrum 3) and C (spectrum 10)). The data were then corrected for the contribution of the 50% pbtrans to yield the pure spectrum of pbcis at pH 2.0 (Fig. 3, A (spectrum 4) for aromatic fluorescence and C (spectrum 11) for chromophore fluorescence). The intensity of fluorescence emission from the aromatic amino acids is increased a factor of 2.0 by the trans to cis isomerization of the pCA in the acid-denatured state of PYP, whereas the FRET emission band at 435 nm is completely lost in pbcis. The chromophore fluorescence from the pCA in pbcis is strongly quenched: fluorescence excitation at 350 nm does not yield an emission band at 435 nm, in contrast with pbtrans (Fig. 3C, spectrum 11). In summary, the pbtrans state exhibits fluorescence properties quite different from pbcis: (i) increased fluorescence of aromatic residues, (ii) strong quenching of pCA fluorescence, and, possibly because of this, (iii) no emission band by FRET from aromatic side chains to the pCA chromophore. This indicates that pCA trans to cis photosomerization results in structural changes in the acid-denatured state of PYP.

The three fluorescence characteristics that clearly distinguish pbtrans from pbcis were used to compare the structure of the kinetic pb photocycle intermediate with that of the equilibrium species pbcis. The fluorescence emission properties of the pb photocycle intermediate were reconstructed from kinetic measurements at pH 3.9 during the pb to pgtrans photocycle transition, observed after switching off actinic illumination. Under the conditions used, essentially all PYP was converted to pb, and changes in fluorescence emission during relaxation to pg were determined. Both the aromatic (Fig. 3B, closed circles) and chromophore (Fig. 3C, closed circles, spectrum 12) fluorescence properties of the kinetic photocycle species pb are highly similar to those of the equilibrium acid-denatured species pbcis, indicating that these two states of PYP have a very similar structure.

Stabilization of the Acid-denatured State by Chromophore trans to cis Photosomerization—To further investigate the effect of pCA isomerization on the acid-denatured state of PYP, the thermal stability of pbtrans and pbcis were determined. Thermal denaturation of pbtrans to the fully unfolded state resulted in a shift in absorbance maximum from 350 to 338 nm, and an increase in extinction coefficient at the absorbance maximum by 23% (Fig. 4). This shift occurred in a single, cooperative unfolding transition with a midpoint temperature of 42 °C.

Conversion of 50% of the pbtrans population to pbcis by UV-A illumination of the sample had a marked effect on the thermal denaturation curve. In this case, two thermal transitions were observed: one at 42 °C, corresponding to pbtrans, and a second at 72 °C, corresponding to the thermal denaturation of pbcis (Fig. 4, closed squares). These data show that the trans to cis isomerization of the pCA chromophore significantly increases the thermal stability of the acid-denatured state (Table III). Quantitative analysis (using Equations 8 and 9) of the denaturation curve of pbcis was complicated by the fact that, even at 100 °C, the thermal denaturation of this state was not complete, but indicates that pCA isomerization stabilizes the acid-denatured state of PYP by ~16 kJ/mol.

The native conformation of PYP has a strong energetic preference for the trans chromophore. Our results show that for the acid-denatured state the opposite is true: the cis conformation is energetically favored. However, when the pG state is acidified, it is partially unfolded to pbtrans, not pbcis. This demonstrates that acid denaturation of PYP is under kinetic control (Fig. 4B); because of the higher activation barrier for the formation of pbcis, the acid-denatured state containing trans pCA is accumulated at low pH. Kinetic control was previously proposed by the Agard group (28, 29) for protein refolding. Here, we report that, upon acidification, native PYP is converted to the local free energy minimum of the pbtrans state, even though the pbcis state has a significantly lower free energy.

The pH Dependence of Protein Folding Kinetics in PYP: Transition State Movement—The results on the pH dependence of the kinetics for the formation of pgtrans from the three blue-shifted states studied here result in the following challenge: which model can describe (i) the pH-independent non-linear size of the pbtrans state and (ii) the transition from linear to nonlinear pH dependence upon chromophore isomerization?

This question is corroborated by a re-analysis that we performed of the published pH dependence of the pb to pg photocycle transition of a set of seven PYP mutants (30–34) on a log-log scale, as we did for wild-type PYP (Fig. 5). Two groups of mutant PYPs could be classified according to the pH-dependent profile of refolding kinetics. The E46Q, M100A, R52A and T50V mutants show a nonlinear pH dependence, as does the wild type (Fig. 5A). The E46D, E46A, and Y42F mutants exhibit a linear pH dependence in the pH ranges studied (Fig. 5B). Apparently, not only chromophore photosomerization, but also these side-directed mutations shift the pH dependence of the barrier for the pb to pg transition from nonlinear to linear. In addition, the pH dependence of the pb to pg transition in the E46D, E46A, and Y42F mutants is characterized by fractional ΔQ values (see Fig. 5B).

We examined if the position of Tyr42, Glu46, Thr50, Arg52, and Met100 in the x-ray structure of PYP (35) provides further insights into the pH dependence of the photocycle kinetics. All of these residues contribute to the first shell of atoms surrounding the pCA chromophore (see Fig. 2 of Ref. 12). The phenolic group of Tyr42 and the acidic side chain of Glu46 are directly hydrogen-bonded to the phenolate oxygen of the pCA chromophore, whereas the side chain of Thr50 is hydrogen-bonded to that of Tyr42. The guanidino group of Arg52 and the sulfur atom of Met100 are placed immediately adjacent to the pCA. Interestingly, only mutants that disrupt the active site hydrogen bonds between the chromophore and Glu46 and Tyr42 result in a linear pH dependence.

Previously, the pH dependence of the kinetics of the pb to pgtrans photocycle transition was described using equations developed for the pH dependence of enzyme catalysis, in which the pH dependence of the reaction is caused by equilibrium changes in the protonation state of groups with specific pKa values (30–34). In this description a log-linear pH dependence

| ΔS° | ΔM° | ΔV° |
|-----|-----|-----|
| J/mol·K | kD/mol | kD/mol·K |
| pbtrans ↔ pG | −187 | 13.5 | −2.49 |
| pb ↔ pgcis | −196 | 9.2 | −2.73 |

*Values taken from Ref. 15.
spectra and intermediate (spectra 8) at pH 4 was reconstructed from each kinetic trace at different wavelengths by taking the values at sloping baseline for the native state and a fixed value for the thermally sloping baselines for the native and denatured state (a), and with one plateau, complicating their analysis. The data were analyzed using two results in a slope of 1 for low pH values and \( Q / H 9004 \) based on such a model. Thus, the fractional values of schematic energy surface of PYP at low pH.

The data for pBcis thermal denaturation (see Fig. 4) did not show a plateau, complicating their analysis. The data were analyzed using two sloping baselines for the native and denatured state (a), and with one sloping baseline for the native state and a fixed value for the thermally denatured state at 95 °C (b).

The kinetic control for protein unfolding to the acid-denatured state of PYP exerted by chromophore isomerization. A, thermal denaturation of pBtrans (filled circles) and a pBtrans/pBcis mixture (filled squares) at pH 2.0 monitored by absorbance at 337 nm. B, schematic energy surface of PYP at low pH.

![Graph](Image)

**Fig. 3.** Probing the structure of different states of PYP by fluorescence spectroscopy. The figure shows fluorescence from aromatic residues in the steady state species pBcis at pH 2.0 (A) and in the transient pB photocycle intermediate (B). The pure pBcis spectrum (spectrum 4) was calculated from the spectra of pBtrans (spectrum 2) and 50% mixture of pBtrans and pBcis (spectrum 3) at pH 2. The spectrum of the pB photocycle intermediate (spectrum 8) at pH 4 was reconstructed from each kinetic trace at different wavelengths by taking the values at \( \pm 4 \). Native PYP fluorescence spectra (spectra 1 and 5) at pH 7 were recorded for direct comparison in the two independent experiments (A and B). Spectra 6 and 7 are the PG spectrum reconstructed from each kinetic trace, and continuously scanned at pH 4, respectively. C, chromophore fluorescence excited at 350 nm (spectrum 9–12) or 440 nm (spectrum 13). The pure pBcis spectrum (spectrum 11) was calculated from pBtrans (spectrum 9) and a 50%/50% mixture of pBtrans and pBcis (spectrum 10). pCA fluorescence spectra of the pB photocycle intermediate at pH 4 were also reconstructed from each kinetic trace at different wavelengths (spectrum 12). Spectrum 13 is a PG fluorescence at pH 4.0.

**Fig. 4.** Kinetic control for protein unfolding to the acid-denatured state of PYP exerted by chromophore isomerization. A, thermal denaturation of pBtrans (filled circles) and a pBtrans/pBcis mixture (filled squares) at pH 2.0 monitored by absorbance at 337 nm. B, schematic energy surface of PYP at low pH.

| Transition | \( \Delta G_m^a \) (kJ/mol) | \( T_m^a \) (°C) |
|------------|----------------------------|-----------------|
| pBtrans    | 6.9                        | 315             |
| pBcis      | a                          | 16.5            |
|            | b                          | 29.4            |
| Average    |                            | 23.0            |

The data for pBtrans and pBcis at pH 2.0, 298 K

The data for pBtrans and pBcis at pH 2.0, 298 K

The data for pBtrans and pBcis at pH 2.0, 298 K

The data for pBtrans and pBcis at pH 2.0, 298 K

of the kinetics is interpreted as the titration of a functionally important group with a \( p K_a \) outside the studied pH range. This results in a slope of 1 for low pH values and \( -1 \) for high pH values. Fractional values for the slope are difficult to understand based on such a model. Thus, the fractional values of \( \Delta G^a \), observed both for the folding kinetics of pBcis of native PYP and for the photocycle kinetics of the E46D, E46A, and Y42F mutants, provide an important clue to the origin of the pH dependence of the kinetics. Because we found that the pBcis to pGtrans transition is a protein refolding event, we investigated an alternative interpretation, based on the analysis developed to describe the dependence of the kinetics of protein folding on denaturant concentration (chevron analysis).

We propose that the fractional \( \Delta G^a \) values for the transitions between pGtrans and pBtrans can be described in analogy with the analysis of a denaturant chevron plot for a two-state transition, with \( m^a \) values for unfolding and refolding that are independent of denaturant concentration. The effect of denaturant concentration on the kinetic of folding provides information on solvent-exposed surface area upon transition state formation. Analogously, the effect of pH reveals changes in charge \( \Delta Q^\ddagger \) that are required to reach the folding transition state and provides a measure of the electrostatic interactions in the transition state (22, 23, 36, 37). In this proposal the transfer of protons between the solvent and the protein occurs only partially during the formation of the transition state for pBtrans refolding. The pH independence of the \( \Delta Q^\ddagger \) values for pBtrans then indicates that the position of the transition state does not depend on pH, with \( \sim -25\% \) (0.30/(0.30 + 0.85)) of the native electrostatic interactions formed in the transition state for refolding. The most straight forward candidate of the major contribution to the observed \( \Delta Q^\ddagger \) values is the pCA chromophore, because the deprotonation of this buried group is an essential step in the formation of the native state.

The curved pH dependence for the kinetics of the pB to pGtrans transition correspond to a curved chevron plot, which has been found for a range of proteins. Such curved denaturant chevron plots can be caused by two different phenomena: (i) the involvement of a folding intermediate (38, 39) or (ii) movement of the transition state in a two-state transition (40). We propose a transition state movement to explain the nonlinear pH dependence of the kinetics for pGtrans. First, this proposal is in line with the absence of indications for intermediates in the pB to PG photocycle transition or pH jump experiments. Second, this model can describe the observed curved pH dependence of the kinetics: a pH-induced transition state movement involves a change in the apparent \( \Delta Q^\ddagger_B \). This change in \( \Delta Q^\ddagger_B \) for refolding from pBcis may reflect the titration behavior of a specific group or may be caused by the pH-induced shift from one barrier to a second barrier. We prefer the latter interpretation, based on the following reasoning. For refolding from pBtrans, the chromophore deprotonation event is likely to result in the linear pH dependence of refolding. The refolding process from pBcis involves an additional process: thermal pCA re-isomerization. This is expected to give rise to an additional energy barrier on the folding landscape of pBcis. In this proposal, a transition state movement occurs from a pH-dependent pCA deprotonation barrier to a pH-independent pCA isomerization barrier (Fig. 6B). The pCA deprotonation then results in a log-linear pH dependence, as observed for pBtrans, whereas the kinetics of pCA isomerization do not depend on pH. A shift between these two barriers results in the observed curved pH dependence. In the case of the mutants with a perturbed hydrogen bond network around the pCA chromophore, the pCA deprotonation process may well be strongly decelerated. This would prevent the isomerization step from becoming the dominant bar-
rier in the studied pH range, resulting in the observed pH-independent slope. An attractive aspect of this analysis of the pH dependence of the PYP photocycle kinetics, is that it offers a tool to disentangle the multiple molecular events that occur during the pB to pG photocycle transition.

Conclusions—A striking feature of the results reported here is that the kinetics of protein refolding for the acid-denatured state of PYP containing cis pCA is exactly same as that of the photocycle step from pB to pGtrans over a wide pH (Fig. 1B) and temperature (Fig. 2) range. Therefore, the final step in the PYP photocycle corresponds to a protein refolding reaction, implying a direct link between transient protein unfolding and photosensory signal transduction (Fig. 6A).

We demonstrate that the isomerization state of the pCA chromophore has a number of significant effects on the acid-denatured state of PYP: (i) the kinetics of refolding to pGtrans are decelerated by 3–5 orders of magnitude for cis pCA; (ii) the fluorescence properties of both the pCA chromophore and the aromatic amino acids are altered, indicating differences in tertiary structure; and (iii) the stability of the acid-denatured state are stabilized by \( \Delta H = 16 \text{ kJ/mol} \) by the presence of cis pCA. The stabilizing effect of the cis chromophore of 16 kJ/mol is likely to contribute significantly to the reduced refolding rate for pBcis. From the finding that both the aromatic fluorescence and the stability of the acid-denatured state are significantly affected by the isomerization of the pCA chromophore, it can be concluded that protein-chromophore interactions play an important role in the acid-denatured state.

Our results demonstrate how PYP provides as a strong model system to study protein folding, in which the folding reactions can be initiated not only by jumps in pH or denaturant concentration but also by pCA photoexcitation. We have used this opportunity to study three general issues in protein folding. (i) We performed a comparison of partially folded species observed in equilibrium and kinetic experiments, indicating a clear resemblance between the equilibrium species pBcis and the kinetic intermediate pB. (ii) We provided a clear example of kinetic versus thermodynamic control in protein unfolding. (iii) We investigated the origin of linear and nonlinear pH dependence of the barrier for protein folding. We formulate a general model that can provide a quantitative explanation for transitions that exhibit a pH-independent fractional value of \( \Delta Q \), which has also been reported for bacteriorhodopsin (41).

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pH Jump Refolding Mimics the Photocycle of PYP

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Byoung-Chul Lee, Paula A. Croonquist and Wouter D. Hoff

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