Modulating Native-like Residual Structure in the Fully Denatured State of Photoactive Yellow Protein Affects Its Refolding*

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Residual structure in the fully unfolded state is a key element for understanding protein folding. We show that the residual structure in fully denatured photoactive yellow protein (PYP) is affected by isomerization of its p-coumaric acid (pCA) chromophore. The exposure of total surface area and hydrophobic surface area upon unfolding was quantified by denaturant m values and heat capacity changes (ΔCp), respectively. The exposure of the buried surface area upon unfolding of the acid-denatured state of PYP containing trans-pCA is ~20% smaller than that of the native state. In contrast, for the partially unfolded pB photocycle intermediate containing cis-pCA, unfolding-induced exposure of the surface area is not decreased. These results show that pCA photoisomerization reduces residual structure in the fully unfolded state. Thus, residual structure in the fully unfolded state of PYP is under direct experimental control by photoexcitation. The sensitivity of the unfolded state to pCA isomerization provides a novel criterion that residual structure in the unfolded state of PYP is native-like, involving native-like protein-chromophore interactions. A largely untested prediction is that native-like residual structure facilitates the conformational search during folding. In the case of PYP, refolding from the less disordered fully unfolded state containing trans-pCA indeed is substantially accelerated. The burial of hydrophobic surface area in the fully unfolded state suggests that a significant part of the hydrophobic collapse process already has occurred in the denatured state.

Protein folding is a universal and essential process in biology in which an initially unstructured protein that is synthesized by the ribosome spontaneously folds into its functional threedimensional structure. A central question is how proteins reach their native state by efficiently searching through the vast number of possible conformations. Experimentally, the “fully unfolded” state of a protein is often populated by incubation with high concentrations of chemical denaturants, particularly GdmHCl and urea. The structural properties of native states of proteins are usually well defined by a narrow energy well, allowing their structural characterization at very high resolution. In contrast, the unfolded state can adopt an astronomically large number of distinct conformations, and its structural properties are much harder to quantify.

A number of experiments indicate the existence of considerable residual structures in the fully unfolded state of proteins (1–7). It is possible that subtle structural preferences dramatically reduce the conformational space accessible to the unfolded state. The number of conformers available to a 100-residue protein has been estimated at 10100 (8). Assuming a typical sample volume of 1 ml and a protein concentration of 1 mg/ml (~1015 protein molecules), this implies that even when only 1 part in 1073 of the entire conformational space is sampled by the protein, each molecule will be in a unique conformation. Thus, the fully unfolded state of a protein can exhibit very strong statistical structural preferences while still appearing to behave like a random coil (9).

The importance of the residual structure in the unfolded state for understanding the process of protein folding is that such residual structures may resemble the structure in the natively folded state. Native-like residual structure can promote protein folding by providing folding nuclei and by reducing the conformational search needed to reach the native state. Thus, the presence of significant native-like residual structures would have important consequences for understanding the protein folding process, and it has been proposed to be the key to solving the protein folding problem (8). Although a significant body of evidence indicates the presence of residual structure in the fully unfolded state of proteins, evidence that this residual structure is native-like is scarce and is largely based on NMR spectroscopy (1, 3–6). Computational studies have provided further insights into the structural properties of unfolded states and their role in protein folding (8, 10). However, the existence and importance of the native-like residual structure in the fully unfolded state remain to be debated (11).

Here, we employ photoactive yellow protein (PYP) as a model system for examining residual structure in the fully unfolded state, and we use the photochemical activity of its

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The abbreviations used are: GdmHCl, guanidinium hydrochloride; pB, blue-shifted PYP photocycle intermediate; pBdark, acid-denatured state of PYP; pCA, p-coumaric acid; PYP, photoactive yellow protein; putra, fully unfolded state of PYP containing trans-pCA; pUtrans, fully unfolded state of PYP containing cis-pCA.
chromophore as a tool to modify this residual structure. PYP is a small (125 residues) water-soluble protein (12, 13) that functions as the photoreceptor for negative phototaxis in the photosynthetic bacterium Halorhodospira halophila (14). PYP has an absorbance maximum (λ_{max}) at 446 nm and exhibits a light-induced photocycle (13) triggered by the photo-isomerization (15) of its ionized trans-p-coumaric acid (pCA) chromophore (16).

The pCA strongly interacts with residues in its binding pocket, and its isomerization triggers large conformational changes that result in the formation of the long lived pB photocycle intermediate. The pB state is characterized by a pocket, and its isomerization triggers large conformational changes in the formation of the long lived pB photocycle intermediate. The pB state is characterized by a pocket, and its isomerization triggers large conformational changes (16). In addition, the pCA isomerization state strongly affects the folding of PYP. Chromophore isomerization converts the two-state folding behavior of PYP from the fully unfolded state containing trans-pCA (pU^{trans}) to the native pG state into three-state folding, with pB occurring as an on-pathway folding intermediate (18).

A second partially unfolded state of PYP is the acid denatured pB state (23, 24), which has a λ_{max} near 350 nm and which meets the criteria of an A state (25). PYP provides an attractive light-triggered model system to study protein folding (Fig. 1). In this respect, it is relevant that the small pCA chromophore strongly resembles the Tyr side chain; its isomerization mimics Pro isomerization, and its protonation is equivalent to changes in the protonation state of active site residues upon protein folding.

The observations that pCA isomerization triggers partial unfolding during the PYP photocycle and strongly alters the folding pathway of PYP (18) prompted us to examine its effect on the fully unfolded state of PYP. Our strategy was to compare the denaturant m and ΔC_p values for the unfolding of the fully folded native pG state with those for the unfolding of the partially unfolded pB and pB_{dark} states. Although the denaturant m value provides a measure for the total amount of protein surface area that is exposed during unfolding (26), the value of the ΔC_p that occurs during protein folding correlates well with the amount of hydrophobic surface area that is exposed during unfolding (26, 27). Denaturant m values are extracted from denaturant titration experiments, whereas the ΔC_p is usually obtained from the temperature dependence of folding. Thus, the denaturant m and ΔC_p values yield two independent measures for solvent exposure upon protein unfolding.

A key difference between the two partially unfolded states of PYP studied here is that pB contains cis-pCA, whereas pB_{dark} contains trans-pCA (15, 28). Because pB and pB_{dark} both are partially unfolded, one would expect the denaturant m values and ΔC_p values for their unfolding to be significantly smaller than those for the unfolding of the native pG state. This prediction would only hold if chromophore isomerization does not significantly affect the residual structure in the pU^{trans} and pU^{cis} states.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Spectroscopy**—PYP was overexpressed in Escherichia coli and purified as described previously (29). All kinetic and equilibrium absorbance experiments on the native state were carried out using 8 μM PYP in 20 mM potassium phosphate buffer (pH 7.3) unless stated otherwise. The pB_{dark} state was studied at pH 2.0 in a buffer of 10 mM potassium phosphate, 25 mM citrate, and 20 mM HCl.

**Thermal Denaturation**—Equilibrium thermal denaturation curves for pG were measured using a Cary300 UV-visible spectrophotometer (Varian) with a thermostated cuvette holder flushed with nitrogen gas. The resulting data were analyzed by Equation 1 as an equilibrium between the folded and unfolded states at various temperatures.

\[
\ln K = \frac{\Delta S_{298K}}{R} - \frac{\Delta H_{298K}}{RT} - \frac{\Delta C_p}{R} \left(1 - \frac{298}{T} \ln \left(\frac{298}{T}\right)\right)
\]

(Eq. 1)

K is the equilibrium constant between the folded and unfolded state with \(\Delta G_m = -RT \ln K\). Equilibrium thermodynamic parameters \(\Delta C_p, \Delta S\), and \(\Delta H\) at 298 K were obtained from the temperature dependence of the equilibrium constant K.

Thermal denaturation of the pB photocycle intermediate was studied by measuring steady state light-induced difference spectra with a Hewlett-Packard 8453 diode array spectrophotometer at a range of temperatures. The PYP photocycle was initiated by illumination of the sample with a 150-watt halogen quartz light source (Cuda) equipped with an optical fiber and a broadband blue filter. Absorbance measurements on the pB intermediate were performed on the photostationary state that was reached after ~30 s of continuous illumination. The temperature was controlled using a Peltier element. The light-induced difference spectra were normalized to equal amounts of pG bleaching at 446 nm. In these normalized difference spectra, pB denaturation was monitored at 375 nm and analyzed using Equation 1.

**Equilibrium Denaturant Titrations**—Equilibrium GdmHCl titrations of PYP with absorbance detection were performed at pH 2.0 in 10 mM potassium phosphate (pH 7.3), 25 mM citrate, and 20 mM HCl at 25 °C using a Cary 300 UV-visible spectrophotometer (Varian) with a thermostated cuvette holder. The observed transitions were analyzed using Equation 2,

\[
A = \frac{(a_u + b_u \cdot [D]) + (a_c + b_c \cdot [D]) \cdot e^{(\Delta C_p^{(p)}(R) - m \cdot [D])}}{1 + e^{(\Delta C_p^{(p)}(R) - m \cdot [D])}}
\]

(Eq. 2)

where A is the measured absorbance at 446 or 340 nm; [D] is the denaturant concentration; \(a_u\) and \(a_c\) are the slopes of the base lines before and after the transition; \(a_u\) and \(a_c\) are the signal intensities of these sloping base lines at zero denaturant; \(\Delta G_m(H_2O)\) is the free energy of unfolding in water; m is the denaturant dependence of free energy per mol of denaturant; R is the gas constant; and T is the temperature in degrees Kelvin.

The ΔC_p for the unfolding of the pB_{dark} state was obtained by measuring GdmHCl titration curves at various temperatures in the range 4–35 °C. The denaturant titration curve at each temperature was analyzed using Equation 2, and the resulting tem-
perature dependence of $\Delta G_p(H_2O)$ was analyzed using Equation 1 to extract the $\Delta C_p$ for $pB_{dark}$ unfolding.

RESULTS

Experimental Strategy—The following four distinct unfolding transitions in PYP are relevant to the experimental approach used here (Fig. 1): (i) the temperature- or denaturant-induced unfolding of the native $pG$ state to the fully unfolded state $pU_{trans}$ at neutral pH; (ii) the acid-induced unfolding of the native $pG$ state to the partially unfolded state $pB_{dark}$; (iii) the temperature- or denaturant-induced unfolding of $pB_{dark}$ to $pU_{trans}$ under acidic conditions; and (iv) the temperature- or denaturant-induced unfolding of the $pB$ photocycle intermediate to the fully unfolded state $pU_{cis}$ at neutral pH. These four transitions can be monitored using changes in the UV-visible absorbance spectrum of the $pC$ chromophore as follows: the native $pG$ state has a $\lambda_{max}$ at 446 nm; the fully unfolded states $pU_{trans}$ and $pU_{cis}$ exhibit a $\lambda_{max}$ at 338 nm, and the partially unfolded states $pB$ and $pB_{dark}$ are characterized by a $\lambda_{max}$ near 355 nm.

The characterization of solvent exposure upon the four unfolding transitions in PYP indicated in Fig. 1 based on denaturant $m$ and $\Delta C_p$ values involves a total of eight values. Three of these have already been determined as follows: the $\Delta C_p$ value for the transition between $pG$ and $pB_{dark}$ (17) and the denaturant $m$ values for the transitions from $pG$ to $pU_{trans}$ (18) and from $pB$ to $pU_{cis}$ (18). In addition, the $\Delta C_p$ for the photoconversion of $pG$ to $pB$ has been reported (30). No conditions have been identified that allow the denaturant-induced transition from $pG$ to $pB_{dark}$; thus, the denaturant $m$ value of this process is not known. This value is not needed in our strategy; we compare the $pG$ to $pU_{trans}$ unfolding transition with the $pB_{dark}$ to $pU_{trans}$ and $pB$ to $pU_{cis}$ transitions.

$\Delta C_p$ for the Unfolding of the Native $pG$ State of PYP Derived from Its Cold Denaturation—To determine the $\Delta C_p$ for the complete unfolding of PYP, we measured the temperature dependence of its stability $\Delta G_{pC}$ (Fig. 2A). As a result of the $\Delta C_p$ for protein folding, the dependence of $\Delta G_{pC}$ on temperature in general is curved, resulting in both heat and cold denaturations (31). Because PYP is very thermostable at neutral pH (12), we destabilized it by the addition of denaturing agents (2.8 M GdmHCl and 7.5 M urea). At both low and high temperatures, the population of the fully unfolded $pU_{trans}$ state with a $\lambda_{max}$ at 338 nm is observed (Fig. 2A). The resulting temperature dependence of the stability of PYP is strongly curved (Fig. 2B), and it clearly contains two folding transitions as follows: cold denaturation and heat denaturation. These data were used to extract the thermodynamic parameters describing the temperature dependence of $\Delta G_{pC}$ (Fig. 2B). This yields $\Delta C_p$ values for the unfolding of PYP of $7.3 \pm 0.2$ kJ/mol-K in the presence of 2.8 M GdmHCl and 7.9 $\pm 0.2$ kJ/mol-K in the presence of 7.5 M urea (Table 1).

Because $\Delta C_p$ values can depend somewhat on solvent conditions (32), the differences in the $\Delta C_p$ values for the unfolding of the native state of PYP in the presence of GdmHCl and urea could reflect a valid solvent dependence of this parameter. In the case of denaturant titrations, a base line is commonly used in the data analysis to account for the dependence of the spectroscopic properties of the unfolded and folded states on denaturant concentration (see Equation 2). However, in the case of the temperature dependence of protein stability both cold and heat denaturation occur, and this is generally analyzed without

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TABLE 1
Thermodynamic parameters for unfolding transitions in PYP

| Transition | Parameter (unit) | Value |
|------------|------------------|-------|
| pG → pUtrans | ΔGp,H2O (kJ/mol) | 36.4 ± 37.6 ± 10.8k |
| (pH 7.3) | mGdmHCl (M⁻¹) | 5.4 ± 0.2 |
| | murea (M⁻¹) | 1.98 ± 0.60 ± 0.17k |
| | ΔS (J/mol) | 115.1 ± 4.92 ± 4.9 |
| | ΔH (kJ/mol) | 33.1 ± 1.3 ± 8.23 ± 1.3 |
| | ΔCp (kJ/mol) | 7.3 ± 0.27 ± 0.2 |
| pG → pBdark | ΔGp,H2O (kJ/mol) | 0.5 ± 0.5 |
| (pH 3.4) | mGdmHCl (M⁻¹) | 4.2 ± 0.2 |
| | murea (M⁻¹) | 185.0 ± 3.7 |
| | ΔS (J/mol) | 66.3 ± 9.3 |
| | ΔH (kJ/mol) | 6.0 ± 1.2 |
| | ΔCp (kJ/mol) | 2.7 ± 0.4 |
| pG → pB | ΔGp,H2O (kJ/mol) | 10.5 ± 4.7 ± 0.9 |
| (pH 8.0) | mGdmHCl (M⁻¹) | 24.8 ± 1.3 ± 3.3 |
| | murea (M⁻¹) | 142.2 ± 7.4 ± 4.36 |
| | ΔS (J/mol) | 43.6 ± 2.21 ± 11.8 ± 3.4 |
| | ΔH (kJ/mol) | 6.8 ± 0.38 ± 0.5 |
| | ΔCp (kJ/mol) | 8.5 ± 0.9 |

* Data were derived from GdmHCl titrations.
* Data are from Ref. 18.
* Data are derived from urea titrations.
* Data are from Ref. 19.
* This was done in the presence of 2.8 M GdmHCl.
* This was done in the presence of 7.5 M urea.
* Data are derived from Ref. 17.
* Data are from Ref. 30.
* This was done in the presence of 1.75 M GdmHCl.
* This was done in the presence of 5.3 M urea.

such base lines (Equation 1). This likely reduces the accuracy of estimates of ΔCp values compared with denaturant m values. We conclude that the ΔCp values for pG unfolding in GdmHCl and urea are essentially indistinguishable, yielding a final estimate of 7.6 ± 0.5 kJ/mol/K for this parameter.

Denaturant Unfolding of the Acid-denatured State pBdark—To measure the denaturant m value for pBdark unfolding, we used UV-visible absorbance spectroscopy to monitor GdmHCl titrations of PYP at pH 2.0 (Fig. 3A). The fully unfolded state of PYP has a λmax at 338 nm and can thus be distinguished from the pB and pBdark states (12, 18, 19, 23). As expected, the absorbance spectrum shifted from 350 nm at low denaturant concentrations to 338 nm at high denaturant concentrations (Fig. 3A). At pH 2.0, a small amount (~10%) of the pG state is still present at low denaturant concentrations, resulting in small signals around 446 nm in Fig. 3A. The transition from the pBdark state to the fully unfolded pUtrans state is monitored most sensitively at 325 nm (Fig. 4A). Comparison of the GdmHCl dependence of the absorbance at 325 and 446 nm shows that analysis of the unfolding of the pBdark state is not affected by the presence of a small amount of pG state at pH 2.0 (Fig. 4B). Quantitative analysis of the data yields an m value of 4.2 M⁻¹ and a ΔGp,H2O of 10.5 kJ/mol (Fig. 3B). These values are 23 and 71% smaller, respectively, than those found for the pG state at pH 7.3. Thus, for the pBdark state, a clear reduction in denaturant m value compared with that of the native pG state was observed.

ΔCp for pBdark Unfolding—To determine the ΔCp for the unfolding of pBdark, we measured the temperature dependence of the absorbance spectrum of PYP at pH 2.0 in the presence of various amounts of GdmHCl. However, this did not yield satisfactory data on the cold denaturation of this state, likely...
because the temperature at which pBdark exhibits maximal stability is too close to 0 °C. We therefore used an alternative strategy in which we performed GdmHCl denaturant titrations at pH 2.0 at a number of different temperatures between 4 and 35 °C (Fig. 3C). The denaturant titration curve at each temperature was analyzed, and the resulting temperature dependence of ΔGp(H2O) was used to extract the ΔCp for the unfolding of pBdark (Fig. 3D). The resulting value of 6.0 kJ/mol-K is 21% lower than the value for the pG state.

**Thermal Unfolding of the pB Photocycle Intermediate**—The thermal denaturation of the pB photocycle intermediate was studied under two different experimental conditions, at 1.75 M GdmHCl and at 5.3 M urea. These conditions were selected to allow the detection of the thermal denaturation of pB without overlap from the thermal denaturation of the pG state (18). The thermal equilibrium between pB and pUcis was probed by the difference in absorbance spectrum between pB and pUcis (18, 19). Conditions that favor pUcis were observed to shift the absorbance spectrum of the photoproduct from 355 to 338 nm (Fig. 5A). The resulting temperature dependence of the ΔGp, for pB denaturation is strongly curved (Fig. 5B). This provides an example of the specific thermal heat and cold denaturation of a kinetic functional intermediate. From these data, the ΔCp for pB unfolding was found to be 6.8 ± 0.3 kJ/mol-K in GdmHCl and 8.5 ± 0.5 kJ/mol-K in urea. In the case of the pB intermediate, the measurements are based on absorbance difference spectroscopy instead of steady state absorbance spectroscopy. This added level of complexity in the measurements is in line with the somewhat larger error for the estimates of the ΔCp values for pB unfolding. These data thus yield a ΔCp value for pB unfolding of 7.7 ± 1.3 kJ/mol-K that is solvent-independent within the error of the experiment.

**DISCUSSION**

**Reversibility of the Observed Thermal Unfolding Transitions**—We report three equilibrium thermal denaturation transitions in PYP as follows: from the pG to the pUtrans state, from the pBdark to the pUtrans state, and from the pB to the pUcis state. A possible complication with such thermal unfolding transitions is (partial) protein aggregation in the unfolded state at increased temperatures. This can cause the transitions to be partially irreversible, introducing errors in the description of the process as an equilibrium transition. Thus, it is important to determine whether the transitions reported here are fully reversible. The following observations are relevant to this issue.

First, in none of the absorbance measurements reported here was an increase in scattering observed, arguing against the occurrence of protein aggregation. Second, in the case of thermal unfolding of the pG and pB states, we performed the measurements in two different solvent conditions. These two independent determinations yielded very similar ΔCp values. In the case of protein aggregation in the thermally unfolded state, the aggregation often proceeds during the course of the experiments and its extent is time-dependent. The finding that the same ΔCp values were obtained under two different experimental conditions argues against a significant perturbation by possible irreversibility. Third, the sum of the ΔCp values for the pG to pBdark transitions (1.6 + 6.0 = 7.6 kJ/mol-K) closely matches the value of 7.6 ± 0.5 kJ/mol-K derived from the pG to pUtrans transition, arguing against artifacts introduced by irreversibility. Finally, we have measured the temperature dependence of the kinetics for the unfolding/refolding transitions between pG and pUtrans by stopped-flow rapid mixing. The sum of the activation ΔCp values for unfolding and refolding match the equilibrium ΔCp value for pG unfolding reported here. Based on these observations, we conclude that the thermal transitions analyzed here are sufficiently reversible to allow the conclusions that we report.

**Exposure of Surface Area upon the Unfolding of Two Partially Unfolded States of PYP**—The results reported here yield estimates for the ΔCp values for the unfolding of the pG and pB states of 7.6 ± 0.5 and 7.7 ± 1.3 kJ/mol-K, respectively. Therefore, within the uncertainty of the experiment the ΔCp for the denaturation of the pB intermediate is essentially unchanged from that found for the pG state. Under the assumption that the structures of the pUtrans and pUcis states are identical, and based on the well-established correlation between ΔCp values and solvent exposure of hydrophobic area (26, 27), the unfolded nature of the pB state should have resulted in a significantly reduced ΔCp value for its unfolding.

In addition, relevant information has been reported regarding changes in heat capacity during the PYP photocycle. The activation the ΔCp value for pB decay is between −2.35 ± 0.1 (17) and −2.7 ± 0.15 kJ/mol-K (21), indicating that the last PYP photocycle transition involves protein refolding. Photothermal techniques have yielded a value of 2.7 ± 0.4 kJ/mol-K for the ΔCp upon pB formation (30). This provides direct evidence for

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the exposure of hydrophobic surface area in the pB state, and it adds further weight to the failure of the expectation that the \( \Delta C_p \) for pB unfolding should have been reduced (to \( 7.6 - 2.7 = 4.9 \) kJ/mol). We also apply the well established approach (26) of using denaturant \( m \) values to quantify the total surface area exposed to solvent upon the unfolding of native PYP and its partially unfolded pB intermediate. Previously, we have reported \( m_{\text{GdmHCl}} \) values of 5.4 \pm 0.2 \text{ M}^{-1} for the pG to pU\text{trans} transition and 6.4 \pm 0.5 \text{ M}^{-1} for the pB to pU\text{cis} transition (Table 1) (18). For \( m_{\text{urea}} \), we found values of 2.0 \pm 0.1 and 2.5 \pm 0.1 \text{ M}^{-1} for pG and pB unfolding, respectively (18). An independent study yielded \( m_{\text{urea}} \) values for pG and pB unfolding by urea that are somewhat higher than those reported here (Table 1) (19). We ascribe this to differences in experimental conditions and in the procedures used for data analysis. In the experiments presented here, care was taken to perform experiments on pG, pB, and pB\text{dark} in parallel to allow for direct comparisons of the unfolding of these states.

Taken together, the data show that the \( \Delta C_p \) and denaturant \( m \) values for the pB to pU\text{trans} transition do not show the significant reduction that would be expected based on its partially unfolded nature. These findings are in striking conflict with the large body of evidence that the pB state is partially unfolded (17–22). This conflict is particularly urgent because it is confirmed by two independent measures for surface exposure as follows: denaturant \( m \) values and \( \Delta C_p \) values.

Analysis of solvent exposure upon the unfolding of pB\text{dark} does follow the expected pattern for a partially unfolded state. The \( \Delta C_p \) for the transition from pB\text{dark} to pU\text{trans} is reduced by 21% compared with the value for the unfolding of the pG state. The sum of the \( \Delta C_p \) values for the transitions from pG to pB\text{dark} (17) and from pB\text{dark} to pU\text{trans} (1.6 + 6.0 = 7.6 \text{ kJ/mol}) closely matches the value derived from the cold denaturation of the pG state, indicating internal consistency in the data. For the unfolding of the pB\text{dark} state by GdmHCl, we find an \( m \) value of 4.2 \pm 0.7 \text{ M}^{-1}. This value is reduced by 22% compared with that for the unfolding of the native pG state.

Implications for the Effect of pCA Isomerization on the Fully Unfolded State of PYP—The following view emerges from these results (Fig. 6), providing strong evidence for an effect of the pCA isomerization state on the residual structure of fully unfolded PYP. In this interpretation, residual structure in the pU\text{cis} state is significantly less than that in the pU\text{trans} state. Thus, denaturation of the pB state to a less structured unfolded state (pU\text{cis}) will result in a degree of solvent exposure that is larger than expected compared with the unfolding of the pG state to a more structured unfolded state (pU\text{trans}). The values reported here indicate that chromophore trans to cis isomerization causes a reduction in the solvent-exposed area in the fully unfolded state that is equivalent to approximately a quarter of the surface area exposed upon denaturation of the native state of PYP (see Fig. 6). This implies that the fully unfolded state pU\text{trans} buries a substantial amount of surface area. By determining both denaturant \( m \) values from denaturant titrations and \( \Delta C_p \) values from temperature dependence studies, we can conclude that pU\text{trans} buries both total and hydrophobic surface areas.

The conclusion that pU\text{trans} buries a significant amount of surface area is in line with evidence indicating that the fully unfolded states of various proteins retain significant residual structure (1–7). An important implication is that the denaturant \( m \) and \( \Delta C_p \) values derived from denaturant and thermal unfolding experiments depend not only on the structure of the folded state but also on the structure of the unfolded state. Experimental conditions, such as the introduction of point mutations, differences in pH or temperature, or in this case the chromophore isomerization state, can alter the amount of residual structure in the fully unfolded state. In this case, the analysis of denaturant \( m \) and \( \Delta C_p \) values in terms of changes in the structural properties of only the native state is no longer valid. The approach developed here to investigate residual structure in the fully unfolded state by determining solvent exposure upon the unfolding of both native and partially unfolded states of a protein is more generally applicable.

Native-like Residual Structure in the Fully Unfolded State of PYP and Its Role in Protein Folding—Isomerization of the pCA chromophore has a strong effect on the conformation of the native state of PYP, triggering large structural changes that involve partial protein unfolding (17–22). Here, we find that pCA trans to cis isomerization also has large effects on the fully unfolded state of PYP, causing an increase in the solvent-accessible surface area. This implies the following: (i) that the unfolded pU\text{trans} state retains interactions with the pCA chromophore, and (ii) that these interactions in the unfolded state depend on the isomerization state of the pCA. Thus, in this respect, the fully unfolded state pU\text{trans} bears a striking resemblance to the native state, indicating that the residual structure of the unfolded state resembles the structure of the native state. This provides a new criterion for detecting the native-like residual structure that can be generally applied to chromophoric protein in which the cofactor undergoes chemical processes such as isomerization or electron transfer. In the case of PYP, chromophore photoisomerization places the
amount of residual structure under direct “chemical control” of the experimentalist.

A central problem in protein folding is how a protein performs its search through conformational space to arrive at the native state. The Levinthal paradox states that the conformational space of a protein is too large for a random search to result in folding on a physiologically relevant time scale (33). Significant structural bias of the ensemble of unfolded states toward the native state would simplify this conformational search and thus speed up protein folding. The main reason for the recent excitement about residual structure in the protein folding field is that it is native-like and thus significantly reduces the highly challenging conformational search during protein folding. However, it has not been experimentally demonstrated that an increase in the degree of unfolding of the denatured state of a protein indeed slows down refolding. In the case of PYP, we have previously found that refolding to the denatured state of a protein indeed slows down refolding. In this case, PYP, an increase in residual structure in the denatured state significantly reduces the highly challenging conformational search during protein folding. In the case of PYP, an increase in residual structure in the fully unfolded state indeed is correlated with faster refolding kinetics.

**Summary**—We report a novel strategy to examine residual structure in the fully unfolded state based on comparing the unfolding transitions of the native state with those of partially unfolded states of the same protein. This led us to describe the specific heat and cold denaturation of a partially unfolded functional intermediate (pB). The results indicate that residual structure in the fully unfolded state of the blue-light receptor PYP is significantly modified by the isomerization of its light-sensitive pCA chromophore. This allows for the photo-regulation of the amount of residual structure in the fully unfolded state, and it provides a novel criterion for the native-like character of residual structure in the fully unfolded state, i.e. native-like protein-chromophore interactions. This strategy can also be applied to other proteins containing photo-responsive cofactors. We probe solvent burial in the fully unfolded state using two independent criteria as follows: total surface area based on denaturant m values and hydrophobic surface area based on ΔCp values. Both probes indicate significant burial of surface area in the fully unfolded state of PYP containing trans-pCA. Burial of the hydrophobic surface area in the unfolded state has implications for key aspects of protein folding models, particularly the hydrophobic collapse process. In principle, native-like residual structure in the fully unfolded state can significantly reduce the highly challenging conformational search during protein folding.

**REFERENCES**

1. Mok, K. H., Kuhn, L. T., Goez, M., Day, I. J., Lin, J. C., Andersen, N. H., and Hore, P. J. (2007) Nature 447, 106–109
2. Robic, S., Guzman-Casado, M., Sanchez-Ruiz, J. M., and Marqusee, S. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 11345–11349
3. Klein-Seetharaman, J., Okawa, M., Grishmash, S. B., Wirmser, J., Duchardt, E., Ueda, T., Imoto, T., Smith, L. J., Dobson, C. M., and Schwalbe, H. (2002) Science 295, 1719–1722
4. Shortle, D., and Ackerman, M. S. (2001) Science 293, 487–489
5. Garcia, P., Serrano, L., Durand, D., Rico, M., and Bruix, M. (2001) Protein Sci. 10, 1100–1112
6. Neri, D., Billette, M., Wider, G., and Wuthrich, K. (1992) Science 257, 1559–1563
7. Shortle, D. (1996) FASEB J. 10, 27–34
8. van Gunsteren, W. F., Bürgi, R., Peter, C., and Daura, X. (2001) Angew. Chem. Int. Ed. Engl. 40, 351–355
9. Fitzkee, N. C., and Rose, G. D. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12497–12502
10. Weinkam, P., Pletneva, E. V., Gray, H. B., Winkler, J. L., and Wolynes, P. G. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 1796–1801
11. McCarney, E. R., Kohn, J. E., and Plaxco, K. W. (2005) Crit. Rev. Biochem. Mol. Biol. 40, 181–189
12. Meyer, T. E. (1985) Biochim. Biophys. Acta 806, 175–183
13. Meyer, T. E., Yakali, E., Cusanovich, M. A., and Tollin, G. (1987) Biochemistry 26, 418–423
14. Sprenger, W. W., Hoff, W. D., Armitage, J. P., and Hellingwerf, K. J. (1993) J. Bacteriol. 175, 3096–3104
15. Kort, R., Vonk, H., Xu, X., Hoff, W. D., Crielaard, W., and Hellingwerf, K. J. (1996) FEBS Lett. 382, 73–78
16. Hoff, W. D., Düx, P., Här, K., de Vries, B., Nugteren-Roodzant, I. M., Crielaard, W., Boelens, R., Kaptein, R., van Beeumen, J., and Hellingwerf, K. J. (1994) Biochemistry 33, 13959–13962
17. Van Brederode, M. E., Hoff, W. D., van Stokkum, I. H., Groot, M. L., and Hellingwerf, K. J. (1996) Phys. Biol. 71, 365–380
18. Lee, B. C., Pandit, A., Crouenquist, P. A., and Hoff, W. D. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 9062–9067
19. Ohishi, S., Shimizu, N., Mihara, K., Imamoto, Y., and Kataoka, M. (2001) Biochemistry 40, 2854–2859
20. Bernard, C., Houben, K., Derix, N. M., Marks, D., van der Horst, M. A., Hellingwerf, K. J., Boelens, R., Kaptein, R., and van Nuland, N. A. (2005) Structure 13, 953–962
21. Hoff, W. D., Xie, A., Van Stokkum, I. H., Tang, X. J., Gural, J., Kroon, A. R.,
Native-like Residual Structure in Denatured PYP

22. Zhao, J. M., Lee, H., Nome, R. A., Majid, S., Scherer, N. F., and Hoff, W. D. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 11561–11666
23. Hoff, W. D., van Stokkum, I. H., Gural, J., and Hellingwerf, K. J. (1997) Biochim. Biophys. Acta 1322, 151–162
24. Craven, C. J., Derix, N. M., Hendriks, J., Boelens, R., Hellingwerf, K. J., and Kaptein, R. (2000) Biochemistry 39, 14392–14399
25. Fink, A. L., Calciano, L. J., Goto, Y., Nishimura, M., and Swedberg, S. A. (1993) Protein Sci. 2, 1155–1160
26. Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) Protein Sci. 4, 2138–2148
27. Murphy, K. P., and Freire, E. (1992) Adv. Protein Chem. 43, 313–361
28. Unno, M., Kumauchi, M., Sasaki, J., Tokunaga, F., and Yamauchi, S. (2000) J. Am. Chem. Soc. 122, 4233–4234
29. Xie, A., Hoff, W. D., Kroon, A. R., and Hellingwerf, K. J. (1996) Biochemistry 35, 14671–14678
30. Khan, J. S., Imamoto, Y., Kataoka, M., Tokunaga, F., and Terazima, M. (2006) J. Am. Chem. Soc. 128, 1002–1008
31. Privalov, P. L. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 281–305
32. Liu, Y., and Sturtevant, J. M. (1996) Biochemistry 35, 3059–3062
33. Levinthal, C. (1968) J. Chem. Phys. 65, 44–45
34. Dill, K. A., Bromberg, S., Yue, K., Fiebig, K. M., Yee, D. P., Thomas, P. D., and Chan, H. S. (1995) Protein Sci. 4, 561–602