Structure/function relations in Photoactive Yellow Protein
van der Horst, M.A.

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Chapter 2

The role of the N-terminal domain of Photoactive Yellow Protein in the transient partial unfolding during signaling state formation

Michael A. van der Horst, Ivo H.M. van Stokkum, Wim Crielaard and Klaas J. Hellingwerf

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2.1 Abstract

It is shown that the N-terminal domain of Photoactive Yellow Protein, which appears relatively independently folded in the ground state of the protein, plays a key role in the transient unfolding during signaling state formation: Genetic truncation of the N-terminal domain of PYP significantly decreases the extent of cooperativity of the titration curve that describes chromophore protonation in the ground state of PYP, which is in agreement with the notion that the N-terminal domain is linked through a hydrogen-bonding network with the chromophore-containing domain of the protein. Furthermore, deletion of the N-terminal domain completely abolishes the non-linearity of the Arrhenius plot of the rate of ground-state recovery.

2.2 Introduction

A major challenge in enzyme catalysis is to define the alterations in spatial structure during functional turnover. This problem can be tackled with e.g. forming complexes of an enzyme with its substrate and/or product at room- or cryogenic temperatures (Verschueren et al., 1993; Schlichting et al., 2000). Nevertheless, these approaches have intrinsic limitations that can be avoided by real-time recording of these structural transitions. This can be done by using a large array of indirect (spectroscopic) techniques to resolve (details of) protein structure, but has become possible in a very powerful direct way trough the development of time-resolved Laue diffraction analysis at atomic resolution (Perman et al., 2000). The latter technique can now be used to record real-time movies of the alterations in protein structure during functional turnover from the ns to the s time domain. Its application so far, however, is dependent on the availability of specific model proteins, like Myoglobin (Srajer et al., 1996) and Photoactive Yellow Protein (PYP) (Genick et al., 1997a; Perman et al., 1998) from the purple-sulphur bacterium Ectothiorhodospira halophila. This protein is a small water-soluble protein, which functions as the blue-light receptor in a behavioral response of this bacterium. The protein can be crystallized in the P63 and P65 space group and through X-ray diffraction it was shown to belong to the family of the α/β-fold proteins (Borgstahl et al., 1995; Van Aalten et al., 2000). It has two hydrophobic cores, a larger one in which the chromophore is buried, on one side of a large 6-stranded β-sheet and a smaller one, which is formed by the two N-terminal α-helices covering the other side of the central β-sheet. Light absorption by this photoreceptor protein initiates photo-isomerization of its anionic 4-
hydroxycinnamyl chromophore, from the 7-trans,9-cis- to the 7-cis,9-trans configuration (Kort et al., 1996a; Xie et al., 1996). This initially leads to the formation of a series of transient intermediates with a red-shifted absorbance maximum (as compared to the ground state pG$_{446}$), of which the most stable one (pR$_{466}$) decays bi-exponentially to a blue-shifted state (pB$_{355}$), the tentative signaling state. In a few hundred ms the ground state (i.e. pG$_{446}$) has recovered (Meyer et al., 1987; Hoff et al., 1994b; Ujj et al., 1998). This change in configuration of the buried chromophore is relayed to the surface of the protein in the form of a conformational transition, to allow activation of a downstream signal transduction partner.

Time-resolved Laue diffraction experiments have revealed the structure of this signaling state of PYP: Upon isomerization, the chromophore is protonated by a nearby glutamic acid side chain and subsequently exposed to solvent by rotation across its carbon-sulphur single bond and the rearrangement of two arginine side chains, one of which specifically was shielding the chromophore from solvent in the ground state (Genick et al., 1997a; Perman et al., 1998). This signaling state subsequently spontaneously relaxes within a second. This description of the sequence of events that lead to signaling state formation in PYP has been challenged by the results of a range of biophysical techniques that were applied to aqueous solutions of PYP, including transient UV/Vis-, FTIR- and NMR spectroscopy and measurements of the rate of H/D exchange with mass spectrometry and NMR (Van Brederode et al., 1996; Rubinstenn et al., 1998; Hoff et al., 1999; Craven et al., 2000; Kandori et al., 2000). From these experiments it was concluded that PYP shows a significant transient unfolding in its signaling state, equivalent to about 30% of the maximal unfolding upon complete acid- or urea-induced denaturation. This value was estimated from the apparent change in heat capacity associated with signaling state formation, which can be deduced from the deviation from linearity of the dependence of the photocycle kinetics of PYP on reciprocal-temperature and from the number of hydrogen atoms protected from H/D exchange in a light-dependent fashion (Van Brederode et al., 1996; Hoff et al., 1999).

A light-induced conformational transition, very localized within the total volume of the protein, would be very unexpected also in terms of a molecular dynamics analysis of PYP in a box of water molecules. This analysis revealed that most eigenvectors of the intrinsic flexibility of the polypeptide chain of PYP describe concerted motion along the entire backbone (van Aalten et al., 1998).

Recently, this apparent controversy regarding the extent of functional unfolding of PYP in its signaling state was resolved through time-
resolved FTIR measurements on a crystal and an aqueous solution of PYP. These experiments revealed that the extent of unfolding in the signaling state pB, as deduced from the extent of the changes in the amide-I region of FTIR difference spectra, is very restricted when the PYP protein is caught in a crystalline lattice, as compared to the situation when PYP is dissolved in aqueous solution (Xie et al., 2001). Therefore, the extent of transient unfolding of PYP is steered by the mesoscopic environment of the protein.

In our NMR experiments on PYP we noted, from the relatively high rates of backbone H/D exchange (Craven et al., 2000), that its N-terminal domain is of low intrinsic stability. We therefore decided to investigate the role of this domain in signaling state formation through an analysis of the properties of N-terminally truncated PYP molecules.

2.2 Materials and methods

PYP, and truncated versions thereof, were produced and isolated as described in (Kort et al., 1996a) as hexa-histidine tagged apoproteins in Escherichia coli. The N-terminally truncated variants of PYP (truncated up until the 25th and 27th residue, and referred to as $\Delta 25$ and $\Delta 27$, respectively), were made using the polymerase chain reaction, according to standard molecular biological techniques (Sambrook et al., 1989). The sequence of primers for $\Delta 25$ was 5’ CGGCGGATCCGATGACGATGACAAACT GGCCTTCCGCGCCATCCAG 3’, 5’ GCGCAAGCTTCTAGACGCGCTTACGAGACCC 3’ and for $\Delta 27$ 5’ CCGCGGATCCGATGACGATGACAAATTCCGCGC CATCCAGCTCG 3’, 5’ GCGCAAGCTTCTAGACGCGCTTACGAGACCC 3’. As a template, 10 ng of pHISP was used (Kort et al., 1996a).

pH-titrations were carried out according to (Hoff et al., 1997b) using protein solutions in 10 mM phosphate/100 mM KCl buffer. $pK_a$ values, and n-values (or: Hill coefficients) expressing the degree of cooperativity, were calculated by fitting the data to equation 1, in which $n$ describes the steepness of the transition.

$$pG = \frac{1}{10^{pH - pK_a}}$$

(1)

Time-resolved UV/Vis spectroscopy was carried out as described by (Hendriks et al., 1999a) using protein solutions in 50 mM Tris-HCl pH 7.5. Protein samples were used with and without prior removal of the hexa-histidine tag. No significant differences between such samples were noted. Thermodynamic parameters were calculated using equation 2, in which $k_i$ is the rate of groundstate recovery, and $h$ and $k_b$ the Planck and Boltzmann constant, respectively.

$$\ln \frac{k_i h}{k_b} = \frac{\Delta S^R(T_o)}{R} - \frac{\Delta H^R(T_o)}{RT_o} - \frac{\Delta C_p}{R} \left( - \frac{T_o}{T} + \ln \frac{T}{T_o} \right)$$

(2)
Temperature induced denaturation experiments were carried out as described in (Van Brederode et al., 1996) using protein solutions in 50 mM citrate buffer.

Concentration profiles of pB_{dark} and pG as a function of temperature were calculated from UV/Vis difference spectra using global analysis (Van Brederode et al., 1996). We used skewed Gaussian shapes to model the spectra of pB_{dark} and pG.

The equilibrium constant K was calculated from the concentration profiles. Below 20°C the pB_{dark} concentration is very low, and the estimate heavily depends on the correctness of the model for the pG spectrum. Therefore we restricted the fit of the equilibrium constant to temperatures above 15°C. The data were fitted to equation 3, from which the thermodynamic parameters were derived.

\[
\ln K = \frac{\Delta N(T_0)}{R} - \frac{\Delta H(T_0)}{RT} - \frac{\Delta C_P}{R} \left( -\frac{T_0}{T} + \ln \frac{T_0}{T} \right) \tag{3}
\]

2.3 Results and Discussion

The N-terminal domain of PYP in a crystalline lattice is folded into two α-helices that range from residue number 11 - 16 (α1) and from 20 - 24 (α2). It should be noted, however, that in solution the second helix displays dihedral angles that classify it as a loop (Düx et al., 1998). For deletion of the N-terminal domain of PYP we decided to delete the first 25 and 27 amino acids, respectively, thus generating the Δ25 and Δ27 protein. In this way, Gly-29 that is in van der Waals contact with Glu-46, is retained. Further truncation results in non-functional PYP (Hamada et al., 1998). The first amino acid in the next element of secondary structure, i.e. β1, is Gly-29.

Both truncated proteins are stably produced as apo-proteins in E. coli and result in functional holo-PYP upon reconstitution with 4-hydroxy-cinnamic acid. The purity index of both proteins is comparable to that of wild-type PYP, but their absorbance maximum is slightly shifted to shorter wavelength (Figure 1).

\[\text{Figure 1: UV-vis absorption spectra of dark-adapted wild-type and truncated PYP.}\]

Spectra were taken at room temperature in 10 mM Tris, pH 7.5. Solid line: WT PYP, dotted: Δ25 PYP, dashed: Δ27 PYP.

Also their temperature stability has significantly decreased compared to wild type PYP (see further below). Both are photoactive, with similar photocycle intermediates as the wild type protein. However, the kinetics of the recovery reaction in the photocycle of both is
strongly decelerated (see further below). As a first characterization of both proteins, the pH-titration of their chromophore with acid was analyzed. In the wild type protein the chromophore titrates highly cooperatively to the protonated form, presumably because an extensive hydrogen-bonding network, in which several protonatable residues are involved, must be disrupted. Figure 2a,b shows this experiment, with wild type PYP for comparison. A pKₐ of 2.8 ± 0.16 is obtained, with a Hill coefficient, expressing this degree of cooperativity, of 1.9 ± 0.05. Both values are in agreement with previous observations (Meyer, 1985; Hoff et al., 1997b). For the two truncated proteins a set of spectra was obtained with a clear isosbestic point at 380 nm, indicating that lowering of the pH gives rise to a well-defined two-state transition for these two proteins too. Figure 2b also shows the corresponding titration curves of the Δ27 and Δ25 truncated PYP proteins. Strikingly, whereas the pKₐ of Δ25 is unaltered (i.e. 2.9 ± 0.16) and the pKₐ of Δ27 has only slightly decreased (2.4 ± 0.06), the degree of cooperativity in the titration has significantly decreased: To 1.3 ± 0.03 and 1.2 ± 0.02, respectively. This result shows that the N-terminal domain is part of the hydrogen-bonding network that has to be disrupted before chromophore protonation can occur at low pH. In agreement with this, we have observed that during formation of the photocycle intermediate with a protonated chromophore i.e. pB, the hydrogen-bonding network between the N-terminal domain and

![Figure 2: pH Titration of the absorption spectra of wild type and truncated PYP.](image)

Spectra were taken at room temperature in 10 mM Tris, 100 mM KCl between pH 7 and pH 1. A: dependence of the absorption spectra of Δ27 PYP on pH; B: the relative amplitude of the absorbance in the respective absorption maximum as a function of pH for wild type PYP and the two truncated variants. Theoretical curves (solid lines) were obtained by fitting the data to equation 1. Symbols: closed circles: wild type PYP; open circles: Δ25 PYP; open triangles: Δ27 PYP.
the central β-sheet is altered too (Kandori et al., 2000). As expected, when PYP is fully denatured with 6 M urea, its 4-hydroxy cinnamyl chromophore titrates with a pK of 8.8 and an n-value of 1 (J. Hendriks, unpublished observation).

To probe the extent of functional unfolding of the two truncated proteins in the signaling state, we analyzed the temperature dependence of the recovery reaction (i.e. the pB to pG transition (Hoff et al., 1994b)) in their photocycle. Both proteins show a recovery reaction (at room temperature and pH = 7), which is considerably slower (up to 100-fold) than the one of wild type PYP. Of the latter, the rate of the recovery reaction can be modulated over a large range of time scales by adjusting the pH (Genick et al., 1997a; Hoff et al., 1997b; Hoff et al., 1999).

To avoid any technical complications in the measurement and comparison of photocycle recovery rates of wild type PYP and its two truncated derivatives, we analyzed their photocycle recovery kinetics at different pH values, to obtain comparable rates (Figure 3). For wild type PYP the pH was therefore adjusted to 3.4. For all three proteins kinetics were obtained that were reasonably well fitted with single exponents. Plotting of the natural logarithm of the rates obtained, against reciprocal temperature (Figure 3), shows the convex curve that is well known for wild type PYP (Meyer et al., 1989; Van Brederode et al., 1996; Hoff et al., 1999). A change in heat capacity associated with the transition from pB to pG of -2.5 kJ/mol/K can be calculated from this degree of curvature, in agreement with the value reported in (Van Brederode et al., 1996). Figure 3 also shows the corresponding curves for Δ27 and Δ25. It is striking that the extent of curvature in these plots for the two proteins has significantly decreased, up to the point that for the Δ25-protein an essentially linear Arrhenius plot is obtained. From these curves, the thermodynamic parameters as shown in Table 1 can be calculated. We interpret these observations as evidence that the transient functional unfolding of PYP in its signaling state has essentially been abolished in the N-terminally truncated derivatives, in particular in the Δ25 protein.

Figure 3: Thermodynamic analysis of the rate of the pB to pG transition in the PYP photocycle. The natural logarithm of the rate constant k is shown as a function of reciprocal temperature. The solid line was obtained by fitting the data to equation 3. Squares: wild type PYP, circles: Δ25 PYP, triangles: Δ27 PYP.
Both truncated proteins, but especially Δ25, already show room temperature-induced unfolding at physiological pH values. In wild type PYP, this room temperature-induced denaturation only takes place at low pH (Van Brederode et al., 1996). In Figure 1, which shows spectra taken at room temperature and pH 7.5, the formation of a pB-like intermediate is already slightly visible in the two truncated variants. We analyzed the thermodynamic parameters of this equilibrium at low pH in both the wild type and in the two truncated proteins. From the temperature dependent spectra, concentration profiles were determined using a global analysis technique, as described in (Van Brederode et al., 1996). The resulting equilibrium constant K has been plotted against reciprocal temperature in Figure 4.

From these curves, the thermodynamic parameters as shown in Table 2 were derived. Again, the changes in heat capacity in the truncated proteins have decreased compared to wild type protein, but by far not as drastically as during the functional unfolding, as measured from the temperature dependence of the recovery rate of the ground state of the three proteins. These results confirm that all three proteins considered in this study show the expected extent of heat capacity change upon temperature denaturation: Only a slight decrease is observed in the two truncated variants, which may partly be due to their decreased size.

<table>
<thead>
<tr>
<th>ΔS° (J/mol K)</th>
<th>ΔH° (kJ/mol)</th>
<th>ΔG° (kJ/mol)</th>
<th>ΔG°₅₀ (kJ/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22 (0.6)</td>
<td>33 (0.2)</td>
<td>26 (0.1)</td>
</tr>
<tr>
<td>Δ25</td>
<td>-14 (1.9)</td>
<td>28 (0.6)</td>
<td>32 (0.3)</td>
</tr>
<tr>
<td>Δ27</td>
<td>-32 (0.8)</td>
<td>16 (0.2)</td>
<td>26 (0.1)</td>
</tr>
</tbody>
</table>

Table 1: Thermodynamic parameters of the recovery step of the PYP photocycle. The values of the thermodynamic activation parameters describing the recovery in both wild type and truncated PYP were calculated from the fits of the data from Figure 3. Values at 298 K are shown. The values between brackets are the standard deviations in the thermodynamic parameter, according to the least squares fit of the data to equation 2.
Unfolding in the N-terminal domain of PYP

<table>
<thead>
<tr>
<th>ΔS (J/mol K)</th>
<th>ΔH (kJ/mol)</th>
<th>ΔG (kJ/mol)</th>
<th>ΔC_p (kJ/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>135</td>
<td>45</td>
<td>61</td>
</tr>
<tr>
<td>Δ25</td>
<td>92</td>
<td>30</td>
<td>2.3</td>
</tr>
<tr>
<td>Δ27</td>
<td>74</td>
<td>27</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 2: Thermodynamic parameters of the equilibrium between the pG and pB_dark form of PYP at 298 K and pH 3.4. The values of the thermodynamic parameters describing the equilibrium in both wild type and truncated PYP were calculated from the fits of the data from Figure 4. The uncertainty in ΔS, ΔH and ΔC_p is about 10%, whereas in ΔG it is about 3%.

In this study we have not addressed the striking deceleration of the recovery rate of the photocycle in the two truncated variants (approximately 10 and 100-fold in Δ27 and Δ25, respectively; Van der Horst et al., unpublished experiments). We assume that the thermodynamic barrier for re-isomerization of the chromophore to the trans configuration is a crucial factor determining this rate. With the currently available information we cannot provide an explanation for the observed differences in recovery rate between PYP and its two truncated variants. Detailed insight into the spatial structure of the latter two will be required for this. Current work focuses on the resolution of the X-ray structure of these two variants.

Although we show in this study that particularly Δ25 has lost its thermodynamic unfolding characteristics, recent probe-binding studies in our group support the notion that even in this truncated protein chromophore exposure to the solvent still occurs (Hendriks et al., 2002). These probe-binding experiments may provide a selective tool to assay the functional dynamic alterations in the structure of PYP near the chromophore-binding site.

PYP has only a single tryptophan (Trp119), which is located far from the chromophore and is clamped between the central β-sheet and the two N-terminal α-helices. Trp emission is enhanced and slightly blue shifted in pB compared to pG, which points to a more non-polar environment for this tryptophan in the signaling state (Th. Gensch et al., unpublished). This further supports the notion that in the conformational changes in the pB state of wild type PYP also the N-terminal domain is involved.

The experiments reported in this paper show the importance of a concerted motion in a large part of the photoactive yellow protein, upon signaling state formation. This motion gives rise to a very unexpected temperature dependence of the rate of catalysis of ground-state recovery. Since large conformational transitions may be abundant in signal transduction (Wall et al., 2000), such temperature dependence may be observable in the activity of other signal-transducing proteins as well.

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