

# The role of the N-terminal domain of photoactive yellow protein in the transient partial unfolding during signalling state formation

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**Abstract** It is shown that the N-terminal domain of photoactive yellow protein (PYP), which appears relatively independently folded in the ground state of the protein, plays a key role in the transient unfolding during signalling 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. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Photocycle kinetics; Arrhenius plot; Cooperativity; Titration curve; Truncated protein; Specific heat capacity; Cooperativity

## 1. 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 temperature [1,2]. 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 protein structures and their details, but has become possible in a very powerful direct way through the development of time-resolved Laue diffraction analysis at atomic resolution [3]. The latter technique can now be used to record real-time movies of the alterations in protein structure during functional turnover from the nanosecond to the second time domain.

Its application so far, however, is dependent on the availability of specific model proteins, like myoglobin [4] and photoactive yellow protein (PYP) [5,6] from the purple sulfur 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 P6<sub>3</sub> and P6<sub>5</sub> space group and through X-ray diffraction it was shown to belong to the family of the  $\alpha/\beta$ -fold proteins [7,8]. It has two hydrophobic cores, a larger one in which the chromophore is buried, on one side of a large six-stranded  $\beta$ -sheet and a smaller one, which is formed by the two N-terminal  $\alpha$ -helices covering the other side of the central  $\beta$ -sheet. Light absorption by this photoreceptor protein initiates photo-isomerization of its anionic 4-hydroxy-cinnamyl chromophore, from the 7-*trans*,9-*cis* to the 7-*cis*,9-*trans* configuration [9,10]. 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<sub>446</sub>), of which the most stable one (pR<sub>466</sub>) decays bi-exponentially to a blue-shifted state (pB<sub>355</sub>), the tentative signalling state. In a few hundred milliseconds the ground state (i.e. pG<sub>446</sub>) has recovered [11–13]. 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 signalling 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–sulfur single bond and the rearrangement of two arginine side chains, one of which specifically shielded the chromophore from solvent in the ground state [5,6]. This signalling state subsequently spontaneously relaxes within 1 s.

This description of the sequence of events that lead to signalling 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, Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy and measurements of the rate of H/D exchange with mass spectrometry and NMR [14–18]. From these experiments it was concluded that PYP shows a significant transient unfolding in its signalling 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 signalling 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 hy-

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**Abbreviations:** PYP, photoactive yellow protein; pG, ground state of the photocycle of PYP, absorbing at 446 nm; pB, blue-shifted photocycle intermediate, which is the presumed signalling state

drogen atoms protected from H/D exchange in a light-dependent fashion [14,16].

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 [19].

Recently, this apparent controversy regarding the extent of functional unfolding of PYP in its signalling 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 signalling 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 [20]. 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 [18], that its N-terminal domain is of low intrinsic stability. We therefore decided to investigate the role of this domain in signalling state formation through an analysis of the properties of N-terminally truncated PYP molecules.

## 2. Materials and methods

PYP and truncated versions thereof were produced and isolated as described in [9] as hexa-histidine-tagged apo-proteins in *Escherichia coli*. The N-terminally truncated variants of PYP (truncated up to the 25th or 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 [21]. The sequence of primers for  $\Delta 25$  was 5'-CGGCGGATCCGATGACGATGACAACTGGCCTTCG-GCGCCATCCAG-3'; 5'-GCGCAAGCTTCTAGACGCGCTTGACGAAGACCC-3' and for  $\Delta 27$  5'-CCGCGGATCCGATGACGATGACAAATTCGGCGCCATCCAGCTCG-3'; 5'-GCGCAAGCTTCTAGACGCGCTTGACGAAGACCC-3'.

As a template, 10 ng of pHISp was used [9].

pH titrations were carried out according to [22] 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 Eq. 1, in which  $n$  describes the steepness of the transition.

$$pG = \frac{1}{1 + 10^{n(pH-pK)}} \quad (1)$$

Time-resolved UV/Vis spectroscopy was carried out as described by [23] 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 Eq. 2, in which  $k_i$  is the rate of ground state recovery, and  $h$  and  $k_b$  are the Planck

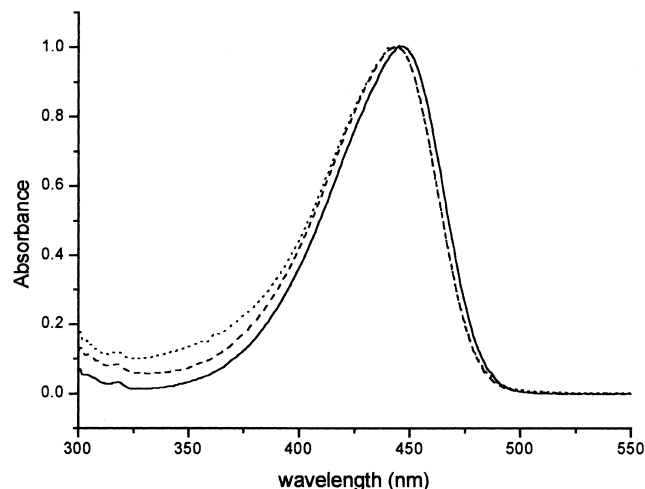


Fig. 1. UV-Vis absorption spectra of dark-adapted wild type and truncated PYP. Spectra were taken at room temperature in 10 mM Tris, pH 7.5. Solid line: WT PYP; dotted line:  $\Delta 25$  PYP; dashed line:  $\Delta 27$  PYP.

and Boltzmann constants, respectively.

$$\ln \frac{k_i h}{k_b T} = \frac{\Delta S_i^\#(T_0)}{R} - \frac{\Delta H_i^\#(T_0)}{RT} - \frac{\Delta C_p^\#}{R} \left( 1 - \frac{T_0}{T} + \ln \frac{T_0}{T} \right) \quad (2)$$

Temperature-induced denaturation experiments were carried out as described in [14] using protein solutions in 50 mM citrate buffer.

Concentration profiles of pB<sub>dark</sub> and pG as a function of temperature were calculated from UV/Vis difference spectra using global analysis [14]. We used skewed Gaussian shapes to model the spectra of pB<sub>dark</sub> and pG.

The equilibrium constant  $K$  was calculated from the concentration profiles. Below 20°C the pB<sub>dark</sub> 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 Eq. 3, from which the thermodynamic parameters were derived.

$$\ln K = \frac{\Delta S(T_0)}{R} - \frac{\Delta H(T_0)}{RT} - \frac{\Delta C_p}{R} \left( 1 - \frac{T_0}{T} + \ln \frac{T_0}{T} \right) \quad (3)$$

## 3. Results and discussion

The N-terminal domain of PYP in a crystalline lattice is folded into two  $\alpha$ -helices that range from residue 11 to 16 ( $\alpha 1$ ) and from 20 to 24 ( $\alpha 2$ ). It should be noted, however, that in solution the second helix displays dihedral angles that classify it as a loop [24]. For deletion of the N-terminal domain of PYP we decided to delete the first 25 or 27 amino acids, thus generating the  $\Delta 25$  and  $\Delta 27$  proteins. In this way, Gly-29, which is in van der Waals contact with Glu-46, is retained. Further truncation results in non-functional PYP

Table 1  
Thermodynamic parameters of the recovery step of the PYP photocycle

	$\Delta S^\#$ (J/mol/K)	$\Delta H^\#$ (kJ/mol)	$\Delta G^\#$ (kJ/mol)	$\Delta C_p^\#$ (kJ/mol/K)
WT	22(0.8)	33 (0.2)	26 (0.1)	-2.5 (0.03)
$\Delta 25$	-14(1.9)	28 (0.6)	32 (0.3)	-0.1 (0.08)
$\Delta 27$	-32(0.8)	16 (0.2)	26 (0.1)	-1.0 (0.03)

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 Fig. 3. Values at 298 K are shown. The values in parentheses are the standard deviations in the thermodynamic parameter, according to the least squares fit of the data to Eq. 2.

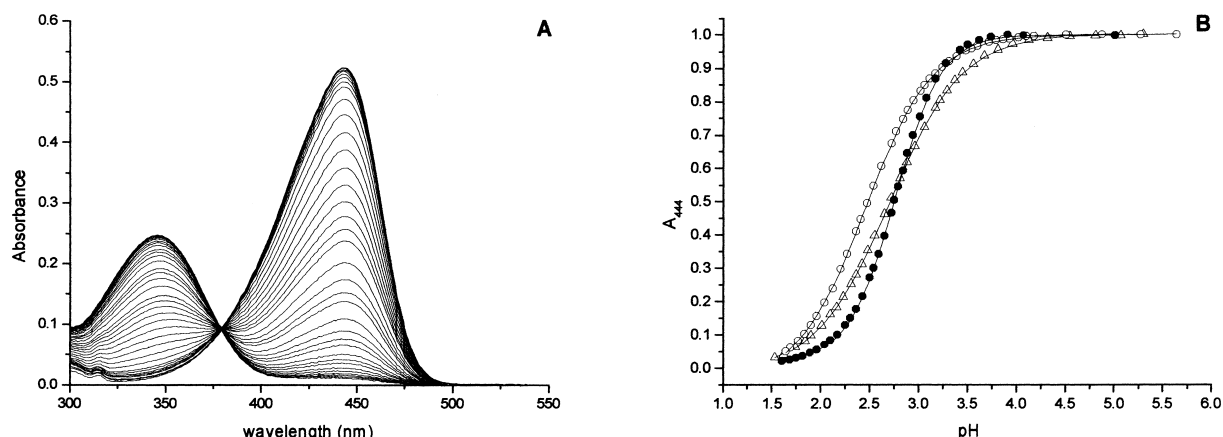


Fig. 2. Titration of the absorption spectra of wild type and truncated PYP. Spectra were taken at room temperature in 10 mM Tris, 100 mM KCl between pH 7 and pH 1. A: Dependence of the absorption spectra of  $\Delta 27$  PYP on pH. B: Relative amplitude of the absorbance in the respective absorption 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 Eq. 1. Closed circles: wild type PYP; open triangles:  $\Delta 25$  PYP; open circles:  $\Delta 27$  PYP.

[25]. The first amino acid in the next element of secondary structure, i.e.  $\beta 1$ , 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 wavelengths (Fig. 1). Also their temperature stability is 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. Fig. 2A,B shows this experiment, with wild type PYP for comparison. A  $pK_a$  of  $2.8 \pm 0.16$  is obtained, with a Hill coefficient, expressing the degree of cooperativity, of  $1.9 \pm 0.05$ . Both values are in agreement with previous observations [22,26]. For the two truncated proteins a set of spectra were obtained with a clear isosbestic point at 380 nm, indicating that lowering the pH gives rise to a well-defined two-state transition for these two proteins too. Fig. 2B also shows the corresponding titration curves of the  $\Delta 27$  and  $\Delta 25$  truncated PYP proteins. Strikingly, whereas the  $pK_a$  of  $\Delta 25$  is unaltered (i.e.  $2.9 \pm 0.16$ ) and the  $pK_a$  of  $\Delta 27$  has only slightly decreased ( $2.4 \pm 0.06$ ), the degree of cooperativity in the titration has significantly decreased: to  $1.3 \pm 0.03$  and  $1.2 \pm 0.02$ , 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 the central  $\beta$ -sheet is altered too [17]. 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 signalling state, we analyzed the temperature dependence of the recovery reaction (i.e. the pB to pG transition [12]) 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 [5,16,22]. 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 (Fig. 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 (Fig. 3) shows the convex curve that is well known for wild type PYP [14,16,27]. A change in heat capacity associated with the transition from

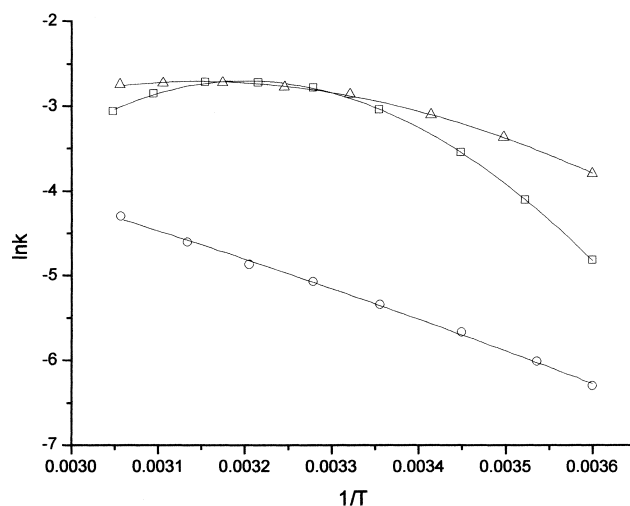


Fig. 3. Thermodynamic analysis of the rate of the pB to pG transition in the PYP photocycle. The natural logarithm of the rate constant  $k$  is shown as a function of reciprocal temperature. The solid line was obtained by fitting the data to Eq. 3. Squares: wild type PYP; circles:  $\Delta 25$  PYP; triangles:  $\Delta 27$  PYP.

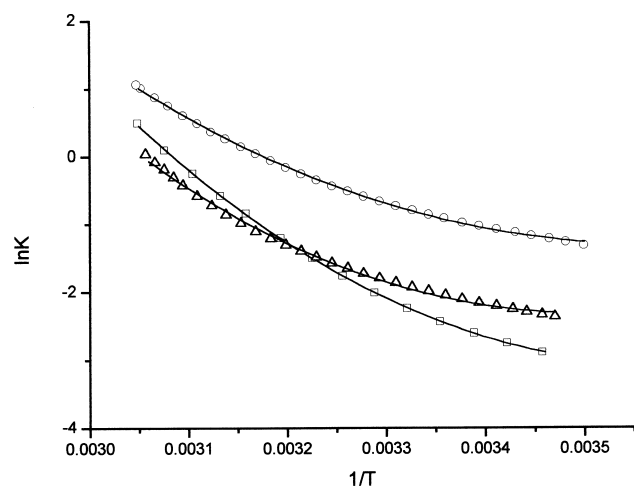


Fig. 4. Temperature dependence of the equilibrium constant that characterizes the reversible thermal denaturation of PYP. The concentration profiles of pG and pB<sub>dark</sub> were used to calculate the temperature dependence of equilibrium constant  $K$ . The solid line was obtained by fitting the data to Eq. 3. Squares: wild type PYP; circles:  $\Delta 25$  PYP; triangles:  $\Delta 27$  PYP.

pB to pG of  $-2.5$  kJ/mol/K can be calculated from this degree of curvature, in agreement with the value reported in [14]. Fig. 3 also shows the corresponding curves for  $\Delta 27$  and  $\Delta 25$ . 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  $\Delta 25$  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 signalling state has essentially been abolished in the N-terminally truncated derivatives, in particular in the  $\Delta 25$  protein.

Both truncated proteins, but especially  $\Delta 25$ , 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 [14]. In Fig. 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 the two truncated proteins. From the temperature-dependent spectra, concentration profiles were determined using a global analysis technique, as described in [14]. The resulting equilibrium constant  $K$  has been plotted against reciprocal temperature in Fig. 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.

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  $\Delta 27$  and  $\Delta 25$ , respectively; M.A. van der Horst, 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  $\Delta 25$  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 (J.J. van Thor et al., unpublished observations). 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 (Trp-119), which is located far from the chromophore and is clamped between the central  $\beta$ -sheet and the two N-terminal  $\alpha$ -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 signalling state (Th. Gensch et al., unpublished observations). 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 PYP, upon signalling 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 [28], such temperature dependence may be observable in the activity of other signal-transducing proteins as well.

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Table 2

Thermodynamic parameters of the equilibrium between the pG and pB<sub>dark</sub> forms of PYP at 298 K and pH 3.4

	$\Delta S$ (J/mol/K)	$\Delta H$ (kJ/mol)	$\Delta G$ (kJ/mol)	$\Delta C_p$ (kJ/mol/K)
WT	135	46	6.1	2.2
$\Delta 25$	92	30	2.3	1.6
$\Delta 27$	74	27	5.2	2.1

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 Fig. 4. The uncertainty in  $\Delta S$ ,  $\Delta H$  and  $\Delta C_p$  is about 10%, whereas in  $\Delta G$  it is about 3%.

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