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Amino Acids in the N-Terminal Region Regulate the Photocycle of Photoactive Yellow Protein[1]

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The spectroscopic properties of photoactive yellow protein (PYP) partially digested by chymotrypsin were studied. Chymotrypsin yielded three major products that were yellow but distinguishable by SDS-PAGE. They were readily separated by DEAE-Sepharose column chromatography. Protein sequencing and mass spectrometry demonstrated that chymotrypsin cleaved the N-terminal 6, 15, or 23 amino acids (T6, T15, and T23). The blue-shifts of the absorption maxima and the increases in the apparent pK_a of the chromophores relative to those of intact PYP were less than 4 nm and 0.2, respectively. The absorption spectra of the near-UV intermediates produced from T6, T15, and T23 were identical to that of intact PYP, but with lifetimes that were 140, 2,300, and 4,500 times longer, respectively. These observations suggest that the recovery of the dark state of PYP from the near-UV intermediate is accelerated by the N-terminal region, and that this region acts as a regulatory factor for the photocycle of PYP.

Key words: chymotrypsin, N-terminus, photoactive yellow protein, photocycle, pK_a.

Photoactive yellow protein (PYP) is a putative photoreceptor protein for the negative phototaxis of purple photosynthetic bacteria (*1*). It was first found in *Ectothiorhodospira halophila* (*2*), and similar proteins were successively found in other bacteria (*3–7*). The chromophore of PYP is a *p*-coumaric acid bound to Cys69 (*8–11*), which is deprotonated and forms a hydrogen-bonding network with Tyr42 and Glu46 (*11*), resulting in the bright yellow color (absorption maximum, 446 nm). Upon absorption of a photon, the chromophore is isomerized from the *trans* form to the *cis* form (*12–15*), and thereafter, subsequent thermal reactions take place to return photo-converted PYP to its original state. This photocycle of PYP has been studied using various techniques (*16–20*). To date, several intermediates have been identified, and the intramolecular event involved in the formation of each intermediate has been clarified.

Among the intermediates of PYP, the last one, PYP_M, (also called I2 or pB) is thought to be physiologically important and is the best characterized. PYP_M is a bleached intermediate with an absorption maximum at 357 nm and an extinction coefficient relative to that of PYP of about 0.3. The chromophore of PYP_M is in a protonated *cis* form although that of PYP is in a deprotonated *trans* form. In addition, a large conformational change in the protein moiety takes place upon the formation of PYP_M in solution (*21*, *22*). The recovery of PYP from PYP_M takes ~100 ms and involves thermal isomerization, deprotonation of the chromophore, and global protein conformational change. Thus, studies of the formation and decay of PYP_M provide a route toward understanding the principle of the protein conformational change essential for function.

In the course of previous experiments, we found that the lifetime of PYP_M in a stale sample is extremely prolonged (Tai, T. *et al.*, unpublished observation). SDS-PAGE analysis demonstrated that the molecular weight of PYP in such a sample is slightly smaller than that of freshly prepared PYP. This observation suggests that several amino acids in a terminal region(s) have a great effect on the recovery of PYP. To reproduce this observation, PYP was partially digested by chymotrypsin and the resulting fragments were separated. Using these samples, the spectroscopic properties of truncated PYPs were studied.

MATERIALS AND METHODS

PYP from *E. halophila* was prepared as reported previously (*23*). Briefly, apoPYP was expressed in *Escherichia coli* and extracted with 8 M urea in 10 mM Tris buffer (pH 7.4). After 2-fold dilution in Tris buffer, holoPYP was reconstituted by adding *p*-coumaric anhydride (*10*), purified by ammonium sulfate precipitation and DEAE-Sepharose column chromatography (Amersham Pharmacia Biotech), and finally suspended in Tris buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4).

Purified PYP (final concentration, 2.3 mg/ml) was digested with bovine pancreas chymotrypsin (Wako, final concentration, 0.027 mg/ml) at 20°C in the dark. During the incubation, small aliquots (5 μl) were taken from the reaction

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Abbreviations: PYP, photoactive yellow protein; PMSF, phenylmethanesulfonyl fluoride.

mixture, supplemented with 1 μl of 100 mM PMSF in ethanol to inactivate the chymotrypsin, and kept at −80°C for SDS-PAGE analysis (*24*).

To prepare truncated PYP on a large scale, 8 mg PYP was digested with 0.08 mg chymotrypsin in 3.0 ml Tris buffer for 16 h. After the addition of PMSF, the mixture was applied to a DEAE-Sepharose column and eluted with a gradient of NaCl (0–200 mM) using a Hi-Load System (Amersham Pharmacia Biotech).

N-termini of digested PYPs were sequenced by a protein sequencer (Procise 492 or 476A, Applied Biosystems). Mass spectra were recorded by MALDI (matrix assisted laser desorption ionization)-TOF (time of flight)-MS (Voyager STR, PerSeptive Biosystems), using sinapinic acid as the matrix. The mass of truncated PYP was calibrated using the mass of intact PYP as the standard ($M+H^+$ = 14,020.8).

Absorption spectra were recorded on a Shimadzu UV2400PC recording spectrophotometer with the temperature of the sample controlled by a refrigerated bath/circulator (RTE-111, NESLAB). For pH-titration experiments, a thin pH electrode (6069-10C, Horiba) was placed in the sample cell, and the sample was continuously stirred by a magnetic stirrer. The pH was changed by adding HCl (0.1–1 N).

The photoreaction of PYP was studied by measuring the transient absorption spectra after photo-excitation using multi-channel CCD spectroscopy system (S2000 system, Ocean Optics). The deuterium lamp of a Beckman DU650 spectrophotometer was used as the monitoring light source. PYP was excited by a short arc xenon flash lamp (SA200E, Eagle) triggered by a delay pulse generator (DG535, Stanford Research Systems).

The spectral and kinetic data were analyzed by IGOR Pro ver. 3.14 for Windows (WaveMetrix).

RESULTS

Our preliminary experiments demonstrated that the lifetime of PYP_M in the truncated form is extremely long. To reproduce this phenomenon, PYP was digested with chymotrypsin. The purified PYP sample was incubated with chymotrypsin at 20°C in the dark. Changes in the absorption spectra of the reaction mixture are shown in Fig. 1a. Intact PYP has an absorption maximum at 446 nm. With time, the absorbance at 446 nm decreased with a slight blue shift, and the absorbance at 350 nm increased. During this reaction, small aliquots of the reaction mixture were removed just prior to spectral measurement, the chymotrypsin was inactivated by the addition of PMSF, and the samples were subjected to SDS-PAGE analysis (Fig. 1b). Intact PYP has an apparent molecular weight of 18,800 on SDS-PAGE, while its mass is 14,000 Da (*25*). Chymotrypsin digestion yielded three major fragments with apparent molecular weights of 17,800, 14,400, and 13,500.

In order to study their spectroscopic properties, truncated PYPs were prepared on a large scale. Eight milligrams of PYP was digested for 16 h under the same conditions as for the experiment shown in Fig. 1. The resulting mixture, which contained the three major fragments but almost no intact PYP, was subjected to DEAE-Sepharose column chromatography (Fig. 2) eluted with a linear gradient of NaCl (0–200 mM). The elution profile was obtained by monitoring the absorbance of the fractions at 446 nm, and clearly showed the truncated PYPs to be separated into three major components and two minor components. The three major components, fractions 35–39 (Fr. 1), fractions 45–48 (Fr. 2), and fractions 68–70 (Fr. 3), were collected and analyzed by SDS-PAGE (Fig. 2, inset). The results demonstrate that the greater the number of cleaved amino acid residues, the earlier the fragment eluted from the column. The absorption spectra of these fragments are shown in Fig. 3a. To determine the cleavage sites, the N-termini of Fr. 1, Fr. 2, and Fr. 3 were sequenced by a protein sequencer (Table I). The fragments agree with sequences starting with Asp24, Ala16, and Gly7 of PYP. MALDI-TOF mass spectrometry demonstrated the masses of Fr. 1, Fr. 2, and Fr.3 to be 11,487.5, 12,347.3, and 13,305.7 Da (Table I), which agree with the calculated masses of Asp24–Val125 (11,488.0 Da), Ala16–Val125 (12,347.0 Da) and Gly7–Val125 (13,306.0 Da), respectively (Table I). Therefore, Fr. 1, Fr. 2, and Fr. 3 lack the N-terminal 23, 15, and 6 amino acids of PYP, and they are hereafter called T23, T15, and T6, respectively.

It is known that the chromophore of PYP is protonated and bleached under acidic conditions (*2*, *26*). Because pK_a is related to the strength of the hydrogen-bonding network around the phenolic oxygen of the chromophore, pH titration experiments of the truncated PYPs were carried out. The pH of the samples was lowered by adding HCl, and the absorption spectra were recorded. The absorbance at 445 nm was then plotted against pH (Fig. 3b). The titration curves for intact PYP, T6, T15, and T23 overlapped at pH 1.5–2.0 while they were separate at pH 2.0–4.0. The slope

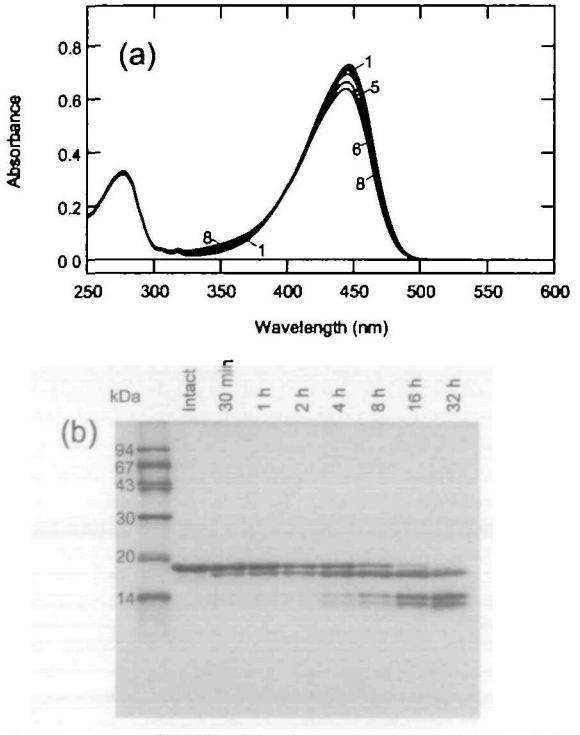

Fig. 1. **Digestion of PYP with chymotrypsin.** (a) 2.3 mg/ml PYP (curve 1) was incubated at 20°C in the presence of 0.027 mg/ml chymotrypsin for 0.5, 1, 2, 4, 8, 16, and 32 h (curves 2–8, respectively). The spectra were recorded with a 1 mm light pathlength. (b) SDS-PAGE analysis of the reaction mixture.

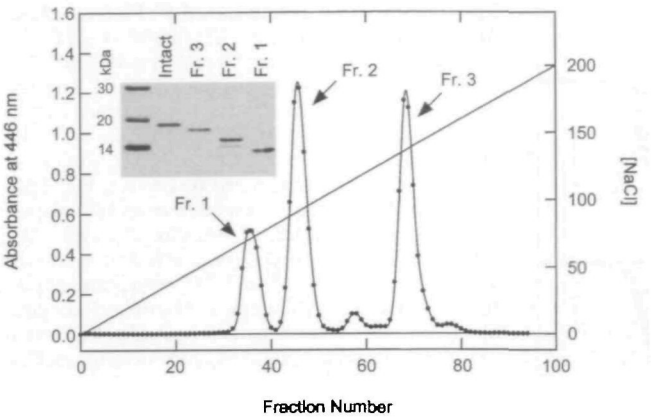

Fig. 2. **Separation of truncated PYPs by DEAE-Sepharose column chromatography.** The reaction mixtute after 16 h of incubation was applied to a DEAE Sepharose-column (2 × 10 cm) and eluted with 0–200 mM NaCl. Each fraction was 1 ml. (Inset) SDS-PAGE of intact PYP and three major components (Fr. 1–Fr. 3).

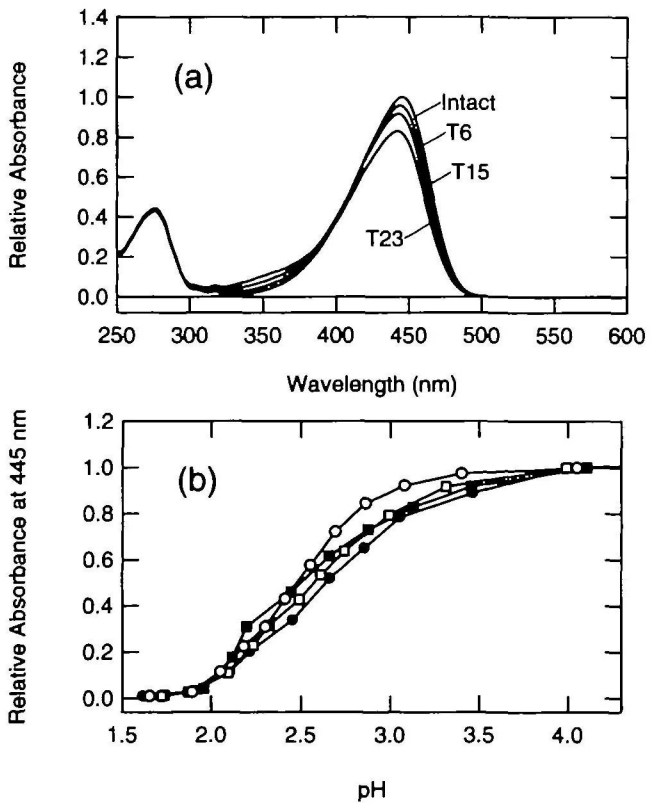

Fig. 3. **(a) Absorption spectra of intact PYP, T6, T15, and T23.** Those of T6 and T15 were normalized at 414 and that of T23 at 397 nm based on Fig. 1a. The absorption maxima of T6, T15, and T23 were 444, 443, and 442 nm, respectively. **(b) pH titration of truncated PYPs.** The absorbance at 445 nm was plotted against pH for intact PYP (open circles), T6 (open squares), T15 (closed circles), and T23 (closed squares).

of the titration curves became gentle in the truncated PYPs: the apparent Hill constant for intact PYP is 2.0, while those of T6, T15, and T23 are 1.6, 1.5, and 1.4, respectively. However, the apparent pK_a, at which the absorbance at 445 nm is 50% of that at neutral pH, was not

TABLE I. **N-terminal sequence and mass of truncated PYP.**

Sample	N-terminal sequence	Mass[a] (M+H$^+$)	Deduced sequence	Calculated mass (M+H$^+$)
Intact	MEHVAFGSED...	14,020.8	Met1–Val125	14,020.8
Fr. 1	DGLAFGAIQL...	11,487.5	Asp24–Val125	11,488.0
Fr. 2	AKMDDGQLDG...	12,347.3	Ala16–Val125	12,347.0
Fr. 3	GSEDIEN...	13,305.7	Gly7–Val125	13,306.0

[a]Calibrated using the calculated mass of intact PYP.

altered greatly by truncation: 2.48 for intact PYP, 2.57 for T6, 2.64 for T15, and 2.50 for T23.

The photocycles of truncated PYPs were then studied by flash photolysis in millisecond-second time scale, in which PYP recovers from its M intermediate. Intact PYP, T6, T15, and T23 were excited by a yellow flash (>410 nm), and the transient difference absorption spectra before and after excitation were recorded at 20°C (Fig. 4). In each data set, the absorption spectrum before excitation was used as the base line. In all samples, a decrease in the absorbance at 446 nm and an increase in the absorbance at 350 nm were observed just after excitation (curves 1), indicating the formation of M intermediates. The shapes of the positive bands are compared in Fig. 4e. Because the shapes are identical, the chromophore/protein interactions in the M intermediates formed from truncated PYPs are similar to that formed from intact PYP. The M intermediates decayed to the original states with clear isosbestic points. The recoveries of the absorbance at 445 nm were plotted against the time after excitation (Fig. 4f), and these were fitted by first-order kinetics to estimate the lifetimes of the M intermediates. As a result, the lifetimes of PYP_M, $T6_M$, $T15_M$, and $T23_M$ were estimated to be 0.13, 18, 300, and 590 seconds, respectively.

DISCUSSION

Chymotrypsin selectively cleaves the N-terminal region of PYP, with cleavage sites at Phe6-Gly7, Leu15-Ala16, and Leu23-Asp24. These are consistent with the knowledge that chymotrypsin cleaves peptide bonds on the C-terminal side of Tyr, Phe or Leu. PYP also has aromatic residues, but these are not accessible to chymotrypsin under our experimental conditions. However, it has previously been reported that chymotrypsin cleaves Tyr42-Asn43 and Phe62-Phe63 (*27*), a difference that may be due to the chymotrypsin concentration and/or reaction time. The electrophoresis pattern shows that T6 is produced first, followed by T15 and T23.

Truncated PYPs were eluted from the DEAE-Sepharose column in order of molecular weight. The NaCl concentration for elution was 70 mM for T23, 90 mM for T15, and 140 mM for T6 (Fig. 2), whereas that for intact PYP was 160 mM (data not shown). PYP has six acidic residues (Glu2, Glu9, Asp10, Glu12, Asp19, and Asp20) and two basic residues (His3 and Lys17) in the N-terminal region. The pK_a of Glu, Asp, His, and Lys are 4.25, 3.65, 6.00, and 10.53, respectively (*28*). Therefore, under our experimental conditions (pH 7.4), the changes in the net charges by truncation are calculated to be 0.96 for T6, 3.96 for T15, and 4.96 for T23. The affinity to DEAE-Sepharose is thus lowered by truncation, resulting in elution at lower concentration of NaCl than that for intact PYP.

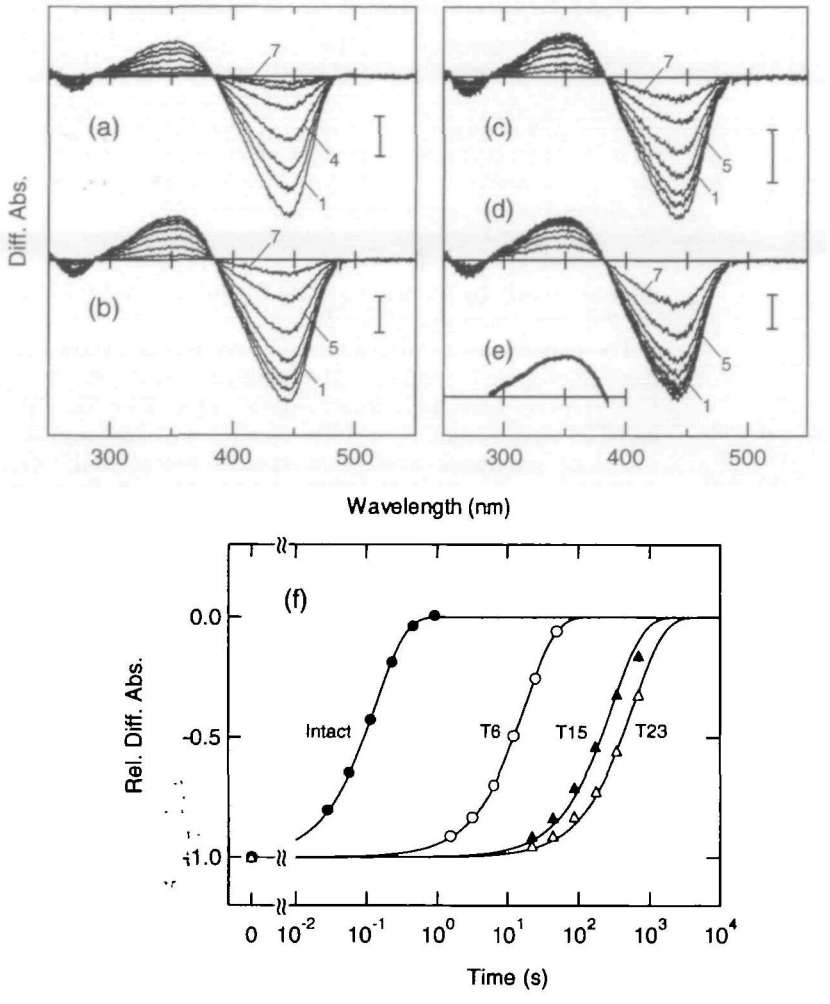

Fig. 4. **Recoveries of truncated PYPs from their M intermediates.** Intact PYP (a), T6 (b), T15 (c), and T23 (d) in Tris buffer (10 mM Tris-HCl, pH 7.4) were excited with >410 nm flash at 20°C and the transient difference absorption spectra were recorded. Scale bars represent 0.02 absorbance. (a) 0, 30, 60, 110, 230, 460, and 920 ms after excitation (curves 1–7, respectively). (b) 0, 1.5, 3.1, 6.1, 12.3, 24.6, and 49.3 s after excitation (curves 1–7, respectively). (c) and (d) 0, 22, 44, 88, 176, 351, and 702 s after excitation (curves 1–7, respectively). (e) Curves 1 for intact PYP, T6, T15, and T23 were compared after normalization at 350 nm. (f) Absorbance changes at 445 nm were plotted against time after excitation in logarithmic scale and fitted by first-order kinetics.

pH titration experiments demonstrated that the apparent pK_a of truncated PYP is almost identical to that of intact PYP. However, the apparent Hill constants, which correlate with cooperativity, differ: the Hill constants of intact PYP, T6, T15, and T23 are 2.0, 1.6, 1.5, and 1.4, respectively. Therefore, at least two reactions are involved in the protonation of the chromophore upon acidification, and those in intact PYP are the most cooperative. Namely, in intact PYP, the native structure of the protein moiety prevents protonation of the chromophore, and, at the same time, the deprotonated chromophore hinders the acid denaturation of the protein moiety, probably due to the hydrogen-bonding network. However, such cooperativity is partially lost when the N-terminal region is truncated. This trend is prominent at pH 2–4 rather than at pH 1.5–2. Under strong acid conditions, the N-terminal region is unfolded (*29*) so that truncation of this region would not affect the protonation of the chromophore.

Although truncation has a small effect on the absorption maximum and apparent pK_a in the dark states, the lifetime of PYP_M is strikingly different. Our data clearly demonstrate that the N-terminal region accelerates the recovery of PYP from PYP_M. PYP has a six-strand β-sheet structure in its central part (*11*), with the chromophore loop containing Cys69 located on one side, and N-terminal region located on the opposite side. Furthermore, the N-terminal 4 amino acids are exposed to solvent in solution (*30*). Therefore, no direct interaction seems to exist between the chromophore and the N-terminal amino acids. In fact, the absorption spectra of the M intermediates as well as the dark states of truncated PYPs are similar to those of intact PYP. This suggests that the electrostatic interaction to the chromophore is not altered by truncation. The N-terminal region is therefore considered to interact with other amino acid residues rather than the chromophore to regulate protein conformational change.

The protein structure of PYP is radically changed upon the formation of PYP_M, resulting in the partially unfolded state (*22*, *31*, *32*). The structure of the N-terminal region of PYP in the crystal (*11*) is different from that in solution (*30*). Recent H–D exchange experiments have suggested exposure of the N-terminal region of PYP during the photocycle (*29*). Therefore, the N-terminal region appears to be highly mobile and partial denaturation of this region to be essential for the formation of PYP_M. The primary structures of PYPs in *Halochromatium salexigens* (*5*), *Rhodothalassium salexigens* (*6*), *Rhodobacter sphaeroides* (*7*), and *Rhodobacter capsulatus* (*33*) have been identified in addition to that in *E. halophila*. In the N-terminal region, Met1, Phe6, Gly7, Asp10, Asn13, and Ala16 are completely conserved among these PYPs. These residues seem to be responsible for the regulation of the photocycle by the N-

terminus. Because no solution structure for PYP_M at high resolution has been reported, the interactions of these amino acid residues in the M state remain unclear. However, they may interact with the amino acid residue at the core of the protein, and this may regulate the protein conformational changes. This issue should be clarified by systematic site-directed mutagenesis.

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