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ARTICLE *in* BIOCHEMISTRY · OCTOBER 1997

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Raman Spectroscopic and Light-Induced Kinetic Characterization of a Recombinant Phytochrome of the Cyanobacterium *Synechocystis*

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Received June 30, 1997; Revised Manuscript Received August 13, 1997[⊗]

ABSTRACT: A phytochrome-encoding cDNA from the cyanobacterium *Synechocystis* has been heterologously expressed in *Escherichia coli* and reconstituted into functional chromoproteins by incubation with either phycocyanobilin (PCB) or phytochromobilin (PΦB). These materials were studied by Raman spectroscopy and nanosecond flash photolysis. The Raman spectra suggest far-reaching similarities in chromophore configuration and conformation between the P_{fr} forms of *Synechocystis* phytochrome and the plant phytochromes (e.g. phyA from oat), but some differences, such as torsions around methine bridges and in hydrogen bonding interactions, in the P_r state. *Synechocystis* phytochrome (PCB) undergoes a multistep photoconversion reminiscent of the phyA $P_r \rightarrow P_{fr}$ transformation but with different kinetics. The first process resolved is the decay of an intermediate with red-shifted absorption (relative to parent state) and a 25-μs lifetime. The next observable intermediate grows in with 300 ($\pm$25) μs and decays with 6−8 ms. The final state (P_{fr}) is formed biexponentially (450 ms, 1 s). When reconstituted with PΦB, the first decay of this *Synechocystis* phytochrome is biexponential (5 and 25 μs). The growth of the second intermediate is slower (750 μs) than that in the PCB adduct whereas the decays of both species are similar. The formation of the P_{fr} form required fitting with three components (350 ms, 2.5 s, and 11 s). H/D Exchange in *Synechocystis* phytochrome (PCB) delays, by an isotope effect of 2.7, both growth (300 μs) and decay rates (6−8 ms) of the second intermediate. This effect is larger than values determined for phyA (*ca*. 1.2) and is characteristic of a rate-limiting proton transfer. The formation of the P_{fr} state of the PCB adduct of *Synechocystis* phytochrome shows a deuterium effect similar as phyA (*ca.* 1.2). Activation energies of the second intermediate in the range 0−18 °C are 44 (in H_2O/buffer) and 48 kJ mol^{-1} (D_2O), with essentially identical pre-exponential factors.

The phytochrome family of chromoproteins act as light detectors in probably all higher plants and have also been identified in various lower plants (Wada & Kadota, 1989; Quail et al., 1994). Phytochromes consist of some 1100 amino acid residues which are arranged in two relatively well separated domains. The front half (up to around position 600) contains the chromophore, phytochromobilin (PΦB),[1] and enables the protein to convert from the red-absorbing P_r form (λ_{max} *ca*. 665 nm) into the far-red-absorbing P_{fr} form (λ_{max} *ca*. 730 nm) upon light absorption. This conversion is complete within several milliseconds and is driven by the energy stored in the primary process, a double-bond $Z \rightarrow E$ photoisomerization of the PΦB chromophore (Schaffner et al., 1990; Braslavsky et al., 1997). The C-terminal portion of phytochrome is essential for the transduction of the light-generated signal, which presumably is accomplished via protein−protein interaction with a hitherto unknown reaction partner (Cherry & Vierstra, 1994; Elich & Chory, 1994; Quail et al., 1995). While for the phytochromes from higher plants a variety of interacting proteins and signal transduction pathways have been proposed, for phytochromes from lower plants a signal transduction mechanism involving a kinase activity of the receptor has been discussed on the basis of sequence alignments (Schneider-Poetsch, 1992; Thümmler et al., 1995). This type of signal transduction, accomplished by transphosphorylation to an interacting protein, is widely distributed among prokaryotes (two-component systems) (Parkinson & Kofoid, 1992; Chang, 1996).

Recently, open reading frames (ORFs) in the cyanobacteria *Fremyella* and *Synechocystis* sp. (PCC 6803) have been reported encoding proteins with similarities to phytochromes and bacterial sensor kinases (Kehoe & Grossmann, 1996; Hughes et al., 1997; Kaneko et al., 1996). The RsaE and PlpA genes in *Fremyella* and *Synechocystis*, respectively, show restricted homologies in the N-terminal region, in particular lacking the highly conserved chromophore-binding motif. The ORF slr0473 from *Synechocystis* (Kaneko et al., 1996) on the other hand shows close similarities in this and numerous other regions. Accordingly, heterologous expression of the latter sequence in *Escherichia coli* yielded an apoprotein which binds phycocyanobilin (PCB) to become red/far-red photoreversible, very much in the manner of phytochrome from higher plants (Hughes et al., 1997). We report here the spectral and kinetic characterization of such recombinant *Synechocystis* chromoproteins, containing either PCB or PΦB as a chromophore.

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: FT, Fourier transform; HOOP, hydrogen out-of-plane; I_{700}, first intermediate of the $P_r \rightarrow P_{fr}$ transformation; LADS, lifetime-associated difference spectrum; ORF, open reading frame; P_r and P_{fr}, red and far-red light absorbing forms of plant phytochromes; PCB, phycocyanobilin; PΦB, phytochromobilin; RR, resonance Raman; RT, room temperature.

S0006-2960(97)01563-8 CCC: $14.00

MATERIALS AND METHODS

Cloning and Expression of Syn_phy cDNA. Recombinant plasmid pF10.His was used to overexpress C-terminally His_6-tagged *Synechocystis* phytochrome apoprotein in *E. coli*. The soluble lysate was purified and concentrated by ammonium sulfate precipitation and nickel-affinity chromatography as described (Lamparter et al., 1997).

Chromophore Preparation, Protein Purification, and Chromophore Binding. PCB and PΦB, which were used for the reconstitution of the *Synechocystis* phytochrome, were prepared as described (Kufer & Scheer, 1979; Cornejo et al., 1992) and crystallized from chloroform−hexane. Autoassembly was performed at room temperature in the darkness with either chromophore.

Deuterium Exchange. Fully reconstituted samples of *Synechocystis* phytochrome were placed onto a Centricon-30 cartridge (Amicon) and washed with 5 vols of deuterated buffer (50 mM Tris, 5 mM EDTA, 2 mM DTT, 2 mM Pefabloc, Merck).

Difference Spectroscopy. Samples of *Synechocystis* phytochrome were irradiated with far-red light (cutoff filter, $\lambda \geq 715$ nm). The sample was then irradiated with red light (interference filter, $\lambda = 658 \pm 7$ nm) for the formation of the P_{fr} state. Irradiation with far-red light regenerated the P_r state. Absorption spectra, recorded with a Shimadzu spectrophotometer (UV 2102-PC) after each irradiation, were subtracted from each other in order to generate the difference spectra.

Flash Photolysis and Data Handling. Double-beam flash photolysis was performed as described (Ruddat et al., 1997). Briefly, samples were adjusted to maximum absorbance of 0.1−0.3. Laser flashes of 15 ns duration at $\lambda = 650$ nm and a repetition rate of 0.02 Hz were chosen. Re-irradiation of the sample with $\lambda \geq 715$ nm between the flashes quantitatively recovered the P_r form prior to each new pulse. Transient absorption changes were detected in the wavelength range 620−740 nm. The temperature was 10 °C, except for the determination of the activation parameters (see text) which was performed between 0 and 18 °C. At least ten single transient absorption traces at each wavelength were averaged for data evaluation. Lifetime-associated difference spectra (LADS) were generated by the global fit function (Scurlock et al., 1993; Ruddat et al., 1997)

$$\Delta A^{\lambda}(t) = \sum_{i=1}^{n} \Delta A_i^{\lambda} \, e^{-t/\tau_i} + \text{constant}^{\lambda}$$

where $\Delta A^{\lambda}(t)$ is the observed absorption change at time t and wavelength λ, and ΔA_i^{λ} is the absorption change associated with λ and lifetime τ_i (which was assumed to remain constant over the whole wavelength range). According to the algorithm used, a negative amplitude refers to a growth of absorbance (i.e. a rise term), whereas a positive amplitude reflects a decay of an intermediate. The constant represents the absorption difference between the ground state and a transient with a lifetime longer than the time window.

Fourier Transform (FT)-Resonance Raman (RR) Measurements. FT-RR spectra were measured with a Bio-Rad FT-Raman spectrometer equipped with a Nd:YAG laser (Spectra Physics, FC-106V, bandwidth < 1 cm^{-1}). The samples were cooled down to −140 °C in a cryostat as described previously (Matysik et al., 1995). Prior to cooling, the samples were converted to the P_r and P_{fr} forms by irradiation with 730- and 667-nm light, respectively. The concentration of the samples corresponded to optical densities (660 nm, P_r) of *ca*. 1.0 and 0.6 for oat phytochrome (phyA) and the *Synechocystis* phytochrome-PCB adducts, respectively, and of 0.1 in the case of the PΦB-assembled chromoprotein. The laser power focused onto the sample was 360 mW. The spectral resolution was 4 cm^{-1}, and the total accumulation time for each spectrum was between 40 and 320 min, depending on the spectral quality of the sample. A triangular function was used for apodization of the interferograms. The raw spectra were corrected for the instrumental response as described in Hendra et al. (1991).

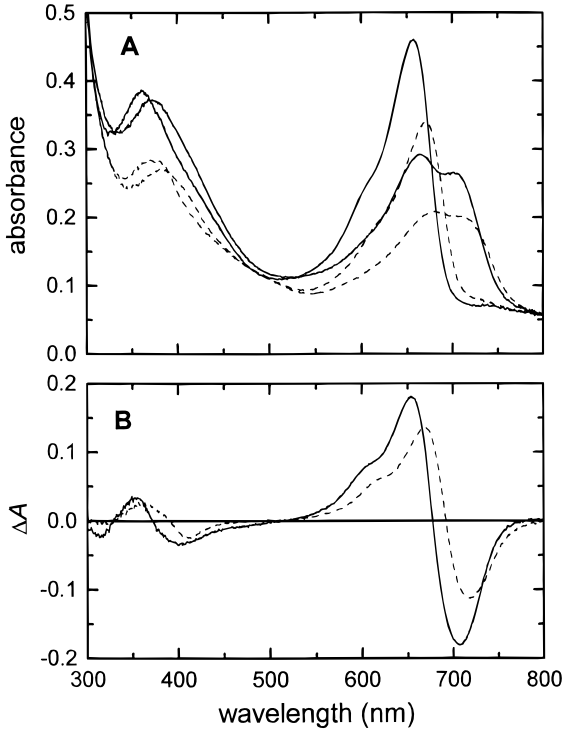

FIGURE 1: (A) P_r and P_{fr} absorption and (B) absorption difference spectra of *Synechocystis* phytochrome, reconstituted with (—) PCB and (- - -) PΦB. The samples were autoassembled and irradiated with far-red light in order to generate quantitatively the P_r form. Subsequently, the samples were irradiated with $\lambda = 658 \pm 7$ nm and with far-red light (cutoff filter 715 nm). The difference spectra were generated from the difference of the absorption spectra recorded after each irradiation.

RESULTS

Preparation of Chromoproteins. C-terminally tagged *Synechocystis* phytochrome apoprotein was expressed in *E. coli* and prepared as described (Lamparter *et al*., 1997). The wild-type protein without a His-tag was enriched from *E. coli* extracts by ammonium sulfate precipitation. Holoprotein was autoassembled *in vitro* with either PCB or PΦB. The samples exhibited λ_{max} values of 654 and 668 nm (±2 nm) for the P_r states with PCB and PΦB chromophore, respectively, and of 706 and 717 nm (±2 nm) after photoconversion with red light to the P_{fr} states (Figure 1). Except for a higher background for the untagged sample, the absorption maxima were identical for the tagged and untagged samples. *In both cases, the photoconversions were fully reversible.*

Resonance Raman Spectroscopy. As shown previously (Matysik et al., 1995; Hildebrandt et al., 1992), excitation with 1064 nm provides a sufficient (pre)resonance enhance-

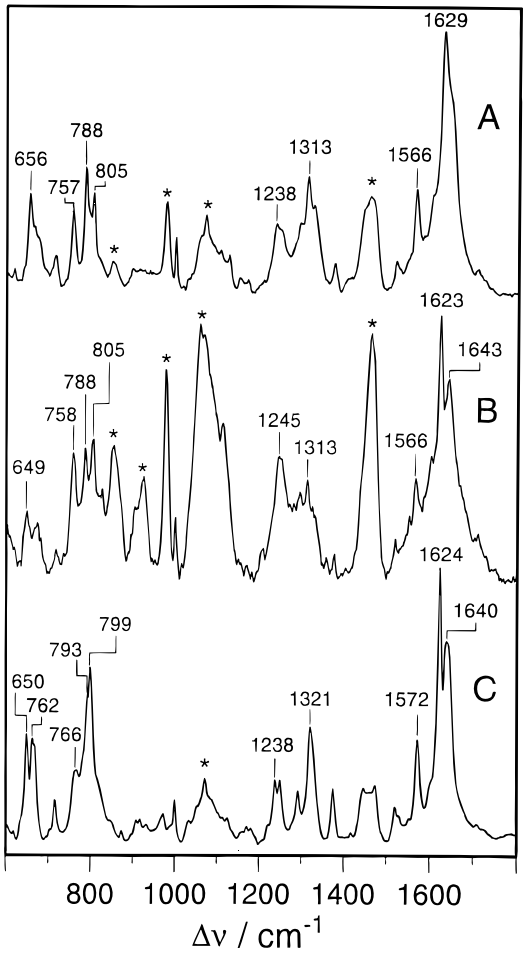

FIGURE 2: RR spectra of the P_r states of (A) *Synechocystis* phy-PCB, (B) *Synechocystis* phy-PΦB, and (C) native oat phyA, excited at 1064 nm. Impurities due to contaminating protein are indicated by asterisks.

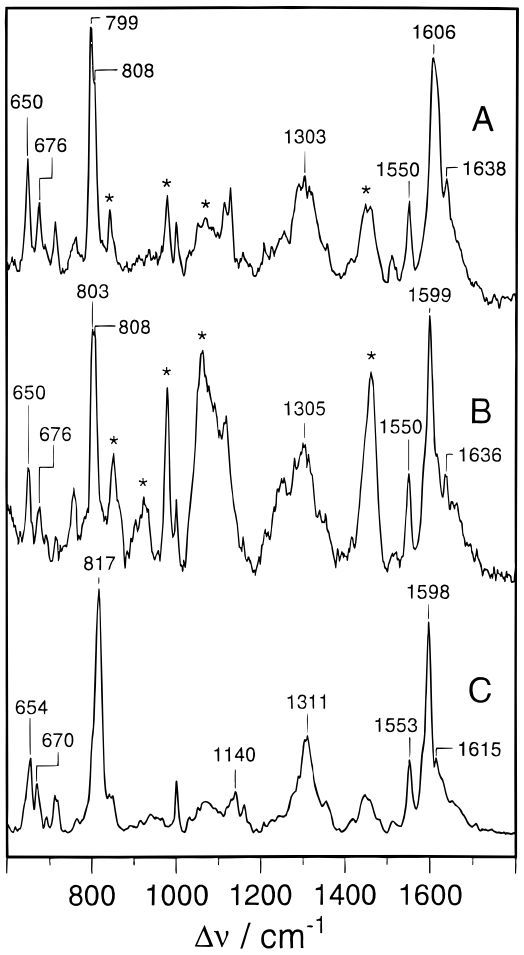

FIGURE 3: RR spectra of the P_{fr} states of (A) *Synechocystis* phy-PCB, (B) *Synechocystis* phy-PΦB, and (C) native oat phyA, excited at 1064 nm. Impurities due to contaminating protein are indicated by asterisks.

ment ensuring that the spectra of phyA are dominated by the RR bands of the chromophore (Figures 2C and 3C). The only band which can be assigned unambiguously to a Raman band of the protein is the 1002-cm^{-1} band originating from the symmetric ring stretching of the phenylalanine residues (Hendra et al., 1991). All other bands can be assigned to the PΦB chromophore. This also holds for the RR spectra of the P_r and P_{fr} forms of the *Synechocystis* PCB adduct (Figures 2A and 3A) except for the 980-cm^{-1} band which stems from residual ammonium sulfate used for precipitation in the protein purification (Matysik et al., 1995). In the case of the PΦB-adduct, however, the RR spectra of P_r and P_{fr} (Figures 2B and 3B) display additional broad and poorly structured peaks (marked by asterisks) which arise from background scattering including Raman bands of the buffer. These features are generally observed in dilute phy samples (as in the PΦB adducts here), whereas their relative intensities are weak in RR spectra of samples of sufficiently high concentration (phyA and PCB adduct of *Synechocystis* phytochrome). These spurious peaks do not obscure those spectral regions which include the most important RR bands of the chromophore. In all cases, His-tagged samples were used for the Raman spectroscopy.

Restricting the comparison of the spectra in Figures 2 and 3 to the RR bands of the chromophores, we note striking similarities in the overall vibrational band pattern of the PCB and the PΦB adducts, and phyA in both the P_r and P_{fr} states. The C═C stretching region between 1500 and 1650 cm^{-1} is sensitive to changes in the conjugated π-electron system and, thus, should reflect changes of the configuration and conformation as well as of the protonation state of the tetrapyrrole (Matysik et al., 1995; Fodor et al., 1990).

In P_r, this region is dominated by a doublet with the strongest component at 1624 cm^{-1} and the somewhat weaker one at ca. 1640 cm^{-1} for both, *Synechocystis* PΦB adduct and phyA (Figure 2). In the PCB form, the low-frequency component is slightly shifted to higher wavenumbers so that the doublet is only poorly resolved. This frequency difference can readily be attributed to the missing vinylic double bond on ring D in PCB. Below 1610 cm^{-1} all spectra exhibit three minor bands with similar RR intensities but some frequency differences. In particular, we note that the 1572-cm^{-1} band of phyA shifts down to 1566 cm^{-1} in the PΦB- and the PCB-*Synechocystis* phytochromes. This band disappears in phyA upon H/D exchange (Matysik et al., 1995). A variety of H/D sensitive bands, which include N−H in-plane bending vibrations, are identified in the region between 1200 and 1400 cm^{-1}. In particular, the 1321-cm^{-1} band of phyA, which also disappears upon H/D exchange, shifts to 1313 cm^{-1} in the RR spectra of both PΦB and PCB adducts.

The region between 850 and 750 cm^{-1} includes bands which result from C−H out-of-plane (HOOP) bending modes of the methine bridges. The RR intensities of these modes are known to be a measure for the torsion of the correspond-

ing C−C single bonds so that this part of the spectra includes further information about the tetrapyrrole conformation (i.e. the coplanarity of the pyrrole rings). In all three spectra three bands of considerable intensity indicate that there are substantial torsions of the various methine bridges. In phyA, these are the strong band at 799 cm^{-1} and the weaker ones at 793 and 766 cm^{-1}. In the *Synechocystis* phytochrome, however, these bands reveal distinct frequency shifts (805, 787, and 758 cm^{-1}) and a redistribution of the RR intensities which appear to be largely independent of the constitution of the chromophore, inasmuch as the spectra of the PΦB and PCB adducts are very similar in this region.

Also in the P_{fr} state, the main RR spectroscopic features are similar for phyA and both *Synechocystis* phytochromes. In the C═C stretching region, the spectra are dominated by a band at 1598 (phyA) and at 1599 cm^{-1} (*Synechocystis* PΦB adducts) which, due to different chromophore constitution, is upshifted to 1608 cm^{-1} in PCB adducts. Particularly interesting is the fact that the 1553 cm^{-1} band of phyA can be found at very similar frequencies in both recombinant *Synechocystis* phytochromes. This band disappears upon H/D exchange in phyA (Matysik et al., 1995). Apparently it has the same origin as the 1572-cm^{-1} band of P_r of phyA which shows distinct frequency shifts in the *Synechocystis* phytochromes. In addition, the RR spectra of the P_{fr} states do not reveal any of the significant spectral differences in the N−H in-plane bending region observed for the P_r states.

On the other hand, the HOOP region displays a remarkable difference since the prominent band at 817 cm^{-1} (with a shoulder at *ca.* 805 cm^{-1}) shifts down by 14 and 18 cm^{-1} in the *Synechocystis* PΦB and PCB adducts, respectively. Apparently, in all cases there is a large torsion around a particular methine bridge, although conformational details may be different in phyA and the *Synechocystis* phytochromes.

Flash Photolysis of PCB Adducts. Absorbance changes in the microsecond time range (performed with a His-tagged *Synechocystis* phytochrome) revealed the rapid *decay* of a first intermediate with a lifetime of 25 μs. This process was followed by a slow *rise* of a species with red-shifted absorption (Figure 4A) and kinetics of 300 (±25) μs. This second observable intermediate had a lifetime of 6−8 ms (Figure 4B). On the millisecond-to-second time scale, the formation of the P_{fr} form, which was found to be thermally stable for long periods, was observed with a biexponential kinetics of 450 (±40) ms and 1 s (Figure 4C; see also first row of Table 1) although the latter component was rather weak.

The corresponding lifetime-associated difference spectra (LADS) in the wavelength range 620−740 nm (Figure 5) were derived from a global fit. The first intermediate (25-μs decay) absorbs maximally around 670 nm, whereas the second species formed within 300 μs has a maximal difference absorption around 700 nm. The final rise, i.e., the formation of the P_{fr} state, shows a maximum between 710 and 720 nm.

The dynamics of the phototransformation were further explored by H/D exchange and by the determination of the Arrhenius parameters. The kinetics of the second component (300-μs rise and 6−8-ms decay at 10 °C) changed strongly with temperature between 0 and 18 °C (Table 2). Even more remarkable, both processes were slowed down by a factor of 2.7 in the deuterated sample. This is the first observation

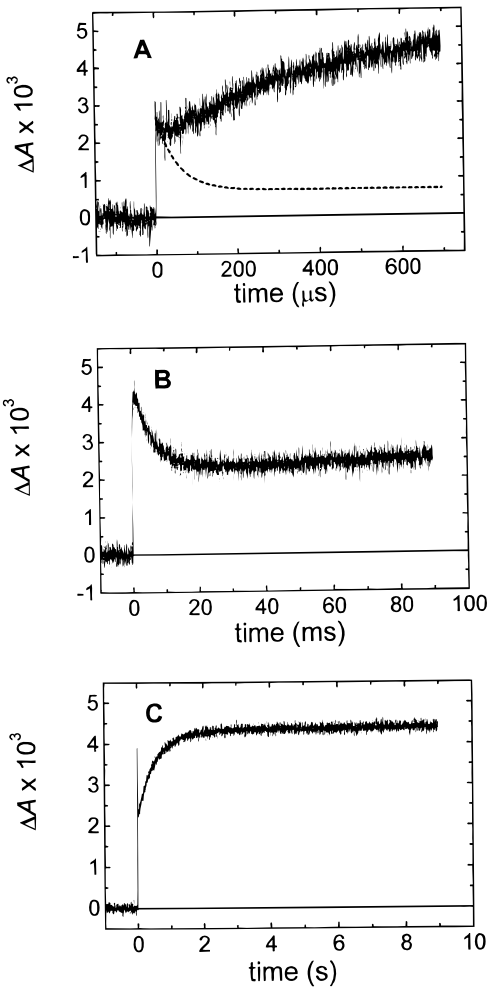

FIGURE 4: Kinetic behavior of *Synechocystis* phy-PCB, detected at (A) λ_{obs} = 690 nm, and at (B, C) λ_{obs} = 700 nm. (A) Absorption changes in the time range of up to 700 μs. For comparison, the decay of the I_{700} intermediate of recombinant phyA-PCB from oat with a lifetime of 90 μs is also shown (- - -); (B) as A, but up to 90 ms; (C) as A, but up to 9 s.

of a strong deuterium effect in a phytochrome. In contrast to this considerable effect, the subsequent formation of the P_{fr} state (450 ms and 1 s at 10 °C) was only moderately affected (similarly to oat phyA) by a factor of 1.2−1.4.

The Arrhenius plot of the rise kinetics (ln k) *vs* the temperature yielded linear fits (Figure 6). A value of log A = 11.6 s^{-1} and an activation energy of E_a = 44 kJ mol^{-1} were determined for (H)-phytochrome (PCB), while for the (D)-phytochrome a nearly identical log A value (11.8 s^{-1}) but a higher activation energy (E_a = 48 kJ mol^{-1}) resulted.

In order to investigate a putative influence of the His-tag on the kinetics, a wild-type *Synechocystis* phytochrome apoprotein without His-tag was also expressed, purified by ammonium sulfate precipitation, and incubated with PCB. Owing to its high expression yield (Hughes et al., 1997), this protein was already rather pure, and it could readily be concentrated to A = 0.15 at its λ_{max}. Flash photolysis of the apoprotein reconstituted with PCB was performed at the few wavelengths which had proved analytically significant in the former experiments (660, 680, and 700 nm), and in the μs to early ms time window. A decay component of 26 μs with small amplitude was followed by a rise term of 540 μs (at 10 °C). The decay of this species, determined to be 6−8 ms for the His-tagged sample, was only slightly retarded and took place within 10 ms.

Table 1: Kinetics of Flash Photolysis at 10 °C of Reconstituted *Synechocystis* Phytochrome (PCB) and (PΦB)[a]

sample	early μs time range	late μs time range	early ms time range	late ms and s time range
PCB adduct	25 (d)	300 ± 25 (r)	6−8 (d)	450 ± 40 (r), (*ca.*1 s) (r)
PCB adduct without $(His)_6$ tag	26 (d)	540 (r)	10 (d)	nd
PΦB adduct	(5) (d), 25 (d)	750 (r)	8 (d)	350 (r), 2.5 s (r), 11 s (r)

[a] Rise and decay type kinetic processes are indicated by (r) and (d), respectively. Small amplitudes are in parentheses. nd, not determined.

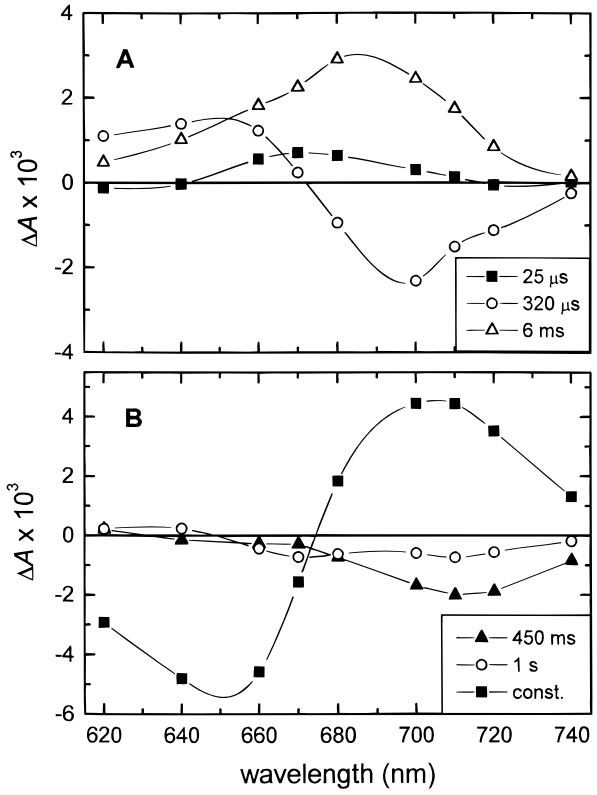

FIGURE 5: Lifetime-associated difference spectra (LADS) of *Synechocystis* phy-PCB. (A) Intermediates in the μs and early ms time range; (B) intermediates in the ms-to-s time range. It should be kept in mind that, except for the constant term, a negative amplitude indicates a rise in absorbance whereas a positive amplitude refers to a decay.

Table 2: Effects of Temperature and Deuterium Exchange on the Kinetics of the 300−μs Rise Component of the *Synechocystis* PCB Adduct

temperature (°C)	H_2O		D_2O		
	τ_H (μs)	ln k_H (s^{-1})	τ_D (μs)	ln k_D (s^{-1})	k_H/k_D
0[a]	731	7.22	2050	6.19	2.8
4[a]	558	7.49	1500	6.5	2.7
8[a]	426	7.76	1150	6.77	2.7
10[a]	320 ± 40[b]	7.93−8.18[b]	nd[d]	nd	nd
12[a]	393	7.84	1080	6.83	2.7
18[a]	227	8.39	575	7.46	2.5
10[c]	7 ms	−	22 ms	−	3.1

[a] Values of the rise kinetics. [b] Values from the global fits of the flash photolysis data at 10 °C. [c] Values of the decay kinetics. [d] nd, not determined.

Flash Photolysis of Synechocystis PΦB Adducts. Flash photolysis of this *Synechocystis* phytochrome revealed, similarly to the PCB adduct, a first intermediate with a lifetime of 25 μs (Figure 7A). The fit of this decay was slightly improved when an even faster component of 5 μs, albeit with very small amplitude was added. The subsequent rise, observed to occur in 300 μs in the PCB adduct, was found here to be much slower, with a time constant of 750 μs. This intermediate decayed with a lifetime of 8 ms (Figure 7B), a kinetics which is nearly identical to the PCB-containing phytochrome. Differences between the PCB and the PΦB adducts became again evident in the long millisecond-to-second rise terms. For the PΦB-containing sample, the fit afforded three kinetic components of 350 ms, 2.5 s, and 11 s, and again, the latter was of only small amplitude.

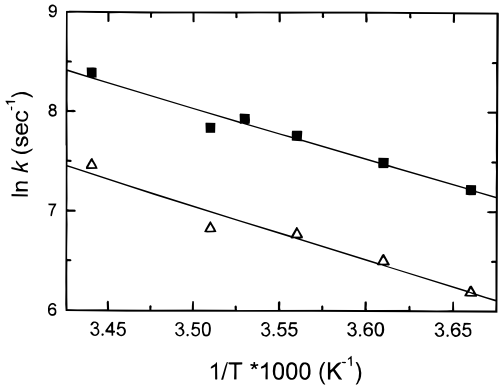

FIGURE 6: Arrhenius plot of the rise component (determined as 300 μs at 10 °C in H_2O/buffer) between 0 and 18 °C for *Synechocystis* phy-PCB in (■) H_2O/buffer and (△) D_2O/buffer.

DISCUSSION

An open reading frame from the cyanobacterium *Synechocystis*, exhibiting peptide motifs similar to phytochromes throughout its length and to the histidine kinase domain of prokaryotic two-component sensor systems, has been expressed in *E. coli*. The purified protein was autoassembled *in vitro* into a chromoprotein upon incubation with either of the open tetrapyrroles, PCB or PΦB. The chromoproteins exhibit a photochromicity similar to that known for phytochromes from higher plants, with a P_r-like form absorbing at around 654 (PCB) and 668 nm (PΦB), and a thermally stable photoproduct (reminiscent of the P_{fr} state) absorbing at around 706 and 717 nm, respectively. The general spectral shifts of 11−14 nm, depending on the chromophore used, are comparable to the absorption shifts encountered in recombinant phyA from oat (Cornejo et al., 1992; Schmidt et al., 1996). Compared to recombinant phytochromes from plants (e.g. phyA from oat), the absorption maximum of the P_r form is found at expected position for both chromophores whereas the P_{fr} maximum is clearly hypsochromic. No explanation for this result can be given. However, the overall difference of the protein sequence (despite the discussed islands of high similarity) readily can be suggested to lead to a change of the chromophore−protein interactions in the P_{fr} state. Despite this smaller red shift between P_r and P_{fr} states, the P_{fr} form was found to be thermally stable in the dark over several hours at ambient temperature (Lamparter et al., 1997).

The capability of *Synechocystis* apophytochrome to incorporate the same chromophores which bind to recombinant

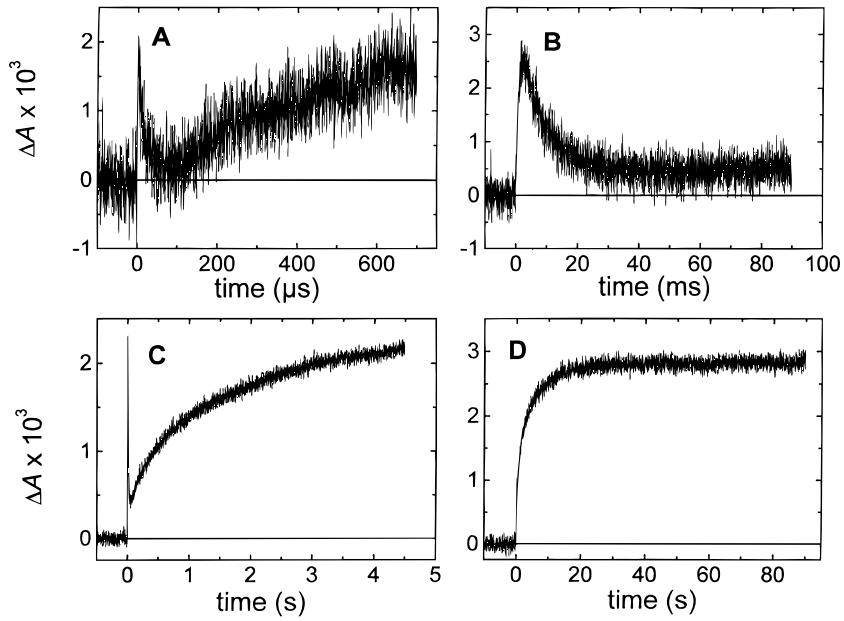

FIGURE 7: Kinetic behavior of *Synechocystis* phy-PΦB, $\lambda_{obs} = 700$ nm. (A) Absorption changes up to 700 μs; (B) as A, but up to 90 ms; (C, D) as A, but up to 4.5 and 90 s, respectively.

apophytochromes from higher plants, and the far-reaching similarities of phyA and phytochrome from *Synechocystis* with respect to spectra and $P_r \rightleftarrows P_{fr}$ photoreversibility make the protein from *Synechocystis* an excellent model for the study of photochromic, protein-bound tetrapyrroles.

The RR spectra of phyA from oat and both PCB and PΦB adducts of *Synechocystis* clearly suggest that the gross chromophore structures are similar, while the RR spectra of the two *Synechocystis* preparations differ in some aspects from those of phyA, irrespective of the chromophore incorporated. These differences which probably indicate slight structural differences of the chromophore-binding site of the protein are more pronounced for the P_r forms. Here, an altered band pattern in the HOOP region apparently reflects modified torsions around the methine bridges induced by changes in the chromophore−protein interactions. Also, the frequency shifts of H/D sensitive bands suggest differences between the *Synechocystis* phytochromes and oat phyA in hydrogen bonding. In the P_{fr} forms, such differences are not apparent, i.e., the P_{fr} states of *Synechocystis* and oat phytochrome (phyA) are more similar than the P_r states, except for a frequency shift of the prominent HOOP mode. In phyA this mode has been attributed to the isomerization site (Matysik et al., 1995; Fodor et al., 1990), implying that this part of the chromophore differs in conformational details in the *Synechocystis* phytochromes. The conclusion of apparently different conformations of the chromophore in the plant and the *Synechocystis* phytochromes is supported from data recently reported by Andel et al. (1996). In this work, strong conformational distortions of the chromophore in particular for the P_{fr} state have been identified from an analysis of the same low-frequency range of the Raman spectrum of native oat phyA which is used here to propose the different chromophore conformation of *Synechocystis* phytochrome with respect to that of native oat phyA. A more detailed analysis of the differences between plant and cyanobacterial phytochrome will add further information to the chromophore conformation and conformatioal changes (Kneip et al., 1997).

Flash photolysis of the phytochrome of *Synechocystis* revealed some similarities but also important differences from the photoconversion of phyA, illustrating that, as outlined recently (Braslavsky et al., 1997), a detailed description of the photochromic cycle of such chromoproteins requires a kinetic analysis of the light-induced conversions supplementary to the absorption spectra of the parent and intermediate states. As recently pointed out, and as shown here, the His tag does not interfere significantly with the kinetics, thus allowing the affinity-purified material to remain in use as an authentic sample. In *Synechocystis* phytochrome (PCB), the first intermediate that can be observed decays with 25 μs, whereas the corresponding intermediate of phyA, I_{700}, decays with a lifetime of 90 μs (Figure 4A). The rapid decay in *Synechocystis* phytochrome is followed by an intermediate with rise kinetics of 300 (PCB) and even 750 μs (PΦB) and an 8-ms decay time. This second intermediate has no equivalent to any other known intermediate of phyA. Whereas the putative I_{700} of *Synechocystis* phytochrome decays faster than the possibly related I_{700} of phyA, the formation of the P_{fr} form is slower.

Despite the faster rates, the course of the early decay kinetics of both PCB and PΦB adducts are somewhat similar to recombinant oat phyA (Schmidt et al., 1996) which decays with unambiguously biphasic kinetics of 8 and 80 μs (phyA−PΦB), whereas the phyA−PCB sample decays monoexponentially with 90 μs. In *Synechocystis* phytochrome (PCB), a putative additional shorter component than the observed 25 μs kinetics would have to be considerably faster or of much lower intensity in order to have escaped detection.

The second intermediate has no equivalent to any intermediate in the phyA's. It is all the more intriguing through its isotope effect (k_H/k_D) of 2.7 (found for its rise and decay), a far higher value than any previously reported for the processes in the $P_r \rightarrow P_{fr}$ conversion. A value of this size results from a direct proton transfer step as the rate-limiting process in this conversion (Northrop, 1997). The subsequent processes of P_{fr} formation are subject to moderate isotope effects only, with a ratio of *ca*. 1.2 similar to that reported

for oat phyA (Aramendía et al., 1987; Westphal, 1994; Lindemann et al., 1993). In contrast to a direct proton transfer process, such a change in the kinetics by ca. 20% could well reflect an overall change of the interactions between the protein and solvent.

For an inspection of the shape of the LADS, one has to keep in mind that the LADS are constructed from apparent kinetic constants without the application of any modeling procedure and thereby simply represent difference spectra between the preceding and the following intermediate. Changes in shape and maximum for rise and decay LADS (e.g. Figure 5A, spectra of 320-μs and 6-ms components) are thus to be expected.

Interestingly, the deuterium exchange in *Synechocystis* phytochrome only perturbs the value of the activation energy, leaving the pre-exponential factor unchanged. This is in contrast to data determined for oat phytochrome kinetics after experimental variation, such as addition of cations, changes in viscosity, or antibody binding. In all these cases, the preexponential factors and the activation energies changed in the same direction, thus leading to a compensation of the disturbances (Aramendía et al., 1987; Westphal, 1994).

In summary, recombinant *Synechocystis* phytochrome resembles plant phytochrome with regard to Raman and visible spectral properties of the P_r and P_{fr} states. However, although the phytochrome of *Synechocystis* undergoes a photoreversible conversion reminiscent of the $P_r \rightleftarrows P_{fr}$ process, time-resolved analysis of this conversion reveals red-shifted intermediates (compared to the absorption of the parent state) with kinetics different from those determined for the phytochrome intermediates. In contrast to all other phytochromes, in a novel intermediate of *Synechocystis* phytochrome a strong deuterium effect of 2.7 has been determined for the rise (occurring within ca. 300 μs) *and* decay (within 6−8 ms) processes.

ACKNOWLEDGMENT

The assistance of S. Russell in the flash photolytic measurements and of G. Koc and T. Huestege in the chromophore preparation and purification is gratefully acknowledged.

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BI971563Z