Heteronuclear Solution-State NMR Studies of the Chromophore in Cyanobacterial Phytochrome Cph1[†]

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ABSTRACT: Precise structural information regarding the chromophore binding pocket is essential for an understanding of photochromicity and photoconversion in phytochrome photoreceptors. To this end, we are studying the 59 kDa N-terminal module of the cyanobacterial phytochrome Cph1 from *Synechocystis* sp. PCC 6803 in both thermally stable forms (Pr and Pfr) using solution-state NMR spectroscopy. The protein is deuterated, while the chromophore, phycocyanobilin (PCB), is isotopically labeled with ^{15}N or ^{13}C and ^{15}N. We have established a simple approach for preparing labeled PCB based on BG11 medium supplemented with an appropriate buffer and $NaH^{13}CO_3$ and $Na^{15}NO_3$ as sole carbon and nitrogen sources, respectively. We show that structural details of the chromophore binding pocket in both Pr and Pfr forms can be obtained using multidimensional heteronuclear solution-state NMR spectroscopy. Using one-dimensional ^{15}N NMR spectra, we show unequivocally that the chromophore is protonated in both Pr and Pfr states.

Phytochrome photoreceptors control numerous developmental processes in all plants. Light is absorbed by a covalently bound tetrapyrrole chromophore, phytochromobilin (PΦB), that is incorporated autocatalytically into a chromophore binding domain in the N-terminal sensory module of the protein. The physiological function of phytochrome is associated with the photochemical production of the Pfr[1] signaling state following light absorption by the Pr ground state ($\lambda_{max} \sim 660$ nm). The Pfr form itself ($\lambda_{max} \sim 730$ nm) is transformed back to the Pr form following appropriate photon absorption. In daylight, this photocycle establishes a dynamic equilibrium between the two forms, their relative abundances containing information on light quality. The difference between Pr and Pfr is attributed to a *Z*−*E* isomerization of a double bond within the chromophore, as suggested from NMR data of isolated chromopeptides (*1*).

With the discovery of Cph1 in the cyanobacterium *Synechocystis* sp. PCC 6803 (*2*−*4*), it was realized that phytochromes are also present in prokaryotes. Subsequently, phytochromes have also been detected in other cyanobacteria and even nonphotosynthetic bacteria (*5*). The architecture of Cph1 is similar to that of plant phytochromes except that a module bearing two PAS domains is missing. In both plant and prokaryotic phytochromes, kinase activity is attributed to the C-terminal portion of the protein. It has been demonstrated that the C-terminal module of prokaryotic phytochromes acts as a histidine autokinase/phosphotransferase in a canonical two-component light sensory system (*3*, *6*). On the other hand, the biological function of phytochrome-mediated phosphorylation in plants remains unclear, especially as it seems that the N-terminal module signals autonomously (*7*). Heterologous expression of Cph1 in *Escherichia coli* yields an apoprotein that is capable of self-assembling with a suitable bilin chromophore to yield a red/far-red photochromic holoprotein. The native chromophore of Cph1 in *Synechocystis* is phycocyanobilin (PCB, Figure 1) (*8*).

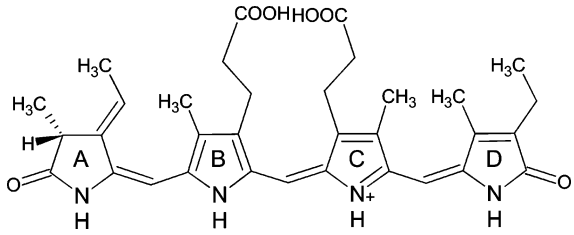

FIGURE 1: Structure of phycocyanobilin (PCB). The incorporation of PCB into phytochromes is accomplished by the addition of a cystein side chain to the exocyclic double bond at ring A. All carbons and nitrogens depicted were labeled with ^{13}C and ^{15}N using the optimized protocol presented here.

A deeper understanding of the initial photochemistry and subsequent molecular rearrangements within the phytochrome molecule requires detailed knowledge of the structure of the chromophore in its binding pocket. While Rüdiger and co-workers (*1*) were able to describe the configuration of the chromophore attached to proteolytic fragments, only ambiguous information regarding the conformation in situ

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[1] Abbreviations: CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; Cph1, cyanobacterial phytochrome 1; Cph1Δ2, N-terminal (residues 1−514) sensory module of Cph1; HMQC, heteronuclear multiple-quantum correlation; NMWCO, nominal molecular weight cutoff; PBS, phycobilisome; PCB, phycocyanobilin; Pfr and Pf, far-red-absorbing and red-absorbing forms of phytochrome, respectively; TROSY, transverse relaxation optimized spectroscopy.

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is available to date: contradicting structural models for the chromophore have been proposed on the basis of resonance Raman experiments (*9*−*11*). It has been shown recently that solution-state NMR is capable of elucidating the structure of the chromophore binding pocket in chromoproteins (*12*): we are employing a similar strategy for Cph1 using the bilin chromophore uniformly labeled with ^{15}N and/or ^{13}C. The 59 kDa N-terminal module (amino acids 1−514, Cph1Δ2) autoassembles to form a holophytochrome photochemically identical to full-length Cph1 (*3*). Highly soluble Cph1Δ2 apoprotein can readily be produced in *E. coli*, while PCB can be extracted easily from cyanobacterial phycobilisomes and presented to apoCph1Δ2 to form holophytochrome. As the apoprotein and chromophore can thus be produced separately and then combined to yield the functionally photochromic holoprotein, differential labeling of the two components is possible. In principle, structural data for the chromophore in situ can be obtained using completely deuterated protein and normal PCB bearing hydrogen atoms. However, as the apoprotein carries ∼100-fold more protons than the chromophore while commercially available D_2O contains at least 2% H_2O, unambiguous chromophore assignment via this procedure is impossible. Labeling of the chromophore with ^{15}N and/or ^{13}C will therefore be essential for a successful structure determination of the chromophore binding pocket.

We have developed a protocol for preparing uniformly labeled ^{13}C and/or ^{15}N PCB from *Synechocystis* sp. PCC 6803 and show for the first time that multidimensional heteronuclear spectra of the chromophore in its native phytochrome environment can be obtained. We extracted chemical shifts from one-dimensional ^{15}N NMR spectra of Cph1Δ2 in both the Pr and Pfr forms, showing unambiguously that the chromophore is protonated in both cases, verifying earlier interpretations of resonance Raman data (*9*), a conclusion with major implications for models of the phytochrome photocycle.

MATERIALS AND METHODS

Preparation of Unlabeled and ^{2}H-Labeled Cph1Δ2 Apoprotein. Preparation of apo- and holoprotein was essentially performed as described previously (*13*). Briefly, overnight cultures of BL21-DE3 (Novagen) and XL1-blue cells (Stratagene) harboring the p929.5 expression plasmid were grown in RB medium to the early exponential phase (OD_{600} ∼ 0.4) at 30 °C and cooled on ice. BL21-DE3 cells additionally contained plasmid pSE111 encoding the *laqI*^{q} and *ArgU* genes. Expression was induced with 20 μM IPTG at 18 °C for 18 h, and then the cells were washed and concentrated in cold TES [50 mM Tris (pH 7.8), 5 mM EDTA, 300 mM NaCl] with 1 mM DTT added before lysis by two passages through a French press at 120 MPa. Before the first passage, phycocyanobilin (PCB) of the desired labeling pattern was added to the cell suspension. The crude extract was clarified at 25000*g*, precipitated with ammonium sulfate, resuspended in 50 mM Tris (pH 7.8) and 300 mM NaCl, and subjected to Ni^{2+}-affinity chromatography. Eluted holo-Cph1Δ2 was further purified by preparative SEC in a Sephacryl S300 (Pharmacia/GE) column in TES.

For the preparation of ^{2}H-labeled apoprotein, the following modifications were made. Overnight cultures of XL1-blue cells were grown in 50 mL of unlabeled RB medium to the late exponential phase (OD_{600} ∼ 0.8) at 30 °C, harvested by centrifugation, and resuspended in 60 mL of ^{2}H OD2 medium (Silantes, Munich, Germany). The suspension was then transferred to 2 L of ^{2}H OD2 medium, and growth continued at 30 °C until the cells reached an early exponential phase (OD_{600} ∼ 0.2), after which expression was induced as described above and allowed to continue for 48 h.

Table 1: Composition of Media Used for the Preparation of Labeled PCB[a]

	unlabeled	^{15}N	^{13}C and ^{15}N
$NaNO_3$	17.65	17.65[c] ($Na^{15}NO_3$)	4.41 ($Na^{15}NO_3$)
K_2HPO_4	0.227	0.227	0.227
$MgSO_4$	0.304	0.304	0.304
$CaCl_2$	0.245	0.245	0.245
citric acid	0.031	0.031	0.031
ferric ammonium citrate[b]	6 mg	6 mg	6 mg
Na_2-EDTA	0.0027	0.0027	0.0027
$NaHCO_3$	−	−	47.6 ($NaH^{13}CO_3$)
CHES	−	−	60
trace elements solution	1 mL	1 mL	1 mL
trace elements solution (stock)			
H_3BO_3	46.26	46.26	46.26
$MnCl_2$	9.15	9.15	9.15
$ZnSO_4$	0.772	0.772	0.772
Na_2MoO_4	1.78	1.78	1.78
$CuSO_4$	0.316	0.316	0.316
$Co(NO_3)_2$	0.17	0.17	0.17

[a] Numbers are in units of millimolar, if not stated otherwise. [b] Undefined stoichiometry. [c] For initial experiments, $Na^{15}NO_3$ was exchanged for $K^{15}NO_3$, for no other reason than the availability of this substance in our lab. To partially compensate for the loss of Na^+, K_2HPO_4 was exchanged for Na_2HPO_4.

Protein concentrations were determined by absorption spectroscopy using an ϵ_{280} of 59 mM^{-1} cm^{-1} . All manipulations of the holoprotein were carried out under green safelight [light-emitting diodes (LEDs), λ_{max} = 520 nm].

Preparation of Labeled PCB. Axenic cultures of *Synechocystis* sp. PCC 6803 cells were cultivated at room temperature (24 ± 2 °C) in a modified BG11 medium (see below and Table 1) (*14*) in standard 2 L laboratory bottles under constant illumination with continuous white light (Sylvania Grolux, Osram, Munich, Germany). Cells were constantly bubbled with air (6 L/min) provided by a standard laboratory pump fitted with a sterile filter (0.2 μm Acro 50, PALL, East Hills, NY) and harvested when they reached stationary phase after ∼3−4 weeks (OD_{750} ∼ 2.5−3). All further manipulations of the cells used for the preparation of PCB were carried out at 18 °C to avoid disintegration of the phycobilisomes (PBS) (*15*). Cells were harvested by centrifugation at 5000*g* for 10 min, resuspended in 30 mL of 0.75 M K_nPO_4 buffer (pH 7), and broken with a French press (two passages, 120 MPa), and cellular debris was pelleted at 25000*g* for 30 min. One milliliter of Triton X-100 was added to the supernatant and shaken for 10 min; 5−6 mL of this solution was applied to a discontinuous sucrose density gradient [7 mL of 0.5 M, 10 mL of 0.75 M, 7 mL of 1 M, and 7 mL of 1.5 M sucrose in 0.75 M K_nPO_4 buffer (pH 7)] in 6 mm × 25 mm × 89 mm centrifuge tubes and spun in the SW32 swing-out rotor for 18 h at 28 000 rpm in an LE 80K preparative ultracentrifuge (BeckmannCoulter, Palo Alto, CA). PBS (A_{622}/A_{280} > 3) accumulated in the 1

M sucrose layer. PBS were washed salt-free with distilled water in an Amicon pressure cell (NMWCO of 10 kDa; Millipore, Bedford, MA), lyophilized, and subjected to methanolysis in a Soxhlett extractor for 24 h. This and subsequent steps were carried out in darkness or subdued light. PCB was concentrated to 2−10 mM with a rotary evaporator and stored in methanol at −80 °C for no longer than 2 months before being used. The concentration of PCB was measured by absorption spectroscopy in a 5/95 (v/v) HCl/methanol mixture, using an ϵ_{690} of 37.9 mM^{-1} cm^{-1} (*16*).

The preparation of ^{15}N-labeled samples used BG11 medium as described in Table 1. The medium was autoclaved directly in the bottles to be used for culturing. For the preparation of ^{13}C-labeled samples, concentrated BG11 medium (Sigma) was diluted with water, 60 mM/L of CHES (Sigma) added, and the pH adjusted to 8.0 with NaOH (the pH of an aqueous solution of CHES is ca. 4). Then, 47.6 mM $NaH^{13}CO_3$ (CIL, Andover, MA) was dissolved, slightly increasing the pH to ∼8.1, and the resulting solution was sterile filtered (Steritop filter unit, Millipore) directly into a sterilized bottle to be used for culturing. CHES was chosen because of its pK_a of 9.41 (*17*) and because it proved to be nontoxic for PCC 6803. The pH of a saturated culture under these conditions ranged from 9.5 to 10. In case of an unbuffered, $NaHCO_3$-containing medium, the cells became chlorotic and subsequently died as the pH of the solution increased beyond 11. For the preparation of ^{13}C- and ^{15}N-labeled samples, BG11 medium was prepared as detailed in Table 1. Subsequent steps were as for the ^{13}C-labeled samples. ^{13}C and $^{13}C/^{15}N$ cultures were agitated with a magnetic stirrer. Saturation was reached at an OD_{750} of ∼1.5−2; 5−10% (v/v) of a saturated culture was used to inoculate the fresh medium solutions, giving an OD_{750} of ∼0.2.

Correct label incorporation and the chemical identity of the isolated product were controlled with electrospray ionization mass spectroscopy (ESI-MS) and analytical HPLC using published procedures (*18*). Both methods revealed a main fraction of correctly labeled PCB and negligible amounts of impurities. Furthermore, PCB prepared using this protocol yields the Cph1Δ2 holoprotein with spectral parameters in agreement with previously published data (*3*).

Sample Preparation for NMR Spectroscopy. Samples for NMR were exchanged into 50 mM Na_nPO_4 (pH 7.8) and 1 mM EDTA using PD10 columns (Amersham Pharmacia/GE) and concentrated using Vivaspin concentrators (NMWCO of 30 kDa; Millipore). Following photoconversion, samples were kept in darkness. For Pr measurements, samples were irradiated directly in the NMR tube with far-red LEDs (λ_{max} = 730 nm). Since our aim was to study the chromophore in both Pr and Pfr states, the sample had to be photoconverted effectively using an appropriate light source. At concentrations suitable for NMR spectroscopy, conversion of Pr to Pfr by simple irradiation of the NMR tube with red light is inadequate since both forms are present and absorb very strongly, preventing the light from fully penetrating into the sample tube. To generate a Pfr-saturated solution despite the absorbance of the solution in the red region of >30, samples were drawn into a 1 m long, 1 mm inner diameter glass capillary and irradiated over the whole solution column with red LEDs (λ_{max} = 660 nm) until photoequilibrium (ca. 70% Pfr) was reached. The glass capillary was constructed like a pipet with a gastight Hamilton syringe connected to the end opposite the pipet tip. Thus, the sample could be drawn out of the NMR tube for irradiation and transferred back into the NMR tube afterward. This procedure resulted in a Pfr/Pr sample containing 70% Pfr and 30% Pr (*19*). The photoequilibrium was checked by taking a small amount from the sample, diluting it ∼100-fold, measuring the 500−800 nm spectrum, and then irradiating with red light for 60 s prior to recording a second spectrum as before. Incomplete photoconversion of the concentrated solution was apparent from the spectral difference signal at 655 and 708 nm. Conversion of Pfr to Pr with far-red irradiation on the other hand is straightforward: under far-red irradiation, photoequilibrium is at almost 100% Pr, and Pr absorption beyond 710 nm is negligible.

NMR Spectroscopy. All spectra were recorded on spectrometers from Bruker (Karlsruhe, Germany). For all two-dimensional spectra, 5 mm sample tubes were used with holophytochrome at ca. 300 μM. The one-dimensional ^{15}N spectra were recorded at 600 MHz (proton frequency) on a DRX600 NMR spectrometer using a 10 mm sample tube in a 10 mm BBO probe, 65 536 complex points, and 200 000 scans, resulting in a measurement time of 15 days each. ^{15}N HMQC (*20*) spectra were recorded on a DMX750 instrument using a cryogenic 5 mm TCI probe equipped with one-axis self-shielded gradients. To achieve water suppression in the case of the ^{15}N HMQC, an 11-echo sequence was implemented into the HMQC (*21*). Both spectra (for Pr and the Pfr/Pr mixture) were recorded with 5120 scans and 512 × 60 complex points, resulting in a measurement time of 128 h each. ^{13}C HMQC spectra were recorded on a DRX600 instrument using a 5 mm TXI probe equipped with three-axis self-shielded gradients. The spectra were recorded in D_2O, and low-power presaturation was used to remove residual signals from HDO. Both spectra (for Pr and the Pfr/Pr mixture) were recorded with 32 scans and 512 × 64 complex points, resulting in a measurement time of 45 min each.

RESULTS AND DISCUSSION

The goal of this investigation is to obtain structural information about the chromophore within the holoprotein using solution-state NMR in conjunction with isotope labeling. Since the chromophore and the apoprotein can be produced separately in the case of Cph1 from *Synechocystis*, a variety of labeling procedures and patterns can be employed. The simplest pattern consists of the deuteration of the protein while the chromophore is left in the protonated form. However, the Cph1Δ2 apoprotein carries ∼4100 protons in relation to 38 in the PCB chromophore; thus, given the limited degree of enrichment of commercially available D_2O, unambiguous identification of chromophore protons is impossible. Consequently, labeling of the chromophore with ^{13}C and ^{15}N is essential in obtaining assignments and structural information.

Production of the Labeled Chromophore. While the production of proteins in the isotopically labeled form is a well-established procedure and while the same is also possible with DNA and RNA, the production of other types of molecules enriched with ^{13}C and/or ^{15}N is usually

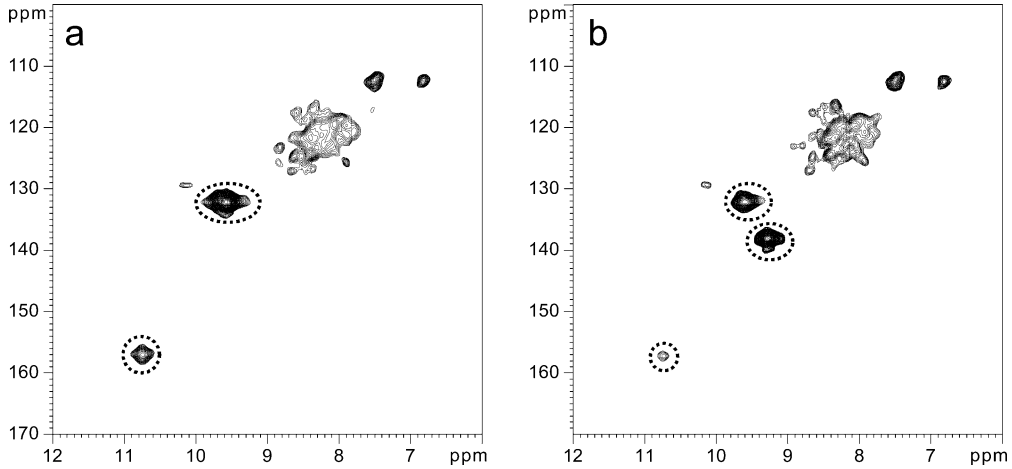

FIGURE 2: ^{15}N HMQC spectra of Cph1Δ2 after the incorporation of ^{15}N-labeled PCB. Both spectra were recorded on a 750 MHz spectrometer equipped with a cryogenic probe at 300 K using an HMQC sequence with 11-echo water suppression; 5120 scans and 60 FIDs were recorded, and signals from the chromophore are circled. The remaining signals result from the natural abundance background of the protein: (a) ^{15}N HMQC spectrum of the protein in the Pr form and (b) ^{15}N HMQC spectrum of the protein in the Pfr/Pr form.

prohibitively expensive. In case of PCB, the chromophore necessary for our investigation, the problem is ameliorated by the fact that PCB is the chromophore of the light-harvesting antenna complex in cyanobacteria. Here, PCB is bound covalently to phycocyanin which comprises up to 50% of the total soluble protein and 95% of the antenna complex (the phycobilisome), which, moreover, can easily be separated from other cell components. We decided therefore to produce isotopically labeled PCB using *Synechocystis* itself. This organism was chosen because the only chromophore in its phycobilisomes is PCB (*22*), so removal of other, potentially interfering chromophore species should not be necessary. Procedures for cyanobacterial growth and chromophore extraction from the phycobilisomes are well-established and could be used with minor modifications. For the production of [^{15}N]PCB, the standard BG11 medium was used, except $^{14}NO_3^-$ was replaced with $^{15}NO_3^-$. For the production of [^{13}C]PCB, the setup was changed. The usual source of carbon for the cyanbacteria is CO_2. Supplementing the medium with labeled CO_2 is experimentally difficult since full control over a closed gas supply to the culture has to be maintained, losses of CO_2 being costly. We therefore grew the cyanobacteria in a closed system in which carbon was supplied as a bicarbonate ($NaH^{13}CO_3$). In principle, this is no problem as PCC 6803 grows best at pH 8−10, a point at which HCO_3^- is the dominant species in solution. However, the carbon form actually taken up by the cells is CO_2 (*23*). Therefore, for each mole of C that is taken up, one mole of OH^- is produced. To compensate for this, the pH of the medium was held in a useful range by including a nontoxic buffer with an appropriate pK_a. As the entire cyanobacterial cell contents are labeled, this protocol should also be useful for investigations of other components.

Two-Dimensional NMR Spectra Recorded Using [^{15}N]PCB. The first spectra were recorded using a sample containing the ^{15}N-labeled chromophore in an otherwise deuterated protein. It should be noted, however, that the purification of the protein and the incorporation of the chromophore were performed in normal H_2O. Most of the exchangeable hydrogens of the protein as well as all of those of the chromophore are therefore protons, not deuterons. The spectra were also recorded in H_2O. The deuteration of the protein will therefore not have any influence on the protonation of exchangeable hydrogens. With a fully labeled sample at 300 μmol, it should be possible to record a ^{1}H−^{15}N correlation within minutes on a 750 MHz spectrometer equipped with a cryogenic probe. Using a sample of ^{15}N-labeled protein, this was indeed the case (data not shown), the spectrum showing a line width typical for a protein of this size. Surprisingly, however, no signal was detectable even after several hours with a sample in which the chromophore was labeled with ^{15}N. Only after an extremely long experiment time could signals of the chromophore be detected (together with signals from the protein in natural abundance) using an HMQC sequence with an 11-echo water suppression (*21*). Signals from the chromophore were, moreover, significantly broader than peaks from the protein. The spectra are shown in Figure 2. The spectrum of the Pr sample shows one strong (132 ppm) and one very weak (158 ppm) chromophore signal; the spectrum of the Pfr/Pr mixture exhibits the same peaks (from the 30% Pr in the sample) and one additional strong signal. To enhance the signal, other types of ^{1}H−^{15}N correlation [TROSY (*24*) and CRINEPT-HMQC-TROSY (*25*)] were tested, albeit without success. The prolonged measurement time necessary to obtain signals from the chromophore results from the fast relaxation of the chromophore which is evident from the large line width. While signals are still present in one-dimensional NMR spectra or NOESY spectra (not shown), the relatively long delays in the pulse sequences of the heteronuclear experiments lead to a severe loss in intensity, in particular with broad lines.

One other reason for the absence of a peak in a ^{1}H−^{15}N correlation could, of course, be that the nitrogen is not protonated. This would, however, not explain the width of the lines, nor would it be likely for more than one nitrogen. As we show below, this is indeed not the reason, since all nitrogens are protonated in both forms of the chromophore.

The increased line width points toward an exchange process that could be either conformational exchange or exchange of the nitrogen-bound protons with water. Since exchange phenomena depend on the temperature as well as the resonance frequency, both were varied to possibly obtain sharper lines for the signals of the chromophore. However,

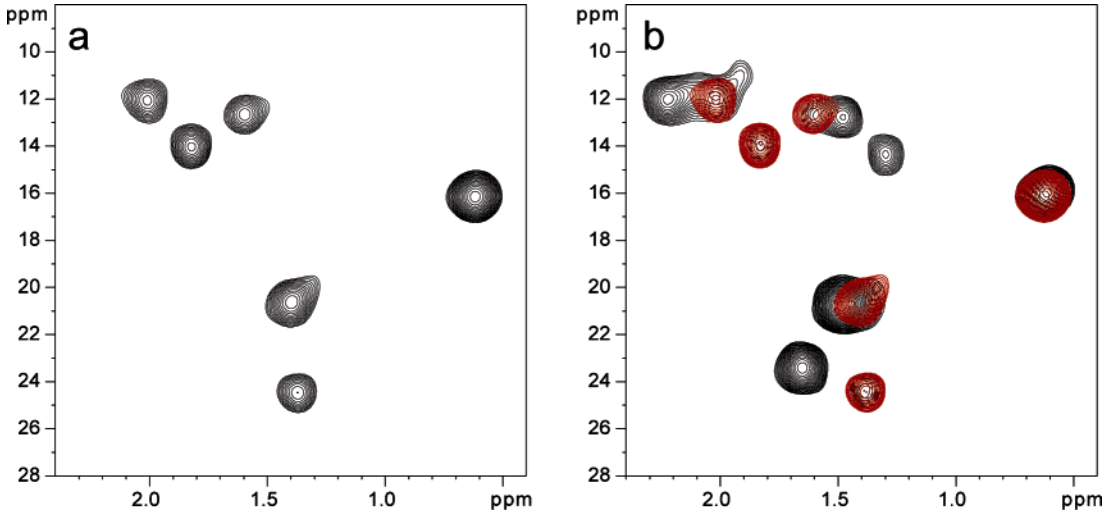

FIGURE 3: ^{13}C HMQC spectra of Cph1Δ2 after the incorporation of ^{13}C- and ^{15}N-labeled PCB. Both spectra were recorded on a 600 MHz spectrometer at 300 K using an HMQC sequence with low-power presaturation to remove residual HDO. Thirty-two scans and 128 FIDs were recorded. Only the region of the methyl groups is shown; no other signals were present. The number of resonances doubles when the sample is converted from the Pr form to the Pfr/Pr form: (a) ^{13}C HMQC spectrum of the protein in the Pr form and (b) ^{13}C HMQC spectrum of the protein in the Pfr/Pr form, where the signals of the Pr form (spectrum in panel a) are colored red.

the spectra did not change significantly at a frequency of 600 or 900 MHz and in a temperature range from 5 to 35 °C (data not shown).

Two-Dimensional NMR Spectra Recorded Using [^{13}C]PCB. To clarify the situation regarding the exchange process, a ^{1}H−^{13}C HMQC spectrum was recorded on a sample containing ^{15}N- and ^{13}C-labeled chromophore. Since an exchange with water is not possible in the case of carbon-bound protons, broadened lines would indicate that a conformational exchange is in fact taking place. Again, a spectrum with sufficient signal to noise would be expected after a measuring time of 1 h using a conventional probe: only methyl groups are present, however, no other signals being detectable. The regions of the methyl groups are shown in Figure 3. Since an HMQC sequence was used, the appearance of methyl resonance is likely since it has been shown that the HMQC experiment is inherently relaxation-optimized for those resonances (*26*, *27*). Increasing the measurement time to 70 h on a spectrometer equipped with a cryogenic probe yields more correlations, again with significantly broadened lines (data not shown). Signals from the natural abundance background of the protein are not likely to appear here, since the protein is deuterated. This confirms that the chromophore indeed shows a mobility different from that of the rest of the protein. Since the line widths of the nitrogen resonances (see below) are all similar and those from the nitrogen-bound protons differ dramatically, hydrogen exchange between water and the nitrogen-bound protons of the chromophore, however, also contributes to the line width of those protons. The fast exchange of the nitrogen-bound protons with water permits tentative conclusions regarding the strength of hydrogen bonds between those protons and partners within the protein. Usually elements of secondary structure can be identified by their slow exchange with the solvent which results from the tight hydrogen bonds present in either helices or sheets. Because of the exchange processes found for the chromophore in Cph1Δ2, strong hydrogen bonds as in regular secondary structure elements are obviously not present. A quantification of dynamics as well as exchange will unfortunately not be possible with NMR experiments commonly employed in this situation because of the low sensitivity that is available.

Six methyl groups are present in a ^{1}H−^{13}C HMQC spectrum recorded with a short measurement time, the signals being clearly separated. The different line width visible for the methyl groups again points to a nonuniform motional behavior. Conversion of the sample from Pr to the Pfr/Pr photoequilibrium mixture doubles the number of signals as shown in Figure 3b. This already indicates that the detection of NOEs from methyl groups will be possible in both forms of the protein and that overlap should not cause difficulties if three-dimensional techniques are employed. In addition, the measurement of residual dipolar couplings should be possible but will require careful optimization of the solution conditions.

One-Dimensional NMR Spectra Using [^{15}N]PCB. To obtain more information using the sample with the ^{15}N-labeled chromophore, one-dimensional ^{15}N spectra were recorded. Because of the low sensitivity inherent in such measurements, the experimental setup was modified: using a broadband probe optimized for detection of heteronuclei, a sample tube diameter of 10 mm, and 80 mg of nondeuterated holoprotein containing ^{15}N-labeled chromophore in 3.5 mL of solution, each spectrum was recorded with a measuring time of >2 weeks on a 600 MHz spectrometer. Spectra of the Pr and Pfr/Pr samples were recorded and are shown in Figure 4. The spectrum of Pr (Figure 4a) exhibits signals from the protein in natural abundance around 115 ppm and four signals from the chromophore at 132.1, 146.2, 157.4, and 158.1 ppm. The spectrum of the Pfr/Pr mixture (Figure 4b) also exhibits signals from the protein in natural abundance around 115 ppm but, in addition, eight further peaks from the chromophore. The same signals as in the spectrum of Pr can be identified, since this represents 30% of the mixture. In addition, other signals with roughly twice the intensity are visible. To separate the signals and obtain a "pure" Pfr spectrum, the spectrum of the Pr form was scaled appropriately and subtracted from that of the mixture. The result is shown in Figure 4c, indeed showing only four chromophore peaks at 137.9, 142.7, 156.1, and 157.9 ppm.

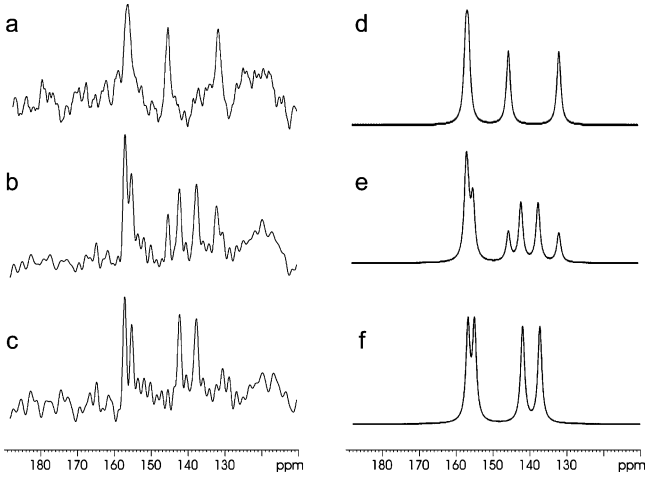

FIGURE 4: One-dimensional ^{15}N spectra of Cph1Δ2 after the incorporation of ^{15}N-labeled PCB. Both spectra were recorded on a 600 MHz spectrometer at 300 K using a 10 mm direct detection probe and were run for more than 2 weeks each. (a) ^{15}N spectrum of the protein in the Pr form. Besides the natural abundance background from the protein around 115 ppm, four signals are present at 132.1, 146.2, 157.4, and 158.1 ppm; two of them nearly overlap at 158 ppm and can be distinguished using only resolution enhancement. (b) ^{15}N spectrum of the protein in the Pfr/Pr form. Besides the natural abundance background from the protein around 115 ppm, signals from the Pr form (see panel a) are present as well as additional signals from the Pfr form. (c) Difference between the spectra from the Pfr/Pr form and the Pr form. After appropriate scaling of the spectrum of the Pr form before subtraction, this results in a spectrum that contains signals from only the Pfr form at 137.9, 142.7, 156.1, and 157.9 ppm. (d−f) Simulated spectra corresponding to the spectra in panels a−c to clarify the peak positions of the chromophore peaks and the extraction of chemical shifts. Simulations were carried out assuming an 80 Hz line width.

Comparison of the one- and two-dimensional spectra can identify those signals with the slowest exchange. In Pr, the signal at 132 ppm shows the strongest correlation in the HMQC, the signals at 158 ppm show a weak correlation, while the peak at 146 ppm is absent. In the Pfr form, the signal at 138 ppm shows the strongest correlation in the HMQC. Whether the signal at 158 ppm only results from the 30% Pr present or also contains Pfr-related signals cannot be decided because of the low resolution inherent in two-dimensional spectra.

The question of whether all nitrogens of the chromophore are protonated throughout the photocycle has been a matter of debate for a long time, although evidence that the chromophore is protonated in both Pr and Pfr forms has accumulated (*9*, *28*, *29*). The chemical shifts measured here can answer this question unequivocally since chemical shifts of nitrogens in deprotonated tetrapyrrole systems exhibit chemical shifts above 220 ppm while those in the protonated state are at least 80 ppm upfield (*30*−*32*). As can be seen in Figure 4, both Pr and Pfr forms show chemical shifts below 160 ppm, indicating complete protonation. This finding has important implications for models of the phytochrome photocycle. First, all models proposing a deprotonated tetrapyrrole in the Pfr state can be ruled out (*33*). Second, the formation of a lactim anion (N═C−O$^-$) on the A-ring of the tetrapyrrole chromophore to explain the red shift of the Pfr form (*34*, *35*) can be excluded, as this would result in a marked shift of one peak in the one-dimensional ^{15}N spectrum.

CONCLUSION

In conclusion, we have presented an efficient method for producing ^{13}C- and ^{15}N-labeled bilins from cyanobacteria and using them to obtain heteronuclear NMR data for Cph1Δ2 phytochrome. One-dimensional ^{15}N spectra of phytochrome with the ^{15}N-labeled chromophore clearly show that the chromophore is protonated in both Pr and Pfr forms, information important in evaluating models for the phytochrome photocycle. The $^{1}H-^{15}N$ HMQC spectrum exhibits significant line broadening due to exchange processes. It shows, however, that hydrogen bonds comparable to those present in elements of regular secondary structure in proteins are not present. The $^{1}H-^{13}C$ HMQC spectra did also show quite broad lines for all nuclei except those from methyl groups. Indeed, it would seem that the chromophore is rather mobile within its binding pocket. Even though this will make the extraction of structurally relevant information more difficult, this will be ameliorated in the case of samples containing carbon-labeled chromophore that allow the acquisition of heteronuclear edited NOESY spectra. Furthermore, the ^{15}N chemical shifts can be used to evaluate structural models via quantum chemical calculations. Work along these lines is in progress and will be presented elsewhere.

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