Structure and Interactions of Phycocyanobilin Chromophores in Phycocyanin and Allophycocyanin from an Analysis of Their Resonance Raman Spectra†

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ABSTRACT: Raman spectra of phycocyanobilin, phycocyanin, and allophycocyanin were obtained at resonance with their visible and near-UV transitions. These spectra were empirically assigned with the help of ^{14}N- and ^{15}N-isotopic substitutions and comparisons with resonance Raman spectra of phycoerythrin. These results confirm the previously suggested assignment of a conformation-sensitive band around 1239–1246 cm^{-1} to a mode involving νC_mH and νCN coordinates. Computer-assisted decomposition of the complex, conformation-sensitive 1580–1670-cm^{-1} region yielded five components that we labeled I–V. The previously described spectral changes observed upon monomerization and denaturation in resonance Raman spectra of phycocyanin and allophycocyanin essentially arise from changes in the relative intensities of these components. Component I (around 1649–1651 cm^{-1}) and component III (1621–1624 cm^{-1}) originate predominantly from $\nu C{=}C$ at C_{15} of the chromophore. Their relative intensity ratio reflects the relative amounts of C_{15}-*Z-anti* and C_{15}-*Z-syn* methine bridge conformations, respectively. Component II (1633–1638 cm^{-1}) is ascribed to a $\nu C{=}C$ mode of pyrrole rings; it is not sensitive to the chromophore conformation. Component IV is also conformation-insensitive and originates from $\nu C{=}N$ and $\nu C{=}C$ coordinates, most likely from ring C. Component V (1591–1594 cm^{-1}) involves a $\nu C{=}N$ coordinate in ring D, coupled to a $\nu C{=}C$ coordinate of the C_{15} methine bridge. The implications of the present assignments on those of resonance Raman active modes of phytochrome are discussed. A consistent set of correlations between chromophore conformations and resonance Raman data is obtained for both phycobiliproteins and phytochrome.

In cyanobacteria and red algae a major part of the light-harvesting function is performed by biliproteins which are organized into macromolecular complexes named phycobilisomes (Gantt, 1981). Phycobilisomes contain three major types of biliproteins: phycoerythrin, phycocyanin, and allophycocyanin. All of them have covalently bound open-chain tetrapyrroles as chromophores (Glazer, 1984).

Although phycocyanin (CPC)[1] and allophycocyanin (APC) contain the same chromophore, phycocyanobilin (PCB) (Figure 1), and involve largely homologous protein sequences (Scheer, 1981), they have remarkably different absorption spectra in their native states. This difference consists of a strong absorption band at 652 nm, which is observed for trimeric APC (MacColl et al., 1980) only and was attributed to excitonic interactions between two of the six chromophores of the APC trimer, on the basis of analysis of its absorption and CD spectra (Csatorday et al., 1984).

Using resonance Raman (RR) spectroscopy, we recently obtained detailed information about the conformations of the chromophores of CPC and APC by analyzing changes induced

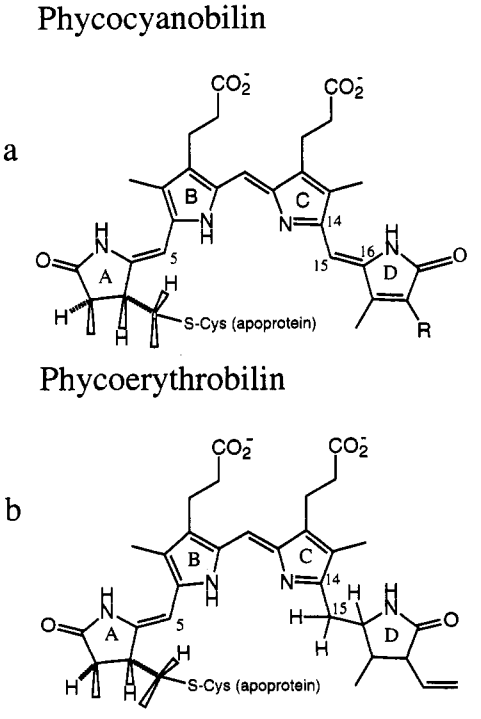

FIGURE 1: (a) Chemical structure of phycocyanobilin, the chromophore of *C*-phycocyanin and allophycocyanin. R = C_2H_5 in phycocyanobilin, and R = C_2H_3 in phytochrome. (b) Phycoerythrobilin, the chromophore of phycoerythrin.

in the RR spectra either by modifying the aggregation states of the proteins or by denaturing them. In agreement with

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[1] Abbreviations: APC, allophycocyanin; BVE, biliverdin dimethyl ester; CARS, coherent anti-Stokes Raman scattering; CPC, phycocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEB, phycoerythrobilin; P_{fr}, far-red-absorbing form of phytochrome; P_r, red-absorbing form of phytochrome; RR, resonance Raman.

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X-ray studies on CPC (Schirmer et al., 1985, 1986, 1987; Duerring et al., 1991) no major heterogeneity was found in the chromophore conformations, in either trimeric CPC or APC (Szalontai et al., 1987, 1989). Further work indicated that chromophore conformations are essentially the same in both trimeric and monomeric APC (Szalontai et al., 1989). In these studies, the frequency of a 1642-cm^{-1} band was shown to constitute a marker of the extended or folded conformations of the chromophores (Szalontai et al., 1987, 1989).

However, further interpretation of these RR data in structural terms necessitates a more precise and more extensive set of assignments than currently available. Indeed, only a few assignments have been proposed so far for open-chain tetrapyrroles. They concerned biliverdin dimethyl ester (BVE) (Margulies & Stockburger, 1979; Margulies & Toporowicz, 1984; Smit et al., 1993), bilirubin (Margulies & Toporowicz, 1988a), phycocyanobilin (PCB) (Margulies & Toporowicz, 1988b; Prenzel, 1993), and phytochrome (Tukotomi et al., 1990; Fodor et al., 1990). Because of the limited amount of experimental data, these assignments generally were based on quantum mechanical, semiempirical calculations.

Phycocyanobilin is almost identical to the chromophore of phytochrome (Scheer, 1982), and in certain conditions (Schmidt et al., 1988) CPC can also show reversible absorption changes. Structural and functional information on phycocyanobilin and phycobiliproteins should be relevant to phytochrome as well. Raman data on phytochrome published so far include a resonance CARS (Hermann et al., 1990) study on the P_r form, as well as UV- and blue-excited RR (Tokutomi et al., 1990; Mizutani et al., 1991), far-red-excited RR (Fodor et al., 1988, 1990), and surface-enhanced RR (Rospendowski et al., 1989; Holt et al., 1989; Farrens et al., 1989) studies of both the P_r and P_{fr} forms. Recently the first Fourier transform resonance Raman (FT-RR) study of both forms of phytochrome has been reported (Hildebrandt et al., 1992).

In the present study, Raman spectra of phycocyanin and allophycocyanin were obtained at resonance with their visible and near-UV transitions. These spectra were empirically assigned with the help of ^{14}N- and ^{15}N-isotopic substitutions and comparisons with phycoerythrin resonance Raman spectra. With the help of computer-assisted decomposition of the conformation-sensitive region of the spectra, an explanation was found for the spectral changes induced by denaturation of the proteins.

EXPERIMENTAL PROCEDURES

Nitrogen isotopic substitution experiments were carried out biosynthetically on the cyanobacterium *Synechococcus* 6301 as follows: Two identical culture media were prepared as in Szalontai et al. (1985). To one of them was added $Na^{15}NO_3$ (97% ^{15}N content; CEA-ORIS, Gif-sur-Yvette, France) as the only nitrogen source; the other one was provided with $Na^{14}NO_3$. The two cultures were inoculated, grown, and harvested in exactly the same way. Phycocyanin, allophycocyanin, and phycocyanobilin were isolated as described earlier (Gombos et al., 1984; Szalontai et al., 1985, 1987).

Denaturation of CPC was achieved by decreasing the pH of the protein solution. Experimental details were described earlier (Szalontai et al., 1989).

The UV-resonant Raman experiments were carried out on a Jobin-Yvon Ramanor HG2S spectrometer using the 363.8-nm line of an argon ion laser (Spectra Physics 171, Coherent Radiation Innova 100). Experiments with visible excitation were performed on a Coderg PHO spectrometer, using the 488-nm line of the same laser. Spectral resolution at 1000 cm^{-1} was 8 cm^{-1} in both experiments. Raman spectra were recorded at low temperature (20–40 K) using a flow of cold helium circulated around the sample in a cryostat (SMC, France).

For isotopic experiments the ^{14}N- and ^{15}N-containing samples were cast on the same sample plate, which could be vertically displaced in the cryostat. Thus a single vertical movement permitted either sample to be probed without changing the setting of the monochromator. This procedure ensured the reliability of the observed isotopic shifts. For the same reason, short (300 cm^{-1}) spectral ranges were recorded from one sample and then from the other, before proceeding to another spectral region (Figures 2–5).

Samples of the free chromophore were stored under an argon atmosphere in sealed ampules and used immediately after opening. The samples were cast under a nitrogen atmosphere onto precooled glass plates. These sample holders were transferred in liquid nitrogen to the spectrometer and were introduced in the cold cryostat.

The resonance Raman spectra presented here are each the result of the summation of 3–8 individually recorded spectra. Spectra were stored in a multichannel analyzer (Tracor Northern 1710) with a resolution of 0.8 cm^{-1} per data point. All spectra were checked individually before summation, and we did not find any sign of photodegradation as a consequence of laser irradiation at these low temperatures.

Lorentzian curve decomposition has been performed on an IBM XT-compatible computer (Amstrad 1512) by a program developed at Szeged. The program fitted Lorentzian curves to the experimental spectra. The number of Lorentzians as well as the initial values of the independent parameters (position, bandwidth, intensity, and background parameters) had to be determined by the operator. It was possible to fix the value of any parameter. The program optimized the fit by changing the free parameters only. The algorithm of the program was a modified least squares method. The fit became unstable if more Lorentzians than minimally necessary were assumed by the operator.

The broad fluorescence backgrounds underlying the RR spectra were first subtracted using a second-order baseline that was fitted over a large part of the spectrum involving Raman band-free regions as well. The Raman bands were fitted with Lorentzian curves after subtracting the above obtained baseline from the spectrum. To find the best fit, the number of Lorentzians to be considered was gradually increased and the last stable fit was accepted.

For the curve fitting shown in Figure 6, the 1500–1750-cm^{-1} spectral region was separately recorded with a higher number of scans (16) and with higher data storage density (0.2 cm^{-1} per data point).

RESULTS AND DISCUSSION

Table 1 presents the frequencies, relative intensities, and isotopic shifts measured from the RR spectra of phycocyanobilin, allophycocyanin, and *C*-phycocyanin; it summarizes the assignments that we propose on the basis of these data and computer analysis. The 488-nm-excited spectra of free phycocyanobilin were not of sufficient quality to support detailed analysis and are not reported here.

Nitrogen-Sensitive Vibrations. Table 1 shows that, in all spectra, RR modes active in the 1070–1200-cm^{-1} region undergo significant shifts upon ^{15}N substitution. They hence involve in part (1073, 1110 cm^{-1}) or predominantly (1090, 1170 cm^{-1}) the νCN and/or δCN coordinates. This is

Table 1: Assignments of Resonance Raman Bands of Bilin Chromophores *in Vitro* and in Phycocyanin and Allophycocyanin[a]

phycocyanobilin	phycocyanin		allophycocyanin		
UV	UV	vis	UV	vis	assignments
		954(0)m/vw		952(1)w/w	
972(2)m/w	976(3)s/s	973(0)m/w	973(3)sh/sh	976(7)m/m	
994(1)m/w	992(1)s/s	993(0)m/w	992(4)s/s	992(7)w/w	νCC +...
		1055(0)m/w		1056(3)m/m	
	1076(3)sh/sh		1073(~3)sh/sh		νCN +...
	1094(6)s/s		1088(~10)m/m		νCN
	1110(4)s/s	1113(2)s/w	1106(1)m/m	1113(7)s/m	νCN +...
1124(4)s/s	1124(5)m/m		1125(5)m/m		νCN +...
		1131(0)s/s		1135(–)s/s	
1165(5)s/s		1169(3)s/s	1158(7)s/s		νCN + νCC
	1176(12)m/s			1170(10)s/vs	νCN
1229(–)–/m					
	1239(2)s/s	1240(5)m/m	1245(?)s/s	1246(6)s/s	$\delta C_mH/\nu$CN +...Δ
1276(2)s/s	1260(1)s/s	1271(?)s/s	1269(~0)m/w		νCC +...
		1281(3)s/s		1284(7?)s/s	
		1298(4)sh/sh			
	1315(0)m/m	1320(0)m/m	1313(~0)m/s	1317(1)m/m	δC_mH
	1343(4)m/m	1345(0)s/s	1339(?)w/–	1344(1)vs/s	
1358(0)m/m	1357(0)s/s		1358(0)m/s		νCC
1367(0)m/m		1370(3)m/m		1370(5)m/m	
1399(0)m/m	1398(0)s/s		1399(0)m/s		νCC
	1422(–)w/–	1420(–)m/–		1427(~7)m/m	
		1434(8)m/m		1437(7)m/m	νCN +...
1445(3)w/w		1456(8)m/m			
		1472(0)m/m	1477(0)w/m	1475(1)s/s	νCC_m
1544(?)w/w	1542(2)m/m	1541(0)m/m			νCC
			1544(5)w/m	1545(8)m/m	νCN + νCC + δNH?
		1591(8)m/m		1594(8)m/m	νCN(ring D) + νCC_{15}[b]
1624(1)s/s					νCC_m
	1642(5)vs/vs	1644(3)vs/vs	1643(7)vs/vs	1643(2)vs/vs	νCC_m, νCC, νCN[c]

[a] Wavenumbers are in cm^{-1}. Numbers in parentheses indicate downshifts (cm^{-1}) of bands upon $^{14}N \rightarrow {}^{15}N$ substitution. Letters after the numbers indicate relative intensities of bands in ^{14}N- and ^{15}N-containing samples, respectively: m, medium; s, strong; sh, shoulder; v, very; w, weak. UV, ultraviolet excited, and VIS, visible excited resonance Raman spectra. Symbols for internal coordinate vibrations: ν, stretching; δ, deformation; Δ, accidentally degenerate band (see text) ~, approximate shift values; C_m, unspecified methine carbon. [b] This band was considered in the decomposition of the 1590–1650-cm^{-1} region and was tagged component V. See Table 2. [c] This band was computer analyzed; the calculated components are tagged I–IV. See Table 2 and text.

consistent with previous isotopic data obtained on chlorophylls (Lutz & Robert, 1988) and on porphyrins (Kitagawa & Ozaki, 1987).

The $^{14}N \rightarrow {}^{15}N$ substitution in bilin chromophores results in significantly different shifts for bands which previously appeared homologous in visible- and UV-excited spectra. In particular, two distinct modes are likely to occur around 1170 cm^{-1}. A lower frequency mode (1158–1169 cm^{-1}) shows moderate (3–7 cm^{-1}) sensitivity to ^{15}N substitution, while a slightly higher frequency mode at 1170–1176 cm^{-1} shows high sensitivity (10–12 cm^{-1}) and hence should arise from nearly pure CN stretching.

The relative intensities of the bands in the 1070–1200-cm^{-1} νCN stretching region change upon denaturation [Figure 5 in Szalontai et al. (1987)]. A complex four-band structure characteristic of the UV-excited RR spectra of both native CPC and APC evolves into a three-band structure upon denaturation. One of the three latter bands (around 1120 cm^{-1}) has about twice the intensity of the other two. Its frequency is close to that of the only major band (1124 cm^{-1}) of the same region in the UV-resonant spectrum of free PCB (Figure 4). This band, which involves the νCN coordinate, hence should constitute a marker of folded chromophore conformations in UV-excited spectra. Conversely, the 1110-cm^{-1} band of the same cluster, which is absent from RR spectra of the denatured proteins and of PCB, may constitute a marker of extended conformations.

The Conformation-Sensitive 1230–1300-cm^{-1} Region. Both native CPC and APC yield a RR band around 1239–1246 cm^{-1} under both UV and visible excitation (Szalontai et al., 1987, 1989). This band is very sensitive to environmental and conformational changes. Its disappearance constitutes the first indication of structural alterations caused by monomerization of these proteins (Szalontai et al., 1987, 1989). Therefore, its correct assignment is of interest.

In UV-excited RR spectra of APC, this band splits upon ^{15}N substitution (Figure 2). One component downshifts from 1245 to 1240 cm^{-1}, while the other part retains its original frequency. The 1239–1246-cm^{-1} band is hence accidentally degenerate, at least for UV-resonant APC. In UV-excited RR spectra of CPC the 1239-cm^{-1} band showed an unambiguous isotopic downshift of 2 cm^{-1}. In visible-excited spectra of APC and CPC, the *ca.* 1240-cm^{-1} band undergoes 6- and 5-cm^{-1} downshifts (Figures 3 and 5, respectively) with no signs of splitting. One of the 1239–1246-cm^{-1} modes involves the νCN coordinate. The other one, which has weaker activity in visible-excited spectra and is more active at UV resonance, is insensitive to ^{15}N and may involve the δC_mH coordinate.

Soret-resonant bands of chlorophyll (Chl) *a* at 1230 cm^{-1} (Lutz, 1984) and of Chl *b* at 1234 cm^{-1} (Lutz & Robert, 1988) were assigned to νCN vibrations on the basis of large downshifts induced by $^{14}N \rightarrow {}^{15}N$ substitutions. On the other hand, for biliverdin dimethyl ester, Margulies and Toporowicz (1984) calculated an in-plane bending mode of methine bridge hydrogens (δC_mH) at 1237 cm^{-1}. The latter assignment agrees with that of the ν^{13} mode of nickel octaethylporphyrin measured and calculated by Kitagawa and Ozaki (1987) (1220 cm^{-1} obsd, 1262 cm^{-1} calcd) and for the less symmetric Nickel protoporphyrin IX (1232 cm^{-1} obsd).

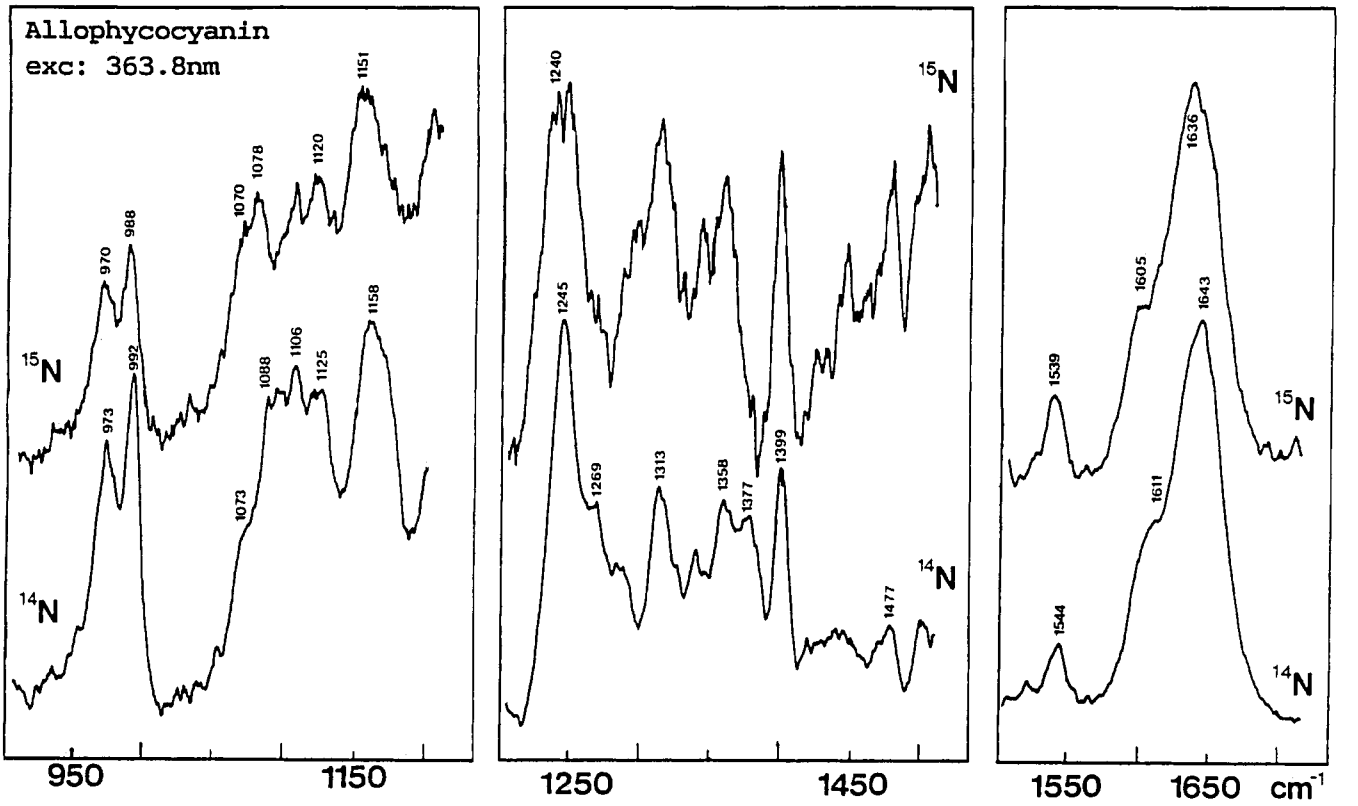

FIGURE 2: UV-excited (363.8 nm) resonance Raman spectra of [^{14}N]- and [^{15}N]allophycocyanin at 30 K. On the ^{15}N-isotopic spectrum only those band wavenumbers that changed upon ^{15}N substitution are indicated.

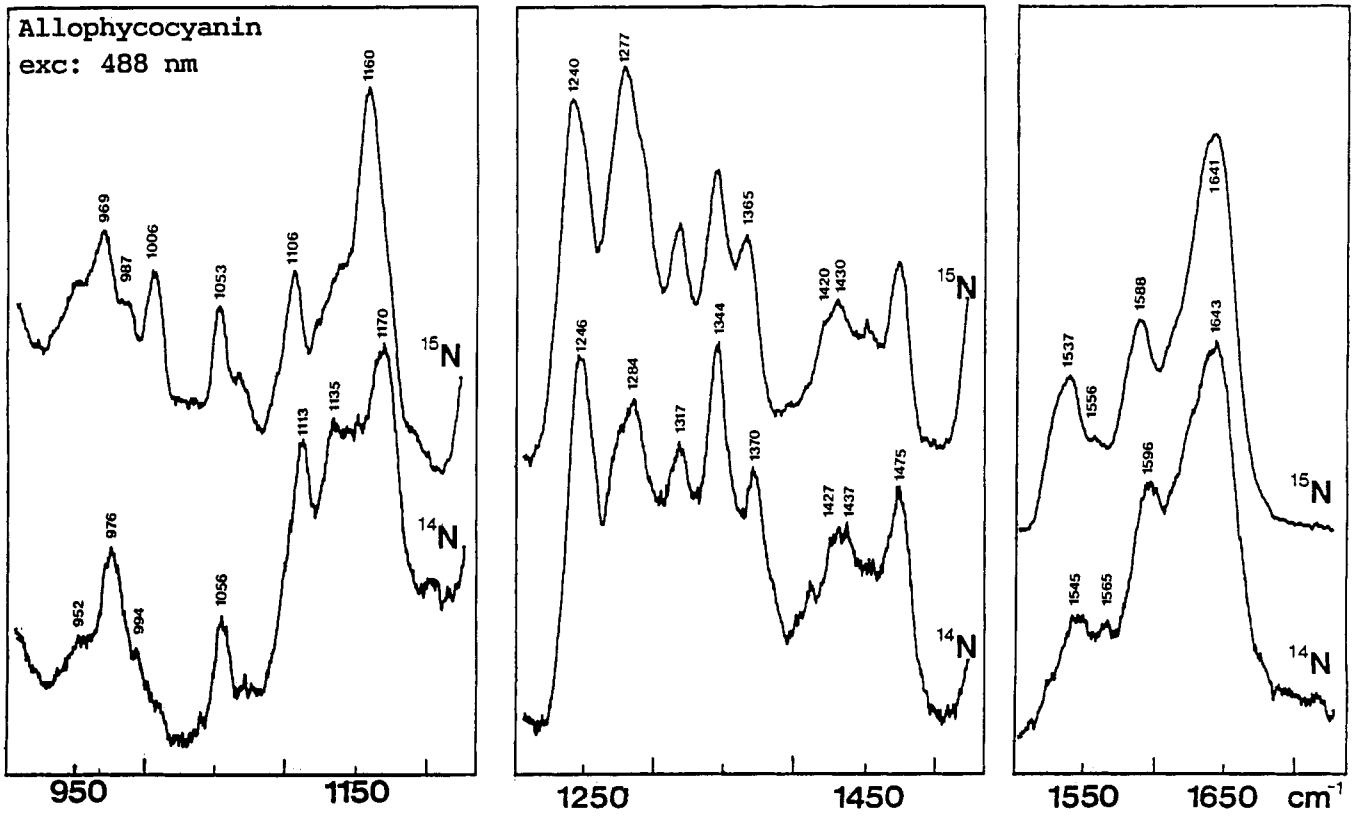

FIGURE 3: Visible-excited (488 nm) resonance Raman spectra of [^{14}N]- and [^{15}N]allophycocyanin at 30 K.

We hence assign the degenerate 1239–1245-cm^{-1} bands of CPC and APC to νCN and δC_mH.

In APC spectra the 1239–1246-cm^{-1} band is considerably stronger than in those of CPC, and its frequency is 5–6 cm^{-1} higher. This and the observed ^{15}N-induced split suggest that, compared to those of CPC, APC spectra involve a larger contribution of the δC_mH, ^{15}N-insensitive component, which should be located around 1245 cm^{-1}. This fact points to differences between chromophore conformations in native CPC and APC.

Upon monomerization and denaturation of both CPC and APC, the 1239–1246-cm^{-1} band disappears and another band

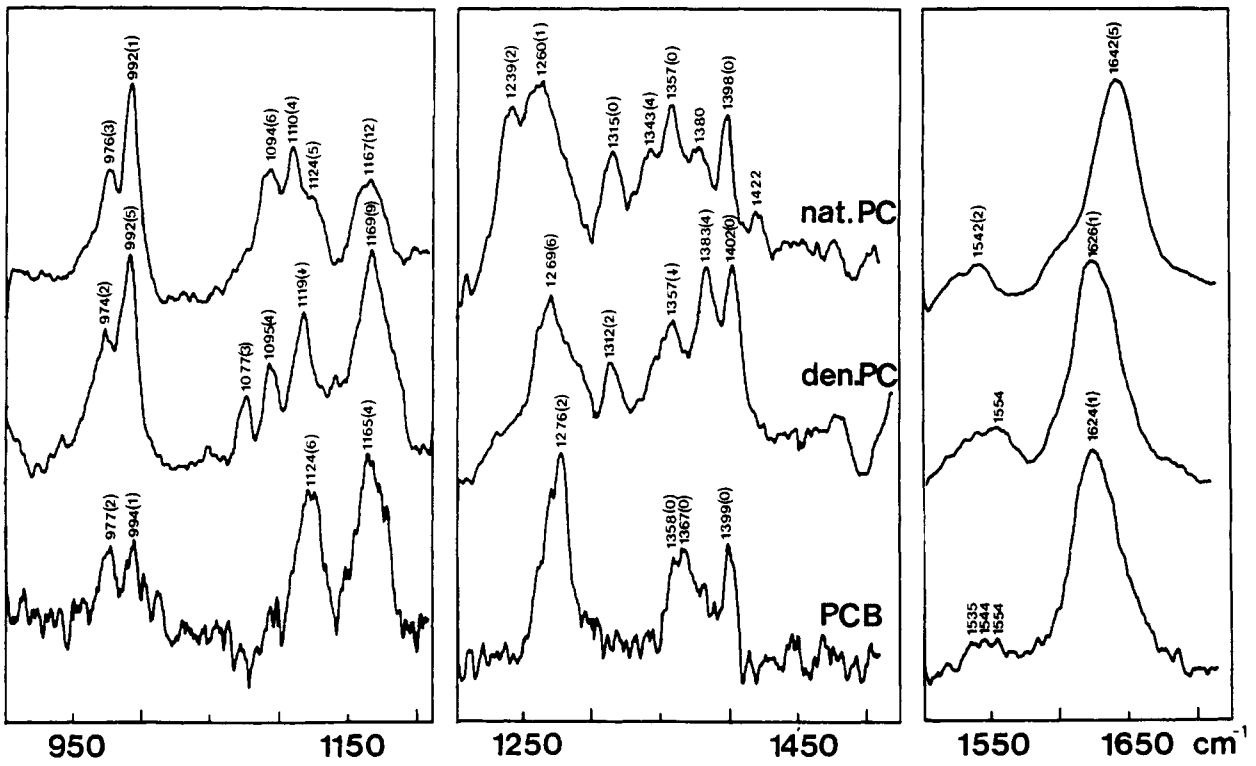

FIGURE 4: UV-excited (363.8nm) resonance Raman spectra of native (nat.PC) and denatured phycocyanin (den.PC) in 20 mM phosphate buffer and of free phycocyanobilin (PCB) in chloroform solution at 20 K. Numbers in parentheses indicate the observed isotopic downshifts. The curves presented here are the ^{14}N-derivative spectra.

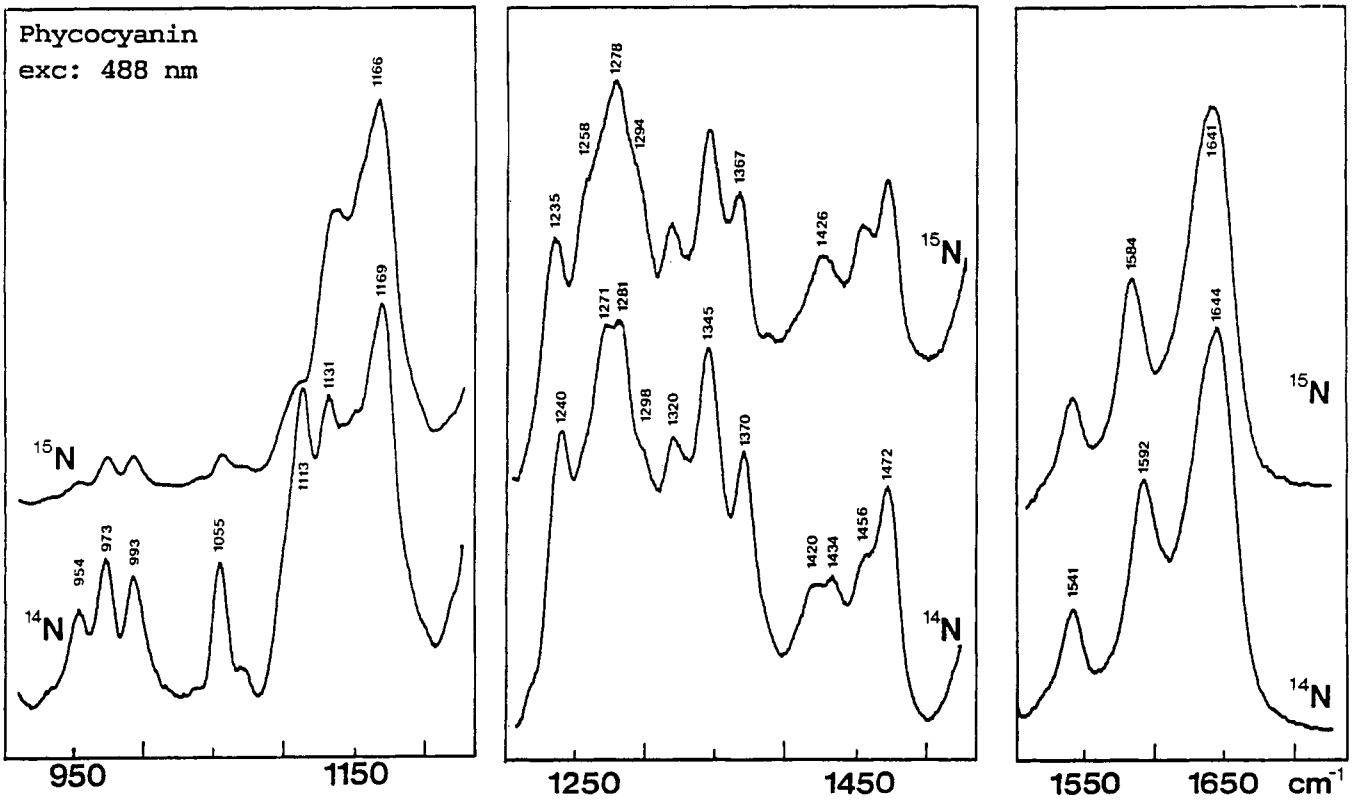

FIGURE 5: Visible-excited (488 nm) resonance Raman spectra of [^{14}N]- and [^{15}N]phycocyanin at 30 K.

gains intensity around 1260–1270 cm^{-1} (Szalontai et al., 1987, 1989). The frequency of this latter band is the same as that of the only band observed in this region for PCB (1276 cm^{-1}) and for BVE (1268 cm^{-1}) (Szalontai et al., 1987). This band showed little nitrogen isotope sensitivity in native proteins and in PCB. Low isotopic sensitivities were also observed for bands of Chl *a* and Chl *b* at 1270 and 1273 cm^{-1} which were assigned to νCC vibrations, respectively (Lutz & Robert, 1988). The vibrational analysis of BVE by Smit et al. (1993) predicted RR-active, side-group modes in this frequency range which, such as ν_{96}, predominantly involve nitrogen-insensitive coordinates.

The 1300–1550-cm^{-1} Region. No bands of the 1300–1550-cm^{-1} region have been used so far in conformational studies

Table 2: Calculated Components of the 1590–1650-cm^{-1} Conformation-Sensitive Region of Native Phycocyanin and Allophycocyanin[a]

phycocyanin		allophycocyanin		
UV	vis	UV	vis	assignments
1651(2) 26%	1649(1) 29%	1647(0) 49%	1649(2) 34%	I: νCC_{15}, *Z-anti*
1638(3) 66%	1636(1) 28%	1633(3) 34%	1636(3) 15%	II: $\nu CC + \nu CN$
	1621(4) 7%		1622(7) 19%	III: νCC_{15}, *Z-syn*
1599(+2) 8%		1604(6) 17%		IV: $\nu CC + \nu CN$ (ring C)
	1591(8) 36%		1594(8) 32%	V: νCN (ring D), νCC_{15}, *Z-anti*

[a] Wavenumbers in cm^{-1}. Numbers in parentheses: $^{14}N \rightarrow {}^{15}N$ isotopic shifts (cm^{-1}). Percentages indicate the relative weights of integrated intensities of the components.

of the CPC and APC chromophores. Possible assignments for this region are given in Table 1. They were based on the present isotopic shifts and on similarities with RR spectra of related compounds.

A band around 1545 cm^{-1} in both the visible- and UV-excited RR spectra of APC showed a 5–8-cm^{-1} downshift upon ^{15}N-isotopic substitution. In contrast, a 1541–1542-cm^{-1} band in the RR spectra of CPC probably was ^{15}N-insensitive. This difference may result from the multiplicity of modes in this range and again points to differences between chromophore conformations in CPC and APC. It is worth noting that a 1552-cm^{-1} band observed in red- and near-IR-excited RR spectra of the P_{fr} form of phytochrome has been proposed to involve νCN and δNH coordinates, on the basis of its sensitivity to deuteration of the chromophore (Fodor et al., 1990; Hildebrandt et al., 1992).

The Marker Band Region (1590–1650 cm^{-1}). A band around 1591–1594 cm^{-1} which is only observed in the visible-excited spectra of APC and CPC (Figures 3 and 5) shows an 8-cm^{-1} downshift upon $^{14}N \rightarrow {}^{15}N$ substitution.

In the UV-excited RR spectra of APC and CPC (Figures 2 and 4) a band around 1642 cm^{-1} significantly shifts (7–5 cm^{-1}) upon ^{15}N substitution. The frequency of this band was shown to constitute a marker of the chromophore conformation (Szalontai et al., 1987, 1989). During denaturation it shifts down to 1626 cm^{-1} and becomes practically insensitive to $^{14}N \rightarrow {}^{15}N$ isotope exchange (Figure 4). Therefore, the denaturation-induced downshift most likely results from relative intensity changes of nearly degenerate components under the contour of the marker band.

The 1624-cm^{-1} marker band of free PCB (Figure 4) also appears nearly insensitive to isotopic substitution. In the visible-excited spectra of APC and CPC (Figures 3 and 5), the 1642-cm^{-1} marker band is clearly less sensitive to isotopic substitution (2–3-cm^{-1} shifts) than it is in the UV-excited ones (5–7-cm^{-1} shifts).

Decomposition of RR Spectra in the 1500–1700-cm^{-1} Region. In order to understand the downshift of the 1642-cm^{-1} marker band upon denaturation of the protein and its apparently variable sensitivity to $^{14}N \rightarrow {}^{15}N$ substitution, a decomposition of this complex part of the RR spectrum was performed.

Computer-assisted decomposition of the RR spectra yielded five components which are noted as I, II, III, IV, and V in the following. Their actual frequencies, bandwidths, and relative weights are listed in the captions of Figures 6–8 and in Table 2.

Downshift of the 1642-cm^{-1} Band upon Denaturation. In order to analyze the denaturation-induced downshift of the 1642-cm^{-1} band, the 1500–1700-cm^{-1} region of the UV-excited RR spectrum of CPC was recorded in the pH 3.0–2.6 range (Szalontai et al., 1989).

The curve-fitting strategy was the following: We started fitting the spectrum obtained at pH 3.0 (i.e., for monomer phycocyanin) with an increasing number of components until

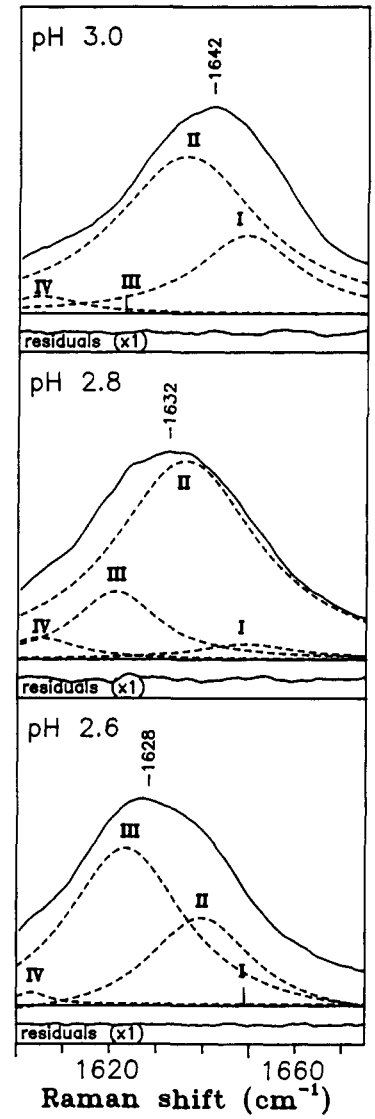

FIGURE 6: Component analysis of the marker band region of UV-excited resonance Raman spectra of phycocyanin. Solid lines indicate measured spectra; dashed lines, the calculated components. pH 3.0, monomer phycocyanin; pH 2.8, denaturation of phycocyanin begins; pH 2.6, denatured phycocyanin. Actual frequencies/widths of the component bands: I = 1649/27 cm^{-1}; II = 1636/38 cm^{-1}; III = 1621/22 cm^{-1}; IV = 1605/19 cm^{-1}.

no further improvements could be obtained. (The criteria for optimal fit are given in Experimental Procedures; for quality of the fits, see also the residuals in Figure 6.) For CPC at pH 3.0 an optimal fit was found with three components, tagged I, II, and IV. An additional component (III) was required for the optimal fit at pH 2.8. This component was stronger at pH 2.6, i.e., for fully denatured phycocyanin. All of these fits were obtained with free parameters; i.e., band positions, widths, and intensities were optimized by the program.

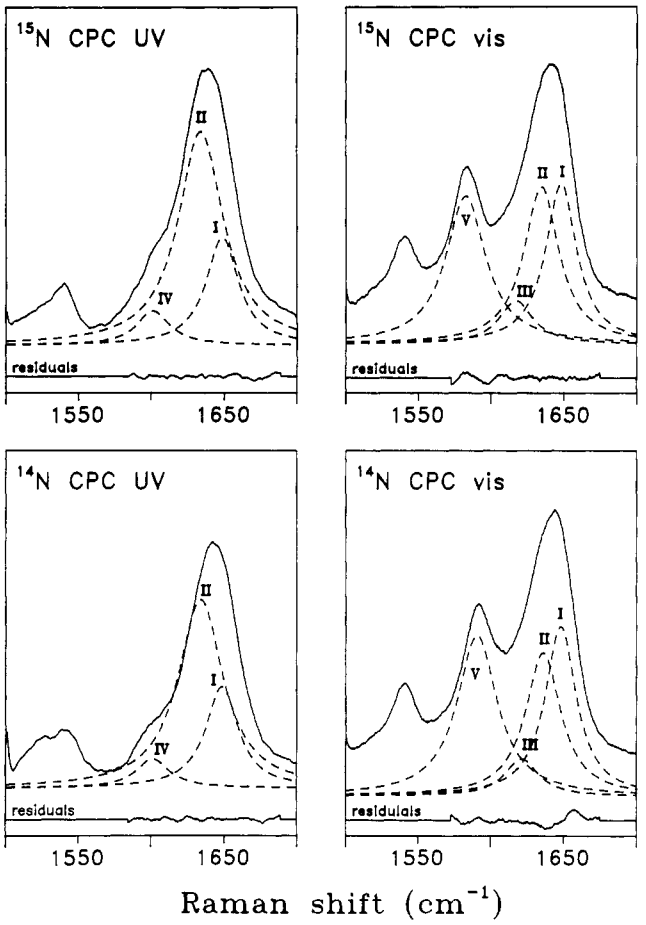

FIGURE 7: Decomposition of the marker band region of phycocyanin upon $^{14}N \rightarrow {}^{15}N$ substitution with both visible and UV excitation. Component band frequencies/widths (downshifts upon ^{15}N substitution) for UV excitation: I = 1651/27 (2) cm^{-1}; II = 1638/37 (3) cm^{-1}; IV = 1599/28 (+2) cm^{-1}. For visible excitation: I = 1649/26 (1) cm^{-1}; II = 1636/29 (1) cm^{-1}; III = 1621/28 (4) cm^{-1}; V = 1591/33 (8) cm^{-1}.

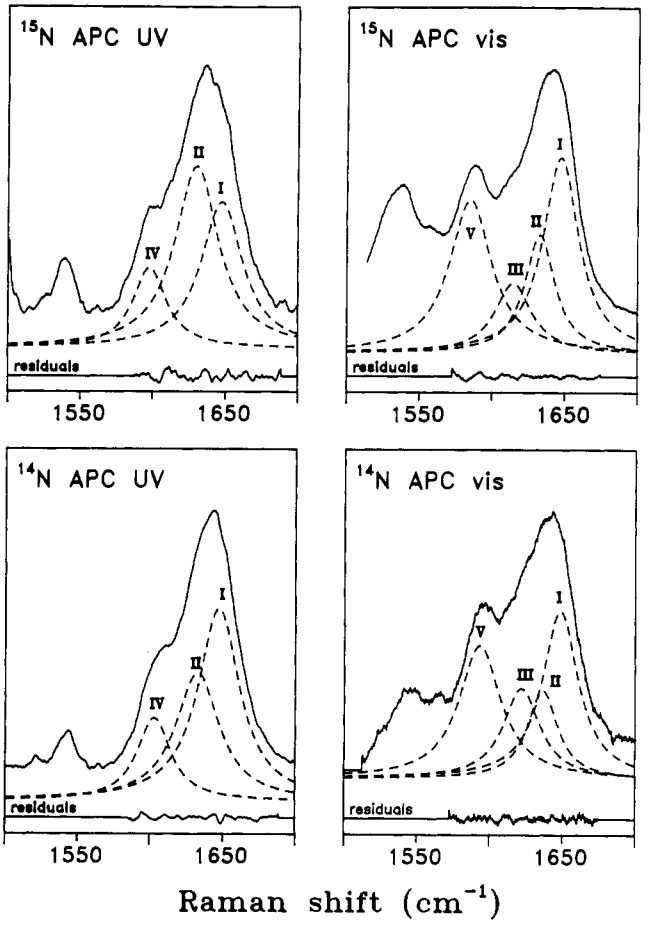

FIGURE 8: Decomposition of the marker band region of allophycocyanin upon $^{14}N \rightarrow {}^{15}N$ substitution with both visible and UV excitation. Component band frequencies/widths (isotopic downshifts) for UV excitation: I = 1647/35 (0) cm^{-1}; II = 1636/29 (1) cm^{-1}; IV = 1604/28 (6) cm^{-1}. For visible excitation: I = 1649/30 (2) cm^{-1}; II =1636/25 (3) cm^{-1}; III = 1622\30 (7) cm^{-1}; V = 1594/35 (8) cm^{-1}.

As denaturation of CPC progressed, component I (1649 cm^{-1}) gradually disappeared and component III (1621 cm^{-1}) emerged, while components II (1636 cm^{-1}) and IV (1605 cm^{-1}) did not change much. None of the frequencies of these components changed during denaturation of CPC (Figure 6).

$^{14}N \rightarrow {}^{15}N$ Substitution-Induced Downshift of the 1642-cm^{-1} Band. Spectra of [^{14}N]CPC and of [^{14}N]APC were fitted with increasing numbers of components until a satisfactory fit was obtained. Three components were sufficient in the case of UV-excited RR spectra, and four were necessary in that of the visible-excited ones (Table 1 and Figures 7 and 8).

In that first step, all parameters (frequencies, intensities, bandwidths) of the components were freely optimized by the program. When applied to the ^{15}N derivatives, the same procedure gave the same numbers of components, but resulted in somewhat different bandwidths. Inasmuch as isotopic substitutions are not likely to alter the distributions of the chromophore conformations within a sample, they should not modify the inhomogeneous broadening of the RR bands. Hence, in order to ensure as accurate isotope shifts measurements as possible, we imposed identical bandwidths on homologous components. All other parameters were freely optimized by the fitting program. The results are shown in Figures 7 and 8 and in Table 2.

It is interesting to note that the relative intensities of the components obtained by this procedure are different for APC and CPC (Table 2). The relative intensity of component I is considerably higher in trimeric APC than in trimeric CPC. In contrast, component II has smaller weight in trimeric APC as compared to trimeric CPC. Components III and IV, which are responsible for shoulders of the visible- and UV-excited RR spectra, respectively, are stronger in APC as compared to CPC. These variations point to previously undetected differences of chromophore conformations or chromophore–chromophore interactions between the two proteins in their native states (Szalontai et al., 1989).

Assignment of Components I, II, III, IV, and V. The analysis of the downshift of the marker band of APC and CPC upon denaturation showed that components I and III were sensitive to the folding of the chromophores. The frequencies of components I and III fall in a range where modes involving stretching coordinates of the methine bridges are expected to occur, both in closed-chain (Lutz, 1984; Hu et al., 1993) and in open-chain tetrapyrroles (Margulies et al., 1988b; Siebert et al., 1990). We proposed earlier (Szalontai et al., 1989) that components of the 1642-cm^{-1} marker band of CPC and APC should also involve these coordinates, which would explain its sensitivity to folding of the chromophores. The present decomposition of the 1642-cm^{-1} band permits assignment of these coordinates more specifically to components I and III, which are the conformation-sensitive parts of the 1642-cm^{-1} band and which exhibit moderate sensitivity to ^{15}N substitution.

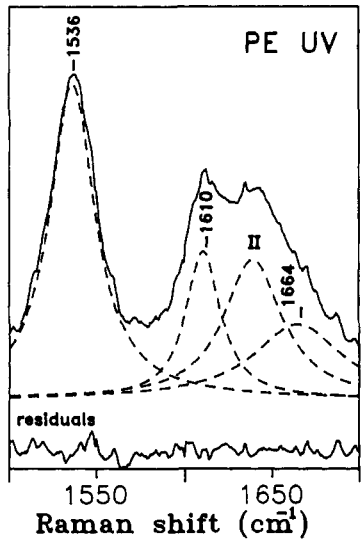

FIGURE 9: UV-excited resonance Raman spectrum of phycoerythrin from *Nostoc* sp. at 30 K. (Other details of the spectra and sample preparation will be described elsewhere.) Solid line, experimental curve; dashed lines, fitted components. Component parameters: frequency/bandwidth, 1664/62 cm^{-1}; component II = 1639/40, 1611/24, and 1536/32 cm^{-1}.

Resonance Raman (Szalontai et al., 1987, 1989), surface enhanced resonance Raman (Debreczeny et al., 1992), and electronic absorption data (Scheer & Kufer, 1977) indicated that denaturation of CPC should proceed through intermediate states, some of which may be stabilized at intermediate denaturing conditions. We also noted earlier (Szalontai et al., 1989) that published crystallographic structures clearly indicated that the ring D ends of the chromophores of CPC quite generally are more loosely interacting with the protein than rings A–C are [*cf.* Table 7 and Figure 11 in Schirmer et al. (1987) and Table 7 in Duerring et al. (1991)]. We thus proposed that early steps (i.e., those reached at pH 3.2–2.6) in the denaturation of the protein should primarily affect the D ends of the chromophores, for which the release of a smaller number of constraints is required for their being allowed to get to the more stable *Z-syn* configuration. It hence may be proposed that the methine stretch coordinates involved in components I and III should arise from the C_{15} bridge connecting rings C and D. This admittedly tentative proposal is consistent with recent calculations (Margulies & Toporowicz, 1988b). It can be strengthened further through the following comparison with RR spectra of phycoerythrin.

A UV-excited RR spectrum of phycoerythrin (PE) in the 1500–1700-cm^{-1} region is presented in Figure 9. Phycoerythrobilin (PEB) (Figure 1) is saturated at the C_{15} bridge connecting rings C and D. The UV-excited spectrum of PE has a strong band at 1536 cm^{-1} which is much weaker in both APC (Figure 2) and CPC (Figure 4) spectra. This band is assigned to νC_aC_b and νC_bC_b vibrations (Table 1). The 1580–1670-cm^{-1} region of the PE spectrum contains three components at 1610, 1639, and 1664 cm^{-1} (Figure 9). The 1639-cm^{-1} one can be safely related to component II observed in APC and CPC spectra (see Table 2). The 1610-cm^{-1} component might be component IV, which is found at 1599 and 1604 cm^{-1} in the CPC and APC spectra, respectively.

On the other hand, the frequency differences between the 1610- and 1664-cm^{-1} components of the RR spectrum of PE and components III and I, respectively, are considerably larger than the frequency jitter of those bands in CPC and APC spectra (Table 1). Therefore, it may be concluded that decomposition of the marker band region of PE yields no components I and III.

Component I is thus absent from the UV-excited RR spectrum of PE and hence should be related to the C_{15} methine bridge connecting rings C and D of PCB. It is located around 1649 cm^{-1} in the UV-excited RR spectra of CPC and APC, which have *Z-anti,Z-syn,Z-anti* chromophore conformations (Schirmer et al., 1987). As the conformation of the C_{15} methine bridge is changing to *Z-syn* during folding of the chromophore, component I disappears (Figure 6). It shows little nitrogen isotope sensitivity (Table 1). Therefore we assign component I predominantly to $\nu C{=}C$ stretching at the C_{15} methine bridge of PCB when it has *Z-anti* conformation.

Component II is present in all APC and CPC spectra (Figures 7 and 8 and Table 1), and it has a rather constant relative intensity. It is also present in the RR spectrum of PE (Figure 9). It hence is probably not related to the C_{15} methine bridge. It is moderately sensitive to nitrogen-isotopic substitution. We therefore assign component II to a predominantly $\nu C{=}C$ pyrrole vibration with some νCN contribution.

Component III appears at low pH in UV-excited RR spectra of CPC (Figure 6) at 1621 cm^{-1} as denaturation progresses and as the chromophores adopt a folded conformation which requires a twist around the C_5 and/or C_{15} methine bridges. During folding of the chromophore the methine bridge(s) involved adopt(s) a *Z-syn* conformation. Because the rise of component III is accompanied by the disappearance of component I, we assign it to the *Z-syn* conformation of the C_{15} methine bridge of PCB in the UV-excited RR spectra of APC and CPC. It shows moderate N-isotope sensitivity and thus probably involves a C_{15} methine $\nu C{=}C$ together with a $\nu C{=}N$ coordinate.

Margulies and Toporowicz (1988b) calculated the frequency of a νC_aC_m mode of the C_{15} methine bridge at 1622 cm^{-1} for the *(syn,syn,syn)* conformer *A*-dihydrobilindione. The present assignment for component III is in agreement with this calculation.

In contrast to what is observed for the UV-excited RR spectra of native APC and CPC, a certain amount of component III is present in their visible-excited RR spectra (Figures 7 and 8 and Table 1). This is not necessarily contradicting the above assignment. Depending on the relative orientation of the transition dipole moment, excitations in different electronic transitions may probe different vibrational modes of the molecule.

Component IV is present in the UV-excited RR spectra of APC and CPC around 1599–1604 cm^{-1}. Its ^{15}N-isotope sensitivity is –6 cm^{-1} for APC (Table 1). The +2 cm^{-1} shift observed for CPC should not be taken too seriously since the weight of this band is low in the CPC spectrum (Figure 7). Component IV hence should involve νCN and νCC coordinates. It remains essentially unaffected during the denaturation process, except for a smaller relative intensity at the lower pH values (Figure 6, pH 2.6). This suggests that it arises from motions of a part of the chromophore which is not involved in the folding. Component IV matches with a 1591-cm^{-1} band of the P_{fr} form of phytochrome, which, as component IV, was resonance-enhanced in UV excitation only (Mizutani et al., 1991). This band was not sensitive to deuterium exchange of phytochrome and hence was ascribed to a mode of ring C, which accordingly was proposed to be nonprotonated in the P_{fr} form by these authors. We propose that component IV of CPC and APC and the 1591-cm^{-1} band of P_{fr} have the same vibrational origin, i.e., that all three involve νCN and νCC coordinates of ring C.

Component IV does not shift or vanish at denaturing pH values, but merely loses some relative intensity (Figure 6). In the frame of the above assignment, this observation implies that at least one of the chromophores of APC or CPC did not protonate, in our experimental conditions, down to pH 2.6. This conclusion is consistent with our previous electronic absorption data on the denaturation of CPC. Indeed, a large 620 to ~660 nm red shift of the visible band has generally been associated with protonation of phycocyanins (Scheer & Kufer, 1977; Brandlmeier et al., 1981; MacColl & Guard-Friar, 1983). Our room temperature spectra of CPC exhibited this shift at pH values lower than 2.0 only (Szalontai et al., 1987). Lowering the temperature in the present RR experiments possibly may have somewhat altered the apparent p*K*s of chromophore(s) within the protein [however, see Friedrich et al. (1981)], thus accounting for the apparent weakening of component IV at pH 2.6.

Component V is present only in the visible-excited RR spectra of APC and CPC, in which it was found at 1594 and 1591 cm^{-1}, respectively. In both proteins it underwent an 8-cm^{-1} downshift upon ^{15}N substitution (Figures 7 and 8). Component V vanishes from CPC and APC spectra at denaturing pH values. These observations suggest that it should arise from a mode involving both C=N stretching and a coordinate sensitive to the conformation of the chromophores. In this frequency range, the calculations of Smit et al. (1993) for BVE predict modes predominantly involving CN stretching at ring D and methine CC stretching at bridge C_{15} (ν_{47} for [^{1}H]BVE, ν_{46} for [^{2}H]BVE). Such modes should account for the behavior of component V (*cf.* above discussion of components I and III), and we tentatively ascribe it this vibrational origin.

Component V may be correlated with a prominent 1595–1600-cm^{-1} band present in the RR spectra of the P_{fr} form of phytochrome when excited in the red or near infrared (Fodor et al., 1990; Hildebrandt et al., 1992). In the P_{fr} form, the chromophore configuration has been shown to be *Z,Z,E* (Rüdiger et al., 1983). Fodor et al. (1990) further proposed an *E,anti* conformation at bridge C_{15}. This proposal was based on the presence of a strong band at 814 cm^{-1}, assigned by these authors to a localized hydrogen out of plane wagging at C_{15}.

The structure of CPC chromophores in the native protein is also C_{15}-*Z-anti* (Schirmer et al., 1987). Their red-excited RR spectra also contain a sizeable 815-cm^{-1} band (Szalontai et al., 1985). We propose that component V constitutes an indicator of the C_{15}-*Z-anti* conformation, inasmuch as it vanishes from CPC and APC spectra when they fold into a C_{15}-*Z-syn* conformation (Figure 6). Consistent with this proposal, the ν_{47} mode of [^{1}H]BVE with which it may correlate also has been considered as Raman-inactive by Smit et al. (1993). It is indeed known that BVE assumes the *Z-syn,Z-syn,Z-syn* conformation (Sheldrick, 1976).

Consequences of RR Band Assignments for APC and CPC on the Interpretation of RR Data on Phytochrome. In the above discussion of the 1642-cm^{-1}, conformation-sensitive region, correlations have been proposed between RR bands of APC and CPC and RR bands of phytochrome. These correlations permit transposition to phytochrome of the ^{15}N-substitution data that we obtained for APC and CPC. Summarizing, we propose that component IV, which we ascribe to νCN and νCC coordinates of ring C, is homologous to the 1591-cm^{-1} band of the P_{fr} form observed under near-UV excitation. We also propose that component V, which involves CN stretching at ring D and methine CC stretching at bridge C_{15}, is homologous to the *ca.* 1595-cm^{-1} band of the P_{fr} form observed under red or near-infrared excitation.

Taken together, these assignments confirm that the UV-resonant 1591-cm^{-1} band of P_{fr} and its red-resonant 1595-cm^{-1} band should actually arise from two distinct modes, as suggested by Mizutani et al. (1991). The disagreement existing in the literature about the protonation state of P_{fr} (Fodor et al., 1990; Siebert et al., 1990; Mizutani et al., 1991; Hildebrandt et al., 1992) is thus solved: The UV-resonant 1591-cm^{-1} mode (component IV), which is not sensitive to deuteration (Mizutani et al., 1991) and does involve a νCN coordinate (this work), most likely from ring C, constitutes a good indicator for an unprotonated ring C in both the P_{fr} and P_r forms. The visible- or near-IR-resonant 1595-cm^{-1} mode (component V), which is sensitive to deuteriation (Fodor et al., 1990; Hildebrandt et al., 1992) and involves a CN stretch at ring D and a methine CC stretch at C_{15} (this work), is an indicator of the *anti* conformation at C_{15} but cannot constitute an indicator of protonation at ring C.

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