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# Chromophore States in Allophycocyanin and Phycocyanin. A Resonance Raman Study†

Balázs Szalontai,*,‡ Zoltán Gombos,§ Vilmos Csizmadia,§ Károly Csatorday,§ and Marc Lutz‖

*Institutes of Biophysics and Plant Physiology, Biological Research Center of the Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary, and Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur-Yvette Cédex, France*

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ABSTRACT: UV-excited, low-temperature resonance Raman spectra were recorded from the trimer and the pH-induced monomer and denatured forms of allophycocyanin from *Synechococcus* 6301 (*Anacystis nidulans*). Monomerization mostly results in changes in relative intensities of Raman bands of the chromophore(s), a likely consequence of changes in their excited electronic states associated with the depletion of the 652-nm excitonic band. Frequency shifts of modes involving motions of the pyrrolic nitrogens indicate that monomerization also results in environmental changes around the chromophores, possibly involving H-bonding of their nitrogens. No such phenomena were observed during monomerization of C-phycocyanin. These events, however, do not include any sizable change in the native, fully extended conformations of any of the two chromophores, as manifested by the absence of any sizable shift or broadening of the 1642-$cm^{-1}$ marker band. Upon monomerization, resonance Raman spectra of allophycocyanin become very similar to those of monomeric or trimeric C-phycocyanin, indicating that the conformations and environmental interactions of the chromophores must be very close to each other in the two proteins. Similarly, in the denatured states, the chromophores of both proteins adopt very similar, cyclohelical conformations, close to those of free chromophores in vitro. Yet the denaturation processes follow significantly different routes for allophycocyanin and C-phycocyanin. In particular, allophycocyanin monomers appear more sensitive to pH-induced denaturation than C-phycocyanin monomers.

Phycobilisomes, the light-harvesting complexes of cyanobacteria and red algae (Gantt, 1981), involve the assemblies of biliproteins. The prosthetic groups of these proteins are covalently bound open-chain tetrapyrrole molecules named phycocyanobilins. The phycobilisomes of *Synechococcus* 6301 (*Anacystis nidulans*) contain two types of biliproteins, C-phycocyanin (CPC)[1] and allophycocyanin (APC), the native forms of which are oligomeric. The monomers are equimolar associations of two nonidentical polypeptide chains, $\alpha$ and $\beta$. The $\alpha$ subunit carries one covalently bound phycocyanobilin molecule in both proteins. In APC the $\beta$ subunit also binds one phycocyanobilin, while the $\beta$ subunit of CPC binds two [for a review see Glazer (1984)]. Both CPC (MacColl et al., 1971) and APC can be monomerized. The monomers are very similar in their absorption and fluorescence spectra (MacColl et al., 1980). Therefore, it is tempting to assume that the chromophore structures as well as the protein environments are also rather similar in CPC and APC monomers (Murakami et al., 1981).

MacColl et al. (1980) discussed the electronic absorption spectra of APC and CPC. They showed that the visible absorption spectrum of native trimeric phycocyanin only differs by a red shift from that of the monomers. In contrast, trimeric

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‡ Institute of Biophysics, Hungarian Academy of Sciences.
§ Institute of Plant Physiology, Hungarian Academy of Sciences.
‖ Centre d'Etudes Nucléaires de Saclay.

[1] Abbreviations: APC, allophycocyanin; CPC, C-phycocyanin.

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APC is characterized by a strong 652-nm absorption, absent from the monomer spectrum. Recently, Csatorday et al. (1984) deconvoluted the absorption and CD spectra of native APC and showed the occurrence of excitonic interactions in the trimers. These authors found that the 652-nm band of native APC originated from the interaction of two of the six chromophores present. This excitonic band was found to have a biphasic decay upon dissociation of the APC trimer (Huang et al., 1987), indicating that the physical separation of the chromophores may be accompanied by, albeit slower, conformational changes in the protein environment as well. Two groups of proposals have favored either origin in excitonic interactions among chromophores (MacColl et al., 1980, 1981; Csatorday et al., 1984) or origin in specific conformations and environments of chromophores within the trimeric form (Sugimoto et al., 1984). These two proposals are obviously not mutually exclusive.

Resonance Raman spectroscopy offers the possibility of selective observations of conformational changes of chromophoric sites in these proteins, at the atomic level. It provides information both on excited-state phenomena (changes in electronic structure, excitonic interactions, etc.) and on ground states (e.g., conformations and molecular interactions) of the chromophores. [For a review on photosynthetic applications see Lutz and Robert (1988).] In the specific case of CPC we have recently shown that resonance Raman spectra yielded information on changes in chromophore geometry induced by monomerization of this protein (Szalontai et al., 1987). Similar results were obtained by coherent anti-Stokes Raman scattering experiments on a CPC (Schneider et al., 1987, 1988).

Here we present the results of the first UV-excited resonance Raman investigation of conformations and environmental interactions assumed by the phycobilins within trimer, monomer, and denatured APC. Similarities and differences between APC and CPC are also discussed.

## Materials and Methods

APC and CPC were isolated from *Synechococcus* 6301 (formerly *Anacystis nidulans*) as described previously (Gombos et al., 1984). The samples used in the experiments were freshly prepared from stock solutions of ammonium sulfate precipitated proteins by overnight dialysis against 20 mM, pH 7.5, phosphate buffer. The pH was changed by 12 h of dialysis against a 20 mM phosphate buffer adjusted to the required pH. The pH of the buffer was adjusted by addition of 1 N HCl. The aggregation states of APC at various pH values were determined by linear (0.1–0.4 M) sucrose gradient centrifugation. Gradients were prepared with buffers of the required pH. Centrifugations were carried out by using a Beckman SW 41 rotor at 40 000 rpm for 16 h. Estimates of aggregation states were obtained by comparison with reference gradients calibrated with known standards.

Electronic absorption spectra were recorded on a Shimadzu UV-160 UV–visible spectrophotometer. Afterward, the samples were concentrated (up to about OD 30–50) with Amincon Centricon 30 microconcentrators. Resonance Raman spectra were recorded on a Jobin Yvon spectrometer (Ramanor HG2S-UV) using the 363.8-nm excitation wavelength from an argon ion laser (Coherent Radiation Innova 100). This excitation wavelength was chosen because of the relative stability of the UV absorption band during the monomerization of the proteins. It was also advantageous that with this excitation high fluorescence backgrounds could be avoided. During the resonance Raman experiments the sample temperature was kept around 20 K by a flow of cold gaseous helium to avoid photodegradation. The spectral resolution was 8 $cm^{-1}$. The signal to noise ratios were improved by summation of six spectra in a multichannel analyzer (Tracor Northern 1710). All these individual spectra were recorded separately, checked for signs of deterioration of the sample in the laser beam, which we never found, and subsequently summed. The only data processing performed on the spectra was a linear base-line subtraction when necessary.

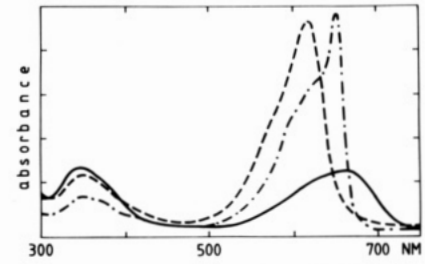


FIGURE 1: Electronic absorption spectrum of allophycocyanin in 20 mM phosphate buffer in the trimer state at pH 7.5 (–·–), in the monomer state at pH 3.5 (- - -), and in a denatured state at pH 1.8 (—). Protein concentration was the same for each curve.

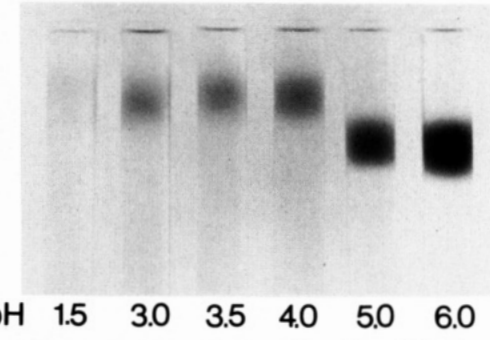


FIGURE 2: Sucrose density gradient centrifugation of allophycocyanin as a function of pH. Gradients obtained at pH 6.0–5.0 contain the trimer, at pH 4.0–3.0 the monomer, and at pH 2.5–1.6 the denatured forms.

## Results

*Electronic Absorption Spectra.* The electronic absorption spectrum of native trimeric APC differs from that of CPC by manifesting a sharp band at 652 nm (Brown et al., 1975; MacColl et al., 1980, 1981), which can be completely abolished upon monomerization/denaturation by urea treatment (Brown et al., 1975) or by addition of chaotropic salts (MacColl et al., 1981). The 652-nm band can be accounted for by strong exciton coupling between two chromophores in this trimeric system (Csatorday et al., 1984).

As in the case of CPC (Szalontai et al., 1987) the lowering of the pH changes the association state of APC. When the pH is decreased from 7.5, the 652-nm band is the first observed to change, starting to weaken around pH 4.0. At lower pH values the visible part of the APC spectrum is dominated by a band around 620 nm (Figure 1). At pH 3.5 the absorption spectrum is that of APC monomers. The absorption spectrum of native monomer APC is very similar to the CPC absorption spectrum (MacColl et al., 1980).

We have checked the aggregation state of APC by sucrose density centrifugation as well, in the pH range 1.5–6.0. According to these experiments (Figure 2) APC is indeed in monomeric state at pH 3.5.

As shown by in vivo (Scheer & Kufer, 1977) and model studies (Burke et al., 1972; Scheer et al., 1982; Falk & Muller, 1983) the electronic absorption spectra of open-chain tetrapyrroles, more precisely, the ratios of peak absorbances at 380 and 660 nm (UV/vis ratios), are sensitive to the conformations

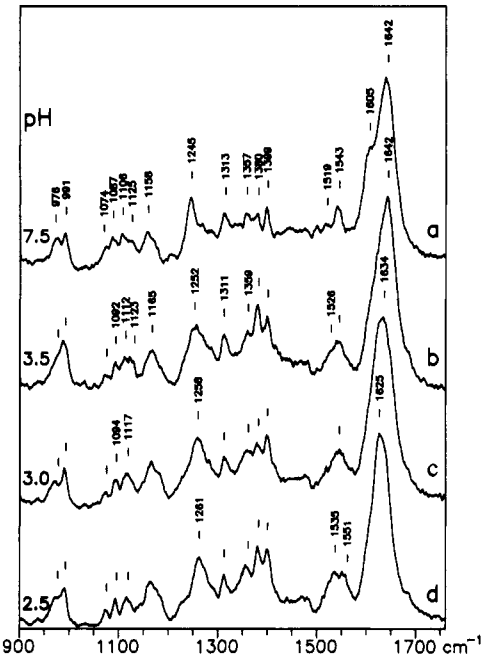


FIGURE 3: Resonance Raman spectra of 363.8-nm excited allophycocyanin in different pH-dependent states: (a) trimer; (b) monomer; (c) disturbed monomer; (d) denatured allophycocyanin.

of these molecules. Low UV/vis absorption ratios have been associated with extended chromophore conformations, while high UV/vis ratios are characteristic of folded, cyclohelical conformations.

We recorded the electronic absorption spectra of APC and CPC and found that in the 7.5–3.5 pH range the values of the UV/vis ratios in CPC and in APC are very close to each other. This indicates that the conformations of phycocyanobilin in CPC and in monomer and trimer APC are rather similar. However, when the pH is lowered further, the UV/vis absorption ratio in APC starts to increase at pH values about 0.3–0.5 higher than observed for CPC. For these proteins the UV/vis ratios did not exceed the 1.60 value in the pH range studied. For free phycocyanobilin the UV/vis ratio can reach values as high as 1.92 (data not shown). This difference indicates that even in denatured proteins the chromophores probably cannot adopt the same, fully folded conformation as, e.g., in chloroform solution, most likely because of constraints exerted by the protein environment.

Obviously, identical UV/vis ratios do not imply that the detailed conformations and environments of the chromophores in CPC and in APC must be identical, but rather their overall degree of folding. Resonance Raman spectroscopy permits these more detailed investigations. Indeed, as shown in the following in addition to differences in chromophore excited-state properties, resonance Raman spectroscopy reveals differences in environments and ground-state structures between chromophores of CPC and APC.

*Resonance Raman Experiments. Native Allophycocyanin.* A Raman spectrum of native trimer APC from *Synechococcus* 6301 at 363.8-nm resonance is shown in Figure 3a. This spectrum exhibits some differences from spectra previously obtained at 514.5-nm resonance (Szalontai et al., 1985). The major ones are the same as those observed between Raman spectra of CPC obtained at UV resonance and at visible resonance (Szalontai et al., 1985, 1987). They essentially consist of the presence in the visible-excited spectrum of a strong band at 1585 $cm^{-1}$ lacking in the UV-excited spectrum and of the presence in the UV-excited spectrum of a medium-intensity band at 1400 $cm^{-1}$ that is missing in the vis-excited spectrum. Such relative intensity differences are expected from the mere fact that the UV- and vis-excited spectra involve resonance at distinct electronic transitions.

Table I: Environment-Sensitive Mode Wavenumbers (900–1750 $cm^{-1}$) in APC and CPC[a]

| CPC, native, pH 7.5 | APC, native, pH 7.5 | APC, monomer, pH 3.5 | $\delta_1$[b] | $\delta_2$[c] | $\delta\nu(^{14}N-^{15}N)$[d] |
|---|---|---|---|---|---|
| 1076 | 1073 | 1074 | −3 | −1 | 4 |
| 1093 | 1087 | 1092 | −6 | −5 | 12 |
| 1111 | 1106 | 1112 | −5 | −6 | 2–7[e] |
| 1124 | 1121 | 1123 | −3 | −2 | 0 |
| 1165 | 1158 | 1165 | −9 | −7 | 7 |
| 1239 | 1245 | 1252 | +6 | −7 | 8 |
| 1259 | 1269 | 1261 | +10 | +8 | 8 |
| 1526? | 1520 | 1526 | −6 | −6 | ? |

[a] Raman spectra at Soret temperature; 363.8-nm excitation. [b] $\delta_1$, wavenumber difference ($cm^{-1}$) between native CPC and native APC. [c] $\delta_2$, wavenumber different ($cm^{-1}$) between native APC (pH 7.5) and monomer APC (pH 3.5). [d] $\delta\nu$, shift ($cm^{-1}$) induced on modes of native APC (pH 7.5) by $^{14}N-^{15}N$ isotopic substitution of the four nitrogens of the chromophores (Szalontai et al., unpublished data). [e] Dispersion on four distint measurements (complex band).

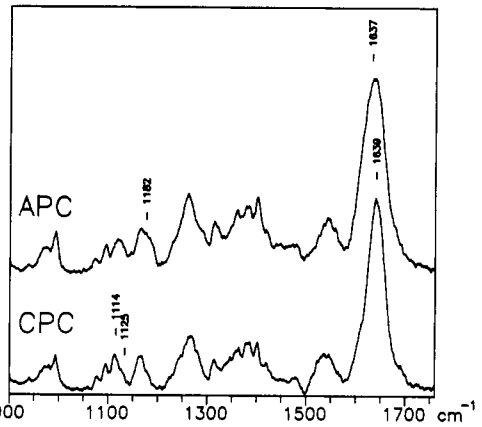


FIGURE 4: UV resonance Raman spectra of monomeric allophycocyanin at pH 3.2 (APC) and of monomeric C-phycocyanin at pH 3.0 (CPC). Excitation was at 363.8 nm.

Resonance Raman spectra of native APC and CPC (Szalontai et al., 1987) obtained in the same conditions under 363.8-nm excitation at pH 7.5 present some differences in relative band intensities as well as in band frequencies. A strong 1245-$cm^{-1}$ band, associated with a weaker 1268-$cm^{-1}$ component, is present in APC spectra, a situation opposite to that occurring in CPC spectra. Taking the 1642-$cm^{-1}$ band as a standard, the 1399 $cm^{-1}$ band of APC is stronger than that of CPC. Frequency differences are listed in Table I. They essentially concern eight bands of the 1050–1200-$cm^{-1}$ range, at least five of which exhibit sizable sensitivity to $^{14}N-^{15}N$ isotopic substitution (Table I; Szalontai et al., unpublished data). On the other hand, the frequencies of the major 1642-$cm^{-1}$ bands are the same for both proteins. Resonance Raman spectra of monomeric APC and CPC are almost identical in nearly all of their relative band intensities, widths, and frequencies (Figure 4). The only notable exceptions concern the structure of a complex band of CPC at 1114–1125 $cm^{-1}$, which occurs at 1112–1122 $cm^{-1}$ in APC spectra, and a 1182-$cm^{-1}$ shoulder that is relatively stronger in APC than in CPC spectra.

*pH Effects on Resonance Raman Spectra of Allophycocyanin.* Resonance Raman spectra were recorded from solutions of APC in phosphate buffer at decreasing pH values starting from pH 7.5. Each sample of different pH was prepared separately, dialyzed from stock solutions of ammo-

nium sulfate precipitated protein, first against 20 mM, pH 7.5, phosphate buffer and then against phosphate buffer of the same ionic strength adjusted to the required pH.

Lowering the pH causes changes in the same regions of the APC and CPC resonance Raman spectra. Therefore, to facilitate the comparison with CPC, we present and discuss APC resonance Raman spectra using the same division into five spectral regions as we did for CPC (Szalontai et al., 1987).

The Raman bands will be identified according to their wavenumbers in the resonance Raman spectra of the native proteins.

*1075–1130 cm$^{-1}$*. The relative intensities of the four bands of this region at 1074, 1087, 1106, and 1125 cm$^{-1}$ vary as compared to each other in the pH range 7.5–1.8. At pH 7.5 the 1087- and the 1106-cm$^{-1}$ bands dominate the 1074- and 1125-cm$^{-1}$ bands that appear as shoulders. In the resonance Raman spectra of the APC monomer at pH 3.5, bands at 1112 and 1123 cm$^{-1}$ dominate the region. In the pH range 7.5–3.5, significant shifts affect bands at 1087 (+5 cm$^{-1}$), 1106 (+6 cm$^{-1}$), and 1158 cm$^{-1}$ (+7 cm$^{-1}$). All of these bands are shifted upon $^{14}N$–$^{15}N$ substitution (Table I). During denaturation, at pH values lower than 3.0, an evolution toward a three-band structure can be observed in this region with bands at 1074, 1094, and 1117 cm$^{-1}$.

*1240–1275 cm$^{-1}$*. At pH 7.5, this region of resonance Raman spectra of APC trimers excited at 363.8 nm is dominated by a strong, sharp band at 1245 cm$^{-1}$, with a shoulder around 1268 cm$^{-1}$. Around pH 4.0–3.5 this shoulder is stronger and the 1245-cm$^{-1}$ band is weaker. This may account, but only in part, for the apparent upshift of this band with decreasing pH, from 1245 cm$^{-1}$ (pH 7.5) to ca. 1252 cm$^{-1}$ (pH 3.5), 1258 cm$^{-1}$ (pH 3.0), 1261 cm$^{-1}$ (pH 2.5), and 1272 cm$^{-1}$ (pH 1.8). The 1269-cm$^{-1}$ shoulder occurs at 1261 cm$^{-1}$ at pH 3.5 (Table I).

*1350–1450 cm$^{-1}$*. In the resonance Raman spectra of APC at pH 7.5 this region contains three bands at 1357, 1381, and 1399 cm$^{-1}$. The relative intensities of these three bands change during monomerization and denaturation. However, these variations do not appear to depend monotonously upon pH and hence are difficult to rationalize. The 1399-cm$^{-1}$ band monotonously shifts to 1404 cm$^{-1}$ as the pH is lowered from 7.5 to 1.8. This band also gains intensity as compared to the 1642-cm$^{-1}$ band as the pH is lowered from 7.5 ($I_{1399}/I_{1640}$ = 0.3) to 2.5 ($I_{1404}/I_{1626}$ = 0.4).

*1520–1560 cm$^{-1}$*. This region contains one complex band located at 1540 cm$^{-1}$ at pH 7.5. A weak feature appears to shift from 1519 to 1526 cm$^{-1}$ when the pH is decreased from 7.5 to 3.5. At lower pH, a two-band structure evolves from this band having maxima at 1535 and 1551 cm$^{-1}$ at pH 2.5. At pH 1.8 (spectrum not shown) the 1535-cm$^{-1}$ band dominates the 1551-cm$^{-1}$ one, which appears as a shoulder.

*1590–1650 cm$^{-1}$*. This region contains the strongest band of resonance Raman spectra of APC excited at 363.8 nm. At sample pH between 7.5 and 3.5, this band occurs at a constant wavenumber, 1642 cm$^{-1}$. It shifts continuously from 1642 to 1625 cm$^{-1}$ as the pH is lowered from 3.5 to 1.8 (Figure 5). To actually be observed as continuously varying with pH, the frequency of the 1642-cm$^{-1}$ band has to be measured on protein samples that have been equilibrated by a long (12 h) dialysis against the buffer adjusted to the required pH value. In earlier experiments with CPC (Szalontai et al., 1987) the pH of the protein solution was adjusted directly and the samples were studied without waiting. In these latter conditions, the 1642-cm$^{-1}$ band was observed to shift in a steplike fashion. These experiments on CPC were repeated by using the same

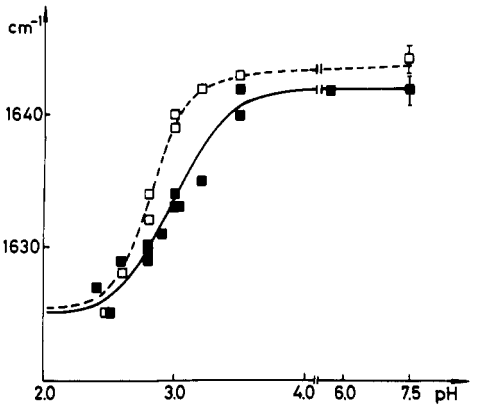


FIGURE 5: pH dependence of the 1642-cm$^{-1}$ resonance Raman band in phycocyanin (□) and in allophycocyanin (■). For details see text.

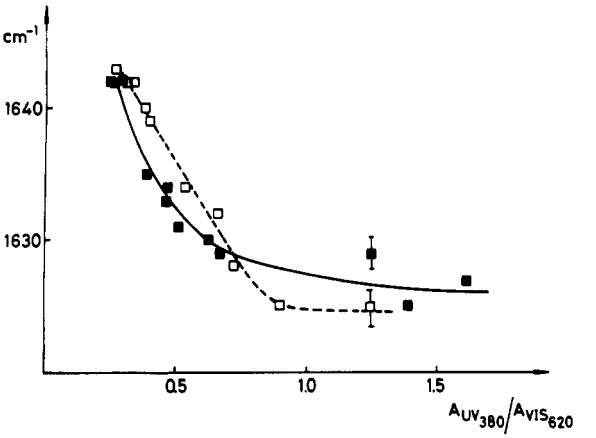


FIGURE 6: Wavenumber of the 1642-cm$^{-1}$ resonance Raman band as a function of the UV/vis absorption ratio for phycocyanin (□) and for allophycocyanin (■). Vertical bars indicate the error of peak wavenumber determination.

equilibrium procedure as for APC, and a continuous shift of the 1642-cm$^{-1}$ band with decreasing pH was observed as well (Figure 5).

To check whether the wavenumber of the 1642-cm$^{-1}$ resonance Raman bands of APC and CPC could constitute reliable markers of the degree of folding of their chromophores, as suggested by these experiments, we plotted these values against those of the ratios of their UV and visible absorption bands during denaturation experiments from the monomeric states obtained around pH 3.5 (Figure 6). The UV/vis ratio is indeed known to characterize the overall degree of folding of the chromophores (Burke et al., 1972). The plots of Figure 6 were limited to the pH range 3.5–1.8 since at higher pH values the 620-nm absorption of APC is only a shoulder on the strong excitonic band at 652 nm (Figure 1).

Another pH-sensitive feature of this spectral region is the 1605–1610-cm$^{-1}$ shoulder observed at pH 7.5, which vanishes from the monomer spectrum at pH 3.2.

Finally, UV-excited resonance Raman spectra of APC and CPC at pH 1.8 are almost identical (Szalontai et al., 1988). At this pH, both proteins are fully denatured, and their resonance Raman spectra both contain 1625-cm$^{-1}$ bands. This indicates that their chromophores assume folded, cyclohelical conformations close to that of free phycocyanobilins in vitro (Szalontai et al., 1987).

## Discussion

According to the sucrose gradient experiments (Figure 2) and the electronic absorption spectra (Figure 1), APC is trimeric at pH 7.5 and monomeric at pH 3.5. When going

from the trimeric to the monomeric state, several relative intensity changes can be observed in the resonance Raman spectra of APC: the weakening of the 1245-$cm^{-1}$ band, the enhancement of the 1268-$cm^{-1}$ band, and the disappearance of the shoulder around 1605–1610 $cm^{-1}$.

In resonance Raman scattering, changes in band intensities may result both from changes in the electronic excited states of the molecule and from changes in its ground electronic structure, e.g., in its conformation and symmetry. Considering the fact that upon monomerization an excitonic interaction is disrupted in APC, as reflected by the disappearance of the 652-nm absorption and 656-nm CD bands (Csatorday et al., 1984), we suggest that the above intensity changes in resonance Raman spectra are largely due to this phenomenon. True, the present Raman spectra were recorded at resonance with an electronic transition at 350 nm, which is slightly perturbed only by the monomerization process. However, resonance Raman intensities are expected to depend, to some extent, on the whole manifold of vibronic states of the molecule; the major perturbations observed on the visible transitions of APC thus may well account for the changes occurring in its UV-excited Raman spectra. Indeed, major relative intensity changes have been observed, at Soret resonance, in Raman spectra of the chlorophylls when excitonic coupling involving the lowest excited level took place (Lutz, 1974).

Resonance Raman spectra of APC also exhibit some bandshifts when the pH is lowered from 7.5 to 3.5 (Table I). Certain of these bandshifts may actually result from relative intensity changes of individual components within complex bands and hence may result from changes in excited-state properties of the chromophores. Such may be the +7-$cm^{-1}$ shift of the 1245-$cm^{-1}$ band. Other shifts are most probably genuine, such as those affecting bands at 991 (−3), 1087 (+5), 1106 (+6), 1121 (+2), 1158 (+7), 1269 (−8), and 1519 $cm^{-1}$ (+7 $cm^{-1}$) (Table I). These shifts must result from changes in ground electronic states of the chromophores, i.e., most probably, from environmental and/or conformational rearrangements. Isotopic experiments showed that, except the 1121- and 1269-$cm^{-1}$ ones, these modes are sensitive to $^{14}N$–$^{15}N$ substitution. Hence, these rearrangements should primarily concern certain of the pyrrole rings, likely through changes in the H-bonding of their nitrogen atoms. No such behavior was observed for phycocyanin, the resonance Raman spectra of which underwent practically no change upon monomerization (Szalontai et al., 1987).

However, no major conformational change, and in particular no sizable folding, may affect the chromophores of APC upon monomerization. This can be ascertained from the fact that the wavenumber of the 1642-$cm^{-1}$ band remains unchanged when the pH is lowered from 7.5 to 3.5. We indeed proposed earlier (Szalontai et al., 1987) that in resonance Raman spectra of CPC the wavenumber of this band constitutes a reliable marker of the folding of the chromophores. The present study on APC and CPC fully confirms this proposal (Figure 5, and discussion below). The possibility that only one of the two chromophores of APC might change its overall conformation during the monomerization process can be excluded as well: in these conditions, a broadening of the 1642-$cm^{-1}$ band would be expected or at least a change in its structure; neither phenomenon is observed in the present spectra (Figure 3).

As the pH is further decreased from 3.5, in addition to the above-mentioned changes, the 1642-$cm^{-1}$ band starts to shift downward. With small pH steps and dialysis (cf. Material and Methods) we could observe any intermediate position of the 1642-$cm^{-1}$ band between the 1642- and 1625-$cm^{-1}$ values that are characteristic for the native and the denatured proteins, respectively.

The downshift of the 1642-$cm^{-1}$ band of APC to 1625-$cm^{-1}$ upon denaturation of the protein indicates that in this state the chromophores assume folded conformations. Indeed, as discussed previously for the denaturation of CPC, this 1625-$cm^{-1}$ frequency coincides with the value 1624 $cm^{-1}$ observed in resonance Raman spectra of free phycocyanobilin, which is certainly folded (Szalontai et al., 1987). Furthermore, Figure 5 shows that a monotonous correlation exists between the frequency of the 1642-$cm^{-1}$ band and the value of the UV/vis absorption ratio, for both APC and CPC. The latter value is known to depend on the conformation of the chromophores (Burke et al., 1972; Scheer et al., 1982).

The 17-$cm^{-1}$ downshift of the 1642-$cm^{-1}$ band of APC when the pH is decreased most probably reflects complex changes in the structure of this multicomponent band. Recent calculations on resonance Raman active modes of phycocyanin indeed suggested that this band might involve at least three stretching modes, namely, those of the carbonyl group at ring D and of the A–B and C–D methine bridges (Margulies & Toporowicz, 1988). Isotopic $^{14}N$–$^{15}N$ substitutions performed on both phycocyanin and APC at resonance with either the visible or UV transitions (Szalontai et al., unpublished results) also confirmed this complexity, but showed that at least one of the modes contributing in both the UV- and visible-excited spectra must involve C=N stretching as well. Further, the analogy that this strong band presents with the ca. 1615-$cm^{-1}$ bands of chlorins and dihydrochlorins in similar conditions of excitation is striking. For these derivatives, these bands have been safely assigned to stretching of the methine bridges (Lutz, 1974; Boldt et al., 1987; Lutz & Robert, 1988). One may thus propose that such a mode, observed at 1615 $cm^{-1}$ in closed tetrapyrroles, might occur at 1625 $cm^{-1}$ in cyclohelical, open-chain tetrapyrroles and at 1642 $cm^{-1}$ in extended open-chain tetrapyrroles. Hence, the sensitivity of the ca. 1642-$cm^{-1}$ mode of APC to the overall conformation of its chromophores, as demonstrated by its dependence on the UV/vis absorption ratio (Figure 5), most likely results largely from its sensitivity to the conformations of their methine bridges.

*Chromophore States in Phycocyanin and Allophycocyanin.* Native forms of APC and CPC at pH 7.5 essentially differ by the presence in electronic absorption spectra of the former of the 652-nm excitonic band that is depleted when APC trimers are disrupted into monomers. Resonance Raman spectra of the native forms also differ slightly, but quite measurably, by a number of relative band intensities and frequencies (see Results and Table I).

The question arises whether these differences can be due to differences in excited electronic and ground states of chromophores within the two proteins or, more plainly, to the presence of an additional chromophore in CPC. This second possibility can be excluded for most of the spectral differences observed between the two native forms by comparing the resonance Raman spectra of the monomeric forms. Figure 4 shows that these spectra are almost identical in most band frequencies and, even, intensities. Only the structures of the complex 1114–1125- and 1165–1182-$cm^{-1}$ bands differ slightly. These limited differences might be due to the specific presence of chromophore $\beta$155 in CPC.

Hence, most of the differences occurring between resonance Raman spectra of the native forms must correspond to differences in excited electronic states and in ground-state conformations and environments of chromophores in the two proteins. Interestingly enough, most of the differences in band

frequencies correspond qualitatively and even quantitatively to those observed to accompany the monomerization of APC between pH 7.5 and 3.5 (Table I). This constitutes a confirmation of the validity of the two sets of spectral comparisons and indicates that, due to specific ground-state interactions in the APC trimer, the local structures and, likely, H-bonding of pyrrole(s) of chromophore(s) differ from those of their homologues in CPC. In contrast, the conformations and environmental interactions of chromophores must be extremely similar in monomeric APC and CPC. This latter conclusion is not unexpected in view of the high degree of homology existing between the polypeptides constituting the two proteins, particularly in their central regions, which accommodate chromophores $\alpha 84$ and $\beta 84$ (Glazer, 1987).

Starting from these very similar monomeric states, denaturation of both proteins when the pH is lowered further from 3.5 to 1.8 results in very similar changes in the UV-excited resonance Raman spectra of the chromophores. For instance [Figure 3 and Szalontai et al. (1987)], the gradual disappearance of a 1245-$cm^{-1}$ band and the concomitant increase of a 1265–1270-$cm^{-1}$ band can be observed in resonance Raman spectra of both proteins. For both proteins, the 1642-$cm^{-1}$ band progressively shifts to the same 1625-$cm^{-1}$ wavenumber. However, as shown in Figure 5 for this latter band, all of these events occur at slightly higher pH values (0.3–0.5 pH unit) for APC than for CPC. This indicates that the APC monomer is slightly, but sizably, less stable than the CPC monomer. This difference in behavior of APC and CPC upon denaturation is also manifested in different dependences of the 1642-$cm^{-1}$-band waenumber on the UV/vis absorption ratios for the two proteins. Figure 6 shows that APC exhibits a smooth curve and that the 1625-$cm^{-1}$ wavenumber value is only reached at UV/vis ratios close to, or higher than, 1.5. For phycocyanin, the 1642-$cm^{-1}$ band shifts more linearly to 1625 $cm^{-1}$. This wavenumber is reached around $A_{UV}/A_{vis}$ = 0.8 and does not change further at higher values of the UV/vis ratio. The different behaviors manifested in Figure 6 indicate that upon denaturation the folding processes of the chromophores from fully extended (1642-$cm^{-1}$ bands) to fully folded conformations (1625-$cm^{-1}$ bands) follow somewhat distinct routes in APC and in CPC. This constitutes a partly unexpected result since (i) chromophore states must be extremely similar in the two proteins in both their monomeric and denatured state (see above) and (ii) there are structural indications (Schirmer et al., 1985, 1986, 1987) that, due to stronger interactions from the proteins on rings A than on rings D, the folding of all chromophores in both proteins should start at the same D ends and should progress similarly toward the other ends.

*Conclusion.* This first structural study of APC using resonance Raman spectroscopy has shown that in the monomeric state of the protein the two chromophores do not exactly assume the same ground-state structures and environmental interactions as in the native trimeric state, although they still are assuming the native fully extended conformations found in the trimer. Hence, formation of the trimeric form, in addition to the proper distances and orientations of chromophores, ensures the proper ground-state interactions and structures which result in the excitonic interaction that is characteristic of the native state.

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