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The chromophore structure and chromophore-protein interactions in C-phycocyanin as studied by resonance Raman spectroscopy

Balázs Szalontai [a,b], Zoltán Gombos [b], Vilmos Csizmadia [b] and Marc Lutz [a]

[a] *Service de Biophysique, Département de Biologie, C.E.N. Saclay, Gif-sur-Yvette (France)*
and [b] *Biological Research Center, Hungarian Academy of Sciences, Szeged (Hungary)*

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We have studied the changes occurring in the electronic absorption and resonance Raman spectra of C-phycocyanin from Synechococcus 6301 (*Anacystis nidulans*) during denaturation of the protein induced by lowering the pH of the medium. Absorption studies showed that phycocyanobilin probably does not change its native, extended conformation in the pH range 7.5–3.0, but folds into a helical conformation as the pH is lowered further. Resonance Raman spectra obtained using ultraviolet excitation at 363.8 nm and at low temperature (40 K) revealed that non-covalent chromophore–protein interactions which are necessary in maintaining the extended conformation of the phycocyanobilin chromophore in the native protein involve the ends of the chromophore. They probably involve strong interactions from the protein on the two lactam C=O groups. These groups indeed become protonated only at pH values lower than 2.7, hence allowing folding of the chromophore. At pH 1.5 a complete denaturation of the protein occurs, but even this is partly reversible upon restoration of the physiological pH values.

Introduction

Phycocyanin, a biliprotein, is a major constituent of the light-harvesting phycobilisomes in blue-green (cyanobacteria) and red algae [1]. Its monomeric form consists of two polypeptide chains, α and β, containing one and two chromophores, respectively. The chromophore is phycocyanobilin (Fig. 1) an open-chain tetrapyrrole molecule, which is bound covalently via a thioether linkage to the protein. There were earlier suggestions for a second linkage as well, to ring C through an ester bond [2,3]. Very recently, however, Bishop et al. [4] produced evidence for the presence of only one thioether bond at ring A for phycocyanobilin in the α subunit and for one of the two chromophores in the β subunit. In the latter, the second chromophore is thought to bind to the apoprotein via the same type of thioether linkage at ring D [4].

Phycocyanobilin is very similar to the chromophore of the R-form of phytochrome (Pr), the sensory pigment of higher plants (for a review see Ref. 5). The only difference between these two chromophores is that the phytochrome chromophore contains a vinyl group on the fourth pyrrole ring as compared to the ethyl group of phycocyanobilin in the same position. Because of this close similarity, phycocyanin can serve as a

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Correspondence: B. Szalontai, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, P.O.B. 521, Hungary.

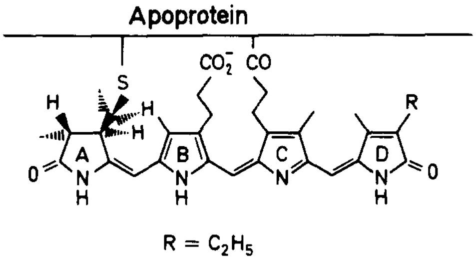

Fig. 1. Structural formula of phycocyanobilin, the chromophore of C-phycocyanin. This formula includes a second covalent bond to ring C. Most recent experiments, however, point to the existence of the thioether linkage only [4]. (In phytochromes, R is a vinyl group.)

very useful model in studying phytochromes and our data should be considered in this respect as well.

Much is known about bilin chromophores in solution. The free chromophore generally adopts a cyclohelical conformation in organic solvents. Theoretical studies using Hückel's method [6] and force field calculations [7] pointed to a direct relationship between the folding of the chromophore and the ultraviolet/visible absorption ratio. Resonance Raman spectra of biliverdin dimethyl ester (Fig. 2), the most common model compound for bilin chromophores, were studied under neutral, mildly and strongly acidic conditions [8]. On the basis of theoretically calculated resonance Raman spectra [9] the involvement of neutral, singly and doubly protonated states of the chromophore was assumed, the ring A carbonyl oxygen and ring C nitrogen atoms being proposed for protonation sites.

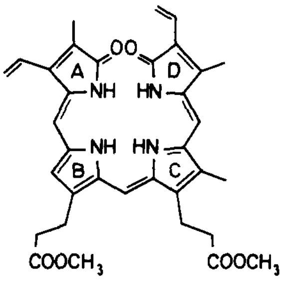

Fig. 2. Structural formula of biliverdin dimethyl ester.

Less work was devoted to the properties of the protein-bound chromophores. It is known, however, that the native protein contains phycocyanobilin in an extended conformation [5]. This implies that apart from the covalent bonds which anchor the rings A or D [4] or A and C [2,3,5] of the chromophore to the protein, additional chromophore–protein interactions must be involved in determining the actual conformation of the protein-bound chromophore. The present work has been aimed at studying these interactions.

Resonance Raman spectroscopy has already proved to be a powerful tool in studying pigment structures in highly complex photosynthetic systems. In particular, it has yielded information on chlorophyll structure in vivo, as well as on chlorophyll–protein interactions. The results obtained in this way have contributed substantially to a better understanding of the primary events in photosynthesis at a submolecular level. (For a detailed review on these applications of resonance Raman spectroscopy see Ref. 10.) The use of resonance Raman spectroscopy to study biliproteins has, however, been rather limited up to now. To our knowledge, the only published report concerns a C-phycocyanin resonance Raman spectrum [11].

We have shown previously [12] that it is possible to obtain resonance Raman spectra of biliproteins and of intact phycobilisomes, the quality of which is suitable for further studies on chromophore–protein and chromophore–chromophore interactions.

For the present study, phycocyanin has been preferred to allophycocyanin because the latter involves extensive excitonic interactions between adjacent chromophores [13,14] and therefore appeared to be less suitable for studying chromophore–protein interactions specifically.

Resonance Raman spectra of biliverdin dimethyl ester, which constitutes a good model for phycocyanobilin, have been studied in detail [9]. Theoretical calculations permitted the characteristics of these spectra to be related to molecular conformations of the chromophores. There are differences, however, which limit the use of resonance Raman data obtained on biliverdin dimethyl ester for the case of protein-bound phycocyanobilin. One of them is that the conjugation is not extended to ring A in phycocyanobilin while this ring is conjugated to the rest of the molecule in biliverdin dimethyl ester. The other fact is that even in denatured phycocyanin the chromophore remains covalently bound to the protein. The third difference is the extended conformation of phycocyanobilin in the protein, a conformation which is not observed in vitro [5].

Partial denaturation of the protein, for example by addition of urea or by heating, may result in reversible alterations of the chromophore [15]. The denatured biliproteins are so similar in their properties to free bile pigments that their chromophores are also expected to assume preferentially cyclohelical conformations.

Hence, in order to 'calibrate' resonance Raman spectra over the whole range of phycocyanobilin conformations we thought it useful to study changes in the resonance Raman spectrum of phycocyanin induced by gradual alteration of the protein conformation and of chromophore–protein interactions. In addition, by following changes in the resonance Raman spectra of phycocyanin during denaturation, one can hope also to be able to separate features characteristic of chromophore–protein interactions from features characteristic of the conformation of the chromophore in the native protein. In order to alter the chromophore conformation and environment gradually, we have chosen to denature the protein by lowering the pH of the protein solution. This choice also allowed us to make optimal use of the previous resonance Raman studies on biliverdin dimethyl ester, which involved pH variations [8,9].

As far as we know, the present work constitutes the first detection of ultraviolet-excited resonance Raman spectra of any biliprotein and the first attempt to analyse chromophore structure and chromophore–protein interaction in these chromoproteins by resonance Raman spectroscopy.

Materials and Methods

Phycocyanin was isolated from Synechococcus 6301 (formerly *Anacystis nidulans*) as described previously [16].

Phycocyanobilin was isolated by the HCl method according to O'Carra et al. [2]. The purification of the protein-free chromophore was carried out by thin-layer chromatography on preparative TLC Silicagel 60 plates (2 mm, 20 × 20 cm, E. Merck, Darmstadt). The solvent system was chloroform/ethylacetate/methanol/acetic acid (100 : 50 : 50 : 20). For higher purity the pigment sample was rechromatographed on analytical silicagel plates (0.1 mm, E. Merck, Darmstadt).

Biliverdin dihydrochloride was purchased from Sigma Chemical Co. It was esterified similarly to phycocyanobilin in 5% cold methanolic HCl for 1 day. The sample was evaporated under reduced pressure, taken up in chloroform and layered onto a silicagel thin-layer plate (0.1 mm, E. Merck) for purification. Solvent system was benzene/light petrol (60-80′)/methanol (50 : 10 : 6) according to Ref. 17.

The phycocyanin samples used in the experiments were freshly prepared from stock solutions of ammonium sulphate-precipitated phycocyanin dialysed against 20 mM sodium phosphate buffer (pH 7.5). During the denaturation experiments the pH adjustments were done by addition of 0.1 M HCl to the dilute solutions (*A* was about 5 at 620 nm) and then the absorption spectra were recorded at room temperature on a Cary 17D spectrophotometer.

Resonance Raman spectra were recorded from solutions having an absorbance of about 30–50 at 620 nm. The protein samples were concentrated up to these values using Amincon Centricon 30 microconcentrators. Solutions of both isolated pigments, phycocyanobilin and biliverdin dimethyl ester, in chloroform were transferred to the Raman spectrometer under nitrogen atmosphere. 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 (Spectra Physics Model 171).

This excitation wavelength was preferred to the 488 nm wavelength used in our previous studies [12] because denaturation of the protein strongly alters the visible absorption band at 620 nm. This change alone would affect the intensities of the Raman modes resonating with this transition. On the other hand, the ultraviolet band wavelength and oscillator strength remain practically the same during denaturation. Therefore, one may safely assume that any resonance Raman spectral variations would arise from changes in chromophore structure rather than from changes in the resonance conditions.

During the measurements the sample temperature was kept at 40 K by a flow of cold gaseous helium, in order to avoid photodegradation. The cooling of the samples was carried out in two steps. The sample droplet on a microscope cover plate was immersed immediately into liquid nitrogen and frozen within seconds. Afterwards it was transferred to the helium cryostat for further cooling. The spectral resolution was 8 cm^{-1} at 1000 cm^{-1}. The signal-to-noise ratios were improved by the summation of 3–6 spectra in a multichannel analyzer (Tracor Northern 1710). The spectra were recorded individually and added later. All single scans were identical within experimental uncertainty, indicating that any photodegradation could be avoided by working at low temperature.

Results and Discussion

Electronic absorption measurements

Since absorption spectra are generally used to characterize the conformations of free and of protein-bound chromophores [18] we have used this method for monitoring the denaturation of phycocyanin as induced by lowering the pH of the medium. Precise knowledge of the absorption spectrum is also essential for resonance Raman studies.

Absorption spectra of C-phycocyanin at different pH values are shown in Fig. 3. Several changes can be observed in these spectra as the pH is lowered. The maximum of the visible absorption band progressively shifts to the blue, from 618 to 612 nm, when the pH is decreased from 7.5 to 4.0. When the pH is further lowered, this maximum shifts back and reaches 618 nm again around pH 2.5–2.0. At still lower pH values an additional, longer-wavelength band also appears around 680 nm. In the 7.5–3.0 pH range both the visible and the ultraviolet absorption bands become weaker in such a way that the ultraviolet/visible absorbance ratio remains practically the same (Fig. 4). Between pH 2.6 and pH 1.9, however, this ratio increases sharply (Fig. 4).

At pH 1.5, in addition to the continuing tendency of the visible band to weaken and of the ultraviolet band to increase, a significant back-

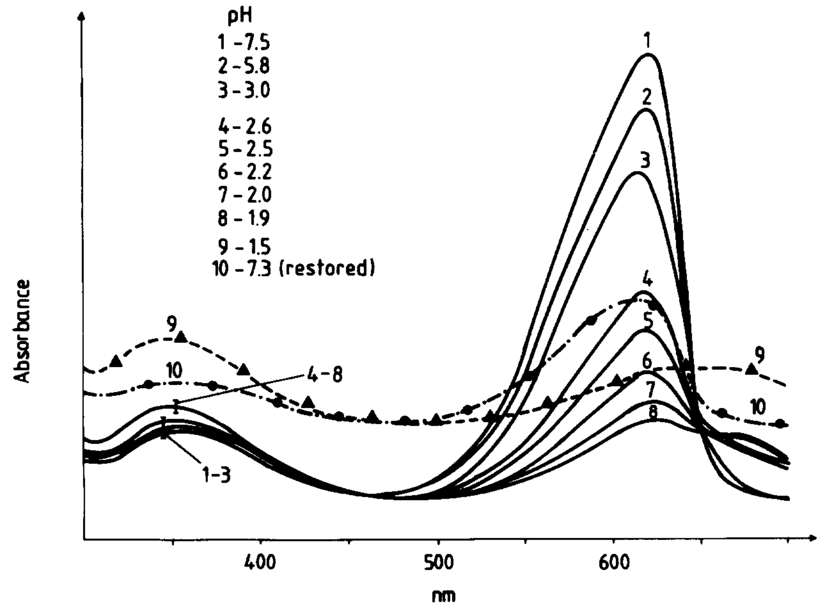

Fig. 3. Room-temperature absorption spectra of C-phycocyanin from Synechococcus 6301 (*A. nidulans*) in the pH range 7.5–1.5. None of the curves was displaced from the others, and the higher background under curves 9 and 10 reflects an irreversibly denaturated protein fraction occurring at pH 1.5 (curve 9) which could not be renatured upon restoring a physiological pH value (curve 10).

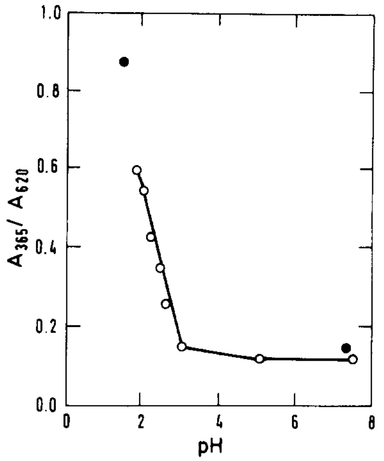

Fig. 4. The ratio of near-ultraviolet (365 nm) and visible (620 nm) absorption maxima of C-phycocyanin as a function of pH. The pH was lowered from 7.5 to 1.5. Filled circles indicate completely denaturated (pH 1.5) and restored (pH 7.3) proteins.

ground appears in the absorption spectrum. This background is probably due to increased scattering from completely denatured proteins (curve 9 of Fig. 3).

If at this point the protein sample is poised back to pH 7.0, the original absorbance ratio of the ultraviolet and the visible bands is restored, but the previously obtained high scattering background remains (curve 10 of Fig. 3).

Scheer and Kufer also studied phycocyanin denaturation by absorption spectroscopy [15]. In these experiments the denaturation was achieved by addition of urea or by heating. These authors showed that in the denatured protein the ultraviolet absorption is high and the visible band is low. As regards the denaturation process itself, a continuous decrease of the visible band was demonstrated with an intermediate state between 4 and 5 M urea concentration, but no such state was observed during heat treatment. Later, Scheer and his co-workers investigated the stability of C-phycocyanin and of its subunits from *Mastigocladus laminosus* [18]. A one-step denaturation was observed during both urea and heat treatments. The denaturation was characterized by a marked, continuous decrease of the visible band and a much smaller increase of the ultraviolet band as urea concentration or temperature were increased.

Denaturation of C-phycocyanin from Synechococcus 6301 induced by lowering the pH proceeds in a way similar to the heat-induced denaturation of C-phycocyanin from *Spirulina platensis* [15] and to both heat- and urea-induced denaturation of C-phycocyanin from *M. laminosus* [18]. Indeed, we observed that down to about pH 3.0 there is very little change in the visible absorbance, but as the pH is lowered further it decreases steadily with no sign of any intermediate state.

The ultraviolet/visible absorption ratio can be reliably related to the conformation of the chromophore. Burke et al. [6] calculated the oscillator strengths of the ultraviolet and visible bands of 16 open-chain tetrapyrrole isomers and found an ultraviolet/visible ratio of 0.15 for the extended conformations. This calculated value increased up to 4.0 if the molecule was folded into a cyclohelical conformation. The increase in the ultraviolet/visible ratio resulted from a monotonous increase in the ultraviolet oscillator strength as the chromophore folded from a linear to a cyclic conformation. Hence, the present observations (Fig. 4) indicate that the protein-bound chromophore retains an extended conformation down to pH 3 and starts folding at lower pH values.

The situation in proteins differs at several points from the one just outlined above for free chromophores, although probably not to such an extent that it would prevent our drawing definite conclusions about the chromophore conformations in the proteins. This difference is most probably also related to the step-like, rather than continuous, folding of the chromophore occurring in the protein.

A second deviation is the slight overall decrease in the ultraviolet and visible absorptions of the protein-bound chromophore in the pH range 7.5–3.0. This slight decrease conceivably may arise from alterations of the protein environment caused by acidification of the medium rather than from changes in the chromophore conformation as confirmed by resonance Raman spectra (see lower).

The blue-shift of the visible band upon lowering the pH to 3.0 may well reflect monomerization of the phycocyanin. A similar shift was observed in phycocyanin in Ref. 13 when lowering the pH from 7.0 to 3.9. In the same study, the phycocyanin hexamers characteristic for pH 7.0 indeed dissociated into monomers at pH 3.9 by analytical ultracentrifugation. Absorption spectra of C-phycocyanin monomers from *M. laminosus* also showed a blue shift of the visible band and a slight increase of the ultraviolet band as compared to the trimers [18].

Relying on absorption and CD measurements, Haidl et al. [19] raised the problem of the presence of aggregates of biliverdin dimethyl ester as well, instead of pure cationic forms, under mildly and strongly acidic conditions [8]. Similarly, in the present experiments the longer wavelength absorption band observed in the visible at lower pH (Fig. 3, curve 9) may indicate the formation of non-specific aggregates of protein-bound phycocyanobilins.

Although absolute conformations of the phycocyanin chromophore cannot be diagnosed from the above results, we may conclude that the relative changes observed in the ultraviolet/visible absorption ratio of phycocyanin when the pH is lowered from 7.5 to 1.5 indicate that the chromophore most probably preserves its native conformation in the protein down to pH 3.0. The chromophore starts to fold only as the pH is lowered further. The possibility of the formation of non-specific aggregates of phycocyanin molecules having cyclic chromophores cannot be excluded.

Resonance Raman experiments

Native phycocyanin

A resonance Raman spectrum of native C-phycocyanin obtained at pH 7.5 with 363.8 nm excitation at 40 K is shown in Fig. 5a.

The 363.8-nm-excited spectrum shows few differences as compared to the previously published [12] 488-nm-excited room-temperature spectrum. Noticeable specifics of the ultraviolet-excited spectrum are the disappearence of the strong 1585 cm^{-1} band observed with 488 nm excitation and the appearance of a medium band at 1401 cm^{-1}. There are minor changes in the relative intensities of other bands as well. All these can be accounted for by differences in resonance conditions in the 363.8 nm and 488 nm experiments due to excitation within two distinct electronic transitions.

Phycocyanin in the 7.5–1.7 pH range

We have found changes in the following regions of the resonance Raman spectra as the pH was lowered (Fig. 5):

1075–1130 cm^{-1}. The relative intensities of the four bands of this region at 1075, 1095, 1115 and 1130 cm^{-1} varied as compared to each other between pH 7.5 and 1.7. At pH 7.5 the 1115 cm^{-1} band is the strongest. As the pH is lowered, the 1095 cm^{-1} band intensity increases, reaching a maximum at pH 4.0 and being then equal to the intensity of the 1115 cm^{-1} band. At pH values lower than 4.0 it decreases again. Interestingly the situation at pH 3.0 for the whole region is almost

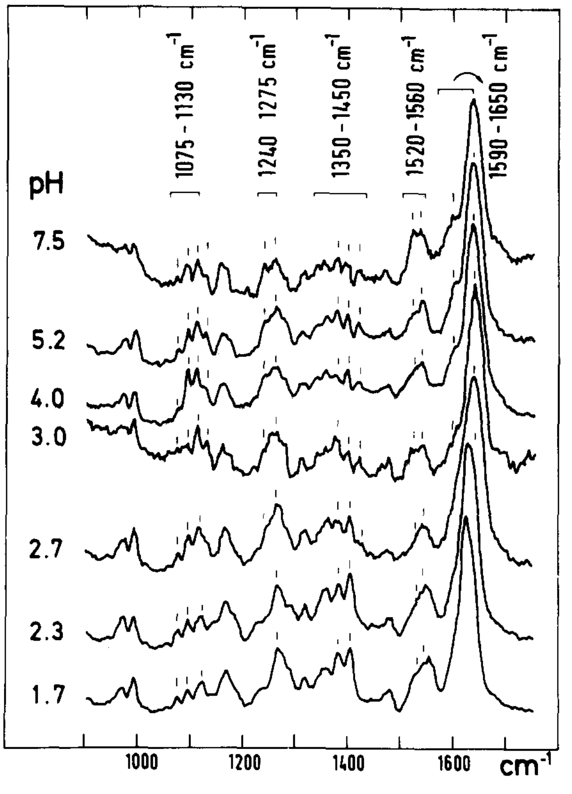

Fig. 5. Low-temperature (40 K) resonance Raman spectra of C-phycocyanin at different pH values excited at 363.8 nm. Spectral bandwidth 8 cm^{-1} at 1000 cm^{-1}; the curves are the summation of 3–6 scans. The regions indicated and the bands marked within are discussed in the text.

identical to that observed at pH 7.5. As the pH is decreased further from pH 3.0, the structure of the 1075–1130 cm^{-1} region is altered into three roughly equal bands, at 1075, 1095 and 1120 cm^{-1}, each having an intensity considerably lower than in the 7.5–3.0 pH range, taking the 1645 cm^{-1} band as a reference.

1240–1275 cm^{-1}. This region is insensitive to a change from pH 7.5 to pH 3.0. Below pH 3.0 a 1245 cm^{-1} band becomes weaker and vanishes completely below pH 2.7. At the lowest pH values this region is thus left with only one band around 1265 cm^{-1}.

1350–1450 cm^{-1}. In the 7.5–3.0 pH range, slight variations are visible in the relative intensities of bands at 1380 and 1425 cm^{-1} as compared to the 1645 cm^{-1} band. Both decrease around pH 4.0 and were found to increase again around pH 3.0. At lower pH values, however, the 1380 cm^{-1} band intensity remains cosntant, while the 1425 cm^{-1} band vanishes. The 1401 cm^{-1} band intensity does not change in the 7.5–3.0 pH range, but it increases gradually as the pH is lowered further.

1520–1560 cm^{-1}. A shoulder at 1525 cm^{-1} has a local minimum in intensity around pH 5.0–4.0. It is more intensive at pH 3.0 and then decreases again at lower pH values. A band observed at 1545 cm^{-1} in the 7.5–2.7 pH range shifts to 1550 cm^{-1} at pH 2.3 and to 1555 cm^{-1} at pH 1.7.

1590–1650 cm^{-1}. A shoulder around 1590–1610 cm^{-1} keeps a constant relative intensity between pH 7.5 and 3.0, but it gradually vanishes at lower pH values. The dominating band of the spectrum is observed at 1645 cm^{-1} between pH 7.5 and pH 2.7. At lower pH values it abruptly shifts to 1625 cm^{-1}. At these low pH values it also reveals a double band character having a shoulder at 1625 cm^{-1} and 1635 cm^{-1}.

It is worth noting the opposite behaviour of the 1095 and 1380 cm^{-1} bands. Whenever the intensity of one of them increases, as for the 1095 cm^{-1} band between pH 7.5 and pH 4.0, the other (1380 cm^{-1}) decreases. When the 1095 cm^{-1} band drops in intensity at pH 3.0, the 1380 cm^{-1} band increases sharply. Finally when the 1095 cm^{-1} band stabilizes between pH 2.3 and 1.7, the 1380 cm^{-1} one also remains constant.

The disappearance of the 1245 cm^{-1} band at pH values lower than 3.0 is very reminiscent of similar changes observed upon formation of a cation of the model compound, biliverdin dimethyl ester. In neutral biliverdin dimethyl ester (Fig. 2) a band is calculated at 1237 cm^{-1} for C-H rocking in bridge C–D [9]. It is observed at 1244 cm^{-1} and disappears under mildly acidic conditions [8].

In the pH 3.0–1.7 range, the 1350–1450 cm^{-1} region of the spectra exhibit an overall enhancement as compared to the 1625 cm^{-1} band. During the analysis of the possible ionic forms of biliverdin dimethyl ester, Margulies and Toporowicz [9] excluded the possibility of a protonation of the oxygen on ring D for singly protonated compounds (structure VI in Ref. 9). Their argument for this exclusion was that theoretical calculations predicted relatively strong bands for that case at 1375 cm^{-1}, 1404 cm^{-1} and 1482 cm^{-1} and that no bands were observed at these frequencies. Interestingly the present pH denaturation experiments resulted in enhancement of bands at 1380 and 1401 cm^{-1} as well as of a weak band at 1480 cm^{-1} (Fig. 5). Hence, we tentatively conclude that the chromophore in phycocyanin may become protonated on the lactam oxygen of ring D (Fig. 1) at low pH values.

The most prominent change observed in the resonance Raman spectra of phycocyanin upon pH denaturation occurs in the 1590–1650 cm^{-1} region. This is the shift of the 1645 cm^{-1} band to 1625 cm^{-1} occurring at pH values lower than 2.7. This band was assigned to the υ(C=C) vibration of the methine bridge at ring A in biliverdin dimethyl ester (Fig. 2) at 1623 cm^{-1}, and was observed as a doublet at 1618 cm^{-1} and 1630 cm^{-1} under strongly acidic conditions [9].

Since we also observe a doublet character for this band at low pH values, with components at 1625 cm^{-1} and 1635 cm^{-1}, we propose that this, as well as the 20 cm^{-1} downshift, reflects the fact that phycocyanobilin is doubly protonated under these conditions. The large downshift of the band can be accounted for by the change in the chromophore from an extended to a cyclic conformation.

The disappearance of the shoulder around 1590–1610 cm^{-1} is completed earlier than the shift of the 1645 cm^{-1} band appears as the pH is

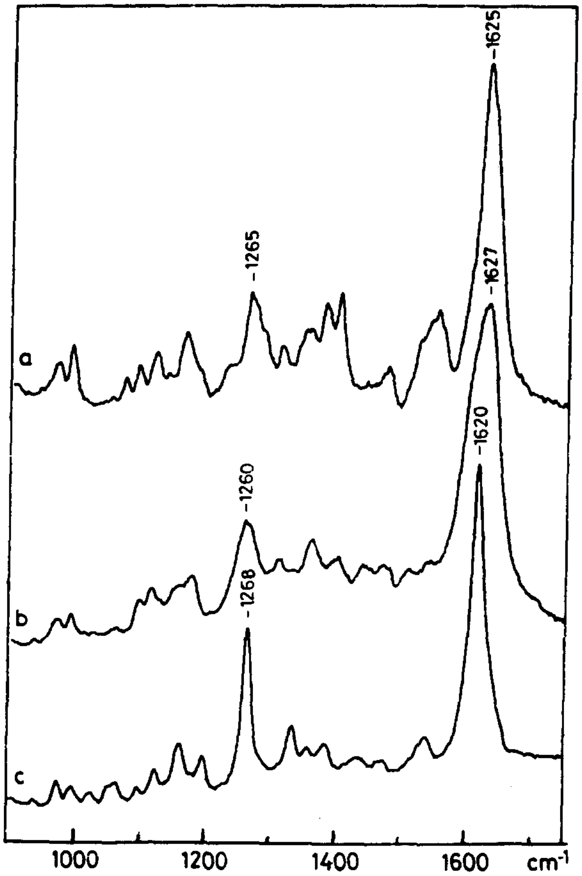

Fig. 6. Low-temperature (40 K) resonance Raman spectra of (a) denatured C-phycocyanin (the spectrum is the same as that shown on Fig. 5 at pH 1.7); (b) phycocyanobilin in chloroform; (c) biliverdin dimethyl ester in chloroform.

lowered from 7.5. In biliverdin dimethyl ester, the C=C stretching vibration of the methine bridge at ring D (Fig. 2) was calculated at 1606 cm^{-1}. The 1590–1610 cm^{-1} region may also involve contributions from C=C vibrations in ring A and ring D (Fig. 2) calculated at 1596 cm^{-1} and 1585 cm^{-1}, respectively [9].

An independent test of the above discussed possible structural changes in phycocyanins is the comparison of resonance Raman spectra of free phycocyanobilin in solution and in pH-denatured phycocyanin. These should be rather similar if our conclusion for a cyclic conformation of the chromophores in denatured phycocyanin holds. Indeed, we have found that the two spectra agree well in those details we have taken as being characteristic for cyclic conformations (Fig. 6).

In particular, the band located at 1625 cm^{-1} in denatured phycocyanin is at 1627 cm^{-1} in phycocyanobilin. The shoulder around 1590–1610 cm^{-1} is missing from both spectra. The 1240–1275 cm^{-1} region is dominated by a single band at 1265 cm^{-1} and 1260 cm^{-1} in phycocyanin and phycocyanobilin spectra, respectively.

A good similarity is further observed between resonance Raman spectra of phycocyanobilin and of biliverdin dimethyl ester excited at 363.8 nm and at 40 K (Fig. 6). This justifies the use of previous assignments of resonance Raman of the latter for those of phycocyanobilin. In particular, both spectra contain a high band in the 1240–1270 cm^{-1} region. This band occurs at 1260 cm^{-1} in phycocyanobilin and at 1268 cm^{-1} in biliverdin dimethyl ester. The positions of the main bands are 1620 and 1627 cm^{-1} for biliverdin dimethyl ester and phycocyanobilin, respectively. This supports our conclusion that the 1625 cm^{-1} position of the same band in denatured phycocyanin reflects a cyclic conformation of the protein-bound phycocyanobilin.

The 1350–1450 cm^{-1} region is missing from the free chromophore spectra (Fig. 6.). These features may arise from Raman-active modes present only in protein-bound chromophores.

The present observations allow us to draw the following conclusions about the structure of phycocyanobilin in native phycocyanin and about the chromophore–protein interactions. A first group of pH-induced changes in the resonance Raman spectra (e.g., the behaviours of the 1095, 1380 and 1525 cm^{-1} bands) shows singularities around pH 4.0, a value around which phycocyanin exists in its monomeric form and still assumes a native structure around the chromophore according to the absorption spectrum.

The bands at 1245 and 1590–1610 cm^{-1} disappear around pH 3.0, a value at which ultraviolet absorption shows that the chromophore starts folding. The corresponding modes involve vibrations at both ends of the chromophore. This indicates that they probably reflect alterations in the chromophore–protein interactions at the ends of the chromophore. These interactions should play predominant roles in maintaining the extended conformations in native phycocyanin.

The relative enhancement of bands in the

1350–1420 cm^{-1} region occurs in the same pH values as the shift of the 1645 cm^{-1} to the 1625 cm^{-1} band. This shift probably reflects the folding of phycocyanobilin into a cyclic conformation. Such a coincidence may point to the fact that the oxygen atoms on rings A and D (Fig. 1) are strongly involved in the chromophore–protein interactions which maintain the extended conformation of the chromophore in phycocyanin. When external protons are able to replace the protein moiety in this interaction, then the resonance Raman spectrum displays all the features of a folded chromophore.

Summarizing, the experiments presented here allow us to propose that, besides the covalent bond, the conformation of phycocyanobilin is largely determined by the chromophore–protein interactions at the ends of the chromophore, most probably predominantly involving the lactam C=O groups. These non-covalent bonds, probably H-bonds, are affected by alterations in the protein structure brought around by lowering the pH of the medium down to 3.0. The folding of the chromophore into a cyclic conformation is then possible and occurs as a subsequent step in the process of denaturation of phycocyanin.

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