CARS Investigation of Changes in Chromophore Geometry of C-Phycocyanin from *Mastigocladus laminosus* Induced by Titration with *p*-Chloromercuribenzenesulfonate

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Titration of trimers of C-phycocyanin with *p*-chloromercuribenzenesulfonate (PCMS) changes spectroscopic and kinetic properties of chromophore B84. The resonance-enhanced CARS spectra of room temperature solutions indicate that these changes are due to a modification in B84 chromophore geometry (movement of ring D) as judged by the disappearance of the 1245 cm^{-1} vibration.

Introduction

In blue-green and red algae, phycobiliproteins act as light-harvesting pigments when organized in form of the so-called phycobilisomes [1]. The efforts to elucidate the efficient energy transfer within the antenna complexes and further down to the reaction centers in the thylakoid membrane have produced important results in recent years. From X-ray studies of crystallized C-phycocyanin (PC) information is available about the geometries of the three different types of tetrapyrrolic chromophores and about the distances and relative orientations of the nine chromophores found in a trimeric unit [2–4]. Different protein environments are predicted from both the X-ray structure and the known sequences of amino acids [5, 6] for the so-called A84, B84 and B155 chromophores of native C-phycocyanin from *Mastigocladus laminosus*. By now it is widely accepted that chromophore-protein interaction causes the fine tuning of the spectroscopic properties of the chromophores and thereby guarantees the well-performance of the biological functions [1]. This implies that small variations in either chromophore geometry or protein environment (*e.g.* induced by aggregation) are sufficient to drastically change spectroscopic properties of individual chromophores and concomitantly the rates of energy transfer. It has been proposed that changes of this kind during preparation or storage of samples are responsible for some of the discrepancies reported in the literature dealing with fluorescence properties of bile pigments [7–9]. Direct evidence for this hypothesis was, however, lacking; it is the main subject of this paper.

Only recently, Siebzehnrübl *et al.* [10] reported that upon titration of a solution of PC-trimers of *Mastigocladus laminosus* with *p*-chloromercuribenzenesulfonate (PCMS), the absorption and CD-spectra are altered gradually. The observation is rationalized by assuming that PCMS is bound to free cystein B111, which is located in the neighbourhood of chromophore B84 [4], and therefore induces measurable spectral changes which are connected with structural modifications of this chromophore (only?). From the CD spectra it is concluded that the chromophore should assume a more helical conformation after PCMS binding. The reduction of fluorescence intensity to about 35% of its original value [10] and the shortening of the fluorescence lifetime [11] are taken as additional evidence for the geometry change of this so-called fluorescing chromophore.

The need for information about chromophore structure in solution has recently stimulated interest in resonance-enhanced Raman spectroscopy of bile pigments [12–14]. The intense fluorescence is, however, a considerable obstacle. It could be overcome only by applying low temperatures (40 K) and high photon energies (λ = 488 nm or 363 nm). We have chosen an alternative method, namely resonance-enhanced Coherent Anti-Stokes Raman Scattering (CARS), to monitor shifts in vibrational frequencies as evidence for structural changes in room temperature solutions.

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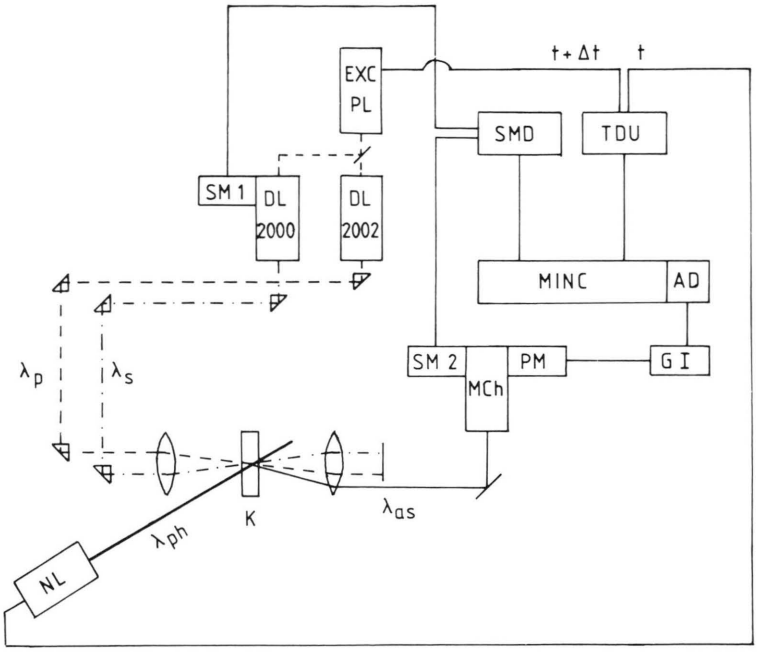

Fig. 1. Experimental arrangement for recording resonance-enhanced Coherent Anti-Stokes Raman Scattering (NL, nitrogen laser; DL, dye laser; EXC PL, excimer pump laser; SM, stepping motor; SMD, stepping motor driving unit; TDU, trigger delay unit; MINC, computer; AD, analog digital converter; GI, gated integrator; MCh, monochromator; PM, photomultiplier).

Materials and Methods

Materials

Objects of this investigation were:

1) Trimers of C-phycocyanin from *Mastigocladus laminosus* dissolved in 100 mM potassium phosphate buffer (pH = 7.0); optical density at 640 nm = 5/cm.

2) Aliquots of sample 1 titrated with PCMS in large excess with respect to monomeric PC. OD (640 nm) = 5/cm.

The samples which were prepared according to procedures described previously [23, 10] were provided to us by Prof. H. Scheer (Munich). The state of aggregation (trimer) was proven by sedimentation coefficients $S_{20,w} = 5.6$ [7].

Methods

The experimental arrangement used for registration of Coherent Anti-Stokes Raman Scattered (CARS) light is displayed in Fig. 1. Pump (λ_p) and Stokes (λ_s) laser beam are each generated by an excimer laser-pumped dye laser. They are focussed onto the sample cell such that they cross under the so-called phase match angle [15, 16]. Its value varies with solvent and solute concentration and also with pumping wavelength.

Forming approximately the same angle with the pump beam, the anti-Stokes beam appears from the sample on the opposite side and can, therefore, easily be separated from the former two by spatial filtering. Nevertheless it proves very important for good S/N ratio, to apply in addition a spectral filtering. This is achieved by passing the anti-Stokes beam through a monochromator whose wavelength setting is tuned perfectly synchronous to the wavelength drive of the Stokes laser. The intensity of the anti-Stokes radiation is measured by a photomultiplier in combination with a gated integrator. Correction for intensity fluctuations of the laser beams and signal averaging over a preset number of shots is performed in a minicomputer, where the data are stored for further evaluation [16].

CARS spectra of metastable, photoinduced intermediates can be recorded by using the nitrogen laser as photolysis laser ($\lambda_{ph} = 337$ nm). Kinetic studies are possible if the delay time between photolysis and analysis pulses is varied [16, 17].

The advantage of resonance-enhanced CARS *versus* spontaneous Raman spectroscopy is its insensitivity to fluorescence. Since the *anti*-Stokes lines are higher in energy than the exciting photons, they can never be covered by fluorescence or stray light.

Being due to a four wave mixing process, the lineshapes in CARS spectra are in a rather complex way dependent on the experimental parameters [15]. A quantitative analysis requires *e.g.* a least-squares fit of at least a section of the spectrum by the function:

$$L(\delta) = \left| R_{NR} + \sum_{j=1}^{n} \frac{R_j + iI_j}{\delta_j - i\Gamma_j} \right|^2 \qquad (1)$$

where R_{NR} = non-resonant background (mainly from the solvent)

R_j, I_j = real and imaginary part of the resonance-enhanced term of tensor $\chi^{(3)}$ due to normal mode j with frequency ω_j,

Γ_j = line width of normal mode j,

δ_j = $\omega_j - (\omega_p - \omega_s) = \omega_j - \delta$,

n = number of molecular vibrations which are close enough to cause interference,

i = $\sqrt{-1}$.

It is obvious from inspection of Eqn. (1) that in case of interfering lines, a small shift in one normal mode frequency ω_j can cause significant changes in line shape. Thus CARS spectroscopy can detect small variations in normal mode frequencies of close-lying lines which could hardly be detected in spontaneous Raman spectroscopy.

Results and Discussion

In Fig. 2, the CARS spectra recorded for trimeric PC in the native state (top) and for trimeric PC fully titrated with PCMS (bottom) are shown for comparison. The wavelength of the pump beam was chosen to be $\lambda_p = 640$ nm, since in the absorption spectra, an isosbestic point is observed at this wavelength [10]. The major advantage of this choice, when doing CARS spectroscopy is that important parameters, which determine the lineshape, are nearly equal and, consequently, the comparison of the spectra is facilitated even without a numerical analysis. In addition, one expects the differences in the resonance enhanced spectra to be largest, when the pump wavelength is close to the maximum of the absorption band of the modified chromophore.

The second, experimental advantage of using $\lambda_p = 640$ nm (lasing medium: Rh 101 in EtOH) is that the interesting frequency range (1000–1800 cm^{-1}) can be covered with only one dye in the Stokes laser (Pyridine 1 in DMSO). Applying a multiplex CARS technique and various dye solutions in the Stokes laser, Mudogo has recorded CARS spectra of phycocyanin trimers down to 400 cm^{-1} [24].

Inspection of Fig. 2 reveals that most bands exhibit a line shape which can be considered to be close to positive Lorentzian [15, 16]. In that case the maxima in the spectra relate directly to the frequencies of the molecular vibrations [15, 18, 19]. A comparison of both spectra reveals that the positions of most peaks coincide within experimental accuracy (± 2 cm^{-1}). This observation can be taken as evidence that the molecular vibrations are not changed by PCMS-binding except for the two, which will be discussed in more detail.

It is evident that possible changes in geometry are connected with rotation(s) around a single or double bond of the methine groups. They should manifest themselves in frequency shifts of the C–C stretching, the C–H bending and rocking and the C=C stretching modes. Estimates by Margulies and Toporowicz [14] yield for these vibrations in biliverdindimethylester (C=C): 1606 and 1623 cm^{-1}, C–H rocking: 1308 and 1247 cm^{-1}.

A large difference is found in the spectra of native and chemically modified PC around 1245 cm^{-1} in that the band observed in the native sample is missing in the modified one (Fig. 2). In order to prove that this difference is not just an experimental artifact, that part of the spectra was repeatedly scanned with smaller increments in the wavelength setting thereby increasing the spectral resolution. The result is shown in Fig. 3; it documents beyond doubt the statement made above. The solid line represents in both cases the best fit by the lineshape function given in Eqn. (1). The calculated molecular frequencies of the persistent bands are the same for both samples, namely:

$\tilde{\nu}_1$ = 1234 cm^{-1}, $\tilde{\nu}_2$ = 1259 cm^{-1} and $\tilde{\nu}_3$ = 1273 cm^{-1}; the frequency of the disappearing band is 1245 cm^{-1}.

In accordance with the conclusions of Siebzehnrübl *et al.* [10], we take the disappearance of the 1245 cm^{-1} band as evidence for a geometrical change of one and only one of the phycocyanobilin chromophores. This interpretation is also supported by the observation of Szalontai *et al.* [13] that upon pH-denaturation of PC from *Synechococcus 6301* the band at 1245 cm^{-1} in the Raman spectrum disappears (despite of the different origin of the biliprotein,

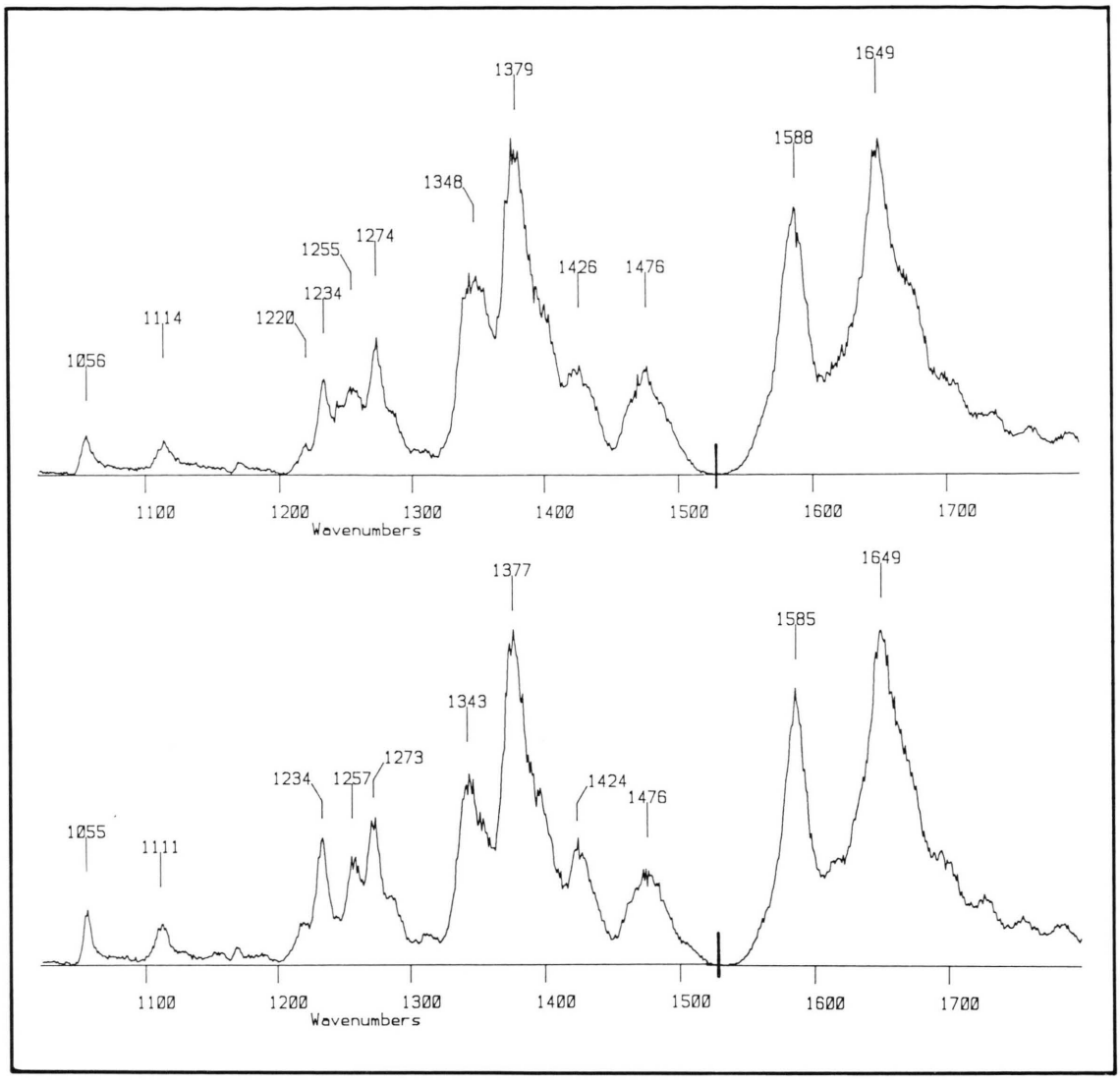

Fig. 2. CARS spectra of trimers of C-phycocyanin from *Mastigocladus laminosus* (λ_p = 640 nm): a) native state (top); b) titrated with PCMS (bottom). For more details see text.

nearly all the frequencies (observed) agree with those derived from our CARS spectra).

The calculated Raman spectra of various geometrical isomers of biliverdindimethylester by Margulies and Toporowicz [12] predict that the 1200–1300 cm^{-1} range is most suitable for the detection of structural changes at the methine bridges since isomerization not only shifts the bands but also changes their intensities. This statement is in accordance with general features of the results of model calculations on tetrapyrrole chromophores [20, 21] which predict for the various isomers different changes in Π-bond order upon S_0–S_1-excitation which should yield different Franck-Condon factors. However, more important could be the geometry dependent coupling between CH-deformation and C–C stretching vibrations. In case of stilbene derivatives [22] as a consequence of this mode coupling the frequency of the CH-deformation mode has been found to be a very sensitive probe for deviation from planarity. The observed disappearance of the 1245 cm^{-1} band in the CARS spectra of PCMS titrated PC trimers must therefore be taken as

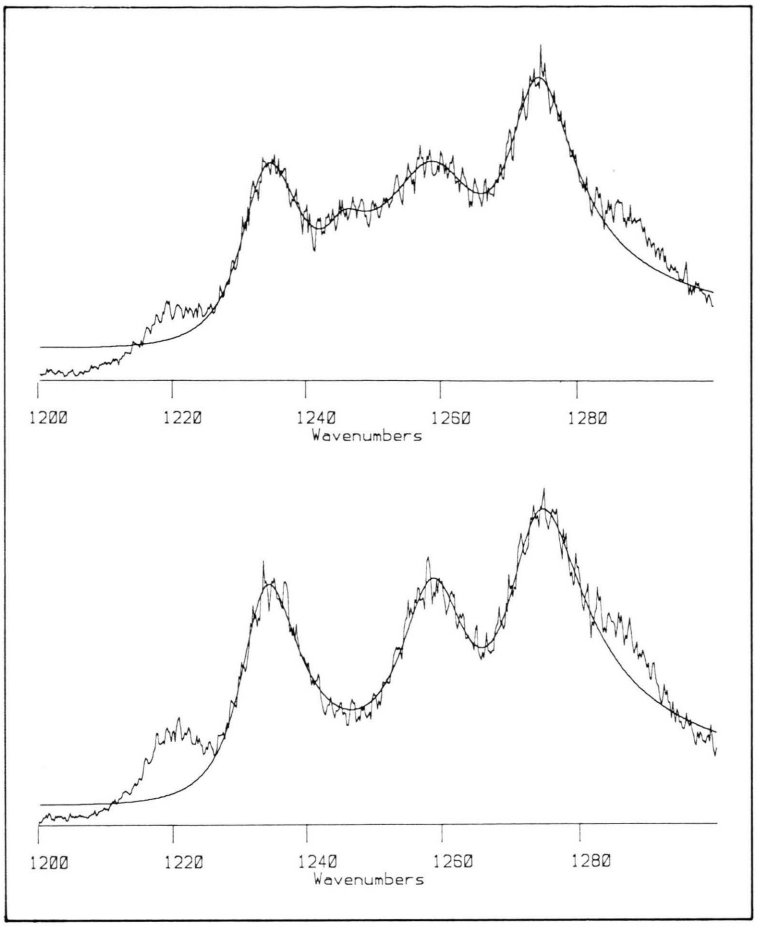

Fig. 3. CARS spectra of CP trimers (λ_p = 640 nm): a) native state (top) and b) titrated with PCMS (bottom). Solid line represents best fit according to Eqn. (1). For more details see text.

good evidence for a rearrangement of ring D of B84. The latter is in the native system in van der Waals contact with cysteine B111 [4]. Binding of PCMS could, therefore, act selectively on this part of the chromophore. Due to the complication of mode mixing, the extraction of information about the structure will necessitate the comparison with spectra of model compounds of known geometry, at least until more precise model calculations can be performed. *E.g.*, we found that in the CARS spectrum of functionally intact phycobilisomes there are two prominent lines in the 1200–1300 cm^{-1} region (F. Baumann, unpublished results); their frequencies $\tilde{\nu}_1$ = 1234 cm^{-1} and $\tilde{\nu}_3$ = 1273 cm^{-1} coincide with the above mentioned values. This dominance of two bands could indicate that in phycobilisomes the geometry of most chromophores is better defined due to the interaction with the linker peptides.

The latter assumption is also suggested by the unusual large width of the CARS band around 1650 cm^{-1}. In the above mentioned CARS spectrum of phycobilisomes, two well-separated bands are observed, the stronger one peaks at 1650 cm^{-1}, the weaker one at 1624 cm^{-1}. Therefore, we have attempted to fit the observed band contours between 1550 and 1700 cm^{-1} by assuming several discrete modes. A good fit of both PC trimer spectra (with and without PCMS) is achieved on the basis of 4 vibrations with frequencies $\tilde{\nu}_1 \approx 1585$ cm^{-1}, $\tilde{\nu}_2$ = 1622 cm^{-1}, $\tilde{\nu}_3$ = 1648 and $\tilde{\nu}_4$ = 1671 cm^{-1}, resp. It appears, however, that not only the relative amplitudes of the four components are different (the contribution of $\tilde{\nu}_2$ drops in the PCMS treated sample to less than one half of that in the native sample), but also the apparent line widths, which have to be introduced in the fit ($\Gamma_2 \approx 14.6$ and 4.5 cm^{-1} for PC with-

out and with PCMS, resp.). In the phycobilisome spectra a $\Gamma_1' \approx 7.5$ cm^{-1} is assigned to the 1581 cm^{-1} band, whereas in native PC trimer, this band, appears broader ($\Gamma_1 \approx 13.0$ cm^{-1}) but not so in the titrated sample. A similar observation is true for the main band around 1650 cm^{-1}. An obvious assumption is that the two bands around 1585 and 1650 cm^{-1} are due to a superposition of two (or more) close lying vibrations. An examination of this hypothesis presupposes that spectra with higher resolution (like Fig. 3) are available and also computer programs which allow line fits on the basis of more than 4 modes. Work in both direction is under way.

Concluding Remarks

The presented CARS spectra document that this technique is a very valuable tool in the attempt of studying conformational changes of chromophores in phycobiliproteins caused either by direct chemical modification, or *via* interaction with the protein. One of the important advantages is its insensitivity towards fluorescence. For this reason the wavelength of the pump beam can be tuned across the red absorption band such that the different chromophores experience different enhancement factors. There is hope that by this technique the structural information, which is needed for a quantitative understanding of energy transfer processes within the antenna complexes, can be collected.

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