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Polarized absorption and fluorescence spectra of single crystals of *C*-phycocyanin

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Polarized optical spectra of thin transparent single crystals of C-phycocyanin from *Mastigocladus laminosus* have been recorded. The isotropic absorption spectra of single crystals differ only at the short-wavelength side of the absorption peak from the corresponding solution spectra. This is presumably due to additional coupling of the β155 chromophores in the crystal. The crystals show dichroism. The position of the absorption maximum shifts between 612 nm (*E* parallel to the optical axis *c*) and 626 nm (*E* perpendicular to *c*) by varying the polarization direction. The observed polarized absorption spectra are compared with theoretical spectra, calculated from the deconvoluted isotropic solution spectra of the individual chromophores (Mimuro, M., Füglistaller, P., Rümbeli, R. and Zuber, H. (1985) Biochim. Biophys. Acta 848, 155–166) and the orientations of the chromophores as determined by X-ray structure analysis (Schirmer, T., Bode, W. and Huber, R. (1987) J. Mol. Biol. in the press). Good agreement is found if the short- and long-wavelength component spectra of the β subunit (β_s and β_f, respectively) are assigned to chromophores β155 and β84, respectively. The fluorescence polarization spectra indicate that the fluorescence originates predominantly from chromophores α84 and β84, thus giving direct evidence that energy transfer is operative in the crystals and that β84 is the long-wavelength chromophore of the β subunit.

Introduction

Phycobilisomes, the supramolecular light-harvesting complexes of cyanobacteria and red algae, are located on the stromal surface of the thylakoid membrane. They are optimized for light absorption and transfer of energy to the photosynthetic reaction centre. C-phycocyanin (C-PC), a protein-pigment complex, is one of their major constituents (for reviews, see Refs. 1 and 2).

C-PC consists of two polypeptide chains, α and β, and bears three open-chain tetra-pyrrole chromophores (phycocyanobilins), which are covalently linked to cysteine residues at positions α84, β84 and β155. The chromophores have been classified as s (sensitizing) and f (fluorescing) type, according to their spectral differences [3]. Energy absorbed by the f chromophores is efficently transferred to the s chromophores (for a recent review, see Ref. 4).

The three-dimensional structure of C-PC from *Mastigocladus laminosus* has been determined at high resolution (2.1 Å) by X-ray structure analysis [12]. In the crystals (space group $P6_3$) the mole-

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Abbreviations: C-PC, C-phycocyanin; p.r., polarization ratio.

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cules are associated to $(\alpha\beta)_3$ trimers [11], an oligomeric form which is also stable in solution (see, e.g., Ref. 5). The crystal unit cell contains two trimers, related by a 2_1 screw axis parallel to *c*. The trimer–trimer contact is rather loose. The chromophores at positions α84 and β84 are far away from the artificial trimer–trimer contact, their nearest neighbours in the crystals being the α84 and β84 chromophores translated by one cell dimension $c = 40.5$ Å. Thus, the β155 chromophores, which extend almost parallel to *c* (see Table I), however, are located near the 2_1 screw axis. The β155 chromophores of two adjacent trimers have a centre-to-centre distance of only 21 Å, pyrrole-ring A approaching ring D of the adjacent chromophore to about 10 Å [13].

In order to determine the path of energy flow the individual spectra of the chromophores have to be assigned to the three chromophores of the C-PC structure. Comprehensive steady-state spectroscopy together with the knowledge of the X-ray structure allowed Mimuro and coworkers [5] to assign β155 and β84 as s ($\lambda_{max} = 594$ nm) and f ($\lambda = 625$ nm) chromophores, α84 having an intermediate excitation energy ($\lambda = 618$ nm). This assignment has been confirmed by spectral analysis of a derivative of C-PC which has a mercury compound bound to cysteine β111 which is adjacent to chromophore β84 [6]. Polarized spectroscopy has been performed on partly oriented phycobiliprotein samples [7–9]. Although single crystals represent the most definite system in this context, only the fluorescence polarization ratio of biliprotein crystals from *Agmenellum quadruplicatum* has been determined so far [10].

In this study we report on the polarized spectra of C-PC crystals and correlate the results with the known arrangements of the chromophores in the crystals.

Materials and Methods

C-PC from the cyanobacterium *Mastigocladus laminosus* Cohn (Genus Fischerella, PCC 7603) has been kindly provided by Dr. W. Sidler and Prof. H. Zuber, Zürich. Its isolation, purification and crystallization have been described elsewhere [14,15].

Spectroscopy of C-PC in solution. C-PC was dissolved in 0.125 M ammonium sulphate (pH 8.5), i.e., almost the same buffer as used for crystallization. For the fluorescence and excitation spectrum measurements the optical density was adjusted to a maximum absorbance of 0.1 absorbance. Absorption spectra were measured with a Perkin-Elmer spectrophotometer 555, fluorescence and excitation spectra with a Perkin-Elmer LS-5 Luminiscence Spectrometer. Cut-off filters were employed to suppress second-order wavelength light. The sensitivity dependence of the analyzer from the direction of the polarization was corrected for. For that purpose the C-PC solution was excited with light polarized parallel to *x* (the direction of the emission beam; for definition of the coordinate system used, see Fig. 3a), in order to generate equal fluorescence intensities I_y and I_z.

Linear dichroism measurements of C-PC crystals. Crystals were grown directly on microscope-slides by the vapour diffusion technique. The salt concentration (ammonium sulphate, pH 8.5) of the droplets, which were spread into thin films on the slides, was 0.05 M ammonium sulphate, that of the reservoir 0.15 M. Thin regularly shaped crystal plates (space group $P6_3$, [11,16]) of light-blue colour (optical density less than 2 absorbance at its maximum) were obtained. Crystals of which the *c*-axes lay parallel (prismatic shape, area dimensions about 0.3 mm (*c*-axis) × 0.15 mm) as well as perpendicular (hexagonal shape, diameter about 0.15 mm) to the slide plane were found.

Polarized absorption measurements were carried out on a Zeiss microspectrophotometer [17]. The diameter of the measured area was about 30 μm. The crystals were not removed form the slides on which they were grown. Drying out was prevented by addition of mother liquor and by covering the assemblies using glass spacers.

Polarized fluorescence measurements of C-PC crystals. A Perkin-Elmer LS-5 luminescence spectrometer with a polarizing filter in the emission light path was used for polarized fluorescence measurements. Long flat crystals (about 0.5 mm (*c*-axis) × 0.2 mm × 0.1 mm) and micro-crystals (about 50 μm × 20 μm × 20 μm) were selected from the batches grown previously for the purpose of X-ray analysis. The crystals were placed on a microscope slide together with a droplet of mother

liquor and covered by a cover slide. The sample was mounted on a goniometer to allow (optical) alignment of the crystal *c*-axis with the vertical *z*-axis and placed at the position of the removed conventional sample holder.

The geometry of the set-up is depicted in Fig. 3a. The slide plane was adjusted at an angle of about 10°–20° with respect to the excitation beam (80°–70° with respect to the emission beam). This arrangement prevents reflection of the excitation beam into the analyzer and also minimizes the effect of self-absorption of the fluorescence emission because the fluorescence, which originates predominantly from the surface of the crystal, has to traverse only a small portion of the crystal to reach the analyzer.

Results

Absorption, excitation and fluorescence spectra of C-PC in solution

The fluorescence spectrum of C-PC in solution (Fig. 1) has its maximum at 637 nm. The absorption spectrum closely superimposes on the excitation spectrum, as well in the visible main band as in the Soret band (Fig. 1). The absorption peak of the aromatic residues of the protein at 275 nm has its counterpart in the excitation spectrum at 280 nm, but the amplitude of the latter is reduced (to about 65% of that of the absorption peak).

An increase in optical absorbance from 0.1 to 0.7 *A* (at the absorption maximum) causes a bathochromic shift of 10 nm of the fluorescence maximum and a flattening of the excitation peak. These effects are most probably due to self-absorption of the fluorescence and to saturation of the excitation, respectively.

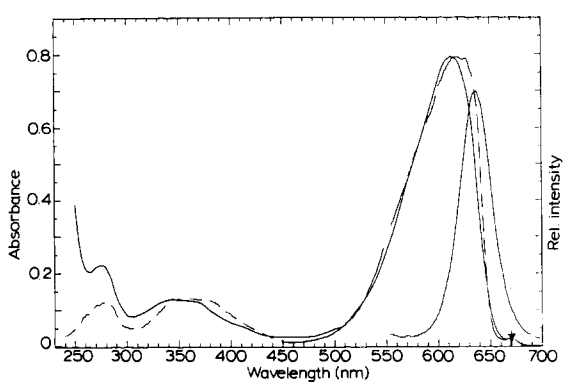


Fig. 1. Absorption (———), fluorescence (———, $\lambda_{ex} = 500$ nm, arbitrary units) and excitation (— — —, $\lambda_{em} = 670$ nm, arbitrary units) spectra of C-PC from *M. laminosus* in 0.125 M ammonium sulphate (pH 8.5).

Polarized absorption of C-PC crystals

Dichroism of thin C-PC crystals which have their *c*-axis lying perpendicular to the polarized white light beam can be observed easily in the microscope. If the polarization direction is parallel to the long axis of the crystal (*c*-axis), the crystals exhibit a violet colour, whereas with the polarization perpendicular to *c* they appear pure blue. Looking down the *c*-axis of the crystals no dichroism can be observed as expected for uniaxial crystals (*c* is the optical axis), the hexagonal plates appearing in pure blue.

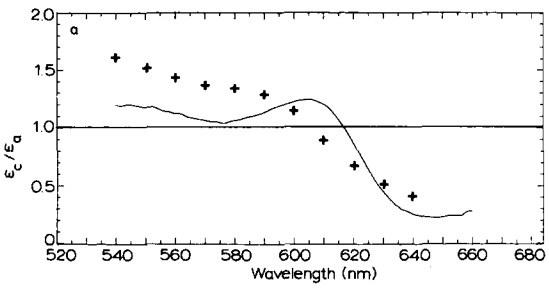
Fig. 2b shows the polarized absorption spectra for different polarization directions. The resulting polarization ratio $\varepsilon_c/\varepsilon_a$ is plotted in Fig. 2a as a function of wavelength.

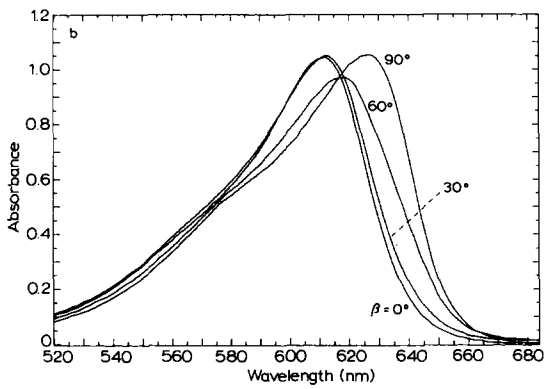
Spectra taken with the beam parallel to the optical axis were identical to those with the beam and the electric vector perpendicular to *c*.

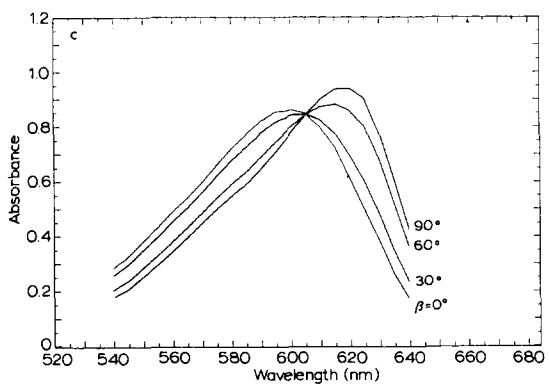
Polarized fluorescence of C-PC crystals

In order to measure the isotropic fluorescence of C-PC crystals with the least as possible artifacts due to self-absorption, microcrystals were investigated. About 20 microcrystals with different orientations (*c* parallel and perpendicular to the slide plane) were placed beside each other on a microslide and excited simultaneously ($\lambda_{ex} = 570$ nm). The position of the fluorescence maximum varied between 643 and 655 nm for different samples. The position of the fluorescence maxima of large flat single crystals varied, however, between 645 and 662 nm, dependent on the crystal size and measuring geometry. This most probably indicates that there is considerable self-absorption even in the case of microcrystals.

Polarized fluorescence spectra of single crystals were recorded with a non-polarized excitation beam ($\lambda_{ex} = 570$ nm), in order to gain increased fluorescence intensity. The set-up of experiment is displayed in Fig. 3a. Fig. 3c shows the fluorescence components I_z and I_y polarized parallel and perpendicular to the crystal *c*-axis, respec-







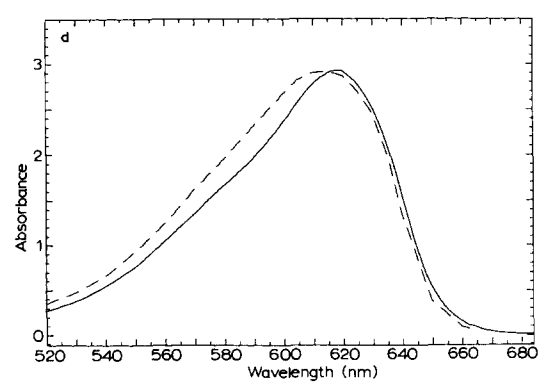
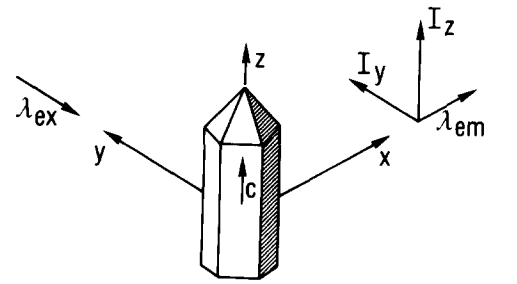
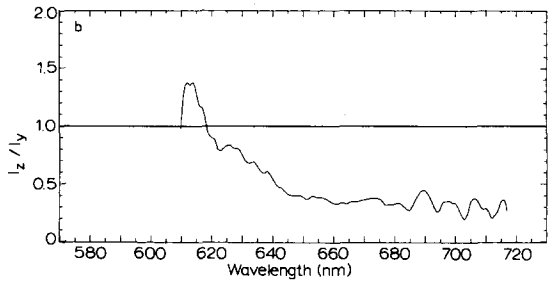


Fig. 2. Polarized absorption spectra of a C-PC single crystal. (a) Dichroic ratio $\varepsilon_c/\varepsilon_a$, calculated from the observed polarized absorption spectra (see Fig. 2b, ———) and calculated from the theoretical polarized absorption spectra (see Fig. 2c, + + +); (b) polarized absorption spectra for $\beta = 0°$, 30°, 60°, 90° (β: angle between electric vector and crystal c-axis; thus, the spectrum for $\beta = 0°$ is proportional to $\varepsilon_c(\lambda)$ and that for $\beta = 90°$ is proportional to $\varepsilon_a(\lambda)$), light ray perpendicular to c; (c) simulation of the spectra of Fig. 2b using the individual spectra of the three chromophores from Ref. 5 and the inclination of the chromophores from the X-ray structure (Ref. 12, see also Table I), for details see discussion; (d) measured isotropic absorption spectrum in solution (- - - - - -) and reconstructed isotropic absorption spectrum of the crystal, $A_{iso} = A(\beta = 0°) + 2A(\beta = 90°)$ (———).





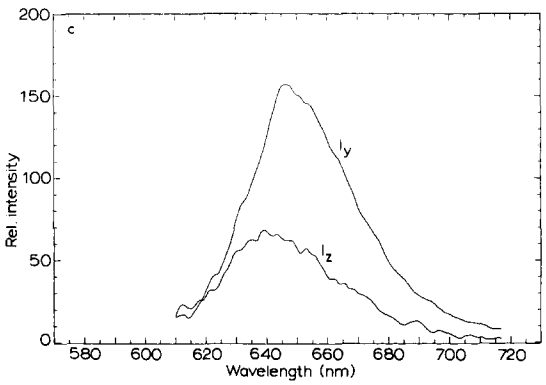


Fig. 3. Polarized fluorescence spectra (accumulated spectra of four measurements) of a single C-PC crystal. (a) Experimental set-up, (b) polarization ratio I_z/I_y, (c) fluorescence components I_z and I_y.

tively. I_z has its maximum at about 639 nm, I_y at about 646 nm. The polarization ratio I_z/I_y is plotted as a function of wavelength in Fig. 3b. The

ratio has a fairly constant value of 0.35 at the long-wavleength side of the fluorescence peaks.

If the crystals are mounted with their *c*-axis parallel to *x*, i.e., with their optical axis parallel to the emitting beam, the two components of the fluorescence are found to be identical within limits of error and – apart from a scaling factor – identical to I_y in the experiment described above.

Discussion

Spectroscopy of C-PC solution

In order to be able to make a meaningful comparison of the spectrum of a C-PC solution with that of the crystals, C-PC was dissolved in a buffer similar to that used for crystallization. The absorption spectrum with its maximum at 613 nm (Fig. 1) does not permit a distinction between monomers or trimers ($\lambda_{max} = 611$ and 615 nm, respectively [5]) or an equilibrium of both under these conditions.

The close resemblance of the excitation and absorption spectrum of the C-PC solution (Fig. 1) indicates efficient energy transfer to the emitting f type chromophore. Not only light absorbed by the visible band (S_1 level), but also that absorbed by the Soret band (S_2 level, $\lambda_{max} = 350$ nm) of the chromophores and – with less efficiency – by aromatic residues of the protein ($\lambda_{max} = 275$ nm) is transferred. For the latter process the coupling of the S_2 level of the chromophore with the S_1 levels of the aromatic residues is probably responsible (see also Ref. 18). The fluorescence spectrum of tryptophan residues overlaps extensively with the S_2 absorption peak of the chromophores.

Many aromatic residues are in the neighbourhood of or even in the Van der Waals contact to the chromophores (Tyr A60, Tyr A74, Tyr A91, Tyr A110, Phe A122, Trp A128, Tyr A129, Tyr B119; Schirmer et al., unpublished data). Note that the only tryptophan residue of C-PC (A128) belongs to this second group.

Linear dichroism

C-PC bears three distinct chromophores. Unfortunately it is not possible to determine the individual polarization ratios for these chromophores because of their large spectral overlap. The directions of the transition dipole moments of the chromophores have been determined recently to a first approximation by fitting the conjugated parts of the X-ray chromophore structures to straight lines [12]. The inclinations α_i of these fitted lines with respect to the *c*-axis are given in Table I. The principal axes of the extinction tensor in the uniaxial crystal system, $\varepsilon_{a,i}$ and $\varepsilon_{c,i}$, are given by (assuming linear molecular extinction coefficients for the chromophores (see Ref. 19):

$$\varepsilon_{a,i} = \tfrac{3}{2}\bar{\varepsilon}_i \sin^2\alpha_i \quad (1a)$$

$$\varepsilon_{c,i} = 3\,\bar{\varepsilon}_i \cos^2\alpha_i \quad (1b)$$

where $\bar{\epsilon}_i$ is the isotropic extinction coefficient. The resulting polarization ratios (p.r.) $\varepsilon_{c,i}/\varepsilon_{a,i}$ are listed in Table I. Note that, due to the different inclinations of the chromophores (α84 and β84 have large components perpendicular to *c*, whereas β155 has a large component parallel to *c*), the p.r. values of α84 and β84 differ very much from that of β155.

The experimental p.r. (Fig. 2a) has its maximum of 1.22 at 605 nm, about the presumed position of the absorption maximum of the β_s chromophores in the crystals (see below), and adopts an almost constant value of 0.25 for wavelengths larger than 640 nm, i.e., for the long wavelength part of the α and β_f chromophore spectra.

Due to the spectral overlap of the chromophores, the experimental p.r. spectrum is flattened considerably with respect to the p.r. of the individual chromophores (Table I). Nevertheless, the location of the p.r. maximum at the blue edge of the spectrum indicates that β155 is the β_s chromophore. On the other hand, the low p.r. at the red

TABLE I

INCLINATION α OF C-PC CHROMOPHORES WITH RESPECT TO THE TRIMER AXIS

The values for α have been taken from Ref. 12.

Chromophore	α	Polarization ratio $= \varepsilon_c/\varepsilon_a = 2\cot^2\alpha$
α84	78°	0.09
β84	119°	0.61
β155	145°	4.08

edge is about the mean vlaue of the individual p.r. values of β84 (= β_f) and α84.

The validity of this assignment was tested by calculating the theoretical polarized absorption spectra $A(\beta)$, where β is the angle between polarization direction and crystal *c*-axis:

$$A(\beta) = \sum A_i(\beta) = cl \sum \varepsilon_i(\beta) \quad (2)$$

where *c* is the concentration, *l* the thickness of the crystal, and the summation is over all three chromophores. The polarized extinction coefficients of the chromophores are given by

$$\varepsilon_i(\beta) = \varepsilon_{a,i} \sin^2\beta + \varepsilon_{c,i} \cos^2\beta \quad (3)$$

where $\varepsilon_{a,i}$ and $\varepsilon_{c,i}$ are defined by Eqn. 1.

The isotropic absorption coefficients $\bar{\varepsilon}_i$ of the individual chromophores were taken from Ref. 5. The resulting spectra $A(\beta)$, calculated with the assignment β_s = β155, β_f = β84 and α = α84, are plotted in Fig. 2c. The spectra are qualitatively very similar to the experimental ones. Note particularly the almost equal maximal absorbances for $A(\beta = 0°)$ and $A(\beta = 90°)$. In contrast, the spectra calculated with the opposite assignment for the β chromophores (not shown) are qualitatively very different from the observed ones.

It is obvious that the experimental spectra (Fig. 2b) are sharper and red-shifted (the isosbestic point is shifted by 12 nm) compared with the theoretical spectra (Fig. 2c). Also, the isotropic absorption spectra of solution and crystal differ somewhat. The isotropic spectrum of the crystal was calculated from its polarized components

$$A_{iso} = A(\beta = 0°) + 2A(\beta = 90°) \quad (4)$$

(see Eqn. 1) and is compared with the solution spectrum in Fig. 2d. The crystals show lower absorbance in the short-wavelength part and the maximum is red-shifted from 613 nm to 618 nm; in the long-wavelength part, however, the spectra are virtually identical.

These differences are most likely due to additional chromophore couplings in the crystal. A red-shift of about 10 nm of the β_s chromophore, which has its absorption maximum at 594 nm in solution [5], and almost unmodified spectra of the β_f and α chromophores would explain most of the differences. This again leads directly to the assignment of chromophore β155 as the β_s chromophore, because the X-ray structure shows that it is the only one for which a strong additional coupling to other chromophores can be expected upon crystallization.

The same chromophore assignment has been proposed by other authors. Mimuro et al. [5], performed steady-state spectroscopy on different C-PC aggregates. Siebzehnrübl et al. [6] modified chemically Cys β111, which is in close proximity to β84, and investigated the concomitant spectral changes. The assignment is also in line with linear dichroism measurements on oriented phycobilisome rods [9], which showed that the short-wavelength transition moment is almost parallel and the long-wavelength almost perpendicular to the rod-axis.

Polarized fluorescence

It is difficult to obtain reliable fluorescence spectra of samples of high optical density. In most cases a correction for the effect of self-absorption is required. Measurements on optically dense (0.7 absorbance) C-PC solutions demonstrated the pronounced red-shift of the fluorescence maximum. For the fluorescence spectra of crystals we tried, therefore, to minimize the pathlength of the emission light in the crystals by an appropriate geometrical set-up. Red-shifts of about 6–25 nm with respect to the C-PC solution fluorescence were observed, depending on crystal size and relative arrangement of the crystal with respect to the emission light path. Thus, most or all of these red-shifts can be attributed to artifacts, namely self-absorption. This would imply that the fluorescing chromophores are almost not affected by the crystal packing. This is in line with the unmodified long-wavelength part of the isotropic absorption spectrum (Fig. 2d). It is possible that the large red-shifts observed by several authors [10,13] for biliprotein crystals are also mainly caused by self-absorption.

The fluorescence maximum of a single crystal at about 640–645 nm (Fig. 3c) has a low p.r. of about 0.35, i.e., it has a large polarization component perpendicular to *c*. The p.r. remains constant for longer wavelengths and compares favourably with the mean value of the individual p.r. values

of α84 and β84 (Table I). Thus, it is most probable that both chromophores are emitting.

The excitation energies of α and β_f do not differ much. It has been pointed out by others (Sauer, K. et al., unpublished data; see also Ref. 20) that there must be considerable back-transfer from β_f to α, therefore. In C-PC trimers (and in the crystals) chromophores α84 and β84 are separated only by 21 Å (centre-to-centre) and observed lifetimes of 10–100 ps have been attributed to energy transfer between these (for a review, see Ref. 2). Therefore, it is probable, that both chromophores are in equilibrium and are emitting.

The non-constant spectrum of the fluorescence p.r. indicates that more than one chromophore is emitting. The increase of the p.r. spectrum towards shorter wavelengths is most probably due to leakage fluorescense of the 'blue' sensitizing chromophore (β_s). Accordingly, its emission dipole must have a much larger component in the *c*-direction than the 'red' fluorescing chromophores. This is consistent with the model of β155 being the β_s chromophore.

Priestle et al. have performed polarized fluorescence spectroscopy on C-PC crystals from *Agmenellum quadruplicatum* [10]. Although these crystals are not isomorphous with the crystals we have investigated here, the inclinations of the chromophores are essentially the same ([12,21]). The fluorescence polarization $p = -0.17$, which they obtained by exciting the crystals with light polarized parallel to *c* and which refers to the major peaks of the polarized component spectra can be converted into a p.r. value of 0.71. The corresponding p.r. of our measurements is 0.44. Considering that in the measurements of Priestle et al. predominantly the β155 chromophores were excited, the agreement is reasonable.

Conclusion

The polarized absorption and fluorescence spectra of C-PC crystals presented in this study and the modification of the isotropic absorbance in the short-wavelength side of the absorption peak upon crystallization can be interpreted consistently with the assignment of the β_s-chromophore to β155 and β_f to β4. Accordingly, in the trimers the net energy flow is directed from the peripheral β155 chromophores to α84 and the central β84 chromophores.

Acknowledgments

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