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PHOTOCHEMISTRY AND PHOTOPHYSICS OF C-PHYCOCYANIN

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Introduction

Phycocyanin (**PC**) belongs to a group of pigments functional for light-harvesting in cyanobacteria, red and cryptophyte algae. In the former two classes of organisms, it is a major constituent of phycobilisomes, the light-harvesting complexes located at the outer surface of the photosynthetic membrane. There, it absorbs light energy in the spectral range between 580 and 640 nm, and transfers it via a second biliprotein, allophycocyanin (**APC**), to the chlorophyll within the membrane. In many species, PC also accepts energy from a third type of biliprotein, e.g. phycoerythrin (**PE**), thus acting as an intermediate carrier in the energy transfer from the latter to **APC**.

The simplest **PC**, which is found in cyanobacteria (**C-PC**), contains three chromophores of the dihydrobilindion type, each of them being attached covalently to the apoprotein via a single thioether bond to cysteine. The same chromophore is present in **APC**, and a chromophore differing only in one of the β-pyrrolic substituents is found in the plant photomorphogenetic pigment, **phytochrome**. According to the different functions of these three pigments, the properties of the chromophores in each of them are quite different from each other, and they all differ considerably from the properties of free pigments bearing this type chromophore (1). The factors responsible for the different adaptations of these structurally so similar chromophores are still only partly understood. From reversible denaturation studies in **C-PC**, it appears that they are mostly due to non-covalent protein-chromophore interactions.

The recent elucidation of the x-ray structures (2,3) of **C-PC**s from two different organisms, has greatly advanced our knowledge of these pigments. It has for the first time in any photosynthetic antenna system become possible to 'look' at the native chromophore structures on a molecular level, and to obtain direct information on their conformations and relative orientations. This renders it possible to test the viability of theoretical models applied in the calculation of the their spectral properties, of the energy transfer pathways, the kinetics among them, etc., by using the structural data as input parameters.

This report summarizes recent work carried out along these lines in München. It contains data on the photochemistry of **C-PC** from the cyanobacterium, Mastigocladus (M.) laminosus, which are compared to the respective properties of the photochromic plant photoreceptor, phytochrome, as well as theoretical and experimental results on the energy transfer in aggregates of different sizes from this chromoprotein.

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Chromophore structure

The three chromophores of **C-PC** are bound to the protein via single thioether bonds to cysteine 84 on the α-subunit (cys α-84), and to cys β-84 and cys β-155 on the β-subunit. A linkage to the C-3 ethyl-substituent of the hydrogenated ring A had been established for chromophores of all type of plant biliproteins including phytochrome (1,4). A more complex binding pattern has recently been proposed, which involves a different linkage and structure for the chromophore β-155 (5). Instead of being hydrogenated at ring A, this chromophore is hydrogenated at ring D and bound via the 'exo' 18-thioethyl substituent.

All chromophores are present in more or less extended conformations (2,3), which account for the observed absorption increase of the red bands, and a concomitant decrease of the near-uv bands (1). It is also likely, that the chromophores have a reduced conformational mobility, which accounts for their high fluorescence quantum yields, and for their inertness to a variety of chemical reagents (metal ions, reducing agents) known to react readily with the free chromophores (see 1 for leading references). The confirmation of this general structural principle in the crystal structure of C-PCs from two different organisms (M. laminosus and Agmenellum (A.) quadruplicatum), makes it likely that the spectrally similar chromophores of **APC** and phytochrome have similar native structures as well.

Chromophore assignment

While the x-ray results supported this general structure principle, they show on the other hand pronounced differences among the details of the chromophore conformations, of the binding sites and of likely interactions with the apoprotein. Such differences account for a variety well documented spectroscopic and chemical results indicating the presence of a set of distinct chromophores in almost any phycobiliprotein (1). In **C-PC**, these allowed the definition of three distinct chromophores, e.g. α-1, β-1 and β-2.

In the case of **C-PC** from M. laminosus, the following data were combined for the spectral resolution: The integral pigment can be separated into two subunits, the α-subunit bearing only one chromophore, and the β-subunit bearing two chromophores. Since the absorption spectra of the two subunits -weighted properly according to the subunit stoichiometry- add up to the spectrum of the monomeric pigment, it is likely that the states of the chromophores remain unchanged during subunit separation, and that strong inter-subunit chromophore-chromophore interactions are absent. This yields directly the required absorption and fluorescence spectra of the α-subunit, and reduces the problem to the resolution of the β-subunit spectrum. The presence of two spectrally distinct chromophores is this subunit is derived from several lines of evidence:

Reversible photochemistry: Native **PC** has a high fluorescence quantum yield. Its photochemistry is characterized by an irreversible bleaching, which has a low quantum yield (0.4%) and proceeds probably via the triplet state because it is slowed down in the presence of oxygen. This irreversible reaction occurs with a similar quantum yield also in phytochrome (Scheer, unpublished results). Addition of urea to **PC** causes a gradual, reversible unfolding of the protein and a concomitant loss of its interactions with the chromophore. At 8 M urea, the protein is completely denatured, and the chromophores then attain the properties characteristic of free bile pigments (1). Here, the fluorescence is greatly reduced ($\phi < 10^{-3}$). The photochemical reactivity is increased, but it is again irreversible, leading to a variety of tri- and tetrapyrroles absorbing at shorter wave lengths. The onset of unfolding at intermediate urea concentrations is characterized by a reduction of fluorescence and the concomitant occurrence of a reversible photochemical reaction, which is maximum at about 5 M urea. Similar reactions have been observed as

well with other denaturants at moderate concentrations, and they have been related to the reversible Z,E-isomerization of the phytochrome chromophore, and to the primary reactions of the less well understood phycochromes (see 1). If the isolated subunits of **C-PC** from M. laminosus are titrated with urea this reaction is negligibly in the α-subunit, but much more pronounced in the β-subunit. The absorption difference spectrum of the latter shows a single negative band in the visible spectral range peaking at 624 nm, which is considerably to the red of the absorption maximum at 606 nm, and its shape is similar to that of a typical bile pigment (6). This suggests, that only one of the two chromophores on the β-subunit is susceptible to this reaction, and that this chromophore absorbs at longer wavelengths than the second, inactive one.

Fluorescence polarization: The fluorescence polarization spectra of nearly all phycobiliproteins show distinct discontinuities, and the anisotropy rises in discrete steps towards longer wavelengths (13, 14, see also 1). Since the red absorption band of bile pigments corresponds to a single electronic transition, this has been interpreted as the result of several distinct chromophores being present, with different orientations and different absorption spectra, among which energy transfer occurs. The fluorescence polarization spectrum of the β-subunit of **C-PC** from M. laminous shows two distinct regions of anisotropy (7). Below 600 nm, it is nearly wavelength independent about 0.2, and then rises sharply to 0.4. There are, therefore, at least two chromophores present absorbing below and above this threshold wavelength. Assuming a similar Stokes' shift for the fluorescing chromophore of the β-subunit (λ_{max}^{fluor} = 643 nm) and the one of the α-subunit (λ_{max}^{abs} = 616, λ_{max}^{fluor} = 641 nm), an absorption around 620 nm can be estimated for the former.

Circular dichroism: The cd spectrum of the α-subunit shows a single positive band in the visible spectral region peaking close to its absorption maximum. The cd-spectrum of the β-subunit also shows a single positive peak. Its intensity is decreased by 40% on a molar basis, increased by 20% on a chromophore basis, and centered well to the blue (590nm) of the absorption maximum. At longer wavelengths, the band trails slightly and indicates the presence of a smaller, much less intense band. This result, which has been reported independently by Mimuro et al. (7), is again, best interpreted as to arise from two different chromophores, one of them is strongly optically active and absorbs around 595 nm, the second one is much less active and absorbs above 610 nm.

Curve resolution of absorption spectrum: To better define the absorption bands of these chromophores, the spectrum of the β-subunit was resolved by computer analysis. It was assumed, that the shape and width of the bands were identical to that of the alpha chromophore, and the starting wavelengths for the analysis were estimated from the aforementioned data. The absorption band was fit best with two bands peaking at 598 and 622 nm, with molar absorptivities of 92 and 60%, respectively, of that of the α-subunit. For an estimation of the individual fluorescence spectra, similar Stokes' shifts and fluorescence lifetime was furthermore assumed. The resulting spectral data are given in table 1 and compared to a similar analysis by Mimuro et al. (7).

Chemical reactivity and assignment to binding sites

Whereas the aforementioned results allowed the distinction of two spectrally defined chromophores on the β-subunit, the correlation between the different chromophores defined above (α-1, β-1 and β-1), and the ones defined by their binding sites (α-84, β-84, β-155), respectively, was still lacking. Following a suggestion by Schirmer, Bode and Huber (2,3), we have been able to make this assignment by treatment of **C-PC** with organic mercurials (8). There is only a single free cysteine present in **C-PC** located at position β-111 (9), which is very close to the chromophore β-84, but more than 22A from chromophores β-155 and β-84 (2,3). This cysteine is the only site to which mercurials were

bound in heavy-atom derivatives of **C-PC** crystals, and it was expected that a binding of the bulky reagent at this position would have a discernible effect on the spectral properties of the neighboring chromophore, and only on this one. In solution, titration of **C-PC** from M. laminosus leads to a decrease of absorption at about 620nm, and a concomitant, albeit smaller, increase around 650nm, and a general absorption increase in the near-uv spectral region (Fig. 1). In the β-subunit, the same spectral changes are observed, but the relative amplitudes are increased. Titration experiments showed, that the reaction is complete after the addition of 1 ± 0.2 moles of the mercurial (p-chloro-mercuribenzenesulfonate, PCMS) per mole of **C-PC** or β-subunit , respectively, and that the reaction can be reversed to more than 80% by addition of thiols. In the α-subunit, the reaction is negligible (Fig.1).

These findings are interpreted in the following way:

1: The reaction site is the single free cys-111 on the β-subunit, similar to the situation in the crystal.
2: No irreversible reaction occurs between mercurials and the chromophores, because the α-subunit is inert.
3: Since the difference absorption maximum is on the red side of the absorption maximum in the β-subunit, it must be related to a spectral change of the long-wavelength absorbing chromophore, e.g. the one defined above spectroscopically as β-1.
4: Due to the spatial relationships, this chromophore is the one bound to cys-β-84, e.g. chromophore β-84 is identical with chromophore β-1.
5: The indirect effect of the mercurial binding on the absorption of chromophore β-84 involves probably a conformational change from the native, extended to a more denatured, cyclic-helical conformation, as indicated by the overall decrease of absorption in the red, and an increase in the near-uv spectral region (1).
6: The resulting spectral data for the three chromophores are summarized in table 1.
7: Due to the inertness of the chromophores to a direct reaction with mercurials, these reagents are suitable to test the accessibility of cys-111 in higher aggregates, and at the same time to identify the absorption of the β-84 chromophores.

	α-84	β-1 = β-84	β-2 = β-155
This work	616(120)	622(72)	598(106)
Mimuro et al. (7)	618(108)	624(103)	594(113)

Table 1: Absorption maxima [λ_{max} (ϵ x 10^{-3})] of the individual chromophores of C-phycocyanin from Mastigocladus laminosus.

The assignment (β-1 = β-84, β-2 = β-155) agrees with the ad-hoc assignment of by Mimuro et al. (7), and there is also a reasonable agreement of the spectral data of the individual chromophores. The most pronounced differences are the position of the β-155 absorption, which is displaced to the blue by appx. 4nm by these authors, and a lower absorptivity of the β-84 absorption in our calculations.

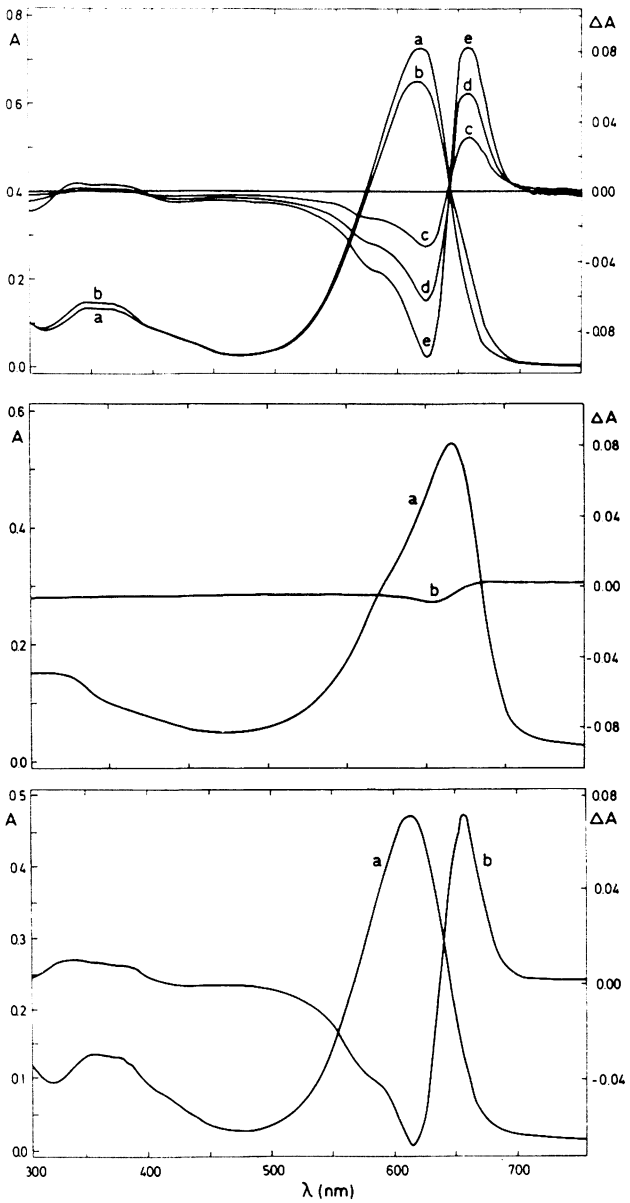

Fig.1: Reaction of trimeric C-phycocyanin from Mastigocladus laminosus (A), its α- (B) and β-subunit (C). Figure (A) shows both the absorption spectra before (a) and after (b) reaction with **PCMS** (1 mole / mole **C-PC**), and the difference spectra during the titration (c,d,e). The other figures show the absorption spectrum before addition of **PCMS**, and the absorption difference spectrum after its addition (1 mole / mole subunit). All reactions in potassium phosphate buffer (50mM, pH 7.5).

Kinetics of energy transfer

Energy transfer in phycobiliproteins has been suggested to occur mainly via the induced dipole or Förster type mechanism. The main arguments to this have came originally from the absence in most biliproteins, including **C-PC** from M. laminosus, of strong, s-shaped signals indicative of strong exciton couplings, and from polarization spectroscopy (see 1). Over the past 5 years, the amount of kinetic data on energy transfer has tremendously increased. It has been found in particular, that the fastest energy transfer processes take place on a time scale in the order of tens of picoseconds or even less (10,11). With all the necessary information at hand, it was then intriguing to test if such fast kinetics could be matched by theory on the basis of a pure Förster transfer mechanism.

The rate constant for energy transfer is described by:

$$k \simeq \frac{\kappa^2}{R^6} \int F_{Donor} \cdot A_{Acceptor}$$

The major variables in this equation are the distance R between the transition dipoles of the donor and acceptor chromophores, the relative orientations factors κ of the two, and the overlap of the fluorescence of the donor (F_{Donor}) with the absorption of the acceptor ($A_{Acceptor}$). The only other variable in the equation, e.g., the refractive index of the medium, is hidden in the proportionality constant. A value of 1.56 has been used throughout. The first two pieces of information have been taken from the x-ray data of Schirmer et al. for **C-PC** from A. quadruplicatum (3). This pigment has a very similar chromophore arrangement as **C-PC** from M. laminosus (2), but it has been resolved to higher accuracy. Moreover, it crystallizes as stacks of hexamers rather than trimers, so that orientations and distances between chromophores on different trimers and hexamers are available. These authors have tabulated the distances of the centers of gravity of the π-systems for all chromophores, as well as the relative orientations of chromophores as defined by the masses of the atoms present in the chromophore π-systems projected on a straight line. These distances and orientations do, therefore, not strictly correspond to the transition dipoles, but rather to their reduced masses. In view of the elongated structure of the chromophores, it is likely, however, that the deviations are reasonable. The overla, integral was finally calculated from the individual absorption and fluorescence bands of the three different chromophores as given in the top row of table 1.

The details of these calculations, which are the result of a continuing cooperation between K. Sauer and our group, are being published elsewhere (12), and a summary is presented in the poster abstracts of this conference. There is a good agreement with most of the currently available experimental data. The calculations show an increased transfer rate with increasing aggregate size. They support, in particular, a preferential energy transfer along the rods of trimer-stacks, as compared to energy transfer within trimers. Such a preferential transfer would greatly facilitate the funelling of energy towards the reaction centers. According to these results, the energy transfer in **C-PC** can be accounted for well by the Förster mechanism, and the flow of excitation energy in these moderately complex aggregates can be analyzed on a molecular basis. Since the data can be transformed readily to mimic a variety of experimental conditions (differen excitation and emission wavelengths, static and dynamic depolarization), a comparison with new data and/or assistance in the choice of experimental conditions are expected to further evaluate this conclusion critically.

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