FIFTH INTERNATIONAL SEMINAR ON

ENERGY TRANSFER IN CONDENSED MATTER

Structure, Conformation and Function of Molecular Systems Largely Related to Photosynthesis

Proceedings

EDITED BY P. PANČOŠKA AND J. PANTOFLÍČEK

Prague, July 8 – 12, 1985 Czechoslovakia

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FLUORESCENCE KINETICS OF C-PHYCOCYANIN FROM *MASTIGOCLAUDUS LAMINOSUS* DEPENDING ON ITS STATE OF AGGREGATION

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Photosynthetic organisms cover most of their energy needs with sunlight. They have consequently developed a variety of adaption mechanisms to compete efficiently for it. In higher plants, a dominant mechanism is the growth towards the light. Aquatic and microorganisms adapt commonly by chromatic adaptation of the photosynthetic antenna. The Chlorophylls a and b are rather inefficient in collecting green light, and several additional pigment systems have evolved to fill this hole in the action spectrum.

The phycobiliproteins are one such group of antenna pigments. They are used in cyanobacteria, red alga and cryptophytes (1). In the former two, they are highly aggregated together with colorless linker polypeptides, in the phycobilisomes. These are microscopic structures situated at the outer surface of the thylakoid membrane, which transfer their excitation energy efficiently (quantum yield ≥95%) to the chlorophyllous reaction centers within the membrane. The phycobilisomes show a remarkable structure. They consist of a central core of allophycocyanin (APC) to which rods of phycocyanin (PC) and phycoerythrin (PE) are attached. This morphology matches the energetic ordering of the chromophores required for an energy transfer chain from PE via PC and APC to the chlorophylls and reaction centers.

This report deals with one major pigment of the phycobilisomes, e.g. PC from two cyanobacteria (Spirulina platensis and Mastigocladus laminosus). Three subjects shall be considered: The molecular structure and covalent protein linkages of the chromophores, the in situ structures of the chromophores as determined by chromophore-protein interactions, and the fluorescence kinetics within PC aggegates of increasing complexity.

Chromophore structure

PC contains like the other phycobiliproteins linear tetrapyrrolic chromophores. They are distinct from the mammalian type bile pigments, e.g. biliverdin, by the presence of one hydrogenated ring (generally ring A) and by covalent linkages to the apoprotein. The thioether linkage shown in fig.1 is well established. Additional linkages have been discussed (see references in 1) which are less stable under the conditions hitherto used for structure elucidation, e.g. chromic acid degradation and proteolysis.

We have recently investigated Ehrlich's diazo-reaction (2) as the key reaction for a milder degradation method. In a three-step sequence (3), PC is first denatured, then reduced with sodium borohydride, and the resulting "phycorubin" finally treated with aromatic diazonium salts. The chromophores are thereby split into halves at the central methylene bridge. Depending on the presence or absence of a covalent linkage, the resulting two azo-dipyrromethenones remain either attached to the peptide chain, or become free and can be separated from the former by solvent extraction or gel chromatography. The sequence has two advantages: Information is gained on the C-5 and C-15 methine substituents (which is lost during chromic acid degradation), and the entire reaction sequence can be carried out below 4°C and at neutral pH. A cleavage of labile bonds, or the formation of new bonds is therefore precluded. A quantitative study has now been performed with PC from the two aforementioned species and their subunits. It was found, that there is always only that one dipyrromethene chromophore attached covalently to the protein, which contains the hydrogenated ring and hence the thioether bond. The fully unsaturated dipyrromethenone chromophore corresponding to the other half of the original PC chromophores was free, and it was identical with the respective ring C,D-fragment of the diazo-product of mesobilirubin. The structure of the PC chromophores is thus the one shown in fig.1. Rapoport and Glazer (4) have reported after the completion of this work, that one of the three chromophores in PC from Synechococcus 6301 is bound to the protein and hydrogenated at ring D. We did not find the expected azo-product in the products derived from S. platensis or M. laminosus, but the sensitivity may not have been sufficient for the detection of one out of three chromophores.

Native state of the chromophores

The spectra and most other properties of PC are quite unusual for bile pigments of the structure shown in fig.1. Free chromophores of this type have only moderate absorptivity in the visible spectral range, and their radiative lifetimes are short (20-70psec for bilipeptides depending on binding sites and isolation procedure). Both properties render them poor candidates for antenna pigments from the biophysycists point of view. These and other properties detrimental to photosynthesis, are greatly improved in phycocyanin. The native pigment has an absorptivity which is almost one order of magnitude larger, and it has a high fluorescence quantum yield (radiative lifetimes ≈1.5nsec). Since these functionally important properties are reversibly abolished by denaturation of the protein, e.g. with 8M urea, they must be entirely due to non-covalent chromophore-protein interactions.

A systematic survey of bile pigments structurally related to I and the effect of various treatments of the chromophores (pH, complexation) on their properties has led to a model (fig.2), in which the chromophores are rigidly fixed in an extended conformation (1). Free bile pigment chromophores are by contrast rather flexible and have predominantly cyclic conformations. The energetically unfavorable conformation of the native PC chromophores, which is essential to their function as light harvesting pigments, has recently been substantiated by the high-resolution x-ray structure of PC from M. laminosus (5).

The picture is further complicated by the fact that PC contains three chromophores at two different subunits. The α-subunit carries one, the β-subunit 2 chromophores. Two of them (α and β1) are at binding sites with similar primary and tertiary structure (5), the third one is distinct with regard to its binding site. It is also possible to distinguish three distinct chromophores spectroscopically from the absorption and circular dichroism properties of PC subunits. The isolated α-subunit absorbs maximally in the red at 618 nm, and a similar component is present in integral PC (fig.3). The β-subunit absorbs at 606 nm, and two chromophore absorptions (≈585 and 615nm) can be resolved by circular dichroism spectroscopy and by fluorescence polarisation (6). The cd-anisotropy of the β-subunit is only 60% of the α-subunit, and its visible maximum shifted by 15 nm to the blue (fig.4). Thus it contains one cd-active, twisted chromophore absorbing at the blue side of the unstructured absorption band, and one cd

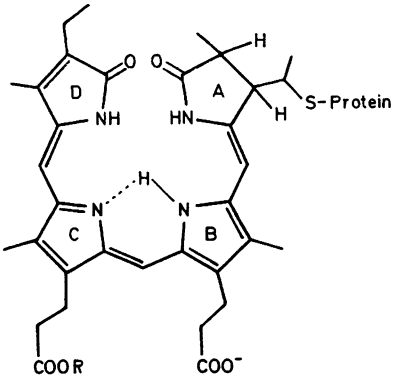

Fig.1: Molecular structure of phycocyanin chromophore drawn in the cyclic-helical conformation characteristic for free bile pigments.

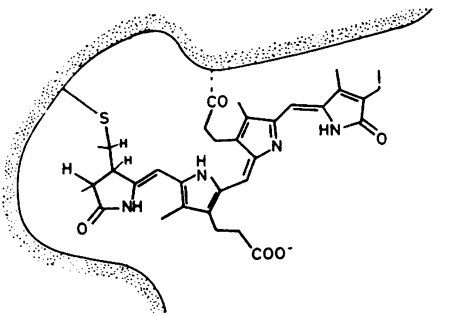

Fig.2: Native conformation of phycocyanin chromophore. Schematic representation of an extended conformation which is suggested from spectroscopic data.

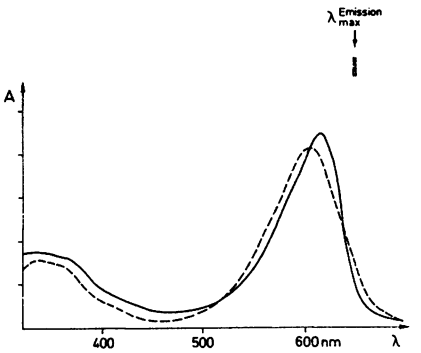

Fig.4: Circular dichroism spectra of subunits of phycocyanin from Mastigocladus laminosus

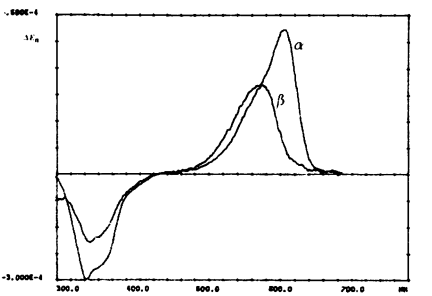

Fig.3: Absorption spectra of subunits of phycocyanin from Mastigocladus laminosus

inactive (planar?) chromophore absorbing at the red side of the absorption maximum. However, a correlation between these two absorption bands of the β-chromophore and the known binding sites is presently not yet possible.

Fluorescence Kinetics

In order to elucidate the fluorescence kinetics of PC and its implications on the energy transfer, we have begun to vary systematically the complexity of PC aggregates and then studied their polarized time resolved fluorescence (7-9). Five different aggregation states have been investigated: The α- and β-subunits in their monomeric states, the (αβ) heterodimer (= monomer), the (αβ) heterohexamer (= trimer) and entire phycobilisomes. In each case, the pigments were excited with a tunable mode-locked dye laser (photon flux at sample $\leq 10^{13}$photons cm $^{-1}$ pulse $^{-1}$, pulse width ≈1psec fwhm, 80 MHz repetition rate). The polarized, time-resolved fluorescences decay curves have been recorded with a synchroscan streak camera. From the decay traces with the emission polarizing filter being oriented parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to the polarisation filter of the exciting light, the isotropic fluorescence ($I_{\parallel} + 2I_{\perp}$) and the anisotropic fluorescence (expressed as difference function $I_{\parallel} - I_{\perp}$, see ref.9) have been caculated.

All decay curves were originally fit under the assumption of a biexponential decay function. Under idential conditions (λ,exc. = 600nm, λ,em. ≥620nm), the isotropic decay rates of the subunits, the monomer and the trimer were similar, but the decay rate is sharply increased in the phycobilisomes due to the presence of an acceptor, e.g. APC. The high rate of transfer to acceptors absorbing at longer wavelengths can be seen directly under conditions conditions of short-wavelength (≤590nm) excitation and long-wavelength detection (≥620nm) (see below and fig.5). It necessitates the introduction of an additional rise-term of the fluorescence. In the anisotropic decay of the increasingly complex aggregates, there is a pronounced increase in the depolarisation rate between monomer and trimer. A second increase is seen between trimer and phycobilisomes, for which the anisotropic decay was beyond the detection limit of the system.

Since rotational depolarization of the chromophores fixed to the proteins (molecular weights bettween 16 and several 100 kDa) is

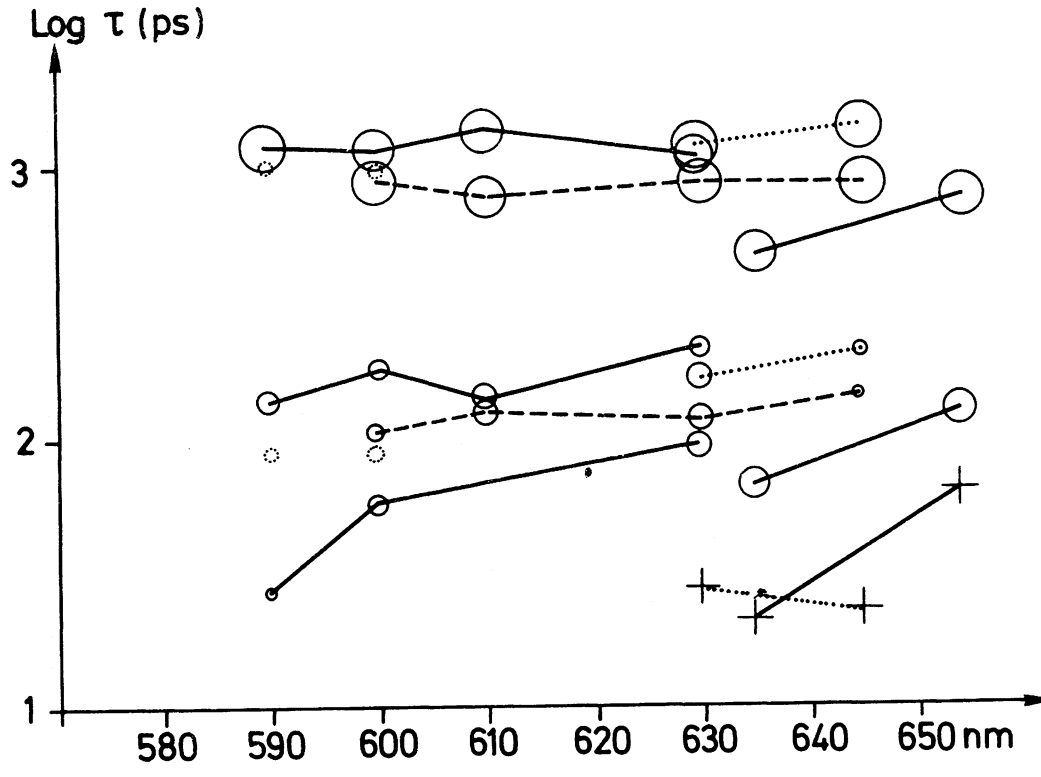

Fig.5: Isotropic fluorescence decay of phycobilisomes from Matigocladus laminosus. Characteristic emission wavelengths (selected by cut-off filter below 630nm, interference filter at longer wavelengths) are given on the abcissa. Excitation wavelengths were at 580nm (———), 590nm (-----) and 610nm (.....). The circles correspond to decay terms, the crosses to rise terms. The area of the circles is proportional to the intergrated intrensities of the respective decaying components. All experimental data were fit under the assumption of a tri-exponential decay law. If bi-exponentials gave an equally good fit, the data are given as dotted circles (590 and 600nm emission, excitation at 580nm).

negligible on the observation time scale, the depolarisation has been interpreted as being due to energy transfer. The increased depolarisation rate in the trimer is rationalized as a result of an increased number of possible acceptors and by the fact, that the closest chromophore-chromophore distance in the trimer does not occur within a monomeric unit, but rather between β- and α-chromophores located on adjacent monomers (5). The absence of a polarized component in the fluorescence of the phycobilisome is then due to an even increased number of acceptors, i.e. essentially no leakage fluorescence from directly excited chromophores is observed. (No data are hitherto available for inter-chromophore distances between trimeric units in the phycobilisome rods.)

Although most decays can be fit well under the assumption of a bi-exponential decay law, more than two components are sometimes necessary. This is in particular true under conditions were rise-terms are encountered, e.g. if the differences between excitation and detection wavelengths ($\Delta\lambda$) become substantial (fig. 5). If a series of distinct energy transfer steps is assumed to occur within a given assembly, a set of constant decay times with varying amplitudes and signs is expected for any selected pairs of excitation and emission wavelengths. An analysis allowing for three exponentials in the fluorescence of phycobilisomes does indeed show a nearly constant rate-constant for the long-lived component, which is probably mainly due to APC (fig.5). However, the short term(s) are variable and increase with increasing $\Delta\lambda$. This indicates, that more than one time constant may be hidden under these terms, whose relative amplitudes change with the probing wavelengths. Experiments with phycobilisomes from a different species have required a fit with four exponentials, whose rate constants are furthermore depndent on the length of the rods (10).

A high complexity of the energy transfer even within isolated phycobiliproteins is indicated by recent results of Holzwarth et al. (11) obtained by single-photon timing techniques. The fluorescence decay data for trimeric aggregates of PC were subjected to a global analysis. It was necessary to use three or, better, even four components, and in particular the fast decaying components were also dependent on the presence or absence of colorless linker polypeptides. This indicates, that even in a relatively simple system, there is already a quite complex energy transfer pattern. It is, however, presently not yet clear which of the energy transfer steps seen in isolated biliproteins are relevant to those observed in integral

phycobilisomes. More work is needed to relate these processes to each other.

Acknowledgements: Work presented here was supported by the Deutsche Forschungsgemeinschaft. The following coworkers were engaged in it: R.Fischer, W.John, W.Kufer, S.Siebzehnrübl, O.Schmid and G.Schmidt (Botanisches Institut), and P.Geiselhart, P.Hefferle and T.Mindl (Institut für Physikalische Chemie).

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