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Spectroscopic properties of the C-phycocyanin-allophycocyanin conjugate and the isolated phycobilisomes from *Spirulina platensis*

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Abstract

C-phycocyanin (CPC) and allophycocyanin (APC) were purified from *Spirulina platensis*, then the CPC was attached covalently to the APC by reacting their ε-amino groups. The excitation energy could be transferred from the CPC to the APC in the CPC-APC conjugate. Intact phycobilisomes (PBS), consisting of CPC, APC, colourless linker polypeptides, and APC B or L_{cm}, were isolated from *S. platensis*. Spectroscopic properties of the isolated PBSs kept at 20 °C for various times showed that the connection between the APC and the APC B or L_{cm} was looser than that between the CPC and the APC in the isolated PBSs. The CPC-APC conjugate was more stable than the isolated PBSs, and the linker polypeptides had a minor influence on the excitation energy transfer characteristic between different phycobiliproteins in the PBS.

Additional key words: absorption and fluorescence emission and excitation spectra, covalent bond, linker polypeptides.

Introduction

Phycobiliproteins (phycocyanin, phycoerythrin, phycoerythrocyanin and APC) are intensively coloured, highly fluorescent, water soluble chromoproteins. They are composed of two or three kinds of subunits (α, β and γ) that carry several open-chain tetrapyrrole prosthetic groups, called phycobilin, covalently bound to cysteine residues *via* thioether bonds. The phycobilins include phycoerythrobilin (PEB), phycourobilin (PUB), phycocyanobilin (PCB), and phycobiliviolin (PXB). All phycobiliproteins have the tendency to form higher aggregates of general structure $(\alpha\beta)_n$, where n is preferentially 2 for cryptomonad phycobiliproteins, while for cyanobacteria and rhodophycean phycobiliproteins *n* is generally 3 or 6 (Glazer 1981).

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In both cyanobacteria and *Rhodophyceae*, phycobiliproteins form supramolecular extra-thylakoidal complexes, the so-called PBSs, which are attached to the stromal surface of the thylakoid membrane. The PBSs possess two morphologically distinct domains, a core and (generally) six rods that radiate from the core. The rod is comprised of hexameric disc-shaped aggregates of phycocyanin and phycoerythrin and of colourless linker polypeptides. The APC, together with the terminal acceptor (APC B or L_{cm}), is in the core (Glazer 1985, Holzwarth 1991). The excitation energy transfer in PBS follows the order: phycoerythrin → phycocyanin → APC. The assembly of phycobiliproteins into PBS is mediated by a group of polypeptides called linker polypeptides which make different phycobiliproteins to be noncovalently attached together. The linker polypeptides are thought not only to contribute to the ordered structure but also to the precise energy transfer characteristics within the PBS rod as well as from the rod to the core (Glazer 1984). However, Bhalerao *et al.* (1991) report that the linker polypeptides have only a minor influence on the energy transfer characteristics of the rod but are mainly involved in determining the length of the rod in response to changing environmental conditions.

The *S. platensis* PBS consists of a core of three contiguous cylindrical aggregates and up to six rods made of several stacked disks (Menon *et al.* 1988). Here we report on the spectroscopic properties of the CPC-APC conjugate, in which CPC was joined to APC by covalent bonds instead of by linker polypeptides, and the intact PBSs that were isolated from *S. platensis*.

Materials and methods

Algae culturing followed that reported by Wang *et al.* (1996).

Purification of CPC and APC: The *S. platensis* cells were washed with filtered seawater, and then immersed in 4 °C distilled water in the dark. After 2 d the cells were autolysed and the autolysate was centrifuged at 1000×*g* for 30 min. The pellet was discarded and the supernatant precipitated with 50 % saturated ammonium sulphate. The precipitate collected by centrifugation was then dissolved in 1 mM Na-phosphate buffer at pH 6.8 and dialyzed against 1 mM Na-phosphate-0.2 M NaCl (pH 6.8) at 4 °C overnight. The dialyzed extract was applied to a column of hydroxylapatite (5×1 cm) equilibrated with 1 mM Na-phosphate-0.2 M NaCl (pH 6.8). The CPC fractions were eluted with 30 mM Na-phosphate-0.2 M NaCl (pH 6.8) and the APC fractions with 0.1 M Na-phosphate-0.2 M NaCl (pH 6.8). The CPC and APC fractions were applied to separate columns of hydroxylapatite again.

Preparation of the CPC-APC conjugate: 20 mg of the CPC and 11 mg of the APC was dissolved in 2 cm^3 of 0.1 M Na-phosphate buffer at pH 6.8. One cm^3 of 21 mM glutaraldehyde solution (also in 0.1 M, pH 6.8, Na phosphate buffer) was added dropwise to the CPC-APC solution under constant stirring. The reaction was allowed to proceed for 16 h at room temperature in dark, and the solution was then dialyzed against 0.1 M Na-phosphate at pH 7.4 overnight (Hurn and Chantler 1980).

Purification of the CPC-APC conjugate: The dialyzed reaction mixture was applied to a column of hydroxylapatite (3 cm^3 settled bed volume) equilibrated with 1 mM Na-phosphate and 0.2 M NaCl (pH 6.8). The column was washed with 15 cm^3 of the starting buffer, and then developed with 30 mM Na-phosphate-0.2 M NaCl (pH 6.8). This lead to a well-defined CPC zone. Elution with the same buffer continued until the zone left the column. The conjugate, which was free from CPC, was eluted with 0.1 M Na-phosphate-0.2 M NaCl (pH 6.8) containing 2 mM NaN_3.

Preparation of intact PBSs: The isolation of the PBS followed the procedure of Gantt and Lipschultz (1974) but a small change was made in the procedure: the sucrose step gradient, on which the supernatant of the 20 000×*g* centrifugation for 30 min was layered, consisted of 1.50, 1.25, 1.00, 0.75, 0.50, 0.25 M sucrose in proportions of 1 : 1 : 2 : 2 : 1 : 1. The gradient was centrifuged at 110 000×*g* for 3 h. The change ensured that the PBS band was more concentrated and discrete. After centrifugation, the PBSs in the 0.75-1.00 M sucrose were removed from the step gradient and then dialyzed against the 0.75 M Na-phosphate buffer containing 2 mM NaN_3 at room temperature overnight. After spectroscopic measurement, the preparation was divided into two parts. One part was diluted 7-fold with distilled water and the other part was kept in the dark at 20 °C.

Spectroscopic measurements: Absorption spectra were recorded at room temperature using a *Shimadzu UV-240* recording spectrophotometer. Fluorescence spectra were recorded at room temperature by a *Hitachi 850* fluorescence spectrophotometer.

Results

Spectroscopic properties of the separated phycobiliproteins and the CPC-APC conjugate: The absorption and fluorescence spectra of *S. platensis* CPC had an absorption peak at 620 nm in the visible region and a fluorescence peak at 650 nm, and those of *S. platensis* APC had an absorption peak at 650 nm and a fluorescence peak at 670 nm (Fig. 1*A,B*). In the CPC-APC conjugate the CPC was covalently joined to the APC by their ε-amino groups. As control, a mixture of CPC and APC in the same ratio as in the conjugate was used. The absorption and emission properties of the conjugate were compared with those of the separated proteins and the control. The absorption spectra of the conjugate and of the control were a sum of the contributions of CPC and APC in the visible region (Fig.1*C*). In the ultraviolet region of absorption spectra there was a difference between the conjugate and the control. The conjugate had an absorption maximum at 266 nm while the control showed a peak at 278 nm which was chiefly attributed to aromatic amino acid residues (Fig. 2). The reaction of proteins with glutaraldehyde gives rise to a chromophore with an absorption maximum at 265 nm (Reichlin 1980). All the facts indicated that the connection of the CPC with the APC was successful.

The fluorescence emission spectrum of the conjugate excited at 620 nm, *i.e.*, at the absorption peak of CPC in the visible region, showed a fluorescence peak at 666 nm. The control excited at the same wavelength showed a fluorescence peak at 662 nm, so there was a small difference between the conjugate and the control in the fluorescence spectra. The APC also absorbed at 620 nm and could also give fluorescence after excitation at 620 nm. Therefore, the control fluorescence peak at

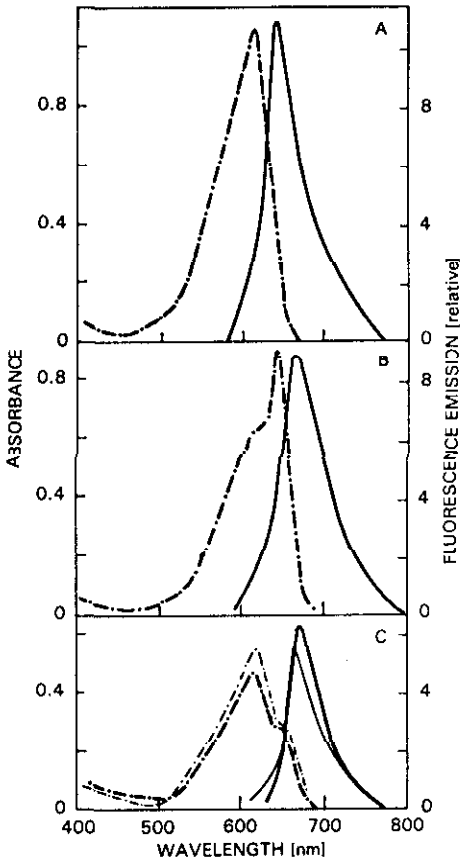

Fig. 1. Absorption (- · - · -) and fluorescence emission (——) spectra of (*A*) *S. platensis* C-phycocyanin, (B) allophycocyanin, or (*C*) the C-phycocyanin-allophycocyanin conjugate (*bold lines*) in comparison with control (*thin lines*).

662 nm might be the sum of the contributions of the CPC at 650 nm and the APC at 670 nm. The fluorescence excitation spectrum of the control showed a peak at 658 nm and a shoulder at 638 nm, and the shape of the spectrum in the visible region was similar to that of the absorption spectrum of the APC. Hence the control fluorescence peak at 662 nm was chiefly attributed to the APC fluorescence at the excitation wavelength of 620 nm (Fig. 3). However, there were two peaks at 638 and 655 nm in the visible region of the excitation spectrum of the conjugate, and the peak at 638 nm was higher than that at 655 nm. Thus the conjugate's fluorescence peak at 666 nm

was chiefly attributed by the energy transfer from CPC to APC, *i.e.*, the CPC absorbed the excitation energy and then transferred it to APC to induce an APC fluorescence emission peak at 666 nm. The results confirmed an energy transfer from CPC to APC in solution only when CPC was attached covalently to APC.

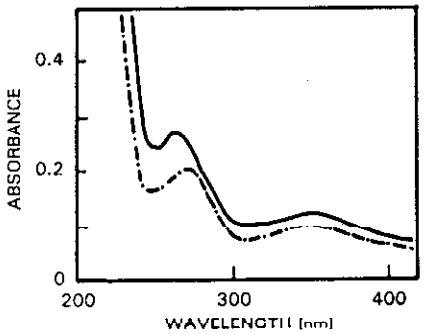

Fig. 2. The ultraviolet region of the absorption spectra of the C-phycocyanin-allophycocyanin conjugate (——) and the control (- · - · -).

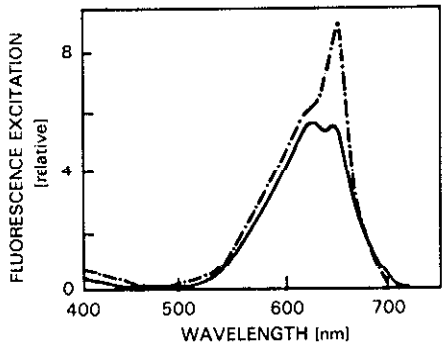

Fig. 3. Fluorescence excitation spectra of the C-phycocyanin-allophycocyanin conjugate (——) (λ_{em} 666 nm) and control (- · - · -) (λ_{em} 662 nm).

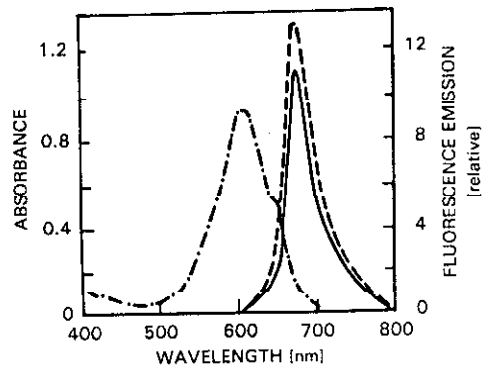

Fig. 4. Absorption (- · - · -) and fluorescence emission (—— λ_{ex} 580 nm; - - - λ_{ex} 620 nm) spectra of the isolated phycobilisomes from *S. platensis*.

Spectroscopic properties of the isolated PBSs from *S. platensis*: In the visible region the absorption spectrum of isolated PBSs from *S. platensis* showed a peak at 615 nm and a shoulder at 650 nm (Fig. 4). The peak at 615 nm was attributed to CPC and the shoulder at 650 nm to APC. The fluorescence emission maximum at room temperature was observed at 680 nm when excited both at 580 or 620 nm (Fig. 4), indicating that the excitation energy absorbed primarily by CPC was effectively transferred to APC ($\lambda_{abs,max}$ 650 nm; $\lambda_{em,max}$ 670 nm) and at last to APC B or L_{cm} ($\lambda_{abs,max}$ 670 nm, $\lambda_{em,max}$ 680 nm), the terminal energy acceptors of the isolated PBSs (Maxson *et al.* 1989). There were two peaks at 638 and 655 nm, respectively, in the visible region of the fluorescence excitation spectrum of the isolated PBSs; the peak at 638 nm apparently represented the CPC and the peak at 655 nm stood for the APC (Fig. 5).

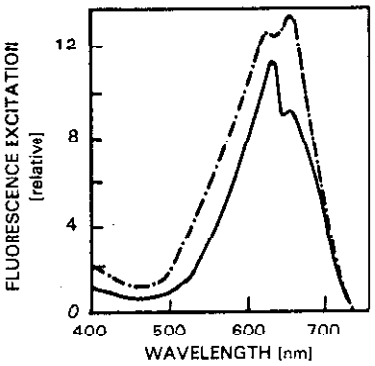

Fig. 5. Fluorescence excitation spectra of the intact phycobilisomes (- · · · -, λ_{em} 680 nm) and the phycobilisomes without the terminal energy acceptors (——, λ_{em} 670 nm).

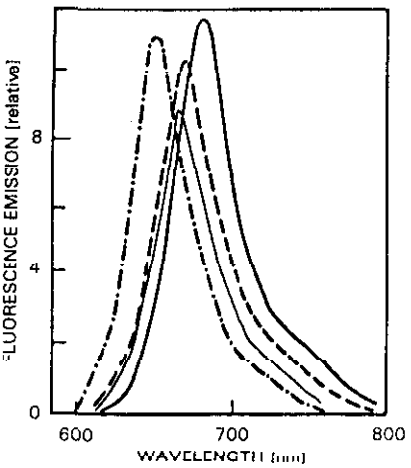

Fig. 6. Fluorescence emission spectra of the isolated phycobilisomes kept at 20 °C for 10 d (——, *bold line*), 15 d (- - -) or 25 d (- · · · -) and of the C-phycocyanin-allophycocyanin conjugate kept at 20 °C for 30 d (——, *thin line*, λ_{ex} 620 nm).

Comparison of stability of the CPC-APC conjugate and of the isolated PBSs from *S. platensis* by their spectroscopic properties: The isolated PBSs were intact in the dark at 20 °C for 10 d because the fluorescence emission maximum remained at 680 nm. When the isolated PBSs were stored in the dark at 20 °C for 15 d, the fluorescence emission maximum was shifted to 670 nm, which indicated that the APC B or L_{cm} was dissociated from the isolated PBSs. After 25 d, the isolated PBSs were completely dissociated (Fig. 6).

The isolated PBSs were dissociated into separate phycobiliproteins after the ion strength of the sample was diluted to 0.1 M Na phosphate buffer with distilled water. The fluorescence emission spectrum of the diluted sample only showed a peak at 650 nm, attributed to CPC, and no peaks of contributions of APC at 670 nm and APC B or L_{cm} at 680 nm. Hence the excitation energy absorbed by CPC could not migrate to APC or APC B or L_{cm} (Fig. 7).

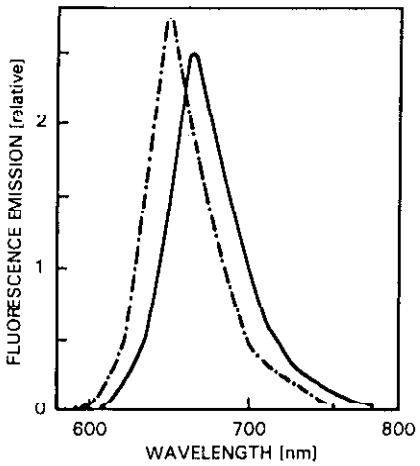

Fig. 7. Fluorescence emission spectra of the isolated phycobilisome solution whose ion strength was diluted to 0.1 M (- · - · -) and of the C-phycocyanin-allophycocyanin conjugate solution whose ion strength was diluted to 0.03 M (——, λ_{ex} 620 nm).

Compared to the isolated PBSs, the fluorescence emission maximum of the CPC-APC conjugate in 0.1 M Na-phosphate (pH 7.0) remained the same after the sample was kept in the dark at 20 °C for 30 d (Fig. 6). When the ion strength of the conjugate solution was diluted to 0.03 M with distilled water, the emission maximum was still at 666 nm (Fig. 7). This indicates that the CPC-APC conjugate is very stable.

Discussion

The CPC and APC are joined noncovalently by linker polypeptides in the PBS of *S. platensis* and the excitation energy can be transferred from CPC to APC, APC B or L_{cm} and at last to chlorophyll *a* in the reaction centre of photosynthesis (Glazer 1985). The CPC-APC conjugate, in which CPC was attached to APC by covalent

bonds instead of by linker polypeptides, did not contain APC B or L_{cm} to act as the terminal energy acceptor. The excitation energy transfer from CPC to APC could occur in the conjugate. The conjugate remained stable both in a low ion strength solution and for a long time while the isolated PBS was dissociated in such conditions. Thus the conjugate was more stable than the isolated PBS.

When the isolated PBS from *S. platensis* was kept in the dark for 15 d, the APC B or L_{cm} was dissociated from it. This meant that the conjunction between APC and APC B or L_{cm} was looser than that between the CPC and APC. The similarity of absorption and fluorescence spectra between the conjugate and the PBS without APC B or L_{cm} may indicate that the linker polypeptides have only a minor influence on the excitation energy transfer characteristics between different phycobiliproteins in the PBS, which is identical with that reported by Bhalerao *et al.* (1991).

Just like the fluorescence excitation spectrum of the conjugate, the peak at 638 nm was higher than the peak at 655 nm in the fluorescence excitation spectrum of the PBS without APC B or L_{cm}. But in the fluorescence excitation spectrum of the intact PBS (with APC B or L_{cm}), the peak at 638 nm was lower than the peak at 655 nm, which indicated that the fluorescence excitation maximum at 655 nm of the intact PBS was attributed not only to APC but also to APC B or L_{cm} (Fig. 5).

In general, the most likely mechanism for energy transfer between different phycobiliproteins in the PBS is the so-called Forster dipole-dipole resonance mechanism (Holzwarth 1991) which needs the overlap of the fluorescence emission maximum of the energy donor molecule and the absorption maximum of the energy acceptor molecule. The fluorescence emission maximum of CPC (donor molecule) is the same as the absorption maximum of APC (acceptor molecule), both are at 650 nm, and thus the excitation energy transfer can occur in the CPC-APC conjugate. However, in previous experiments we conjugated the *Polysiphonia urceolata* R-phycoerythrin ($\lambda_{em,max}$ 580 nm) to *S. platensis* APC ($\lambda_{abs,max}$ 650 nm), and there also existed the excitation energy transfer in the conjugate (Wang *et al.* 1996). Therefore, the donor and the acceptor molecules are joined to result in short distance (<2 nm) between them, the excitation energy can rapidly transfer from the donor to the acceptor. The mechanism for the excitation energy transfer in these conjugates may be the so-called exciton interaction (Holzwarth 1991).

The fact that the spectroscopic properties of the CPC-APC conjugate were similar to those of the *S. platensis* PBS that did not contain APC B or L_{cm} indicated that the conjugate could imitate the process of the excitation energy transfer of the isolated PBS. The conjugate can be useful in studying the protein-protein interactions, chromophore orientation, and the excitation energy transfer from one chromophore type to another.

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