PHOTOSYNTHETICA 36 (1-2): 163-170, 1999

Phycobilisome from *Anabaena variabilis* Kütz. and its model conjugates

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Abstract

The model conjugates phycocyanin-allophycocyanin (C-PC-APC) and phycoerythrocyanin-phycocyanin-allophycocyanin (PEC-C-PC-APC) were synthesized by using a heterobifunctional coupling reagent N-succinimidyl-3-(2-pyridyldithio)propionate. The rod-core complex $(\alpha\beta)_6{}^{PC}L_{RC}{}^{27}(\alpha\beta)_3{}^{APC}L_C{}^{8.9}$ and phycobilisomes were separated from *Anabaena variabilis*. Energy transfer features for the conjugates and the complexes were compared. The absorption and fluorescence emission spectra indicated that the linker-peptides mediate interaction of phycobiliproteins and prompt energy transfer. The energy transfer in the conjugates was detected by fluorescence emission spectra and confirmed by the addition of dithiothreitol. The conjugates may be used as models for studying the energy transfer mechanism in phycobilisomes.

Additional key words: allophycocyanin; dithiothreitol; energy transfer; N-succinimidyl-3-(2-pyridyldithio)propionate; phycocyanin; phycoerythrocyanin; rod-core complex $(\alpha\beta)_6{}^{PC}L_{RC}{}^{27}$ $(\alpha\beta)_3{}^{APC}L_C{}^{8.9}$.

Introduction

Phycobilisomes (PBSs) are supramolecular aggregates of phycobiliproteins which function as the major light-harvesting antennae in cyanobacteria and red algae (Canaani *et al.* 1980). These phycobiliproteins include phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC), and allophycocyanin (APC). Steady-state spectral analysis of isolated PBSs shows that the absorbed energy is transfered from PE (or PEC) to PC and finally to APC (Glazer *et al.* 1976). In the intact PBSs,

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Received 16 March 1998, *accepted* 23 November 1998.

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Abbreviations: APC, allophycocyanin; C-PC, C-phycocyanin; DTT, dithiothreitol; PBS, phycobilisome; PEC, phycoerythrocyanin; PMSF, phenylmethylsulfonyl fluoride; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionate.

Acknowledgement: This research was supported by National Natural Science Foundation of China (NNSFC) No. 29773049.

the phycobiliproteins are linked together by special polypeptides termed linker-peptides, which are uncoloured on Na-dodecylsulphate polyacrylamide gels (Canaani and Gantt 1982). Because of the presence of these linker-peptides, the energy transfer efficiency in PBSs is more than 95 %. If the linker-peptides are replaced by other materials to link the phycobiliproteins together, some PBS models can be constructed. These models may be used in studying the energy transfer mechanism in phycobilisomes (Frąckowiak and Naser 1997, Wang *et al.* 1997). The role of linker-peptides can also be deduced by comparing the properties of native PBSs and model systems. Futhermore, the phycobiliprotein conjugates may be used in fluorescence-activated cell sorting or cytometric analysis. They allow simultaneous excitation of several different tags at the same wavelength. This simultaneous excitation is important for multiparameter analyses with a single laser (Glazer 1994).

Materials and methods

N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and dithiothreitol (DTT) were purchased from *Sigma Chemical Co. Sephadex G-150* and *G-25* were obtained from *Pharmacia*. All other chemicals were of analytical grade and purchased from *Beijing Chemical Factory*.

The culture of *A. variabilis* Kütz. strain ATCC 29413, obtained from Prof. Lu Ronghao, was grown in the Allen and Arnon medium under "white light" at 30 °C with constant bubbling of air.

PBS were isolated according to the procedure of Maruthi Sai *et al.* (1992). For obtaining PEC, C-PC, and APC, the intact PBSs were dialyzed overnight at 4 °C against 5 mM NaH_2PO_4-K_2HPO_4 buffer, 0.5 M NaCl, pH 7.0. The dissociated PBSs were applied onto a column of hydroxylapatite equlibrated with the same buffer, then eluted with 35 mM NaH_2PO_4-K_2HPO_4 buffer, 0.1 M NaCl, pH 7.0. The eluate contained most of the C-PC and PEC. The APC remained on the top of the column and could be eluted with 0.1 M NaH_2PO_4-K_2HPO_4 buffer, 0.1 M NaCl, pH 7.0. The PEC-containing fraction was applied to a column of DEAE-cellulose (*Whatman DE 32*) of 3×10 cm equilibrated with 5 mM NaH_2PO_4-K_2HPO_4 buffer, pH 7.0 at 15 °C, then the column was washed with the same buffer. The PEC and C-PC were separated roughly into two fractions. The two fractions were applied onto a *Sephadex G-150* column (3.3×120 cm) and eluted with 50 mM phosphate, 0.1 M NaCl, pH 7.0, respectively. Using the above procedures, pure PEC and C-PC were obtained. The fraction containing APC was brought to 50 % saturation by the addition of solid $(NH_4)_2SO_4$. The resulting precipitate collected by centrifugation was resuspended in 5 mM phosphate buffer, 0.1 M NaCl, pH 7.0, and dialyzed against the same buffer over night at 4 °C. The dialyzed sample was applied again onto a column of hydroxylapatite equilibrated and eluted with 5 mM phosphate buffer, then washed with 70 mM phosphate buffer, 0.1 M NaCl. The eluate was also applied onto a column of *Sephadex G-150* and the pure APC was obtained.

The separation of the rod-core complex $(\alpha\beta)_6^{PC}L_{RC}^{27}(\alpha\beta)_3^{APC}L_C^{8.9}$: The intact PBSs were dialyzed at 4 °C against 5 mM NaH_2PO_4-K_2HPO_4 buffer (1 mM PMSF), 2 mM EDTA, and 1 mM NaN_3 for 24 h. Then the dialyzed solution was applied onto a column of DEAE-cellulose *DE 52* (3.0×34 cm), and developed with a linear phosphate gradient of 5-205 mM, pH 7.0. The third fraction containing C-PC, APC, L_{RC}^{27}, and $L_C^{8.9}$ was dialyzed against 700 mM phosphate, pH 7.0 for 24 h. T'hen the dialysate was applied to a column of *Sephadex G-150* (3×120 cm) and washed with 700 mM phosphate buffer. The second fraction was the pure rod-core complex $(\alpha\beta)_6^{PC}L_{RC}^{27}(\alpha\beta)_3^{APC}L_C^{8.9}$ (Zhang *et al.* 1997).

Preparation of the thiolated C-PC: To 1.5 cm^3 of 5.4×10^{-8} M C-PC in 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4, we added 20 mm^3 of 5.4×10^{-7} M SPDP in anhydrous ethanol. After 90 min, 20 mm^3 of 1 M DTT in the pH 7.4 buffer was added to the reaction system. After further 60 min, the reaction mixture was applied to a column of *Sephadex G-25* (1×20 cm) and eluted with 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4. This procedure yielded the thiolated C-PC.

Reaction of PEC with SPDP: To 1.5 cm^3 of 5.7×10^{-8} M PEC in 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4, we added 20 mm^3 of 5.7×10^{-7} M of SPDP in anhydrous ethanol. After 90 min, the reaction mixture was applied to a column of *Sephadex G-25* (1×10 cm) equilibrated and eluted with 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4. The frontally eluted colour fraction was phycoerythrocyanin-s-s-pyridyl derivative.

Reaction of APC with SPDP: To 1.5 cm^3 of 5.4×10^{-8} M APC in 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4, we added 20 mm^3 of 5.4×10^{-7} M SPDP in anhydrous ethanol. After 90 min, the reaction mixture was applied to a column of *Sephadex G-25* (1×20 cm), equilibrated, and eluted with 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4. The frontally eluted blue fraction was allophycocyanin-s-s-pyridyl derivative.

Preparation of the C-PC-APC conjugate: Thiolated C-PC in 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4 was mixed with the allophycocyanin-s-s-pyridyl derivative in the same buffer. Afer 20 h, the reaction mixture was concentrated by polyethylene glycol 6000 to half of its original volume, then applied to a column (1.5×50 cm) of *Bio gel P-300* equilibrated with 50 mM Na-phosphate. The column was washed with 50 mM Na-phosphate buffer. The first fraction was the C-PC-APC conjugate.

Preparation of the PEC-C-PC-APC conjugate: Thiolated C-PC in 0.1 M Na-phosphate, 0.1 M NaCl, pH 7.4 was mixed with the allophycocyanin-s-s-pyridyl derivative in the same buffer. After 6 h, the phycoerythrocyanin-s-s-pyridyl derivative was added into the system and allowed to react for further 20 h. After reaction, the reaction mixture was concentrated by polyethyleneglycol 6000 to one third of its original volume, then applied to a column (1.5x50 cm) of *Bio gel P-300*, and developed with 50 mM Na-phosphate buffer. The first fraction was the PEC-C-PC-APC conjugate.

Spectroscopic measurement: Absorption spectra were obtained on a *Hewlett Packard 8511A* spectrophotometer. Fluorescence spectra were recorded on a *Hitachi 850* fluorescence spectrometer.

Results

Absorption spectra of PEC, C-PC, APC, conjugate C-PC-APC, PBS model conjugate PEC-C-PC-APC, rod-core complex $(\alpha\beta)_6{}^{PC}L_{RC}{}^{27}(\alpha\beta)_3{}^{APC}L_C{}^{8.9}$, and the isolated native PBS are shown in Fig. 1. The PBS possesses a single maximum at 620 nm,

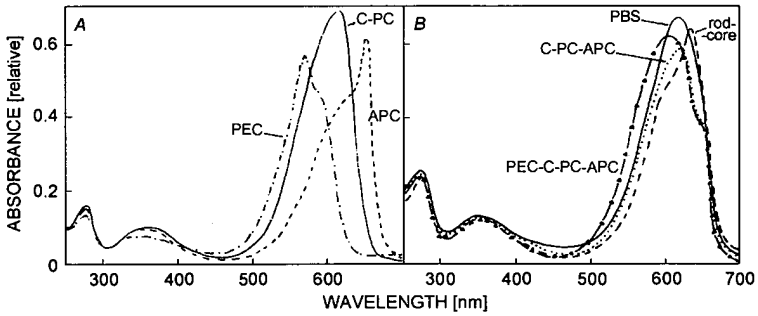

Fig. 1. Absorption spectra of (*A*) PEC, C-PC, and APC, and (*B*) of PBS, rod-core complex, PEC-C-PC-APC, and C-PC-APC. For abbreviations see the text.

similarly as the C-PC-APC complex. Maxima of single pigments are at 570 (PEC), 614 (C-PC), and 652 (APC) nm. Thus the spectral analysis shows that the PBS is mainly made up of C-PC and APC. The absorption maximum of the rod-core complex is at 640 nm, which is between the maxima of C-PC and APC but the shape of its absorption spectrum is very similar to that of APC. The absorption maximum

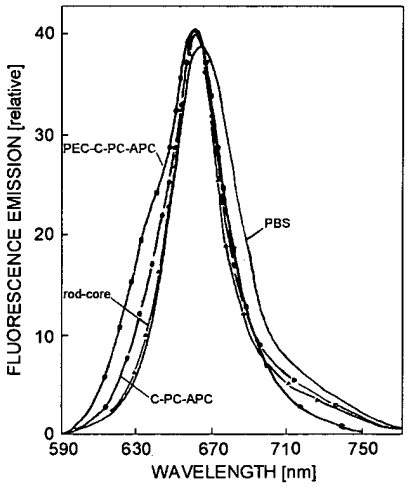

Fig. 2. The fluorescence spectra of PBS, rod-core complex, PEC-C-PC-APC, and C-PC-APC. For abbreviations see the text.

of the conjugate PEC-C-PC-APC is at 604 nm with a strong shoulder caused by APC at 650 nm. Comparing the absorption spectra of it with that of PBS, the shape is very different and the maximum is blue shifted by 16 nm. Conjugate C-PC-APC is a model of the rod-core complex but their absorption spectra are very different. The

absorption maximum of the conjugate is at 620 nm identical to that of PBS and with a strong shoulder similar to that of conjugate PEC-C-PC-APC. The molar ratios of phycobiliproteins in the conjugates were calculated using equations of MacColl and Friar (1987). They are 1.7 : 1 for C-PC-APC, 1 : 2.2 : 1.3 for PEC-C-PC-APC, and 2.2 : 1 for rod-core complex.

Fluorescence spectra (Fig. 2): The emission maximum of the PBS was at 665 nm, but the maxima of the two conjugates and the rod-core complex were at 660 nm. The emission spectra of the two conjugates showed a shoulder in the range of 634 to 640 nm, but that of the conjugate C-PC-APC was very weak. The emission spectra of PBS and the rod-core complex did not show any shoulder in that range. The fluorescence emission spectra indicate that all the conjugates and the complexes are capable of an intramolecular energy transfer. To prove this property, DTT was added to the conjugate solutions and the subsequent spectral variations were recorded. When PBSs were disolved in 0.1 M phosphate buffer, after 15 min the emission maximum shifted to 648 nm and the emission intensity increased (Fig. 3). When DTT

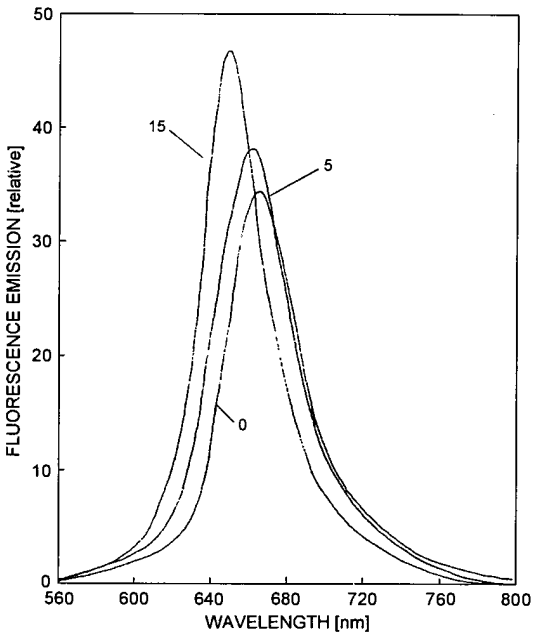

Fig. 3. The fluorescence emission spectra of PBS in 0.1 M phosphate buffer after 0, 5, and 15 min. The excitation wavelength was 510 nm.

was added into the solution of conjugate C-PC-APC, the emission intensity at 660 nm was decreased while the shoulder at 640 nm increased (Fig. 4*B*). For the conjugate PEC-C-PC-APC, the fluorescence intensity decreased at 660 nm but increased at 636 nm with the addition of DTT (Fig. 4*A*).

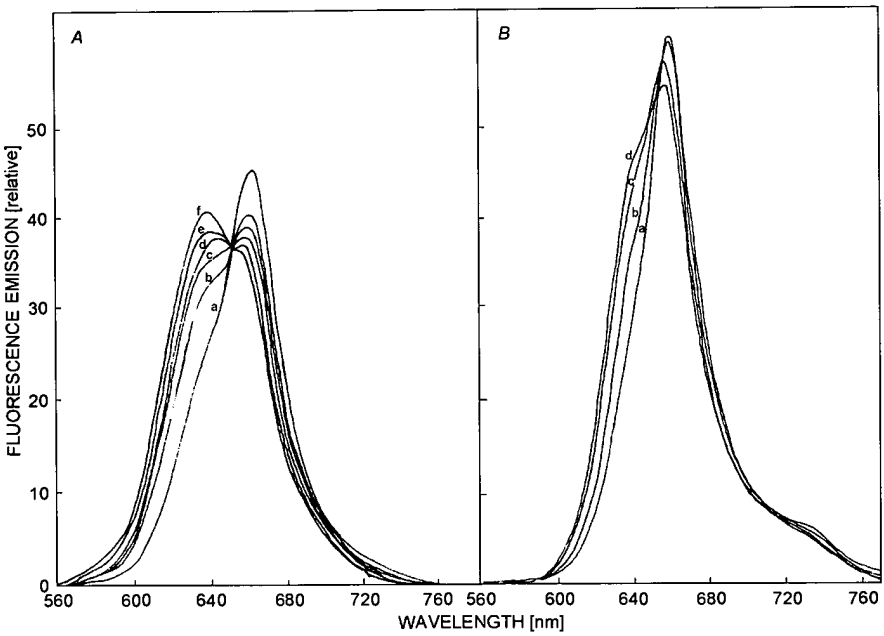

Fig. 4. (*A*) Cleavage of PEC-C-PC-APC by DTT leads to an increase of 636 nm emission and decrease of 660 nm APC emission. The excitation wavelength was 510 nm and the DTT concentration was 50 mM. *a*: 0 min; *b*: 10 min; *c*: 20 min; *d*: 40 min; *e*: 60 min; *f*: 100 min. (*B*) Cleavage of C-PC-APC by DTT leads to an increase in the 644 nm C-PC emission and to a decrease in the 660 nm APC emission. The excitation wavelength was 530 nm and the DTT concentration was 50 mM. *a*: 0 min; *b*: 10 min; *c*: 30 min; *d*: 50 min.

Discussion

Although both the conjugate C-PC-APC and the rod-core complex are made up of C-PC and APC, their absorption and fluorescence emission spectra are very different. Fig. 1 shows that the absorption spectrum of the conjugate C-PC-APC is a simple sum of those of C-PC and APC. This agrees with other synthesized tandem phycobiliprotein conjugates (Glazer and Stryer 1983, He and Jiang 1995, Zhao *et al.* 1997). This idea was confirmed by the fact that when C-PC and APC were mixed together, the shapes of absorption spectra changed with the molar ratio of C-PC to APC. Furthermore, when the molar ratio was the same as that in the experiment in which the conjugate was synthesized, the absorption spectra in both states were almost identical. Therefore, the molar ratio of C-PC to APC in the conjugate can be calculated from the absorption spectra. But the absorption spectrum of the rod-core complex is not the case. In the rod-core complex, the linker peptides L_{RC}^{27} and $L_C^{8.9}$ are also important in determing the absorption spectrum. Therefore, the absorption spectrum shape is very similar to that of APC though the ratio of $n_{C\text{-}PC}$ to n_{APC} is 2.2 : 1.0 (Zhang *et al.* 1997). The linker peptides mediate the interaction of

phycobiliproteins and thus the rod-core complex posseses its characteristic spectrum. For the same reason, the energy transfer efficiency from C-PC to APC is higher in the rod-core complex than in the conjugate C-PC-APC. This can be concluded from their fluorescence emission spectra although quantitative results are difficult to obtain from the steady-state spectra. The emission spectrum of the rod-core complex showed no shoulder in the range of 630-640 nm which indicates that the energy transfer efficiency was almost 100 %. However, only a part of energy was transfered from C-PC to APC in the conjugate C-PC-APC because the fluorescence spectrum exhibited a weak shoulder which was from the emission of C-PC.

The absorption spectrum of conjugate PEC-C-PC-APC was the simple sum of PEC, C-PC, and APC, while the absorption spectrum of PBS did not contain any strong shoulder. The fluorescence spectrum of conjugate PEC-C-PC-APC also possessed a strong shoulder, which indicated that the energy transfer efficiency was lower than in conjugate C-PC-APC and much lower than in PBS. The emission maxima of the conjugates and the rod-core complex were blue-shifted compared with that of PBS. The reason may be that the C-PC-APC does not couple as well as in PBS. The fact that the fluorescence emission maximum of dissociated PBS was also blue-shifted confirms the idea. Hence the linker-peptides may be very important for prompting energy transfer in PBS.

In the synthesized model conjugates, phycobiliproteins are linked by disulfide bonds without the mediating function of linker-peptides. So the phycobiliproteins in the conjugates are not coupled well, and the distances between donors and acceptors are large. Therefore, the energy transfer efficiencies in the conjugates are low. In spite of the lower energy transfer efficiencies, the energy transfer exists in the conjugates. The fact was proved by the addition of DTT into the conjugate solutions. DTT is able to reduce the disulfide bond linking the phycobiliproteins. When DTT was added into the solution, the disulfide bond was cleft, and the energy transfer was stopped. Therefore, the fluorescence intensity at 660 nm decreased and that at 620~644 nm (*e.g.,* at 636 nm) increased with time. The emission peak at 636 nm in Fig. 4*A* is the result of combination of C-PC and PEC whose emission maxima are at 623 and 644 nm, respectively, because both the C-PC and PEC were excited with the excitation wavelength of 510 nm.

We conclude that there is an intramolecular energy transfer in the synthesized conjugates, though the efficiency is not as high as that in the native PBSs. However, the conjugates are still suitable for being used as the models of PBS to study the energy transfer mechanism, because they are stable in phosphate buffer of all concentrations.

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