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Molecular structure, localization and function of biliproteins in the chlorophyll *a*/*d* containing oxygenic photosynthetic prokaryote *Acaryochloris marina*

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Abstract

We investigated the localization, structure and function of the biliproteins of the oxygenic photosynthetic prokaryote *Acaryochloris marina*, the sole organism known to date that contains chlorophyll *d* as the predominant photosynthetic pigment. The biliproteins were isolated by means of sucrose gradient centrifugation, ion exchange and gel filtration chromatography. Up to six biliprotein subunits in a molecular mass range of 15.5–18.4 kDa were found that cross-reacted with antibodies raised against phycocyanin or allophycocyanin from a red alga. N-Terminal sequences of the α- and β-subunits of phycocyanin showed high homogeneity to those of cyanobacteria and red algae, but not to those of cryptomonads. As shown by electron microscopy, the native biliprotein aggregates are organized as rod-shaped structures and located on the cytoplasmic side of the thylakoid membranes predominantly in unstacked thylakoid regions. Biochemical and spectroscopic analysis revealed that they consist of four hexameric units, some of which are composed of phycocyanin alone, others of phycocyanin together with allophycocyanin. Spectroscopic analysis of isolated photosynthetic reaction center complexes demonstrated that the biliproteins are physically attached to the photosystem II complexes, transferring light energy to the photosystem II reaction center chlorophyll *d* with high efficiency.

Keywords: *Acaryochloris marina*; Chlorophyll *d*; Phycobiliprotein; Photosystem II-biliprotein complex; Energy transfer

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1. Introduction

Phycobiliproteins (PBP) are major light-harvesting pigments of cryptophytes, red algae and cyanobacteria. Four major classes of PBP can be distinguished: phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC) and allophycocyanin (AP). The basic unit of the PBP is a heterodimer composed of an α- and a β-subunit with molecular masses between 15 and 22 kDa [1]. In cyanobacteria and red algae PBP are aggregated in trimers of the structure $(\alpha\beta)_3$, which in turn form hexamers $(\alpha\beta)_6$ by a tight face-to-face association. They are organized as phycobilisomes (PBS), large complexes (molecular mass 5000–30 000 kDa) normally constructed of three-cylindrical core units from which several peripheral rods radiate [1,2]. The core cylinders contain AP while the peripheral rods are composed of PC, either alone or with PE or PEC. For reviews on PBS structure, see [2–4]. PBS are shown to be located at the cytoplasmic or stromal side of the thylakoid membranes where they are attached to photosystem II (PS II) [5–7]. In cryptophytes, on the other hand, PBP aggregates consist of PE or PC alone and are located in the thylakoid lumen [8,9]. Their supramolecular organization is not yet well understood.

Light energy absorbed by PBS is transferred to reaction center chlorophyll (chl) *a* through the peripherally located PE or PEC (if present) to PC and then to AP located in the PBS core, adjacent to the thylakoid membrane [10,11]. In this pathway, AP and the core-membrane linker polypeptide (L_{CM}) of the PBS play both structural and functional roles in final transfer of excitation energy to the photochemical reaction centers [12,13]. In cryptophytes, no component is found that may act as a bridge in the energy transfer from PE or PC to chl *a* of the reaction centers. Instead, these PBP may transfer excitation energy directly to chl *a* [14,15].

Acaryochloris marina is a prokaryotic photosynthetic organism with a unique pigment composition. Chl *d* is the predominant photosynthetic pigment whereas PBP and chl *a* are only minor components [16,17]. The thylakoids are stacked, similar to those of prochlorophytes but in contrast to those of most cyanobacteria. PBS are not detectable by electron microscopy [16]. Recently, we isolated from this organism native PBP aggregates as rod-shaped structures (26.0×11.3 nm) which contained PC as the major PBP species and an AP-like pigment as a minor component [18]. The location of these PBP aggregates in relation to the thylakoid membrane, however, remained unknown. In vivo action spectroscopy showed that light energy absorbed by *A. marina* PBP is ultimately utilized for photosynthesis [19], but the exact pathway of energy transfer remained unclear. In this communication, we present further details on the composition, molecular organization, and function of PBP in this enigmatic prokaryote. First attempts are also made towards localization of PBP with respect to thylakoid membrane and photosynthetic reaction center complexes.

2. Materials and methods

2.1. Culture conditions and isolation of crude PBP fractions

A. marina was grown in K medium [20] at a light intensity of 25 μmol m^{-2} s^{-1}, at 25°C with gentle aeration. Cells were harvested by centrifugation, and resuspended in 20 mM Bis-Tris buffer (pH 6.8) containing 20% glycerol (v/v), 10 mM $CaCl_2$, 10 mM NaCl, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by a Bead-Beater (Edmund Buehler, Germany) at 4°C, using 0.2 mm glass beads. The supernatant was separated from the membrane fractions by centrifugation at $6600\times g_{av}$ for 20 min in a Hitachi RP83T rotor, followed by fractionation on a Superdex 200 gel filtration column (Pharmacia Biotechnology). The latter procedure resulted in a crude PBP fraction that was purified further by ion exchange chromatography (Mono Q HR 5/5, Pharmacia Biotechnology). Samples were eluted from the ion exchange column by a linear gradient of 0–200 mM $LiClO_4$ at a flow rate of 1 ml min^{-1} and elution profiles were monitored at 280 nm. Two major PBP fractions eluting at 90 and 110 mM salt were collected and concentrated by ultrafiltration (Centriplus 50 concentrator, Amicon, USA). The purity and molecular size of PBP fractions were determined by HPLC size-exclusion analysis using a SuperoseTM 6 column (Pharmacia Biotechnology). The column was calibrated using low and high molecular weight marker proteins

from Pharmacia Biotechnology. The mobile phase was passed through the column at a flow rate of 0.5 ml min^{-1} and the elution profiles were monitored at 280 nm.

2.2. Determination of sedimentation coefficients of native and dissociated PBP aggregates

Native PBP aggregates of *A. marina* and those from *Rhodella violacea* were isolated as previously described [18] and dialyzed for 4 h at 15°C either against 750 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.1 mM PMSF to retain intact aggregates or against 37.5 mM phosphate buffer to cause a partial dissociation of the aggregates. Sedimentation coefficients and molecular masses were determined by density gradient centrifugation according to Martin and Ames [21] with linear 5–20% (w/v) sucrose gradients in 750 mM phosphate buffer. Each gradient had a volume of 12 ml. Approx. 100–200 μl of samples were loaded and the gradients were centrifuged at $130\,000 \times g_{av}$ at 17°C for 18 h. Identical gradients were calibrated with the following proteins of known sedimentation coefficients: thyroglobulin (porcine), 19.4 S; β-galactosidase (*Escherichia coli*), 16.0 S; catalase (beef liver), 11.3 S. Biliprotein fragments from *Rhodella* PBS served as additional markers. Upon dissociation these PBS yield the following units [22]: $(\alpha\beta)_3$ C-PC units, 5.8 S; $(\alpha\beta)_6$ C-PC or B-PE units, 11.3 S; $2\times(\alpha\beta)_6$ B-PE/C-PC units, 18.0 S; $3\times(\alpha\beta)_6$ C-PC/B-PE units, 22.2 S. The gradients were eluted from the bottom with the aid of a peristaltic pump and the eluent was monitored in a microflow cell at 280, 545 or 620 nm. Molecular masses were determined from sedimentation coefficients using the formula given by Martin and Ames [21]. For *Rhodella* biliproteins, the molecular masses calculated with this formula corresponded well with the masses calculated from the polypeptide composition [22].

2.3. Isolation of photosystem I and photosystem II complexes

Photosystem I (PS I) complexes were prepared from *A. marina* thylakoids as previously described by Hu et al. [23]. Isolation and purification of PS II complexes were carried out at 4°C and whenever possible in the dark. Unless otherwise indicated, the buffer system was composed of 20 mM Bis-Tris (pH 6.8), 20% glycerol (v/v), 10 mM $CaCl_2$, 5 mM NaCl, 5 mM $MgCl_2$, 2 mM benzamidine, and 2 mM PMSF. The membranes were solubilized with 0.6% (w/v) dodecyl-β-D-maltoside on ice for 1 h and 0.5 ml of the extracts was layered onto 10–30% (w/v) linear sucrose gradients of 10 ml volume. The gradients were centrifuged for 18 h at $198\,600 \times g$ (40 000 rpm in a Hitachi P40ST rotor). As a result, PS II complexes were resolved as a green band well separated from the PS I band in the gradient. Further purification of the complexes was performed by ion exchange chromatography (Mono Q HR 5/5, Pharmacia Biotechnology). The purity of the PS I and PS II complexes was assessed by SDS-PAGE, immunoblotting, and N-terminal amino acid sequencing.

2.4. Electron microscopy

For ultrathin sections, *A. marina* cells were washed four times in 0.1 M sodium cacodylate buffer, pH 7.2 and prefixed for 4 h with 4% (v/v) glutaraldehyde in cacodylate buffer. After six washes in buffer the prefixed cells were postfixed with 2% OsO_4 in cacodylate buffer for 2.5 h, dehydrated by increasing the concentration of ethanol (30–96%, v/v) and embedded in Epon 812 (Serva, Heidelberg, Germany). Ultrathin sections were cut with an Ultracut microtome (Reichert, Vienna, Austria) and contrasted with uranyl acetate and lead citrate. For immunogold labeling the cells were fixed with 1% glutaraldehyde in culture medium for 1 h, dehydrated and cooled to −35°C in increasing concentrations of ethanol (30–96%, v/v). The samples were then embedded in Lowicryl KM4 (Lowi, Waldkraiburg, Germany) at approx. 35°C overnight, followed by UV polymerization at −30°C for 24 h. Ultrathin sections were incubated for 30 min in 0.01 M Na/K-phosphate buffer (pH 7.4), 0.15 M NaCl (saline phosphate buffer, SPB) containing 5% (w/v) bovine serum albumin (BSA) and for 1 h in antiserum diluted 1/500 in SPB with 1% (w/v) BSA. For the in situ labeling of PBP an antibody raised against C-PC from the red alga *Cyanidium caldarium* was used. After ten washes in SPB containing 0.2% (v/v) Triton X-100 and one wash in SPB with 1% (w/v) BSA, the samples were incubated with goat anti-rabbit IgG immunogold

conjugates (British Biocell, Cardiff, UK) for 1 h to detect binding sites of the primary antibody. The size of the gold particles was 10 nm. The labeled ultrathin sections were washed ten times in SPB with 0.2% (v/v) Triton X-100, once in SPB with 1% (w/v) BSA and four times in double distilled, membrane filtered (pore size 0.2 mm) water. The immunogold labeled samples were stained with uranyl acetate. Electron microscopy was performed with a Philips 301 G electron microscope (Philips, Eindhoven, The Netherlands).

2.5. Other methods

SDS-PAGE and Western blotting were performed according to Marquardt and Rehm [24]. Gels were either 175 mm (length)×130 mm (width)×1 mm (thickness) or 75 mm (length)×140 mm (width)×1 mm (thickness) in size. They were stained with Coomassie blue R-250 or silver-stained according to Wray et al. [25]. The antibodies used were specific against AP and C-PC from the red alga *C. caldarium* [26]. N-Terminal amino acid sequences were analyzed using a protein sequencer (model 473A, Applied Biosystems, USA). Absorption spectra were recorded with a Hitachi U-3000 spectrophotometer (Hitachi, Japan), and 77 K fluorescence measurements were made on a Jasco FP-777 spectrofluorometer (Jasco, Japan) or as described previously [18].

3. Results

3.1. Localization of biliprotein aggregates in the cells

Native PBP aggregates have been isolated from *A. marina* [18], but their localization within the cell re-

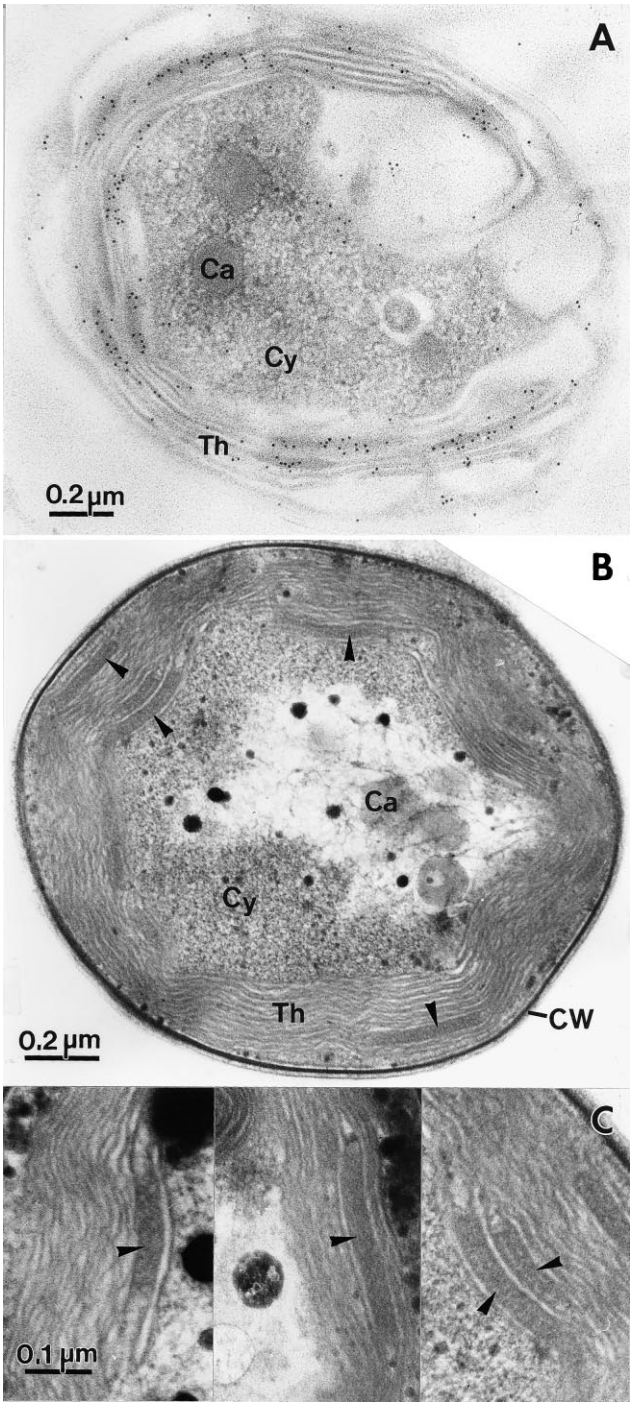

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Fig. 1. (A) Electron micrograph of an *A. marina* cell, immunogold labeled with an antiserum against PC from *C. caldarium*. Black dots caused by gold particles (10 nm diameter) indicate the site of the PBP. (B,C) Electron micrographs of ultrathin sections of *A. marina* contrasted with uranyl acetate and lead citrate. (B) Thin section of an entire cell. Thylakoids usually are in close proximity. In certain regions of the thylakoids the distance is increased and the space between the membranes filled with electron dense material (arrowheads). (C) Higher magnification of thylakoid portions, showing the localization of the electron dense material on the outer surface of the thylakoids. Ca, carboxysome; Cy, cytoplasm; CW, cell wall; Th, thylakoids.

mained unclear. In order to localize the aggregates with respect to the thylakoid membrane ultrathin sections were immunogold labeled with an antibody against PC from *C. caldarium*. As shown in Fig. 1A the gold particles were distributed predominantly over the thylakoid membrane region and sparsely over the cytoplasmic matrix. This confirms our previous suggestion [18] that the PBP aggregates are membrane-bound. Moreover, the labeling appeared to be distributed non-uniformly throughout the thylakoid membrane but concentrated in certain regions. Due to the limits of resolution of the labeling technique, however, it was not possible to verify whether the PBP aggregates are attached to the cytoplasmic side of the membranes or located within the lumenal space of the thylakoids as is the case of cryptomonads. To address this question, we used ultrathin sections of the cells contrasted with uranyl acetate and lead citrate. As shown in Fig. 1B, two domains can be distinguished in the thylakoid region, i.e. stacked regions with closely appressed thylakoids and unstacked regions where thylakoids are clearly separated from one another. The distance between the thylakoids in unstacked regions is approx. 30 nm. Here electron dense material can be visualized, facing the cytoplasmic and absent from the lumenal side of the membranes. This is particularly evident at higher magnification where electron dense material can be easily seen between two vicinal thylakoids (Fig. 1C). Generally, PBP appears as an electron dense material in thin sections of the thylakoid regions of cyanobacteria, red algae and cryptomonads [2,8,9,27]. Furthermore, the distance between the unstacked thylakoids matches rather well the size of isolated native PBP aggregates. From these observations we conclude that the dense material represents the PBP aggregates of *A. marina* which are located on the outer surface of the thylakoid membrane, predominantly in unstacked regions.

3.2. Isolation and characterization of PC and AP complexes

A crude PBP fraction was prepared by gel filtration of the soluble protein fraction of broken cells. Ion exchange chromatography of this PBP fraction resolved several sub-fractions, which could be divided into two classes according to their absorption

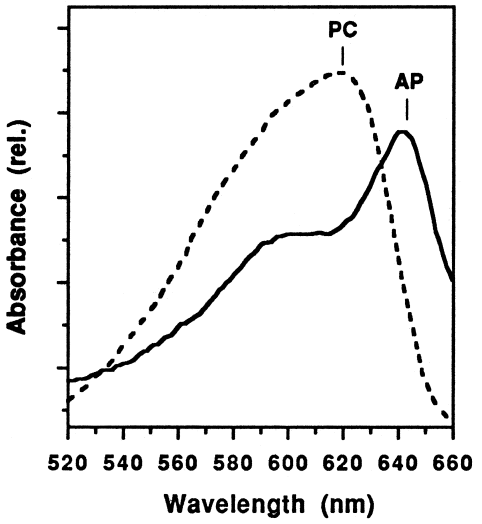

Fig. 2. Absorption spectra of isolated PC (- - -) and AP (———) fractions from *A. marina*.

characteristics (Fig. 2). The most prominent fractions were blue in color and had absorption maxima at 614–618 nm, corresponding to C-PC [28]. Another fraction had a maximum at 640 nm with a shoulder at about 600 nm, similar to AP [29]. In addition, a minor component reddish in color was detected with an absorption maximum at 568 nm. The presence of bilin chromophores in this component, as indicated by light reddish protein bands resolved on unstained SDS-PAGE, might suggest the existence of a PE/PEC-like biliprotein, but insufficient quantities of this fraction prevented further identification.

Two major blue fractions were resolved by ion exchange chromatography: one was eluted at a $LiClO_4$ concentration of about 110 mM, and the other at 140 mM. The purity of the two fractions was checked by a second ion exchange chromatography, and molecular masses of 120 and 220 kDa were determined by size-exclusion HPLC. The two fractions had identical absorption spectra with peaks at 616 nm and identical 77 K fluorescence spectra with maxima at 641 nm (Fig. 5B). Their molecular mass values corresponded well to those reported for trimeric and hexameric PBP aggregates [30,31]. The α- and β-subunits of both trimeric and hexameric PC were fractionated by SDS-PAGE and subjected to N-terminal sequencing. Table 1 compares the N-terminal sequences of the *A. marina* subunits with those of cyanobacteria, red algae and cryptomonads.

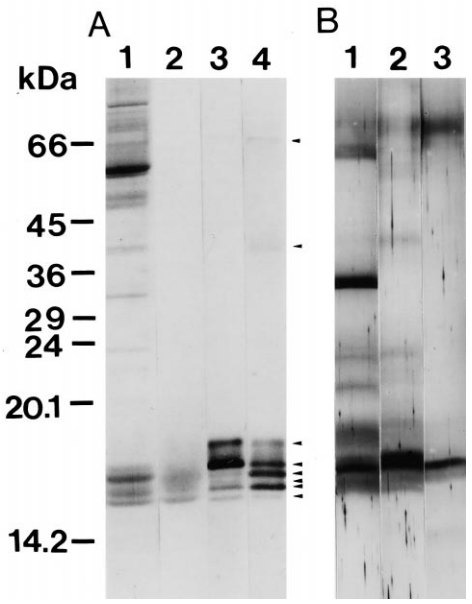

Fig. 3. (A) SDS-PAGE and Western blots of PBP aggregates isolated from *A. marina*. Coomassie-stained gel of 175 mm length (lane 1) and Western blots before labeling (lane 2) and after immunodecoration with antisera against AP (lane 3) or PC (lane 4). The bands recognized by the antisera are marked with arrowheads. (B) SDS-PAGE of undissociated PBP aggregates (lane 1) and of hexameric (lane 2) and trimeric PBP fragments (lane 3) obtained after dialysis and sucrose gradient centrifugation. Silver-stained gels of 75 mm length.

Clearly the PC β-subunit of *A. marina* is similar to the β-subunits of *Pseudanabaena* sp., *Synechococcus* sp. and *Porphyridium cruentum* with some 80% identity within 26 residues at the N-terminus. The PC α-subunit of *A. marina* also shows similarity with the corresponding subunits of *Synechococcus elongatus*, *Synechococcus* sp. and *Pseudanabaena* sp. with over 70% identity within 39 N-terminal residues. In contrast, little sequence similarity was found between PC α- and β-subunits of *A. marina* and Cr-PC645 of *Chroomonas* sp.

3.3. Composition and structure of PBP aggregates

Native PBP aggregates were isolated after cell disruption in a high osmotic strength buffer, followed by detergent extraction and sucrose density gradient centrifugation. When subjected to SDS-PAGE the aggregates yielded several polypeptides. Major polypeptides had apparent molecular masses of 15.5–17.2 kDa and 58 kDa (Fig. 3A, lane 1). Minor bands appeared at about 22, 24, 26, 32, 40, 50, and above 60 kDa. Upon transfer to a nitrocellulose membrane polypeptides of 15.5–18.4 kDa appeared as bluish or reddish bands (Fig. 3A, lane 2), indicating that these polypeptides bind bilin chromophores. Immunodecoration with an antibody against AP (Fig. 3A, lane 3) preferentially labeled the 18.4 and 17.2 kDa polypeptides with weaker signals at 16.6, 16.2, 16.0 and 15.5 kDa. An antibody against PC showed a strong cross-reaction with polypeptides of 17.2, 16.6 and 16.0 kDa and a weak reaction with the polypeptides at 18.4 and 15.5 kDa. Additionally, it produced faint signals with bands at about 40 and 70 kDa (Fig. 3A, lane 4). In order to check for the presence of a homologue to the core-membrane linker polypeptide L_{CM} of PBS, the immunoblot was also screened with antibodies against the cyanobacterial protein. However, no specific immunolabeling could be detected (data not shown).

The size and architecture of PBP aggregates were studied by comparing the sedimentation behavior of PBP aggregates with substances of known *S*-values, including PBP aggregates obtained by dissociation of PBS from the red alga *R. violacea*. Sedimentation coefficients were determined using the calibration curve shown in Fig. 4. Intact native aggregates from *A. marina* yielded two colored fractions. The major fraction (band 1, insert in Fig. 4) had a sedimentation coefficient of 25.5 S, corresponding to a molecular mass of about 1000 kDa, approximately the size for four PBP hexamers. These data correspond well to the structure and size of isolated PBP aggregates analyzed by electron microscopy [18]. A minor fraction (band 2, insert in Fig. 4) is most probably a dissociation product of the larger aggregates. With a sedimentation coefficient of 14.5 S (460 kDa), it corresponded most closely to dodecameric units. An SDS-PAGE of the 25.5 S fraction revealed major polypeptides in the 15.5–17.2 kDa range and at about 33 kDa and 59 kDa. Additionally, there were some minor bands at about 20–24 and at 40 kDa (Fig. 3B, lane 1). Some of the polypeptides found in the original PBP aggregate fraction (Fig. 3A, lane 1) were missing in the 25.5 S fraction, indicating that they are not components of the aggregates, but co-migrate in the first sucrose gradient.

After dissociation the native PBP aggregates yielded two fractions of 11.0 (band 3, insert in

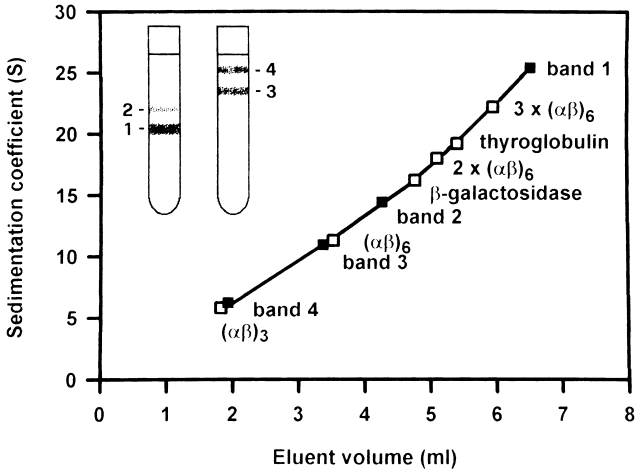

Fig. 4. Schematic representation of 5–20% sucrose gradients loaded either with intact (left) or partly dissociated (right) PBP aggregates from *A. marina* and calibration curve for the calculation of sedimentation coefficients. *S*-values are plotted versus the eluent volume between the respective gradient band and the upper edge of the gradient; open squares represent marker substances, closed squares the bands 1–4.

Fig. 4) and 6.3 S (band 4, insert in Fig. 4), corresponding to 230 and 130 kDa, respectively. Apparently they represent hexameric and trimeric PBP units. Of these, the hexameric PBP unit showed remarkable spectral properties (Fig. 5). Its absorption maximum was at about 614 nm, and the spectrum looked rather similar to that of C-PC. In the second derivative, however, an additional peak appeared at 641 nm that pointed to the presence of AP (Fig. 5A). This peak is significantly stronger than the AP peak in the second derivative of the absorption spectrum of entire aggregates [18], thus indicating an enrichment of AP in these hexamers. The presence of AP was also confirmed by polypeptide composition and fluorescence emission spectra. Upon SDS-PAGE these PBP hexamers showed a polypeptide profile similar to that of entire aggregates. However, the 33 and 59 kDa bands were missing and the bands at 40 and most conspicuously, the 17.2 kDa polypeptide were enriched (Fig. 3B, lane 2). On the other hand, the PBP trimers showed predominant polypeptide bends of 16.0 and 16.6 kDa, while the 17.2 and 18.4 kDa polypeptides which reacted most strongly with the antibody against AP were at least strongly reduced (Fig. 3B, lane 3). The trimeric gradient band had an absorption maximum at 614 nm and a 77 K fluorescence maximum at 641 nm, typical for PC (data not shown). At room temperature the PBP hexameric gradient band had an emission maximum at 665 nm with a shoulder at 653 nm (Fig. 5B) similar to entire native aggregates [20]. The emission maximum of the short wavelength component must be attributed to PC, while the fluorescence peak at 665 nm must be due to AP. The 77 K fluorescence spectrum has a prominent maximum at 670 nm (Fig. 5B). This maximum is about 30 nm red-shifted when

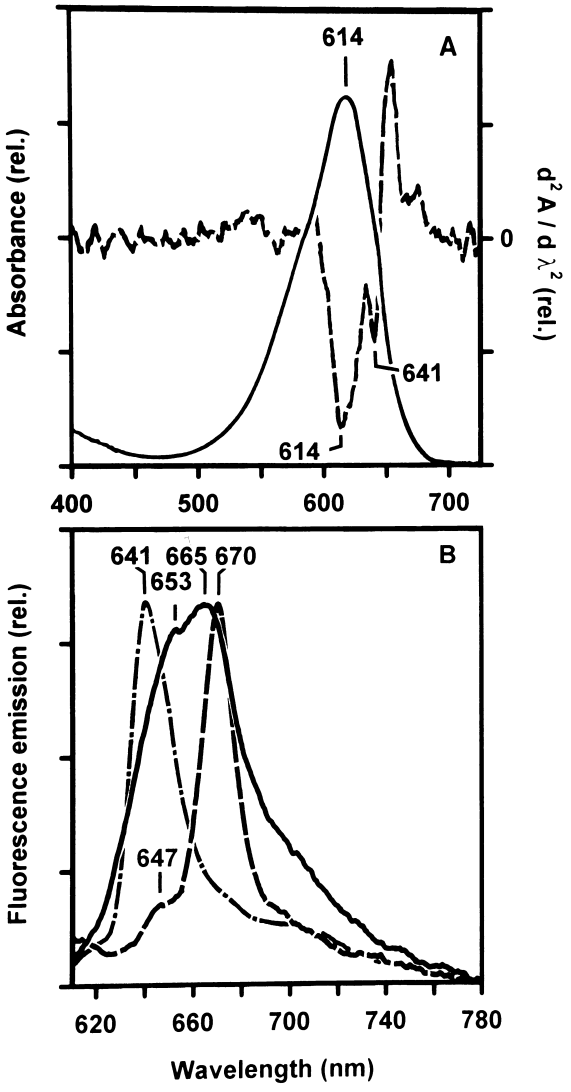

Fig. 5. (A) Absorption spectrum (———) and second derivative thereof (- - -) of PC/AP heterohexamers. (B) 77 K fluorescence spectrum of PC trimers and hexamers (– · –) and room temperature (———) and 77 K fluorescence spectrum (- - -) of PC/AP heterohexamers. The emission spectra were obtained by exciting at 580 or 600 nm.

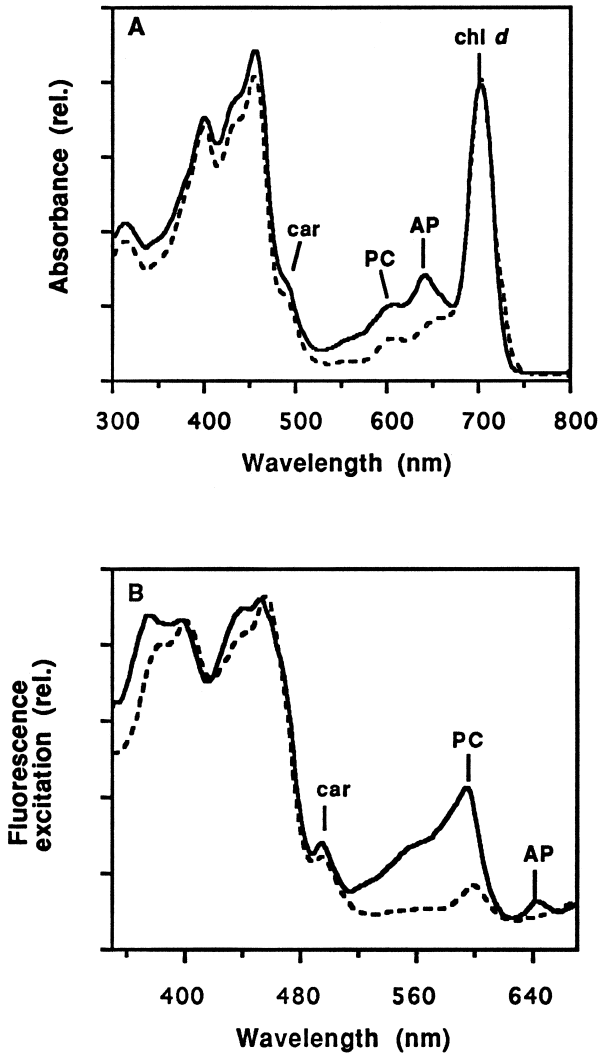

Fig. 6. (A) Absorption spectra of isolated PBP-PS II complexes (——) and PS I complexes (· · ·). (B) 77 K fluorescence excitation spectra of isolated PBP-PS II complexes (——), and PS I complexes (· · ·). The fluorescence emission was recorded at 728 nm.

compared with the 77 K emission maximum of pure PC hexamers. The PC fluorescence emission at about 647 nm was rather weak, indicating that most of the excitation energy was transferred from PC to AP. Because of the presence of both PC and AP the particles are referred to as PC/AP heterohexamers.

3.4. Coupling of PBP with PS II reaction centers

It has been shown that PBP are predominantly associated with PS II in cyanobacteria, red algae and cryptophytes [1,6,32]. On the other hand, efficient energy transfer has also been detected in a PS II-free mutant of *Synechocystis* sp. PCC 8603, implying a possible attachment of PBP to PS I as well [33]. Because of the unique properties of *A. marina*, it was unknown how PBP would be organized in this organism: whether they physically attach to PS I and/or PS II reaction center complexes. An attempt was made to isolate photosystems that retained the PBP by fractionating thylakoids using a mild detergent (dodecyl β-D-maltoside) in a high osmotic strength buffer system, followed by sucrose gradient centrifugation. The isolated PS I complex retained more than ten subunits but none of them was a PBP subunit, as confirmed by SDS-PAGE and N-terminal amino acid sequencing [23]. In contrast, the PS II complexes contained, in addition to the essential components

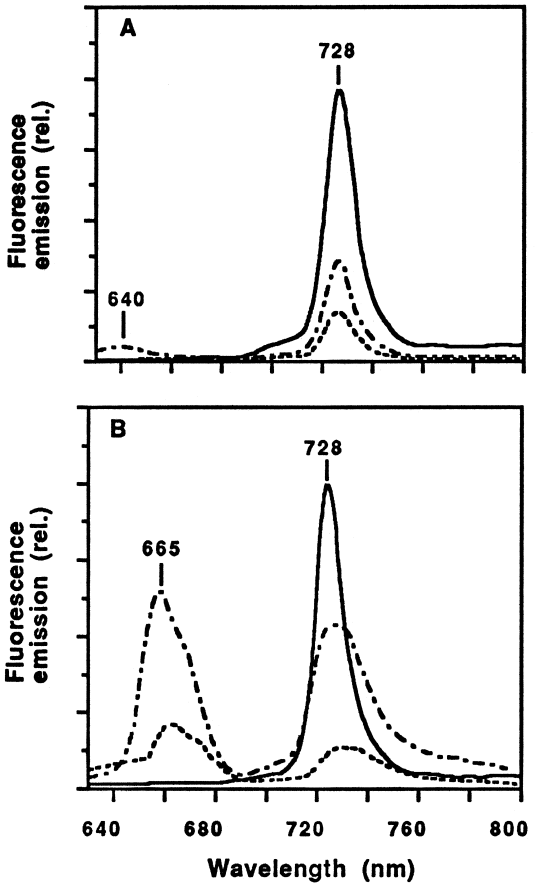

Fig. 7. 77 K fluorescence emission spectra of intact (A) and partially dissociated (B) PBP-PS II complexes. The excitation wavelength was at 400 nm (——), 600 nm (– · –) and 640 nm (· · ·).

Table 1
N-Terminal amino acid sequences of *A. marina* C-PC subunits with known sequences

Source	Proteins	N-terminal amino-acid sequence
		1 10 20
A. marina	C-PC β-subunit	MYDAFAKVVS QADARGAFLS DAQIAAAL
Pseudanabaena sp.	C-PC-1 β-subunit	MYDAFAKVVS QADSRGAYIS AAQIDA
Synechococcus sp.	C-PC β-subunit	-FDAFTKVVA QADARGEFLS DAQLDASL
Porphyridium cruentum	R-PC I β-subunit	MLDAFAKVVA QADARGEFLS NTQIDA
Chroomonas sp.	Cr-PC645 β-subunit	MLDAFSRVVT GADSKAAYVG GADLQA
		1 10 70 80
A. marina	C-PC α-subunit	MKTPLTEALA AADDQG ... TTQMT GSNYASTPEG KAKKVRRI
Synechococcus elongatus	R-PC α-subunit	MKTPITEAIA AADTQG ... TTIMQ GSQYASTPEG KAKCARDI
Synechococcus sp.	C-PC α-subunit	MKTPLTEAVS AADSQG ... TTQME GSNYSTTPEG KAKCSRDV
Pseudanabaena sp.	C-PC-1 α-subunit	MKTPLTEAVS AADSQG ... TTSMT GANYASTQTG KDKCVRDI
Chroomonas sp.	Cr-PC645 α-subunit	KNGDLRTPVI TIFDAR ETAIN VPQIAGKTKK Y

such as the D1/D2 heterodimer, the β-subunit of cytochrome b_{559}, and CP47, also the α- and β-subunits of PC as well as several minor polypeptides with apparent molecular masses between 15.5 and 18.6 kDa (not shown). Fig. 6 shows the absorption spectra of the isolated complexes. In the spectrum of the PS II preparations PC appeared with an absorption maximum at 616 nm (Fig. 6A, solid line). An increased absorption at 640 nm indicates the presence of AP. In contrast, no absorbance originating from PBP was observed in the spectrum of isolated PS I preparations (Fig. 6A, broken line). A small shoulder around 600 nm is due to a vibrational band of chl *d*. These results suggest that PBP are structurally associated with PS II, and not with PS I complexes in *A. marina*. The isolated PS II complexes therefore are referred to as PS II-PBP complexes.

Fig. 6B shows 77 K fluorescence excitation spectra of isolated PS I and PS II complexes monitored at an emission wavelength of 728 nm. It reveals that the isolated PS II but not PS I complexes have peaks in the range of 600–640 nm which correspond to the absorption bands of PC and AP. Clearly, light absorbed by these PBP species contributes to the PS II fluorescence emission. The 77 K fluorescence emission spectra of intact and partially dissociated PBP-PS II complexes are shown in Fig. 7. A single fluorescence emission peak at about 728 nm was obtained when the intact preparation was excited either at 400 or 456 nm, i.e. with light absorbed by chl *d* (Fig. 7A). When excited at 600 nm (light absorbed by PC), a small secondary emission at around 640 nm appeared besides the major fluorescence peak at 728 nm. It was assumed to be due to functionally decoupled PC. Excitation at 640 nm (mainly absorbed by AP) resulted in a single emission peak at 728 nm. Since the emission maximum always occurred at 728 nm, independent of the excitation wavelength, PC and AP obviously transfer their excitation energy to chl *d* with high efficiency. The pattern of the fluorescence emission spectra, how-

ever, dramatically changed when the PBP-PS II complexes were transferred to a buffer in which glycerol was omitted (Fig. 7B). This treatment resulted in a large increase in a fluorescence band at 665 nm, and a concomitant decrease of the 728 nm fluorescence peak upon excitation at 600 and 640 nm, indicating an incomplete energy transfer between PBP and PS II reaction center pigments. Note that the PBP components might still be structurally associated to each other, since we always find, independent from the excitation wavelength, a major fluorescence emission band at 665 nm that must be attributed to AP.

4. Discussion

A. marina, like most cyanobacteria and rhodophytes, contains at least two different types of PBP, i.e. PC and AP. The absorption spectrum of the *A. marina* AP-type PBP differs somewhat from that of other cyanobacteria which usually have maxima between 650 and 655 nm [34]. An AP absorption maximum at about 640 nm, however, has been reported for another marine cyanobacterium [35]. Additionally, the AP-type character of the *A. marina* pigment is confirmed by its emission characteristics. Its fluorescence maximum is clearly in the range of AP fluorescence maxima which are between 660 and 680 nm, depending on the aggregation state and linker species connected, while PC aggregates have emission maxima between 640 and 650 nm [34]. Moreover, our heterohexamers showed short-wavelength absorbance while emitting long-wavelength fluorescence, clearly pointing to the presence of two different types of biliproteins. Another indication for the presence of both AP and PC is given by the immunoblotting experiments, where the antibody against AP reacted predominantly with two polypeptides of 18.4 and 17.2 kDa. Of these polypeptides the larger one showed only a weak cross-reactivity with the antibody against PC. Probably it represents the α-subunit of AP which usually has the least sequence homology with PC. In contrast, the smaller polypeptide that shows higher cross-reactivity with the PC antibody is supposed to be the β-subunit. This subunit has some epitopes in common with the β-subunit of PC [34]. The antibody against PC strongly labeled two other bands with apparent molecular masses of 16.6 and 16.0 kDa. They are assumed to represent the α- and β-subunits of the major form of PC. There are also some additional minor bands recognized by the antibodies, which may represent AP or PC heterogeneity. Cyanobacteria often possess several copies of genes for PBP subunits. They encode for isoproteins varying slightly with respect to their amino acid composition but with identical bilin composition and are expressed differently under different environmental conditions [4].

The PBP of *A. marina* are organized in trimeric and hexameric units, as in cyanobacteria and red algae [34]. In contrast, a dimeric $(\alpha\beta)_2$ structure was proposed as the basic unit in cryptomonads [37]. As indicated by their appearance in the electron microscope [18] and by their sedimentation coefficients the PBP aggregates of *A. marina* are composed of four hexameric units and resemble the peripheral rods of cyanobacterial PBS. They consist of homohexamers containing PC alone and of heterohexamers which contain both PC and AP, and show efficient energy transfer between the two pigments. These heterohexamers seem to be more stable than pure PC hexamers and become enriched after long-term dialysis against low ionic strength buffer. We suggest that the PC/AP heterohexamers form the basal part of the aggregates and funnel excitation energy to intrinsic membrane complexes. Also it has been postulated for some cyanobacteria that AP is not restricted solely to the PBS core. Instead, AP hexamers are also thought to form the basal units of additional peripheral rods [36]. The presence of both, PC and AP, however, within one hexamer seems to be a unique feature of the PBP aggregates of *A. marina*.

Our spectroscopic analysis of isolated photosynthetic reaction center complexes confirms that the PBP complexes are physically and functionally associated with PS II. The PS II-PBP complexes seem to be highly organized since light energy absorbed by PBP is efficiently transferred to chl *d* of PS II. The structural basis of the coupling between PBP and PS II, however, is still unknown. Although no evidence was found for an immunological homology of any polypeptide of *A. marina* PBP aggregates to the core-membrane linker protein (L_{CM}) of PBS, we cannot exclude the presence of such a protein in *A. marina*. In cyanobacteria and red algae only a small portion

of the protein functions in anchoring of PBS, while the larger part is involved in the organization of the PBS core [34]. Since such a structure has not be detected in *A. marina*, a PBP-membrane linker protein could be much shorter, and the epitomes recognized by the antiserum might be missing. In fact, there are two candidates: the 40 kDa and the 70 kDa subunits of native PBP aggregates that were recognized by the PC antisera. While the former is approximately the size expected for a biliprotein domain plus a REP (repetitive linker domain) domain, the latter is about two REP domains plus a biliprotein domain. Note that the 40 kDa subunit was specially enriched in the AP/PC heterohexamers.

PBP were not bound to PS I under our experimental conditions. However, there are no data on the occurrence of the state transitions in *A. marina*, and we cannot exclude that the coupling of PBP with PS II reaction centers might be affected under certain circumstances. As shown by electron microscopy, the PBP aggregates are located on the cytoplasmic side on the thylakoids, and predominantly found in non-stacked areas. Taken into consideration that PBP are predominantly bound to PS II, this points to a lateral heterogeneity of the thylakoid membranes. We conclude that there is either a spatial separation of PS I and PS II centers or there is a PS II heterogeneity where only a part of the PS II centers may be coupled to PBP.

Taken together, our data on the composition, location and protein sequences indicate that the PBP of *A. marina* resemble those of cyanobacteria and red algae but not cryptomonads. The structure of the native PBP aggregates is far less complex than the structure of most cyanobacterial or red algal PBS. But whether the *A. marina* PBP aggregates are primitive precursors of PBS or highly reduced PBS descendants that concomitantly developed the chl *d*-based light-harvesting antenna system remains to be established.

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