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In Vitro Oligomerization of a Membrane Protein Complex

LIPOSOME-BASED RECONSTITUTION OF TRIMERIC PHOTOSYSTEM I FROM ISOLATED MONOMERS*

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Jochen Kruip‡, Navassard V. Karapetyan‡§, Irina V. Terekhova§, and Matthias Rögner‡¶

From the ‡Faculty of Biology, Plant Biochemistry, Ruhr-University Bochum, D-44780 Bochum, Germany and §A. N. Bakh Institute of Biochemistry, Russian Academy of Sciences, 117071 Moscow, Russia

Many membrane proteins can be isolated in different oligomeric forms. Photosystem I (PSI), for example, exists in cyanobacteria either as a monomeric or as a trimeric complex. Neither the factors responsible for the specific trimerization process nor its biological role are known at present. In the filamentous cyanobacterium *Spirulina platensis*, trimers in contrast to monomers show chlorophyll fluorescence emission at 760 nm. To investigate the oligomerization process as well as the nature of the long wavelength chlorophylls, we describe here an *in vitro* reconstitution procedure to assemble trimeric PS I from isolated purified PS I monomers. Monomers (and trimers) were extracted from *S. platensis* with *n*-dodecyl β-D-maltoside and further purified by perfusion chromatography steps. The isolated complexes had the same polypeptide composition as other cyanobacteria (PsaA–PsaF and PsaI–PsaM), as determined from high resolution gels and immunoblotting. They were incorporated into proteoliposomes, which had been prepared by the detergent absorption method, starting from a phosphatidylcholine:phosphatidic acid mixture solubilized by octylglucoside. After the addition of monomeric PS I (lipid:chlorophyll, 25:1), octylglucoside was gradually removed by the stepwise addition of Biobeads. The 77 K fluorescence emission spectrum of these proteoliposomes displays a long wavelength emission at 760 nm that is characteristic of PS I trimers, which indicates for the first time the successful *in vitro* reconstitution of PS I trimers. In addition, a high performance liquid chromatography analysis of complexes extracted from these proteoliposomes confirms the formation of structural trimers. We also could show with this system 1) that at least one of the stromal subunits PsaC, -D, and -E is necessary for trimer formation and 2) that the extreme long wavelength emitting chlorophyll is formed as a result of trimer formation.

Photosystem (PS)[1] I is one of the two light-driven electron pumps involved in oxygenic photosynthesis of green plants and cyanobacteria (1, 2). It mediates the electron transfer between luminal plastocyanin (or cytochrome c_6) and stromal ferredoxin (or flavodoxin), both soluble electron carriers. Cyanobacterial PS I consists of 11 different subunits named PsaA-PsaF and PsaI-PsaM (3). It can occur in two different oligomeric states, a monomeric and trimeric form (4, 5). The molecular organization of trimeric PS I from the thermophilic cyanobacterium *Synechococcus elongatus* was recently solved by x-ray analysis down to 4 Å resolution (6). The location of the primary electron donor P700, the primary electron acceptor A_o (chlorophyll *a*), and the secondary acceptors A_1 (phylloquionone), F_X, and F_A/F_B was determined. Furthermore, the location of 83 antenna chlorophyll molecules (Chl) out of about 90 was resolved in that study.

Although the PS I complexes of green plants and cyanobacteria carry out the same function and show a similar organization, there are several differences between them. First, they differ in their subunit composition with PsaG and PsaH being present in higher plants and green algae and absent in cyanobacteria (2, 7). Second, higher plants contain an additional membrane-integral light-harvesting complex, which increases the number of PS I antenna Chls to about 200 in comparison with about 100 in cyanobacteria. Third, higher plants contain only monomeric PS I, whereas PS I complexes of cyanobacteria are organized as monomers and trimers (5, 8–10). It could be shown that PsaL is responsible for this specific trimerization of cyanobacterial PS I (11) and that the ratio between trimers and monomers within the detergent-free cyanobacterial thylakoid membrane can be regulated through the ionic strength of the medium (5, 12). Presumably, electrostatic interactions between the complexes are altered, causing the transformation of trimers into monomers at high salt and the reverse reaction at low salt conditions.

The reason for the existence of PS I trimers in cyanobacteria is not yet clear. In general, cyanobacteria contain relative high amounts of the so called "red" Chls that show absorption at a longer wavelength than the reaction center P700 itself. The amount of red Chls in PS I varies from species to species. *Synechocystis* PCC 6803, for example, contains two Chls absorbing at 708 nm/monomeric complex (13), whereas PS I from *S. elongatus* contains more red Chls, which absorb at 708 (4–6 molecules) and 719 nm (4–6 molecules) (14). PS I trimers and monomers from *Spirulina platensis* differ in the composition of red Chls; although monomers and trimers have a similar set of Chl forms (12), only the trimers contain an extreme red shifted Chl, which absorbs at 735 nm (Chl 735). This Chl gives rise to a 760 nm fluorescence emission peak (F760) at 77 K. Under the same conditions, the monomer of *S. platensis,* which is similar to all other cyanobaceria, shows only emission peaks at 725–730 nm. Moreover, the intensity of the 760-nm emission band, which is visible only under reducing conditions, reflects directly

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Dedicated to Prof. Dr. Dr. hc mult. Achim Trebst on the occasion of his 70th birthday.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: PS, photosystem; Chl, chlorophyll; DM, *n*-dodecyl β-D-maltoside; HIC, hydrophobic interaction chromatography; HPLC, high performance liquid chromatography; IEC, ion exchange chromatography; P700, primary electron donor of photosystem I; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; F760 (F730), fluorescence band with maximal emission at 760 (730).

the redox state of P700 (9, 12). The general role of these red Chls in photosynthesis is still completely unknown. It was suggested that the red Chls funnel light energy to P700 (14–16), increase the cross-section of light absorption (17), or are involved in radiationless dissipation of excess energy into heat, thereby protecting PS I against photodestruction (18).

One of the approaches to investigate the mechanisms of trimer formation is the establishment of an *in vitro* reconstitution procedure. As *n*-dodecyl β-D-maltoside (DM)-solubilized monomers do not trimerize and DM-solubilized trimers do not dissociate into monomers (19), it is reasonable to assume that for such an oligomerization to occur the existence of a lipid bilayer is necessary. Up to now several studies have reported the incorporation of PS I into liposomes for functional analysis. However, no study dealt with membrane protein oligomerization, although this is an important feature in the course of PS I biogenesis and possibly also in light adaptation processes of cyanobacteria.

Here we report the liposome-based reconstitution of PS I trimers starting from isolated monomers of *S. platensis*. The reconstituted trimers show the same 760-nm emission band as PS I trimers *in vivo* indicating the functional intactness of these trimers. In addition, the appearance of the long wavelength emission band in proteoliposomes confirms the suggestion that the responsible Chls are present also in monomers and are located at the monomer periphery yielding a profound change in fluorescence upon trimerization. We also will show that the trimerization depends on the presence of the stroma-exposed subunits PsaC, -D, and -E. The implications for the trimerization, the possible role of trimers, and the origin of red Chls are discussed.

EXPERIMENTAL PROCEDURES

Isolation of PS I Complexes—Cells of *S. platensis* were grown in Zarouk medium (20) and stored at −70 °C until use. Trimeric and monomeric PS I complexes were isolated according to Ref. 3 with major improvements. Briefly, cells were disrupted with a RibiCell Fractionator (Sorvall), cell debris was removed by centrifugation, and membranes were pelleted by centrifugation (200,000 × *g*, 2 h, 4 °C). To remove phycobilins, membranes were washed two times by centrifugation (200,000 × *g*, 1 h, 4 °C). Membrane proteins were solubilized by the addition of solid DM (final concentration 1%). Remaining insoluble material was removed by centrifugation (200,000 × *g*, 1 h, 4 °C). Pure, homogeneous PS I could be isolated by two rapid perfusion chromatography steps. An ion exchange chromatography (IEC) on Poros 50 HQ (PerSeptive Biosystems, Wiesbaden, Germany) was performed followed by a hydrophobic interaction chromatography (HIC) on Poros Butyl (PerSeptive Biosystems, Wiesbaden, Germany). For HPLC, a Waters system (2 pumps, model 510, fitted with preparative pump heads, pump control module, Rheodyne Injektor, model 9125) coupled to a Waters diode array detector (DAD 996) and operated under the Millenium software package was used. HPLC columns were kept at 10 °C in an oven compartment (Beckman Instruments). Solubilized membrane proteins were filtered (0.45 μm, Schleicher & Schuell, Dassel, Germany) and immediately loaded on a IEC column (Poros 50 HQ, bed volume 17.3 ml). Elution was done by a linear NaCl gradient (flow rate, 6.7 ml/min; 10-300 mM NaCl within 10 min). The fractions containing either monomeric or trimeric PS I were pooled and adjusted to 1.6 M ammoniumsufate. Final purification was achieved on an HIC column (Poros Butyl, bed volume 18.7 ml) using a linear gradient for elution (flow rate, 6.7 ml/min; 1.5-0 M ammonium sulfate within 10 min). Upon concentration to 1 mg of Chl/ml (Centriprep 100, Millipore, Germany) and buffer exchange (20 mM Tris, pH 7.8, EconoPac 10DG, Bio-Rad) monomeric or trimeric PS I was stored at −70 °C until use.

Removal of PsaC, -D, and -E—Purified monomeric or trimeric PS I (200 μg of Chl/ml) was incubated for 30 min in 6.8 M urea (20 mM CAPS, pH 10) similar to Ref. 21. Excess urea and detached subunits were removed by repeated ultrafiltration (Microcon 100, Millipore, Germany).

Biochemical Standard Techniques—SDS-PAGE was done according to Schaegger and von Jagow (22). Electroblotting to a polyvinylidene difluoride membrane (Immobilon, Millipore, Germany) was as described by Matsudaira (23) followed by immunodetection using a secondary phosphatase-coupled antibody as in Ref. 24. Primary antibodies against PsaL were kindly provided by Dr. P. Chitnis (Iowa State University). Chlorophyll concentrations were determined using an extinction coefficient of 74,000 M^{-1} cm^{-1} at 679 nm.

Preparation of Proteoliposomes—Proteoliposomes were prepared according to methods described in Refs. 25 and 26 with some modifications. Soybean phosphatidylcholine and phosphatidic acid (molar ratio 9:1) were dispersed in Tris-buffer (25 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM NaCl) to a final concentration of 20 mg/ml. The mixture was sonicated (Transonic 460, Faust, Germany) for 3 min until the solution was optically clear. The resulting liposomes were completely dissolved by the addition of *n*-octyl β-D-glucopyranoside (3% (w/v)). PS I complexes were added to the solubilized liposomes at different Chl:lipid ratios ranging from 20 to 100 (w/w). After incubation of the mixture for 5 min at room temperature the detergent was removed by direct addition of SM-2 Bio-beads in three steps. 80 mg of wet beads/ml were added to the assay; after 1 h another 80 mg of wet beads/ml were added followed by a final 160 mg of beads/ml after an additional hour. The suspension containing proteoliposomes was pipetted off and stored at 4 °C.

77 K Fluorescence Emission Spectra—77 K fluorescence emission spectra of cells, PS I complexes, and proteoliposomes were measured with an Aminco Bowman Luminescence spectrometer (SLM-Aminco Spectronic Instruments). Chlorophylls were excited at 440 nm, and emission was measured perpendicular to the exciting beam. The raw fluorescence spectra were corrected for the wavelength sensitivity of the detection system. Measurements were done at a Chl concentration of 1 μg/ml for cells and 6 μg/ml for isolated PS I. Before fluorescence measurements a sample preparation of trimeric PS I involved dilution in Tris buffer (200 mM Tris, pH 10.6), reduction by dithionite (20 mM), and after a short incubation the addition of glycerol (final concentration 60% (v/v)). The sample was transferred to a quartz tube (4 mm, inner diameter) and slowly frozen under illumination to reduce P700 and the acceptor side of PS I (12). Because incubation of PS I monomers with dithionite had no effect on the fluorescence spectra, PS I monomers were frozen in the dark without a dithionite addition. Proteoliposomes were centrifugated (10,000 rpm, 4 min) and resuspended in Tris buffer (200 mM, pH 10.6) to a Chl concentration of about 5 μg/ml. After the addition of dithionite and glycerol, proteoliposomes were frozen under illumination. To follow the effect of high salt treatment on PS I complexes in proteoliposomes, they were incubated with 150 mM $MgSO_4$ or 300 mM NaCl for 5 min at room temperature and then dithionite and glycerol were added, and the samples were frozen under illumination.

HPLC Analysis of Proteoliposomes—Proteoliposomes were solubilized for 10 min with 1% DM (w/v). The remaining insoluble material was removed by centrifugation (13,000 rpm, 5 min), and the soluble fraction was applied on an analytical IEC column (Poros 50HQ, bed volume 1.8 ml). A linear NaCl gradient (10-300 mM; flow rate, 3.5 ml/min) was used for elution. The peak assignment as PS I monomers or trimers was based on size exclusion chromatography on a TSK-4000_{SWXL} column.

Materials—*n*-Dodecyl β-D-maltoside was purchased from Biomol (Germany). SM-2 Bio-beads were from Bio-Rad; soybean phosphatidylcholine (Type III-S) and phosphatidic acid were from Sigma. All other chemicals were of analytical grade and were purchased mostly from Fluka (Deisenhofen, Germany).

RESULTS

Isolation and Characterization of PS I Trimers and Monomers—Fig. 1 shows the purification of monomeric and trimeric PS I from the filamentous cyanobacterium *S. platensis*. The purification procedure was based on perfusion chromatography, which enables easy scaling up and works at extremely high flow rates without loss of resolution. We already published a purification procedure for PS I from the unicellular cyanobacterium *Synechocystis* PCC 6803, which included an IEC perfusion chromatography step (27). In this report we further succeeded in speeding up the purification protocol by replacing the second HPLC step with a perfusion chromatography. This enables us to purify large amounts (over 20 mg of protein) of pure and homogeneous monomeric and trimeric PS I from the filamentous cyanobacterium *S. platensis* within 2 days. Fig. 1 shows the elution profiles of the different columns used for purification. The first IEC column yields two peaks (Fig. 1*A*), which represent a monomeric (first peak) and a trimeric (sec-

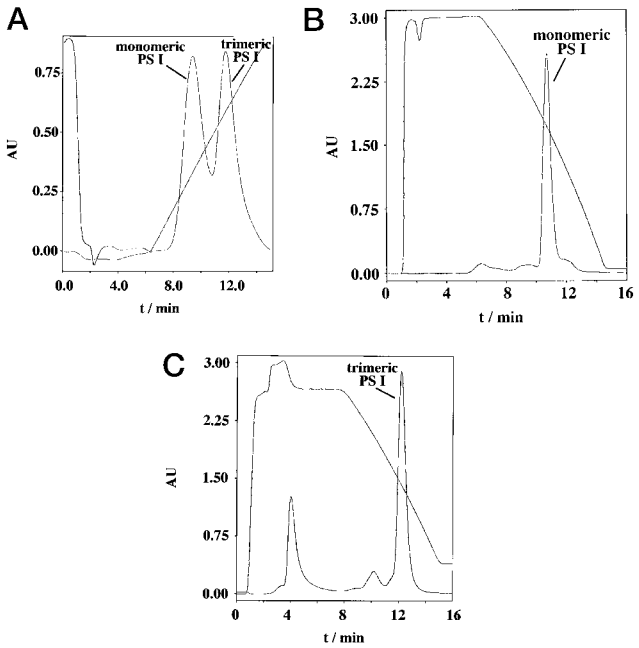

FIG. 1. **Purification of monomeric and trimeric PS I from *S. platensis.*** *A,* elution profile of a membrane extract from an IEC perfusion chromatography column (Poros 50 HQ, PerSeptive, Germany). Elution was done with a NaCl gradient (10–300 mM) at 10 °C and a flow rate of 6.7 ml/min. A separate trace shows the conductivity. *B,* elution profile of monomeric PS I (after IEC) from a HIC column (Poros Butyl, PerSeptive, Germany). A linear gradient of ammonium sulfate (1.5–0 M) was used for elution at 10 °C and a flow rate of 6.7 ml/min. *C,* elution profile of trimeric PS I (after IEC) from a HIC column (Poros Butyl, PerSeptive, Germany). Elution conditions identical to Fig. 1*B*. *AU*, absorbance units.

ond peak) form of PS I. Monomeric PS I elutes at around 100 mM NaCl; trimeric PS I elutes at around 180 mM. With a second purification step, consisting of a HIC column, extremely pure and homogeneous monomeric (at around 900 mM ammonium sulfate, Fig. 1*B*) or trimeric (at around 650 mM ammonium sulfate, Fig. 1*C*) PS I could be obtained. As the monomeric PS I after IEC is already quite pure, only traces of impurities like PS II are removed by the second perfusion step (Fig. 1*B*). In contrast, the trimeric fraction after IEC still contains some residual monomeric PS I as well as higher aggregates that are separated by HIC (Fig. 1*C*). The purity and identity of the trimeric and monomeric preparations were confirmed by HPLC size exclusion chromatography (data not shown).

Fig. 2*A* shows typical SDS-PAGE profiles of trimeric (*left*) and monomeric (*right*) PS I preparations. All of the major PS I subunits are present in both monomeric and trimeric PS I from *S. platensis.* The reaction center subunits PsaA and -B are evident at the top of the gel, whereas other major subunits were assigned by immunodetection as (from *top* to *bottom*): PsaD, -F, -L, -E, and -C. Differences in staining intensities of subunits E, F, and C are within variations of various preparations and not representative of monomers or trimers. Subunit C shows routinely a diffuse, weak staining band.

Smaller subunits are also visible but were not assigned in this report. Of special interest is the PsaL subunit, which is required for trimer formation. By using immunodetection it can clearly be seen that this subunit is present in roughly equal amounts in monomers and trimers (Fig. 2*B*). SDS-PAGE analysis of trimeric preparations sometimes shows a band around 30 kDa, which, according to immunodetection, must be attributed to PsaL. In these preparations the normal band for PsaL, just beneath PsaF, is reduced in staining intensity.

Fig. 3*A* shows the 77 K steady state fluorescence emission

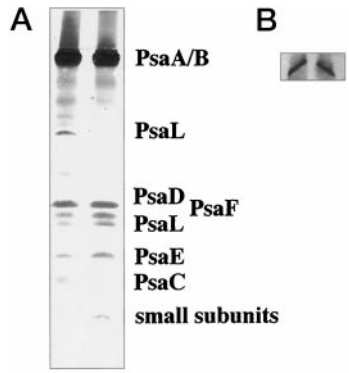

FIG. 2. **SDS-PAGE analysis of purified PS I from *S. platensis.*** *A,* trimeric (*lane 1*) and monomeric PS I (*lane 2*). The gel was stained with Coomassie Blue. *B,* immunoblot analysis of purified trimeric (*lane 1*) and monomeric PS I (*lane 2*). An antibody against the PsaL subunit was used for detection.

spectra of purified monomeric and trimeric PS I upon excitation at 440 nm. The spectrum of monomeric PS I contains only one emission peak at about 730 nm (Fig. 3) and no PS II typical emission around 695 nm, indicating the high purity and homogeneity of the sample. The trimeric PS I sample also shows an emission band around 730 nm, but the spectrum is dominated by the strong emission at 760 nm. This band is characteristic for trimers and completely absent in monomers. In addition, both particles show almost no emission at 680 nm, indicating the absence of uncoupled Chl. This was also confirmed by comparison of fluorescence emission spectra upon excitation of Chls at 440 nm or carotenoids at 500 nm, because energy absorbed by carotenoids migrates only to coupled Chls (data not shown).

Whole *Spirulina* cells frozen under illumination in the presence of dithionite show a high fluorescence emission band around 760 nm at 77 K similar to the emission spectrum of isolated trimeric PS I (Fig. 3*A*). This indicates that around 90% of PS I in intact cells grown at moderate light intensities is present as trimers (Fig. 3*B*), confirming earlier reports about a (mainly) trimeric *in vivo* organization of PS I in cyanobacterial cells (5, 9). The emission spectrum of cells without dithionite (*dashed line*) shows that some PS I trimers in dark incubated cells contain P700, which has already been reduced by internal reductants. Fluorescence peaks at 685 and 695 nm, indicative of PS II, appear more prominent in the *dashed curve* as this one has been normalized with the maximum peak of the full line curve and not with the 730 nm peak.

For a detailed study of the factors controlling the reversible process of PS I trimerization, it is necessary to develop an *in vitro* system. We therefore tried to reconstitute PS I trimers from monomers in an artificial lipid membrane, *i.e.* in proteoliposomes.

Reconstitution of PS I Trimers from Monomers in Liposomes—Based on the work of Rigaud *et al.* (28), we developed a procedure to incorporate PS I in large liposomes. In principle, the protein of interest is added to completely solubilized lipids, and proteoliposomes are generated by selective removal of excess detergent using Bio-beads. Among different detergents tested for solubilization of lipids, *n*-octyl β-D-glucopyranoside turned out to be the most effective one (data not shown). After incorporation of pure monomeric or trimeric PS I, proteoliposomes were characterized by fluorescence emission spectra at 77 K. Fig. 4*A* shows fluorescence emission spectra of proteoliposomes after reconstitution with monomeric PS I. An additional emission band around 760 nm can be seen, which indicates the formation of PS I trimers out of monomers within the proteoliposomes. According to the F760:F730 ratio for purified

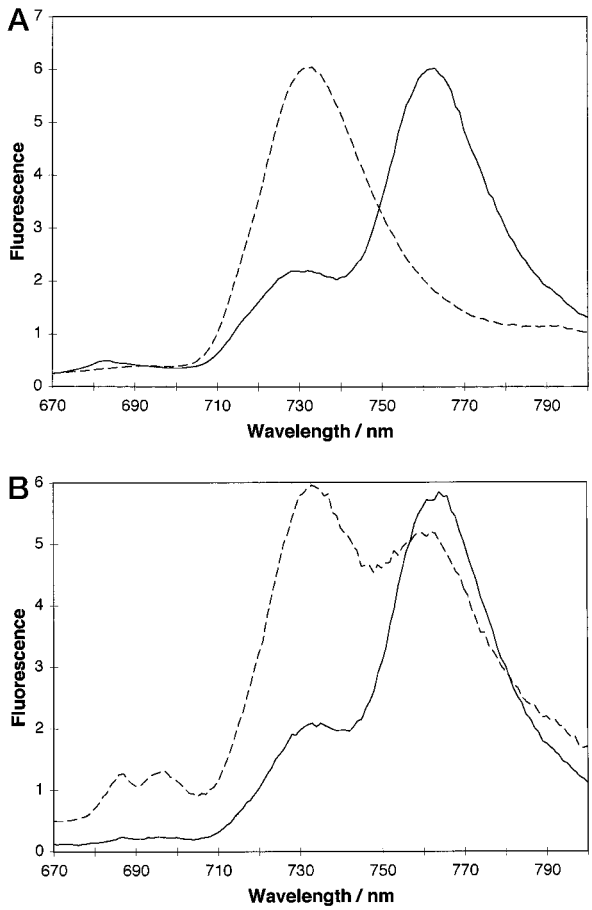

FIG. 3. **77 K fluorescence emission spectra of cells and of purified PS I from *S. platensis*.** *A,* fluorescence emission spectra of purified trimeric (*solid line*) and monomeric (*dashed line*) PS I particles after excitation at 440 nm. Chl concentration was 6 μg/ml (see "Experimental Procedures" for a detailed sample preparation protocol). *B,* fluorescence emission spectra of cells incubated and frozen in the dark without the addition of dithionite (*dashed line*) or frozen in the presence of 20 mM dithionite under illumination (*solid line*). Chl concentration was 1 μg/ml. Both spectra were normalized to the maximal signal, and fluorescence is presented in relative units.

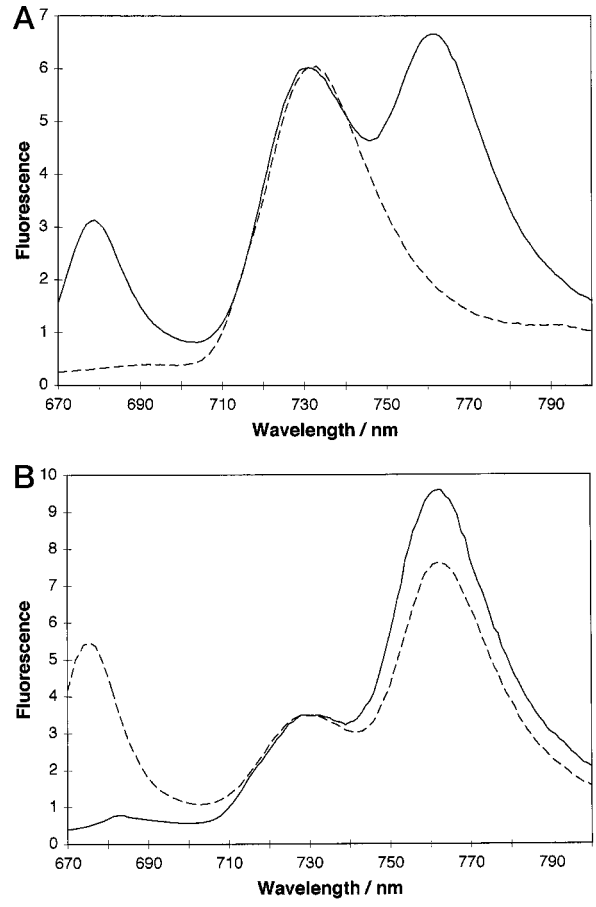

FIG. 4. **77 K fluorescence emission spectra of proteoliposomes.** *A,* emission spectra of the purified PS I monomers before (*dashed line*) and after incorporation into liposomes (*solid line*). Excitation was at 440 nm; the sample contained 5 μg of Chl/ml. *B,* emission spectra of purified PS I trimers before (*solid line*) and after (*dashed line*) incorporation into liposomes. Conditions are identical to *A* (see "Experimental Procedures" for the incorporation procedure). All spectra were normalized at 730 nm, and fluorescence is given in relative units.

(Fig. 3) and for reconstituted (Fig. 4*A*) trimers, up to 40% of the monomers were oligomerized into trimers. If trimeric PS I is incorporated into liposomes, the 760 nm emission is still the predominant peak in the spectrum, but emission at 730 nm has increased (Fig. 4*B*). This indicates the desintegration of some trimers into monomers. Both emission spectra show an additional peak at about 680 nm, which could be attributed to uncoupled Chl. This Chl uncoupling is caused by the high concentration of *n*-octyl β-D-glucopyranoside used during the reconstitution procedure (data not shown).

To confirm the formation of stable structural trimers, the proteoliposomes were extracted with DM. The extract was analyzed immediately by IEC perfusion chromatography (Fig. 5). The purity of the starting material was checked (Fig. 5*A*). Both the monomeric and the trimeric PS I preparations are homogeneous without any trace of other oligomeric forms. In addition it was shown that the preparations are stable and that the oligomeric nature is not altered by time or by salt treatments for 5 days (data not shown). A HPLC analysis of proteoliposomes prepared from monomers (Fig. 5*B*) shows a monomer peak and a smaller trimer peak in addition to uncoupled Chl. This confirms the conclusion that PS I trimers have been reconstituted from monomers. In the case of trimers (Fig. 5*C*), HPLC analysis of proteoliposomes also shows three peaks: uncoupled Chl, monomeric PS I, and trimeric PS I (main peak), confirming the spectroscopic finding that some trimers dissociate into monomers within the proteoliposomes.

To investigate the effect of different lipid:protein ratios on the reconstitution process, two different ratios have been tested. The trimer:monomer ratio in the proteoliposomes was monitored by noninvasive fluorescence spectroscopy (Fig. 6) and is reflected by the ratio of the fluorescence bands at 760 and 730 nm. In proteoliposomes prepared from monomers this ratio was 1.6-fold higher when the protein content in the reconstitution mixture was increased by a factor of five (Fig. 6*A*). In conclusion, the amount of trimers formed depends on the protein concentration. A higher protein concentration increases the amount of trimers. If proteoliposomes were prepared from trimers, a 5-fold increase in the protein concentration had no effect on the ratio of the emission bands at 730 and 760 nm, indicating that the dissociation of trimers is independent of the protein concentration at least within the concentration range tested.

Influence of Salt on the PS I Oligomerization in the Membrane—The monomer:trimer ratio can be shifted in isolated thylakoid membranes by simple salt incubation as published before (12, 24). To test whether this is possible with the reconstituted complexes, we incubated the proteoliposomes with different salt concentrations and determined the monomer:trimer ratio directly by measuring the fluorescence emission spectra (Fig. 7). Proteoliposomes made from monomers show a distinct decrease of the 760 nm peak upon a shift from 10 to 150 mM

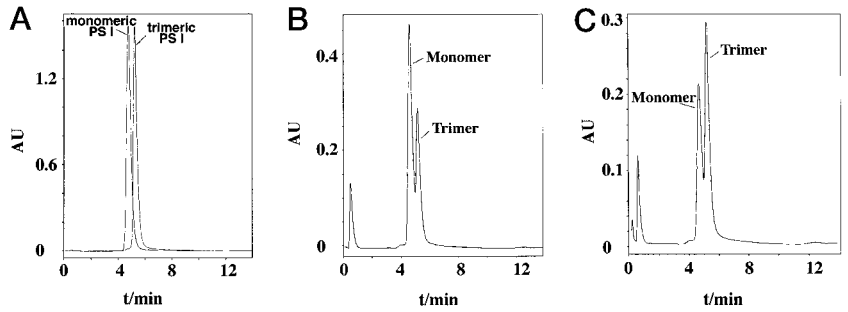

FIG. 5. **HPLC analysis of proteoliposomes.** *A,* elution profile of purified monomeric PS I complexes superimposed on an elution profile of trimeric PS I complexes. Both particles have been used for reconstitution. An analytical IEC column (Poros 50 HQ, PerSeptive, Germany) was used to discriminate between monomers and trimers. Elution was done with a linear NaCl gradient (10-300 mM, 20 mM Tris-HCl buffer, pH 7.8) and a flow rate of 3.5 ml/min. *B,* elution profile of a proteoliposome extract when proteoliposomes were made from pure monomeric PS I complexes. Separation conditions are as in *A*. *C,* elution profile of a proteoliposome extract when proteoliposomes were made from pure trimeric PS I complexes. Separation conditions are identical as in *A*. *AU*, absorbance units.

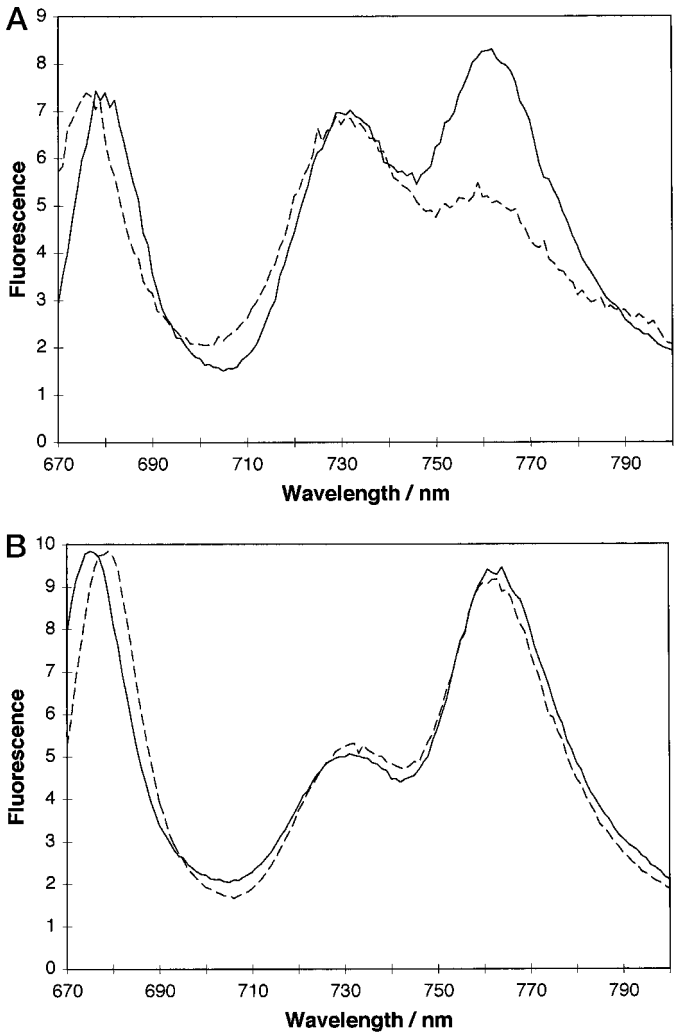

FIG. 6. **Effect of lipid:protein ratio on the reconstitution process.** *A,* 77 K fluorescence emission spectra of proteoliposomes made from pure monomeric PS I. The lipid:Chl ratio was changed from 100:1 (*dashed line*) to 20:1 (*solid line*) with the lipid concentration remaining constant. Excitation of samples containing 5 μg of Chl/ml was done at 440 nm. *B,* 77 K fluorescence emission spectra of proteoliposomes made from pure trimeric PS I. The lipid:Chl ratio was changed from 100:1 (*dashed line*) to 20:1 (*solid line*). Conditions are as in *A*. All spectra were normalized at 730 nm, and fluorescence is given in relative units.

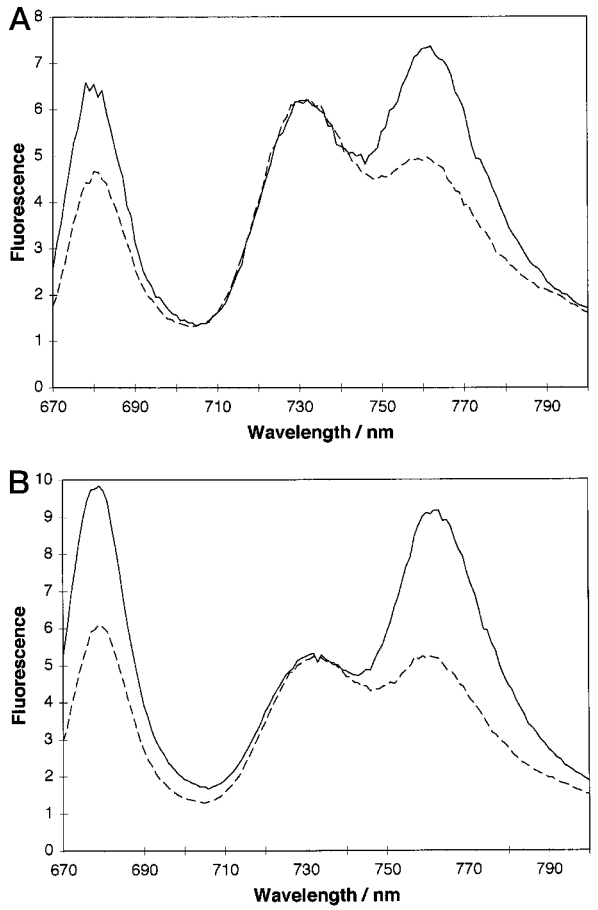

FIG. 7. **Effect of salt treatment on proteoliposomes.** *A,* 77 K fluorescence emission spectra of proteoliposomes prepared from monomers before (*solid line*) and after (*dashed line*) incubation in 50 mM Tris-HCl buffer containing 150 mM $MgSO_4$. *B,* 77 K fluorescence emission spectra of proteoliposomes prepared from trimers before (*solid line*) and after (*dashed line*) incubation in 50 mM Tris-HCl buffer containing 150 mM $MgSO_4$. Excitation wavelength is 440 nm, and Chl concentration is 5 μg/ml. All spectra were normalized at 730 nm, and fluorescence is given in relative units.

$MgSO_4$ (Fig. 7*A*). Conversely, an increase of the fluorescence emission intensity at 730 nm relative to the decreasing intensity at 760 nm can be seen in proteoliposomes prepared from trimers upon increasing the salt concentration from 10 to 150 mM (Fig. 7*B*). The reduction of the 760 nm emission peak and the increase of the 730 nm emission peak indicate the salt-induced dissociation of trimers. Other salts like the monovalent NaCl (up to 300 mM) induced a similar behavior of the photosystems (data not shown).

Influence of Stromal Subunits on PS I Oligomerization—To determine the decisive factors for the trimerization, we first investigated the role of the stromal subunits PsaC, -D, and -E.

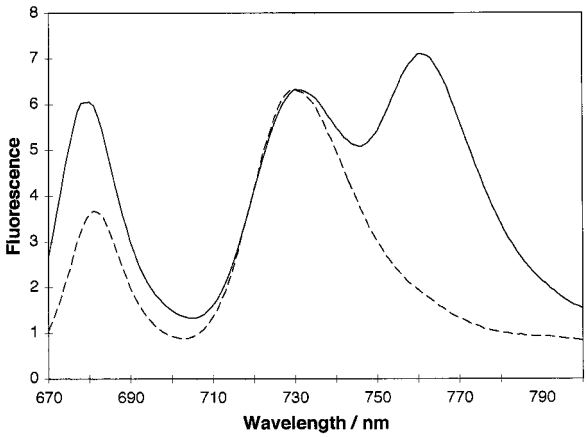

FIG. 8. **Effect of the stromal subunits on the reconstitution process.** 77 K fluorescence emission spectra of proteoliposomes with excitation at 440 nm. Proteoliposomes were prepared either from monomeric PS I containing all subunits (wild type) (*solid line*) or from monomeric PS I lacking the three stromal subunits PsaC, -D, and -E (because of urea treatment, see "Experimental Procedures") (*dashed line*). Spectra were normalized at 730 nm, and fluorescence is given in relative units.

A central role of PsaD, for instance, is suggested, because it had been shown that PsaD can be cross-linked to PsaL and extends to the trimer center (3). The three stromal subunits were removed by urea treatment. The selectivity and effectiveness of the urea wash was confirmed by SDS-PAGE (data not shown). Furthermore, we made sure that the isolated monomeric and the trimeric PS I particles lacking the stromal subunits had the same fluorescence characteristics as the intact complexes, *e.g.* trimeric PS I without PsaC, -D, and -E still showed emission at 760 nm. However, when monomeric PS I without the stromal subunits was used for reconstitution and the resulting proteoliposomes were probed by 77 K fluorescence spectroscopy, no emission at 760 nm could be observed (Fig. 8). This clearly shows that the trimer formation depends critically on the presence of the stromal subunits.

DISCUSSION

Purification of PS I—In this paper we introduce a new type of chromatography, HIC based on porous material allowing perfusion chromatography, for the purification of PS I. With this improvement we created a fast and reliable purification method to obtain mg quantities of pure PS I suitable for x-ray analysis in a very short time. Although the general advantages of perfusion chromatography have already been pointed out by us (3), we show here that hydrophobic perfusion chromatography can even be used to purify highly hydrophobic membrane proteins. Instead of being bound irreversibly to such a hydrophobic column, transmembrane protein complexes like photosystem I can be eluted functionally intact at moderate salt concentrations. This could also be shown by us for the PS II and the cytochrome b_6f complex (29, 30).

Compared with the PS I purification procedure developed by us for *Synechocystis* (3, 24), the new procedure for *S. platensis* is quite similar. Major differences include purification at pH 8.0 instead of pH 6.5, which may be because of the highly alkaline growth environment (pH $>$ 10) of *S. platensis*. Moreover, for the elution in IEC, monovalent anions (NaCl) instead of divalent ions ($MgSO_4$) had to be used. This reflects the unique charge density distribution of PS I from different species.

Characterization of Isolated PS I—Whereas several reports deal with the spectral properties of PS I from *S. platensis* (12, 16, 20), only limited information is available on the biochemical level (9, 31). Here we show that PS I from *S. platensis* contains the same major subunits (PsaA, -B, -C, -D, -E, -F, and -L) that were already found in *Synechocystis* (24) and *Synechococcus* (32). Several smaller subunits appeared below 8 kDa, possibly PsaK, -I, -J, and -M, but they were not assigned because of the lack of specific antibodies. One particular feature of *Spirulina* PS I is the occurrence of a band during SDS-PAGE that can be attributed to an oligomeric form of PsaL. This band at about 30 kDa (monomeric PsaL appears at about 14 kDa) may represent a dimer or a trimer of PsaL. Because PsaL is the very subunit necessary for trimer formation (11), we favor the interpretation of a trimeric aggregate. This is no contradiction to the apparent mass of 30 kDa as hydrophobic membrane proteins often show anomalous migration behavior during SDS-PAGE. It would also imply that the trimers of *Spirulina* are extremely strong, which is confirmed by our reconstitution experiments, and may also contribute to the special spectroscopic features of the trimeric PS I particles from *S. platensis*. In the course of the purification procedure, no traces of a putative dimeric PS I complex could be found. This also indicates that the trimerization is a very specific process. The low resolution x-ray structure of the thermophilic cyanobacterium *Synechococcus* (6) shows the delicate connection domain built by PsaL ruling out an unspecific aggregation, which might result in dimer formation.

In accordance with the literature, isolated monomers and trimers from *S. platensis* show distinct 77 K fluorescence spectra (12, 20). Only the trimeric form exhibits a strong emission at 760 nm, which can be used as an internal spectroscopic marker for the presence of trimers. As this signal prevails in intact cells it must be concluded that the trimeric form is the predominant species of PS I in the living cell. Recently a 740–750 nm emission was also reported at room temperature (33) giving further evidence for the *in vivo* existence of trimers and far red absorbing Chls, which are termed the "red Chls" hereafter.

Reconstitution of PS I into Liposomes—There are a few reports about PS I reconstitution that focus on the function (induction of a light driven proton gradient) (26) or on the spectral properties of PS I (protein-pigment interactions) (34). In contrast, we used reconstitution to study the oligomerization of PS I in a strictly controlled environment. To incorporate homogeneous monomeric or trimeric PS I into liposomes, we modified a reconstitution protocol from Rigaud and co-workers (26).

We used PS I isolated from *S. platensis* for our studies, which proved to be the best model system to study the trimerization process. Only *Spirulina* trimers differ from monomers spectroscopically. We therefore have two independent methods to quantify the oligomeric state of PS I, 77 K fluorescence spectroscopy and HPLC. In addition, as discussed above, these trimers seem to be extremely stable. Although other well characterized cyanobacterial PS I preparations exist from *Synechocystis* PCC 6803 (3, 19, 24) or *S. elongatus* (43), for instance, they do not offer these possibilities. Especially with *Synechococcus* it is extremely difficult to isolate monomers with the complete set of all subunits, particularly the PsaL protein (35). In *Synechocystis*, which can be easily genetically modified, the trimers seem to be less stable. Under identical conditions as for *Spirulina*, only traces of trimers ($<$5%) could be detected with our reconstitution assay.

If we use pure monomeric PS I for the reconstitution process, the proteoliposomes exhibit the typical trimer emission at 760 nm, whereas starting with trimeric PS I yields a reduced F760 and an enhanced F730 intensity in proteoliposomes. Taken together, these data indicate a dynamic equilibrium between

monomers and trimers in the membrane. They show for the first time that no additional proteins or external factors are necessary for the specific formation of trimers. This trimerization process is an excellent example for self organization of a membrane protein. Thus the argument that the trimerization is artificially induced by the detergent treatment during extraction (36) is highly improbable. On the contrary, when isolated monomers are incubated with detergents (DM or *n*-octyl β-D-glucopyranoside) in the absence of a lipid phase, nothing but the denaturation of the pigment-protein complex can be observed after some time.

Reconstitution of trimers from monomers depends on the protein:lipid ratio (on a weight basis) in the reconstitution assay. A higher ratio increases the percentage of assembled trimers out of monomers. In contrast, variation of this ratio had virtually no effect if trimers were used for the reconstitution. The amount of trimers dissociating into monomers upon reconstitution is constant. The first case could be explained by a concentration dependent effect. If the amount of monomers within the liposome increases, the probability for trimer formation will be higher, which is what we observe. There seems to be an optimal monomer:trimer ratio that is established when the reconstitution starts with trimers. This explains why the protein:lipid ratio does not influence the F760 intensity of proteoliposomes made from pure trimers. In the case of monomers, some of them may not be able to form trimers because of loss of the PsaL subunit (in up to 15% of all monomers). For this reason an excess of monomers must be present to establish the optimal monomer:trimer ratio.

The monomer:trimer ratio can also be manipulated by the ionic strength of the medium after incorporation of PS I into liposomes. Higher ionic strength (adjusted either by NaCl or $MgSO_4$) leads to a monomerization of trimers. Such a "salt effect" was first reported for isolated thylakoid membranes from *Synechocystis* and later from *Spirulina* (5, 12). This effect may be explained by charge screening, which weakens the attractive forces between the monomers within the trimer; it is independent of the type of salt used and will be analyzed quantitatively in future experiments.

To understand the structural determinants of the trimerization process, a combination of an *in vitro* system with the possibility to carry out site-directed mutagenesis is necessary. Whereas the *in vitro* system has now been realized, the molecular biology of *Spirulina* has still to be established. Results obtained with *Synechocystis* clearly indicate that the PsaL subunit is absolutely necessary for trimerization as a deletion mutant was unable to form trimers (3, 11). Beyond this, our *in vitro* system also allows the determination of factors that are necessary for the first steps of trimerization. If the stromal subunits PsaC, -D, and -E are removed from monomers by urea treatment, no trimer formation is possible. On the other hand, if these stromal subunits are removed from already assembled trimers, these trimers remain stable (24). This indicates that the stromal subunits are necessary for the process of trimer formation but are not needed to stabilize assembled trimers. The drastically reduced trimer content in a strain of *Synechocystis* in which the PsaD subunit had been deleted confirms our observation (3). These results also point to PsaD as the key component of the stromal subunits for the trimer formation, which is further supported by structural studies using electron microscopy and x-ray analysis (3, 6). Whereas PsaC and PsaE have been mapped toward the outside of the trimer, only PsaD is located near the center close to PsaL, which could also be shown by cross-linking studies. We therefore attribute the drastic effect of the stromal subunits on PS I trimerization to the PsaD subunit alone. A more detailed characterization of this effect will be possible when genetic manipulation of *Spirulina* becomes feasible.

What can we learn about the spectroscopic properties of PS I from our reconstitution experiments? An unresolved question in PS I biophysics is the occurrence of red Chls. Both their function and their location are unknown. Deconvolution of 77 K absorption spectra of monomers and trimers showed a slightly higher content of red Chls in trimers (12). One possibility could be that the Chls responsible for the 760 nm emission of trimers have been lost in monomers. However, our data clearly show that these Chls are present in monomers too, although they do not show such a long wavelength emission there. In conclusion, trimerization should either lead to a conformational change or to a direct interaction of Chls located on different monomers. In both cases the environment of these Chls is altered in such a way that long wavelength emission can occur. We suggest that these red Chls are located near the trimer center. Indeed, x-ray analysis shows several Chls close to the trimer center (6), which is also in line with a PsaL deletion mutant showing a slightly reduced Chl content[2] and a mathematical simulation model, which places the extreme red Chls (Chl735) of *S. platensis* trimers about 42 Å apart from the P700 reaction center (37). In summary, all results clearly favor a location of the extreme red Chl of *Spirulina* close to the trimer center.

Many membrane proteins of different function and origin like bacteriorhodopsin (38), cytochrome oxidase (39), and the glycophorin A receptor (40) have been reported to occur either as trimers (PS I and bacteriorhodopsin) or as dimers (all other cases). The reason for this oligomerization is unknown. For some receptors dimerization is required for their signaling mechanism (41), but in the case of photosynthetic membrane protein complexes such an explanation can be ruled out. Oligomerization may enable cooperation of two complexes during the catalytic cycle as in the case of the cytochrome bc_1 complex or provide enhanced stability against proteolysis or heat stress. The reconstitution system presented in this report provides an excellent model system to analyze specific factors in detail in a defined lipid environment without interference of other (protein) components. These studies will finally help to understand the functional implications of membrane-protein oligomerization. In the case of the glycophorin A receptor, the dimerization of membrane proteins is well documented down to the molecular level (40, 42). Screening by site-directed mutagenesis revealed a dimerization motif formed by few specific residues, which is sufficient to drive the specific dimerization and provides enough energy to stabilize the dimer. A similar approach, combined with our reconstitution system, should enable us to analyze the trimerization-specific factors of PS I on a molecular level once the tools for genetic manipulation of *S. platensis* are established.

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