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Photosystem I from *Synechococcus elongatus*: preparation and crystallization of monomers with varying subunit compositions

Petra Jekow [a], Petra Fromme [b], Horst T. Witt [b], Wolfram Saenger [a,*]

[a] *Institut für Kristallographie, Freie Universität Berlin, Takustr. 6, 14195 Berlin, Germany*

[b] *Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Str. d. 17. Juni 135, 10623 Berlin, Germany*

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Abstract

Monomers of Photosystem I (PS I) from the thermophilic cyanobacterium *Synechococcus elongatus* were prepared and characterized. Using both the zwitterionic sulfobetain 12 and the nonionic β-dodecyl maltoside as detergents, PS I was extracted in trimeric form from the thylakoid membrane. The trimers dissociate into monomers during gel-filtration, apparently due to a change in the osmotic pressure. The PS I monomer prepared in β-dodecyl maltoside exhibits the same subunit composition as the trimeric PS I. The preparation in sulfobetain 12, however, leads to the removal of the two hydrophobic subunits K and L from the monomer. Further treatment of this sample with sulfobetain 12 detaches the subunits F, J and M, associated with the disappearance of a 706 nm band in the absorption spectrum. Both the PS I monomer with complete subunit composition and the monomer lacking subunits K and L were crystallized using batch and vapor diffusion techniques with poly(ethylene glycol) as the precipitating agent.

Keywords: Photosystem I; Crystallization; Thermophilic cyanobacterium; Monomer preparation; Subunit composition

1. Introduction

In cyanobacteria and higher plants, the initial step of photosynthesis is a light-induced transmembrane charge separation. This occurs in two protein complexes (Photosystems I and II) embedded in the thylakoid membrane. In PS II, the light reaction transfers the electron across the membrane from water to plastoquinone (for overview see [1]). PS I is responsible for the light-induced transmembrane transport of electrons from plastocyanin or cytochrome c_6, respectively, via ferredoxin to $NADP^+$-reductase (for review see [2]).

From *Synechococcus* sp., now classified as *Synechococcus elongatus* [3], the most stable form of PS I in vitro is a trimer [4], which may also represent the existing form of PS I in the native membrane [5,6]. The trimers could be dissociated and separated to yield dimers and monomers [7]. The latter contain one P700 each and therefore represent the functional unit of PS I.

At least 13 genes, labeled *psaA* through *psaM*, have been identified encoding for the protein subunits A to M of PS I [8]. Of these genes 12 are present in plants and encode for the subunits A to L. Cyanobacterial PS I consists of at least 11 protein subunits, which are named A to F and I to M. In *Synechococcus elongatus* the genes encoding for all 11 subunits have been identified [9], but some uncertainty still remains as to whether subunit I is expressed and incorporated into the protein complex. Apart from the protein subunits, the PS I monomers contain about 80 to 100 chlorophyll *a*, three $[Fe_4S_4]$ clusters, two vitamin K-1 and 12–15 β-carotenes [8]. The total molecular mass of the monomeric protein complex is about 340 kDa, deduced from the code of the isolated genes [9] and the content of chromophores.

Abbreviations: PS I, PS II, Photosystem I and II; Chl *a*, chlorophyll *a*; SB12, *N,N*-dimethyl-n-dodecylamino-3-propanesulfonate, sulfobetain 12; β-DM, n-dodecyl β-D-maltoside; Tris, tris(hydroxymethyl)aminomethane; P700, primary electron donor of Photosystem I; PEG, poly(ethylene glycol); Mes, 2-(*N*-morpholino)ethane sulfonic acid; A_x, buffer containing 20 mM Mes (pH 6.4) and $MgSO_4$; the index *x* denotes the $MgSO_4$ concentration in mM; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; cmc, critical micellar concentration.

* Corresponding author. Fax: +49 30 8386702.

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Methods of preparation for monomers of PS I from various sources have been published [4,7,10–14]. PS I monomers have been extracted from the thylakoid membrane by treatment with Tris-buffered Triton X-100 solutions [10,11] or alternatively by treating the PS I trimers with sodium dodecyl sulfate [4], sodium cholate [13] or β-octyl glucopyranoside at elevated temperatures [7]. After extracting carotenoids from the membrane using n-heptane, the remaining PS I molecules were observed to be in monomeric form [11]. Extraction of PS I from the membrane of the mesophilic cyanobacterium *Synechocystis* PCC 6803 at high salt concentrations nearly exclusively yields PS I monomers [14]. Mutants of this organism which do not contain the gene encoding for subunit L are no longer able to form trimeric PS I, and only monomers are observed [15].

The trimeric form of PS I from *Synechococcus elongatus* has been crystallized [16,17]. The crystals diffract X-rays to a resolution of 4 Å and the structure has been published at 6 Å resolution [18]. These crystals contain about 80% solvent by volume, which may explain why the attainable resolution of the X-ray diffraction does not extend beyond 4 Å even if synchrotron radiation is employed. The X-ray structure analysis shows that each monomer of the trimeric PS I consists of a catalytic domain and a smaller domain in close proximity to the threefold axis which connects the monomers into trimers (Fig. 1). This connecting domain is presumed to contain subunit L [15,18].

Because trimeric PS I from different sources crystallizes in unit cells comparable to that of *Synechococcus elongatus* [10], we initiated the preparation and crystallization of monomeric PS I, which has been reported to crystallize in a more closely-packed fashion [10]. To further improve the crystal packing, we aimed at obtaining monomers of PS I more regular in shape in particular by removing the protruding connecting domain, subunit L, from the complex. Preparations using two alternative detergents led to photosynthetically active monomers with diverging subunit compositions, both of which could be crystallized.

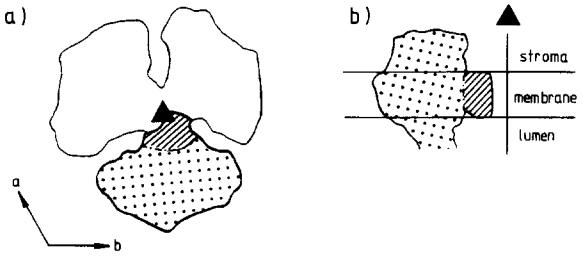

Fig. 1. (a) A view of the projection-surface of a PS I trimer onto the crystallographic *a,b* plane, which is parallel to the membrane layer. One monomer is dotted, the assumed connecting domain is hatched. The threefold axis of the trimer is indicated by a black triangle. (b) View of one monomer (dotted in a) along the crystallographic *b*-axis. Separation lines between the monomer and the connecting domain are drawn arbitrarily. The figure is taken from [18] with the permission of Norbert Krauß and has been slightly modified.

2. Material and methods

Synechococcus elongatus cells were grown and harvested, and the thylakoid membranes isolated as described in [19]. The membranes were suspended to a chlorophyll *a* concentration of 1 mM in buffer containing 20 mM Mes (pH 6.4), 10 mM $MgCl_2$, 20 mM $CaCl_2$ and 500 mM mannitol, and the PS I trimers were extracted with either 0.6% (w/v) SB12 (Serva) or 0.6% (w/v) β-DM (Anatrace) in the same buffer. Sucrose-density gradient centrifugation of the extracts [7] removed most of the phycobilisomes and free carotenoids.

All eluting buffers A_x (where the subscript denotes the $MgSO_4$ concentration in mM) used in the following PS I trimer-dissociation and monomer-purification procedure contained 20 mM Mes (pH 6.4) and *x* mM $MgSO_4$ and were filtered using 0.22 μm sterile filters. The detergent concentration in these buffers was slightly above the respective critical micellar concentration of the detergent.

2.1. Protocol for preparation and purification of PS I monomers using β-DM

The PS I trimers dissociated into monomers when passed through a G50 gel-filtration column. The eluting buffer A_{25} contained 0.02% (w/v) β-DM. For subsequent crystallization trials, highly purified PS I monomers had to be created on a preparative scale. Purification of the monomers and separation from PS II, ATP-synthase, still undissociated PS I trimers, etc. was performed on three ion-exchange columns with increasing separating power:

Step I: The gel-filtrate was applied to a Q-Sepharose Fast Flow column (Pharmacia), and the protein was eluted with a step gradient of A_{25}, A_{50} and A_{200} and 0.02% (w/v) β-DM. The separation performance of this column was not sufficient to separate the PS I monomer fraction entirely from the other membrane proteins. It removed remaining phycobilisomes and free carotenoids. The photosystem fractions eluted with the buffer of highest ionic strength.

Step II: Further purification was achieved by diluting the sample to a final $MgSO_4$ concentration of 30 mM using A_0-buffer (0.02% (w/v) β-DM) and applying this to a Q-Sepharose High Performance column (Pharmacia) connected to a HPLC (Knauer). The sample was eluted using a linear gradient of $MgSO_4$ from 25 to 200 mM in 20 mM Mes (pH 6.4) and 0.02% (w/v) β-DM; PS I monomers were detected at a $MgSO_4$ concentration of approx. 70 mM. This column removed the PS I trimers and most of PS II. The sample still contained about 5% ATP-Synthase and inactive PS II, and pure PS I Monomer

was obtained after the following Mono Q HR 10/10 column.

Step III: After diluting the protein solution to a ratio of 1:10 using A_0-buffer (0.02% (w/v) β-DM), the monomer fractions were finally purified on a Mono Q HR 10/10 column (Pharmacia), which was connected to the same HPLC referred to above. Employing a gradient of A_0 to A_{100} with 0.02% (w/v) β-DM, the PS I monomer eluted with approx. 40 mM $MgSO_4$. The purified protein sample was concentrated in an ultrafiltration chamber (Amicon) under nitrogen pressure of up to 2 bar with YM100-membranes or in Centricon 100 microconcentrators (Amicon) at 2000 RPM in a SS34-rotor (Sorvall). To lower the salt concentration, the sample was washed twice in buffer A_0 with 0.02% (w/v) β-DM.

Protocol for preparation and purification of PS I monomers using SB12 (only deviations from the protocol for β-DM-extracted protein are supplied)

The elution buffer used to dissociate the trimers on the gel-filtration column contained 0.2% (w/v) SB12 instead of β-DM. The first purification step on the Q-Sepharose Fast Flow column was also performed with 0.2% (w/v) SB12 instead of β-DM in the elution buffers. In order to avoid additional removal of subunits from the protein complex during the subsequent process of crystallization, the zwitterionic detergent SB12 was replaced by the non-ionic β-DM. For this purpose, 10% (w/v) β-DM in A_0-buffer was added to the protein solution to give a final concentration of 0.2% (w/v) β-DM. The solution was incubated for at least 12 h at 4° C, before the protein was applied to the Q-Sepharose High Perfomance column (step II). All the following steps were carried out according to the procedure described for β-DM-extracted monomers.

2.2. Characterization of PS I monomers

Two analytical columns were used to monitor the dissociation and purification steps. Monomeric and trimeric PS I can be distinguished on the basis of their respective molecular weight and surface charge. Separation according to the molecular weight has been performed using size exclusion chromatography. On the analytical size exclusion column TSK 4000 SW (Beckmann) run with A_{25} (0.02% (w/v) β-DM) as elution buffer and a flow rate of 0.8 ml/min, PS I was eluted with volumes of 9.4 ml for trimers and 10.7 ml for monomers (data not shown). On ion exchange chromatography columns (Mono Q HR 5/5, Pharmacia), the protein complexes were separated according to their various surface charges.

A Phastsystem electrophoresis unit (Pharmacia) with ready-to-use high-density gels (resolving from 100 to 1.5 kDa) was used for SDS-PAGE; the gels were silver-stained.

Photosynthetic activity and the concentration of P700 were measured by flash-light induced absorption changes of the photooxidation of P700 at 703 nm [20]. The content of chlorophyll *a* was determined as described in [7]. The absorption spectra were performed on a Spectronic 3000 array spectrophotometer, Milton Roy.

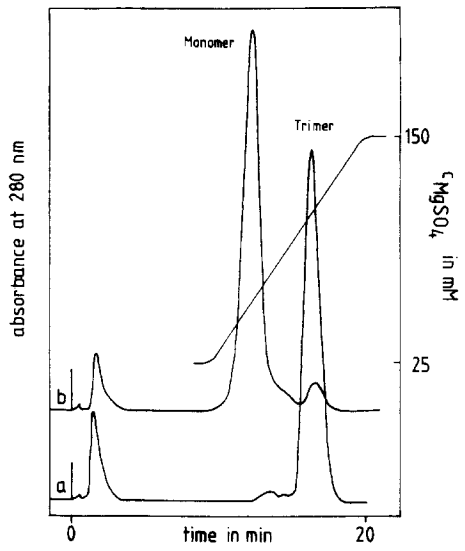

Fig. 2. Chromatograms of analytical FPLC-runs with a Mono Q HR 5/5-column. The eluting buffer was 20 mM Mes (pH 6.4) and 0.02% β-DM with a linear gradient of 25 to 150 mM $MgSO_4$. (a) Trimeric PS I after sucrose density gradient centrifugation. (b) Monomeric PS I, after elution of sample (a) from a G50 gel-filtration column.

2.3. Crystallization of PS I monomers

For the crystallization experiments, batch and vapor diffusion techniques were used.

The SB12-extracted monomers were crystallized according to [10] using batch methods with a precipitating buffer containing 20 mM Mes (pH 6.4), 50 mM $MgSO_4$, 0.02% (w/v) β-DM and 7 to 9% (w/v) poly(ethylene glycol) (PEG) 6000. The protein (5 μl samples with a protein concentration of approx. 10 mg/ml) was mixed with the precipitating buffer in a volume ratio of 1:2 in siliconized glass tubes of 6 mm diameter and 36 mm height. The tubes were closed with parafilm and left undisturbed at 18° C. Due to the fragility of the crystals obtained, the protein-precipitating buffer mixture was also applied directly into X-ray quartz capillaries of 1 mm diameter.

Crystallization of both PS I monomer preparations was achieved by vapor diffusion methods with a precipitating buffer containing 20 mM Tris (pH 8.5) and 7 to 10% (w/v) PEG 4000. These conditions were found by screening tests with different precipitating solutions according to the method described in [21]. For vapor diffusion in the 'hanging drop' mode Linbro trays (model FB-16-24-TC, Flow Laboratories) were used. The wells were filled with 700 μl precipitating buffer and closed with siliconized microscope cover slips (20 mm diameter), to which drops of 1 to 3 μl PS I sample mixed with the precipitating buffer in a volume ratio of 1:2 (final protein concentration approx. 5 mg/ml) had been applicated. Sealing the rim of

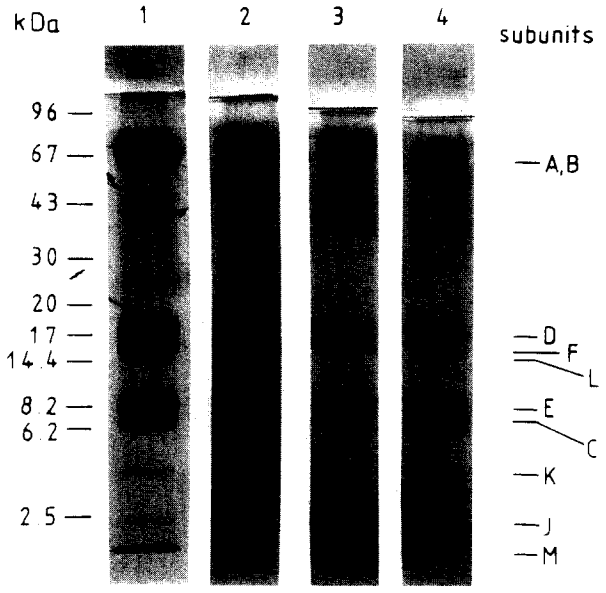

Fig. 3. SDS-PAGE patterns. Lane (1) PS I trimer; (2) PS I monomer, extracted and prepared in β-DM; (3) PS I monomer, extracted and prepared in SB12 (PS I {−K, −L}); (4) PS I monomer as in (3), further incubated with 5% (w/v) SB12 for 24 h at room temperature. On the left of the pattern, bands of a molecular mass marker are indicated, and on the right, bands are assigned to PS I subunits. (Silver stained Phastsystem High Density Gel).

the wells with silicone vacuum grease ensured an airtight compartment once the cover slips were placed on top.

X-ray diffraction experiments were performed with a MAR Research Image Plate detector using graphite monochromatized CuK α-radiation from an ENRAF-NONIUS FR571 rotating anode generator operated at 40 kV, 80 mA, focus 0.25×0.25 mm^2.

3. Results and discussion

Using both SB12 and β-DM, PS I was extracted from the thylakoid membranes as a trimer. After sucrose density gradient centrifugation, the sample contained about 95% trimeric PS I in both preparations (Fig. 2, chromatogram a). Gel-filtration of these samples led to the dissociation of trimeric PS I into monomers. For both extracts, the amount of PS I monomers obtained by this method corresponds to 85% of the total protein (Fig. 2, chromatogram b).

We assume that the described dissociation of PS I trimers into monomers on a simple gel-filtration column is caused by osmotic pressure. The sample applied to the G50 column contained 0.6% (w/v) of the respective detergent and a sucrose concentration close to saturation.

Our working hypothesis for the dissociation process may be summarized as follows: the detergent micelle encloses the PS I trimer and covers the solvent accessible cavities between the individual monomer units. Initially these cavities are in equilibrium with the surrounding solution and are consequently filled with sucrose. During gel-filtration the sucrose outside these cavities is removed instantaneously. Whereas the detergent shell hinders the passage of sucrose molecules out of the cavities, water is free to diffuse inward. The resulting osmotic pressure forces the dissociation of the trimers. The dissociation in the described way is also possible on one of the Q-sepharose columns described below or by dialysis.

We observed that three conditions were critical for successful dissociation: (1) The detergent micelle enclosing the PS I trimer should be continuous and homogeneous. If this detergent shell is not continuous (detergent concentration close to the cmc) or contains different detergent molecules (mixed micelle), the sucrose can diffuse out of the cavities and no dissociation occurs. (2) The sucrose concentration in the sample must be close to saturation. (3) The protein concentration should be above 1 mM Chl *a* (about 0.3 mg/ml of PS I). This protein-detergent-micelle concentration ensures that the exchange of detergent molecules to and from the micelles occurs predominantly between neighboring protein-detergent-micelles and not between the micelle and the surrounding solution. In this way the detergent shell will not be thinned during the dissociation procedure.

Following dissociation, the monomers were further purified using three anion-exchange chromatography columns in the order of increasing separating power. The samples were desalted and concentrated for subsequent characterization and crystallization.

This mild method of PS I monomer preparation provided samples with a molar ratio of 85 (±5) Chl *a*/P700 for the β-DM-extracted and 65 (±5) Chl *a*/P700 for the SB12-extracted protein, as determined by measurements of photooxidation of P700 [20]. The significantly lower value of Chl *a*/P700 for the SB12-extracted protein implies a loss of chlorophyll *a*, which we assume to be due to the treatment with the zwitterionic detergent SB12.

The subunit composition of both monomer preparations was analyzed and compared to the subunit composition of

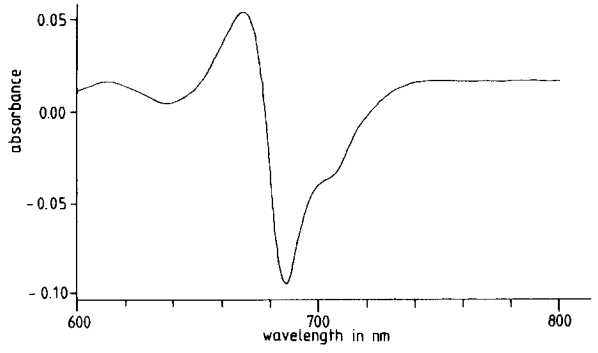

Fig. 4. Difference absorption spectrum at room temperature from 600 to 800 nm: PS I monomer prepared in SB12, further incubated with 5% (w/v) SB12 for 24 h at room temperature, minus intact PS I monomer with complete subunit composition (compare Fig. 3, lane 2); subunits J, K and L were completely, subunits F and M partly, removed (compare Fig. 3, lane 4).

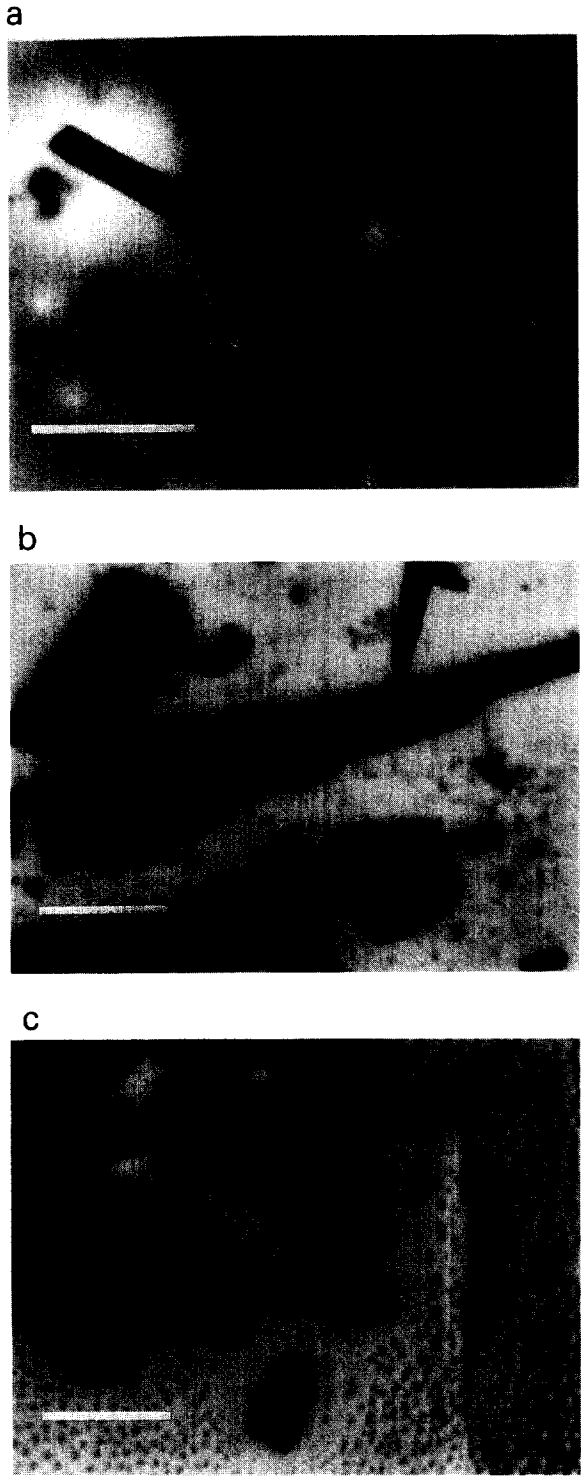

Fig. 5. Crystals (a) from PS I {−K, −L} (compare Fig. 3, lane 3), grown with PEG 6000 in batch method. (b) From PS I {−K, −L} (compare Fig. 3, lane 3), grown with PEG 4000 in vapor diffusion method. (c) From intact PS I monomer with complete subunit composition, grown with PEG 4000 in vapor diffusion method. All crystals are dark-green to black depending on thickness. The bar represents 0.2 mm.

PS I trimers. The SDS-PAGE pattern of monomers isolated with β-DM as detergent (Fig. 3, lane 2) exhibited all protein bands that could also be detected on silver stained gels obtained from trimers (Fig. 3, lane 1), indicating that the subunit composition of this PS I monomer is identical to the composition of the trimer described in [9]; this monomer preparation will henceforth be referred to as 'intact PS I monomer'. In contrast, two bands are missing in the SDS-PAGE pattern of SB12 prepared PS I monomers (Fig. 3, lane 3). One of these bands corresponds to the gene product of *psa K*, a hydrophobic subunit of 8.5 kDa [9], while the other subunit with an apparent molecular mass of about 15 kDa runs slightly in front of subunit F in the gel. This subunit has recently been identified by peptide specific antibody-binding to be the hydrophobic subunit L (Haehnel, W. (Freiburg), personal communication). Both subunits K and L are predicted to be intrinsic membrane proteins, spanning the membrane by α-helices [8]. Subunit L is assumed to be part ofthe connecting domain in the PS I trimer [15,22] and has also been found in other cyanobacteria [23] and in plants [24]. This suggests that the SB12-extracted monomer no longer contains the connecting domain, and we call this preparation PS I {−K, −L} in the following. The absence of the connecting domain may result in a more globular shape of PS I {−K, −L}, which in turn may faciliate crystallization. Since there is no evidence for any cofactors being attached to subunits K and L, the loss of chlorophyll *a* in the SB12-extracted protein sample cannot be explained by the removal of these subunits. It may be due to dissociation of chlorophyll *a* loosely bound to subunits A or B.

Additional investigations on the influence of SB12 on the protein complex were performed by further treatment of PS I {−K, −L} with this detergent. The sample was incubated with 5% (w/v) SB12 in A_0-buffer for 24 h at room temperature, followed by washing in Centricon 100 microconcentrators with A_0-buffer containing 0.02% (w/v) β-DM. This treatment further removed subunit J and partially subunits F and M (Fig. 3, lane 4) from PS I {−K, −L}. The eluate of this washing step contained the detached subunits, as detected by SDS-PAGE, and free chlorophyll *a*. Subunits J and M are highly hydrophobic and predicted to contain a membrane-spanning α-helix each [8]. Located on the lumenal side of the membrane, subunit F is presumed to be involved in the binding of plastocyanin or cytochrome c_6. There is some evidence that the hydrophobic part of this protein is anchored within the membrane [8]. None of the subunits F, J or M has been shown to be associated with any cofactors.

It similarly proved possible to sequentially remove the subunits F, J, K, L and M by incubating the intact PS I monomer with 0.5 to 5% (w/v) SB12 in A_0-buffer. The incubation was stopped after varying periods of time as described above. By comparing the SDS-PAGE patterns of these samples, it was possible to deduce the order in which the subunits were lost (data not shown). While subunit L is removed immediately even at very low concentrations of SB12, subunits K and then J are the next to be removed. Finally, subunits M and F were detached simultaneously,

though only partially, their complete removal not being possible under these conditions.

The absorption spectra of SB12-treated sample of PS I differ significantly from those of the intact PS I monomer at equal chlorophyll *a* concentrations. While the absorption spectrum of PS I from *Synechococcus elongatus* with complete subunit composition exhibits a shoulder at 706 nm, this absorption band disappears, if the sample is incubated with 5% (w/v) SB12 for 24 h at room temperature. In addition, the absorption maximum of the intact monomer sample at 680 nm is shifted to 676 nm if the sample was treated with SB12 (Fig. 4). These changes in the absorption spectra are not apparent on removal of subunits K and L, but are observed only after the detachment of subunits F, J and M from the protein complex. It was, however, not possible to ascribe the disappearance of the 706 nm band and the shift in the peak maximum in the absorption spectra to the loss of any one of these three subunits in particular. None of these subunits exhibited an absorption maximum at 706 nm. We, therefore, assume the changes in the absorption spectra to be correlated to the loss of chlorophyll attached to subunit A or B after the detachment of subunits F, J or M. Further investigations into this phenomenon are at present being undertaken.

PS I {−K, −L} was crystallized using batch methods and PEG 6000 as the precipitating agent. Within 1 to 7 days dark-green thin needles were observed with a size of approx. 1 mm × 0.2 mm (Fig. 5a). They were mechanically very unstable and diffracted X-rays to a resolution of about 8 Å. Since the crystals decayed in the X-ray beam within 15 min, unit cell constants and space group could not be determined. Using these crystallization conditions it was not possible to crystallize intact PS I monomers including the subunits K and L.

To obtain more stable crystals, we tested different precipitating solutions according to [21] employing 53 solutions with different combinations of 5 buffers, 9 salts and 5 nonvolatile and volatile precipitating agents, which were used to spread a so called sparse matrix of crystallization conditions based on the incomplete factorial method [25]. This screening test yielded crystallization conditions for intact PS I monomers as well as for PS I {−K, −L}. Crystals were obtained with vapor diffusion techniques ('hanging drop') using as precipitant a Tris-buffered PEG 4000-solution with a pH of 8.5. The platelets of dimensions 0.3 × 0.15 × 0.04 mm^3 obtained from PS I {−K, −L} under these conditions are far more stable mechanically and in the X-ray beam, but the diffraction limit was also about 8 Å (Fig. 5b). Since crystals of intact PS I monomer grew only to a size of approx. 0.11 × 0.06 × 0.02 mm^3 under these conditions (Fig. 5c), it appears that the removal of the connecting domain (in PS I {−K, −L}) faciliates the crystallization of PS I monomers. This work shows that it is possible to crystallize PS I monomers with complete subunit composition as well as monomers deficient in subunits K and L. Attempts continue to grow crystals with improved diffraction qualities from both PS I monomer preparations.

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