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Photosystem I from the unusual cyanobacterium *Gloeobacter violaceus*

Dedicated to Prof. Wilhelm Menke on the occasion of his 91st birthday

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Abstract

Photosystem I (PS I) from the primitive cyanobacterium *Gloeobacter violaceus* has been purified and characterised. Despite the fact that the isolated complexes have the same subunit composition as complexes from other cyanobacteria, the amplitude of flash-induced absorption difference spectra indicates a much bigger antenna size with about 150 chlorophylls per P700 as opposed to the usual 90. Image analysis of the PS I preparation from *Gloeobacter* reveals that the PS I particles exist both in a trimeric and in a monomeric form and that their size and shape closely resembles other cyanobacterial PS I particles. However, the complexes exhibit a higher molecular weight as could be shown by gel filtration. The preparation contains novel polypeptides not related to known Photosystem I subunits. The N-terminal sequence of one of those polypeptides has been determined and reveals no homology to known or hypothetical proteins. Immunoblotting shows a cross-reaction of three of the polypeptide bands with an antibody raised against the major LHC from the diatom *Cyclotella cryptica*. Electron microscopy reveals a novel T-shaped complex which has never been observed in any other cyanobacterial PS I preparation. 77 K spectra of purified PS I show an extreme blue-shift of the fluorescence emission, indicating an unusual organisation of the PS I antenna system in *Gloeobacter*.

Abbreviations: PS – photosystem; Chl – chlorophyll; DM – dodecyl-β-D-maltoside; ECL – enhanced chemiluminescence; HPLC – high performance liquid chromatography; IEC – ion exchange chromatography; GF – gel filtration; LHC I – light harvesting complex associated with Photosystem I; P700 – primary electron donor of photosystem I; SDS – sodium dodecyl sulfate; SQDG – sulfoquinovosyl diacylglycerol; PAGE – polyacrylamide gel electrophoresis; F760 (F730) – fluorescence band with maximal emission at 760 nm (730 nm)

Introduction

Photosynthetic electron transfer in chloroplasts and cyanobacteria is dependent on the action of two light-driven electron pumps, one of them being Photosystem I (PS I). PS I mediates the trafficking of electrons from the luminal to the stromal side of the thylakoid membrane, where they are passed on to the soluble electron carrier ferredoxin (or alternatively to flavodoxin in cyanobacteria) and ultimately to $NADP^+$ to generate NADPH (Chitnis 1996). Typically, cyanobacterial PS I shows a highly conserved subunit pattern consisting of 11 different polypeptides named PsaA–PsaF and PsaI–PsaM (Kruip et al. 1997). In cyanobacteria, PS I can occur in two different oligomeric states, a monomeric and a trimeric form (Boekema et al. 1987), which seem to be at least partly interchangeable, with a majority of trimers being present *in vivo* under moderate light conditions (Kruip et al.

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1994, 1999). Trimerisation appears to be dependent on the electrochemical situation in the lipid bilayer and to require the presence of the stroma-exposed subunit PsaD and the membrane integral subunit PsaL (Kruip et al. 1999; Chitnis and Chitnis 1997; van der Staay et al. 1998). PS I complexes from a whole range of cyanobacterial strains have been studied extensively in the past years. The structure of trimeric PS I from *Synechococcus elongatus* has been resolved at 2.5 Å by X-ray analysis, making cyanobacterial PS I the first reaction centre of oxygenic photosynthesis for which a high resolution structure is available (Jordan et al. 2001).

Although PS I in cyanobacteria and chloroplasts have the same function and share a lot of properties, there are some characteristic differences (Chitnis 1996). First, cyanobacterial PS I has fewer subunits: It does not have PsaG, PsaH and PsaN. Furthermore, chloroplast PS I is associated with membrane integral light-harvesting complexes (LHC I), which were so far assumed to be absent in cyanobacteria. Only recently, PS I from *Synechococcus* and *Synechocystis* has been shown to associate with a membrane intrinsic antenna when grown under iron limitation (Boekema et al. 2001; Bibby et al. 2001). Because of their sequence homology to CP43 these polypeptides had been originally attributed to PS II (Riethman and Sherman 1988; Park et al. 1999). Commonly, light harvesting in cyanobacteria is mediated through membrane extrinsic antenna polypeptides, the phycobilisomes. Unlike LHC I in chloroplasts, phycobilisomes are mobile and shared by PS I and PS II. By shifting phycobilisomes from one photosystem to the other, cyanobacteria are believed to accomplish state transitions (Mullineaux et al. 1997).

Although the PsaL subunit, which has been demonstrated to be essential for PS I trimerisation in *Synechocystis*, is present in chloroplast PS I, the latter does not show trimerisation. This is probably due to the additional subunits or LHCs surrounding the chloroplast PS I reaction centre that obstruct the strict spatial organisation necessary for PS I trimerisation. Interestingly, the cryptophytes have both, a PS I associated LHC and a phycobilisome antenna (Wolfe et al. 1994; Bathke et al. 1999).

Gloeobacter violaceus (called *Gloeobacter* hereafter) is a cyanobacterium which, according to 16S rRNA data, diverges very early from the common cyanobacterial phylogenetic branch, thus suggesting that it is a very early representative of oxygenic photosynthesis (Nelissen et al. 1995). It is the only cyanobacterium which does not contain any internal thylakoid membranes (Rippka et al. 1974). Instead, it contains a green plasma membrane, carrying both the photosynthetic and the respiratory electron transport chain. This fact raises interesting questions concerning the molecular organisation of *Gloeobacter*'s extraordinary membrane and the evolution of oxygenic photosynthesis in general.

The photosynthetic apparatus of *Gloeobacter violaceus* has been shown to be different from most cyanobacterial systems in more than one respect: it lacks the long wavelength chlorophyll fluorescence that all investigated cyanobacterial strains have been shown to emit at 77 K (Koenig and Schmidt 1995), indicating that *Gloeobacter* must have an unusual PS I organisation. A similar observation has been made for *Prochlorococcus* (Garczarek et al. 1998), which has recently also been classified as belonging to the cyanobacterial taxon (Matthijs et al. 1994). Furthermore, the phycoerythrin subunits contain two different chromophores in *Gloeobacter*, phycoerythrobilin and phycourobilin, the latter of which is typical for rhodophytes and among the cyanobacteria only found in few marine strains such as *Synechococcus* WH 8103, WH 8020 and WH 7803 (Bryant et al. 1981). Also, the membrane lipid composition of its only cellular membrane is exceptional among photosynthetic organisms as it does not contain sulfoquinovosyl diacylglycerol (SQDG). It has been proposed that the lack of SQDG might be the reason for the extremely high doubling time of *Gloeobacter* (Selstam and Campbell 1996).

The physiological relevance of both the long wavelength fluorescence emission at 77 K and the ability of cyanobacteria to form PS I trimers has been a point of speculation over the past years. Long wavelength absorbing chlorophylls (Chls) have been proposed to focus excitation energy to the reaction centre of PS I (Shubin et al. 1995), to be an intermediate energy trap (Woolfe et al. 1994), to facilitate light absorption (Trissl 1993) and to serve a photoprotective purpose for PS I (Karapetyan et al. 1999). The trimerisation of PS I *in vivo* has been suggested to mediate state transitions in cyanobacteria (Kruip et al. 1994). It has been proposed in the case of *Spirulina platensis* (called *Spirulina* from here on) that the absence of the far red fluorescence emission at 77 K goes along with an inability to form PS I trimers (Karapetyan et al. 1997). This hypothesis, however, cannot be generalised for other PS I trimer forming species as they do not show this very long wavelength emission (Gobets et al. 2001). *In vitro* reconstitution data further showed

that the long wavelength emission of trimers is a direct consequence of the trimerisation process which leads to an altered environment of a cluster of special chlorophylls (Kruip et al. 1999).

After the long wavelength emission at 77 K had been reported to be absent in whole cells as well as in plasma membranes of *Gloeobacter* (Koenig and Schmidt 1995), the question arose, whether PS I showed unusual structural features. A detailed analysis of PS I from *Gloeobacter* required the isolation of intact PS I. The purification procedure reported here was adapted from purification protocols published earlier (Kruip et al. 1999; Wenk and Kruip 2000). It became evident that *Gloeobacter* PS I exists primarily in trimeric form, with a comparatively high Chl:P700 ratio, as well as with a higher molecular weight than in other cyanobacteria. Moreover, the cross-reactivity of as yet unidentified polypeptides in the preparation with antibodies against the major light harvesting complex from the diatom *Cyclotella cryptica* (Rhiel et al. 1997) was shown.

Materials and methods

Source of cyanobacterial strains

Gloeobacter violaceus PCC 7421 was obtained as strain SAG 7.82 from the culture collection of algae at the University of Goettingen. *Spirulina platensis* cells were a kind gift from N. Karapetyan (Moscow) and the *Synechocystis* strain was made available by H. Pakrasi (St. Louis, Missouri).

Cell culture and membrane isolation

Gloeobacter violaceus cells were grown photoautotrophically in Allen's medium (Allen 1968) as 1-litre cultures in continuous white light (4 μmol photons m^{-2} s^{-1}) without disturbance. The growth temperature was 21 °C. Cells were harvested after 6 months while still in exponential growth phase by centrifugation at 10 000 *g*, 20 °C for 10 min. Cells were then washed with 30 mM Na-phosphate buffer, pH 7.0, pelleted again (10 000 *g*, 20 °C, 10 min) and resuspended in cold 30 mM Na-phosphate buffer, pH 7.0. All subsequent steps were carried out at 4 °C in near darkness. The cell suspension was then mixed with twice the volume of glass beads (∅ 0.5 mm) and treated with a glass bead homogeniser (Vibrogen, Bühler, Tübingen) in cycles of four 1.5-min pulses with 1 min cooling intervals. The resulting homogenate was filtered through two layers of Miracloth and washed from the glass beads with 30 mM Na-phosphate buffer, pH 7.0. The homogenization was repeated once, using smaller glass beads (∅ 0.15 mm). Again, the suspension was filtered through Miracloth and washed from the glass beads with 30 mM Na-phosphate buffer, pH 7.0. This crude cellular extract was then centrifuged for 15 min at 5000 *g*, 4 °C. The resulting pellet containing unbroken cells and cellular debris was discarded and the supernatant centrifuged for 2 h at 40 000 *g*, 4 °C. The supernatant containing mainly phycobilisomes and other soluble cellular components was discarded and the pellet resuspended in 50 ml 30 mM Na-phosphate buffer, pH 7.0, supplemented with one complete^{TM} proteinase inhibitor cocktail tablet (Boehringer, Mannheim). This crude membrane extract was frozen in liquid nitrogen and stored at −80 °C until needed.

PS I purification

For solubilisation the membrane suspension was thawed at room temperature in the dark and diluted with the same volume of low salt anion-exchange chromatography (IEC) buffer (20 mM Tris/HCl, pH 8.0; 10 mM $MgCl_2$; 5 mM $CaCl_2$; 10 mM NaCl). Solid dodecyl-β-D-maltoside (DM) was then added to a final concentration of 1.2% (w/v), yielding a typical Chl:DM ratio of 1:2500. The suspension was stirred in the dark at 4 °C for 30 min, then centrifuged for 15 min at 150 000 *g*, 4 °C. The supernatant was filtered (Schleicher & Schuell, 0.45 μm) and immediately loaded onto a Poros HQ 50 column (Applied Biosystems) equilibrated with low salt IEC buffer supplemented with 0.03% (w/v) DM. Elution from the column was done with a linear gradient of NaCl up to a concentration of 300 mM at a flow rate of 6.75 ml min^{-1}. The elution profile was recorded with a photodiode array detector (Waters) between 250 and 800 nm. Chlorophyll-containing fractions were pooled and concentrated in an Amicon ultrafiltration cell (Millipore, YM100 membrane, cut-off 100 kDa) at 4 °C in the dark. The resulting fractions were then loaded onto a Superose 6 HR 10/30 gel filtration column (Amersham) equilibrated with gel filtration buffer (20 mM Tris/HCl, pH 8.0; 10 mM $MgCl_2$; 5 mM $CaCl_2$; 150 mM NaCl; 0.03% (w/v) DM) at a flow rate of 0.5 ml min^{-1}. The elution profile was recorded at 280 and 435 nm. For calibration of the Superose 6 column pure preparations of trimeric

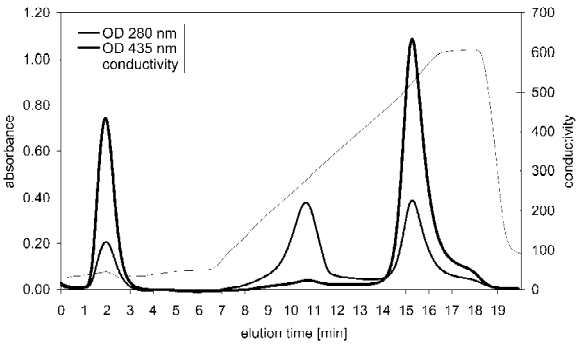

Figure 1. Typical elution profile of a *Gloeobacter violaceus* membrane extract from an anion exchange column (POROS HQ 50) using a flow rate of 6.75 ml min^{-1}. Elution was achieved by a linear NaCl gradient and recorded at 280 nm and 435 nm. For buffer details see 'Materials and methods'.

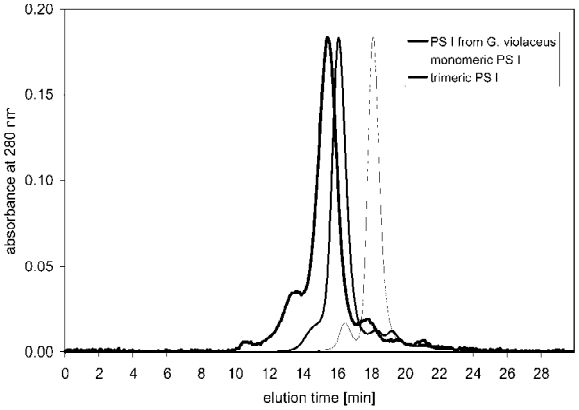

Figure 2. Elution profile of the purified PS I fraction from a size exclusion column (Superose 6) at a flow rate of 0.5 ml min^{-1}. Absorption was recorded at 280 nm (bold line). For calibration monomeric PS I (300 kDa, thin line) or trimeric PS I (800 kDa, medium line) from *Spirulina platensis* were used.

and monomeric PS I from *Synechocystis* or *Spirulina platensis* were used (Kruip et al. 1997, 1999).

Biochemical standard techniques

Electrophoretical protein subunit analysis was done with the Tris/Tricine system according to Schägger and Jagow (1987), the acrylamide concentrations of the separating, spacer and stacking gels being 16% T/3% C, 10% T/3% C and 4% T/3% C. Gels were run at 30 V/$\leq$300 mA for 1 h, then at 25 mA/$\leq$300 V for 18 to 24 h at room temperature and stained either with Coomassie Brilliant Blue or silver. For immunodetection proteins were transferred to a PVDF membrane (Immobilon, Millipore) by semi-dry blotting at 1 mA/cm^2 for 2 h. Antibodies against PsaC and PsaD from spinach were kindly provided by R. Berzborn (Bochum), antibodies against the major LHC of *Cyclotella cryptica* were made available by E. Rhiel (Oldenburg). As a secondary antibody, an anti-rabbit peroxidase conjugate (Sigma) was used. Detection was done by chemiluminescence using luminol as a substrate. N-terminal sequencing was done in the laboratory of M. Yoshida (Tokyo Institute of Technology) by Edmann degradation.

Fluorescence spectroscopy

77 K fluorescence spectra were recorded with a luminescence spectrometer equipped for 77 K measurements (Aminco Bowman). Chlorophyll-containing fractions collected from anion-exchange chromatography were adjusted to a chlorophyll concentration of 2 μg ml^{-1} in 200 mM Tris, pH 10.0, then mixed with equal volumes of glycerol and frozen in liquid nitrogen. For reducing conditions, the buffer contained 20 mM dithionite. Fluorescence was excited at 435 nm and fluorescence emission was recorded between 600 and 800 nm using a slit width of 4 nm. Spectra were corrected for the wavelength sensitivity of the instrument.

Difference spectroscopy

Flash-induced absorption changes (Hiyama and Ke 1972) were measured in the region of 650 to 750 nm using a chlorophyll concentration of 3.45 μM in a half-micro cuvette (800 μl sample volume, path length 1 cm). The buffer contained 50 mM ACES, pH 6.5, 50 mM KCl, 30 μM PMS and 5 mM sodium ascorbate. A halogen lamp in combination with a monochromator (Bausch & Lomb, 2 nm slit width) was used as light source. The sample was protected from higher-order scattering light generated at the monochromator by using appropriate cut-off filters (Schott RG filters, ITOS, Mainz). Detection was done with a photomultiplier (Thorn-EMI 9658, Electron Tubes), protected by a band pass filter/cut-off filter combination (Schott DAL and Schott RG, respectively), which were adjusted to the measuring wavelength. Saturating blue light flashes for the excitation of PS I were obtained by a xenon flash lamp (10 μs pulse width) with a combination of optical filters (4 mm Schott BG 39 and 2$\times$Balzers DT Blue). Signals from 64 flashes (given at a frequency of 1 s^{-1}) were averaged for each measured wavelength. Each sample was used at four different wavelengths, no age-

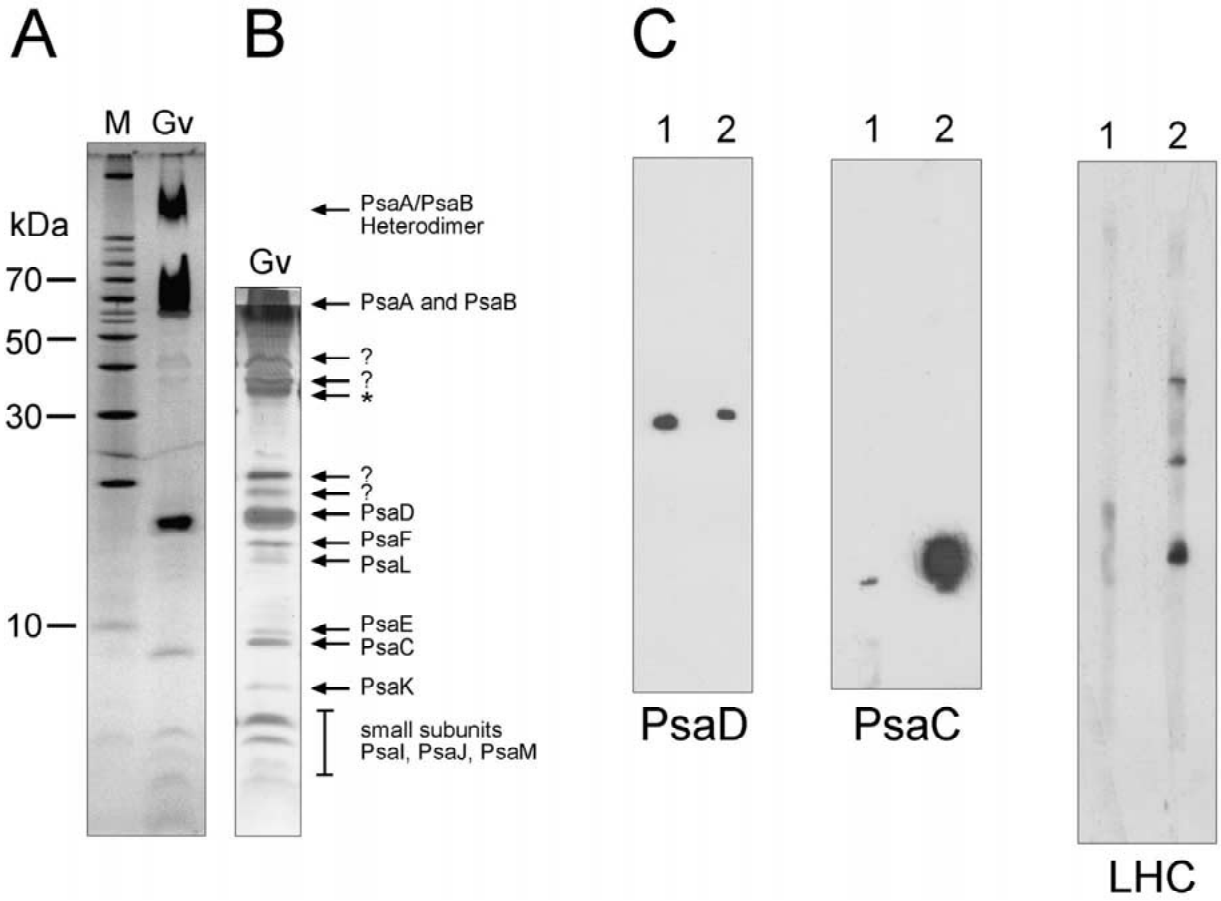

Figure 3. Tris/Tricine PAGE analysis of the purified PS I fraction. Lane M represents a molecular weight standard; lane Gv shows the *Gloeobacter* PS I preparation. (A) A Coomassie Blue stained and (B) a silver stained gel are shown. Identified subunits are labelled in the figure. The N-terminally sequenced polypeptide band has been marked by an asterisk. (C) Comparison of *Synechocystis* PS I (lane 1) and *Gloeobacter* PS I (lane 2). Immunological identification of PsaD and PsaC subunit using polyclonal antisera raised against spinach proteins and evidence for the existence of a light-harvesting protein in *Gloeobacter violaceus* which is immunologically related to an LHC protein from *Cyclotella cryptica*.

ing effects were observed during this procedure and sample stability allowed for several hundred flashes without detectable loss of signal amplitude.

Electron microscopy and image analysis

Transmission electron microscopy was performed with a Philips CM10 electron microscope at 52 000× magnification and 100 kV. Negatively stained specimens were prepared with a 2% solution of uranyl acetate (Lelong et al. 1996) on glow-discharged plain carbon-coated copper grids by diluting the samples 5× with buffer plus 0.03% DM but without NaCl. From 34 digitised negatives a total of about 4200 particle projections was selected, with a window size of 72×72 pixels, compatible to 35×35 nm. Image analysis was carried out with IMAGIC software (van Heel 1989). The projections were analysed following an aperiodic (single particle) procedure, including repeated cycles of alignment procedures (Harauz et al. 1989) and treatment by multivariate statistical analysis (van Heel and Frank 1981) and classification (van Heel 1989).

Results

Purification of PS I

A membrane extract from *Gloeobacter violaceus* shows a typical elution profile when purified by perfusion chromatography on an anion-exchange material (Figure 1). Three major protein-containing peaks are

visible. The first peak represents non-binding proteins and has an orange colour due to carotenoids. The second peak with its intense pink colour represents mainly phycobilisomes with traces of chlorophyll detergent micelles which show an absorption maximum at 671 nm (data not shown). The third peak contains the PS I fraction as indicated by the green colour. It has a typical PS I absorption spectrum with a characteristic maximum at 680 nm (data not shown). This latter peak could be further analysed by size exclusion chromatography and was found to contain mainly trimeric PS I together with traces of monomeric PS I and higher aggregates (Figure 2). When the column was calibrated using monomeric and trimeric PS I from other cyanobacteria, it was clearly evident that the trimers of *Gloeobacter* had a higher apparent molecular mass of around 1100 kDa in comparison to 800 kDa for PS I from *Spirulina*. If the PS I preparation was either concentrated or frozen, the number of aggregates increased drastically. In concentrated and frozen samples, only aggregates were detected which could not be resolved on the Superose 6 column (data not shown). Apart from aggregation, all PS I particles retrieved from the IEC column are extremely stable and even the aggregates show normal function with respect to flash-induced turnover (see below).

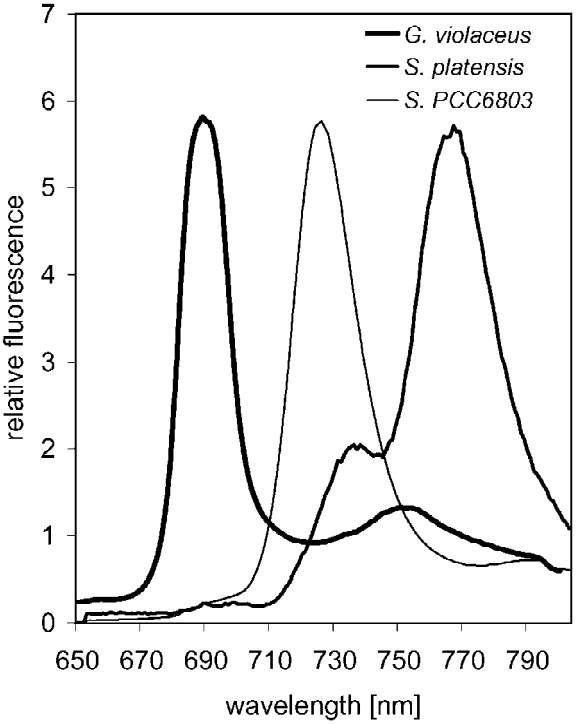

Figure 4. 77 K Fluorescence emission spectra of purified PS I trimers from *Gloeobacter violaceus*. Comparison with emission spectra of trimeric PS I from *Synechocystis* PCC 6803 and *Spirulina platensis*. All spectra were recorded under reducing conditions (for details see 'Materials and methods').

Subunit composition, immunochemical analysis and N-terminal sequencing

Purified PS I fractions were applied to high-resolution acrylamide gels and stained with Coomassie Brilliant Blue or silver (Figures 3A and B). The corresponding subunit pattern shows typical PS I related protein bands starting with the smeared band of the PsaA/B heterodimer at the top of the gel. Below that, bands can be attributed to the subunits PsaA and -B (both around 66 kDa), -D (17 kDa), -F (16 kDa), -L (15 kDa), -C and -E (both around 9 kDa) and several smaller subunits by analogy to PS I preparations from other cyanobacteria. The identity of PsaD, PsaC (Figure 3C), PsaF and PsaL (data not shown) has also been shown by detection with specific antibodies. Besides these typical PS I subunits, several additional protein bands are visible in the gels. The N-terminal sequence of one of the protein bands has been determined as SDVQHLPFGGSTPLFGGITD. So far, database searches have not revealed any sequence homology to known or hypothetical proteins. To test whether one of the unknown proteins might be related to light-harvesting proteins, the sample was probed with an antiserum against the major LHC from *Cyclotella cryptica* (Figure 3C). Clearly, three immunologically related proteins were detected in *Gloeobacter*, whereas no cross-reaction could be found for *Synechocystis*. The corresponding polypeptides from *Gloeobacter* have apparent molecular masses of about 9, 15 and 23 kDa, respectively.

77 K fluorescence characteristics

Fluorescence emission spectra of purified PS I complexes were recorded at 77 K upon excitation with blue light of 435 nm (Figure 4). The typical long wavelength emission is missing in the purified *Gloeobacter* PS I, which confirms earlier data obtained with whole cells and isolated plasma membranes (Koenig and Schmidt 1995). Instead, a broad emission band centred around 690 nm dominates the spectrum besides a weaker band around 750 nm. It is known that the PS I fluorescence spectrum of some cyanobacteria can be influenced by the redox conditions. Trimeric PS I from *Spirulina* for instance shows an extreme

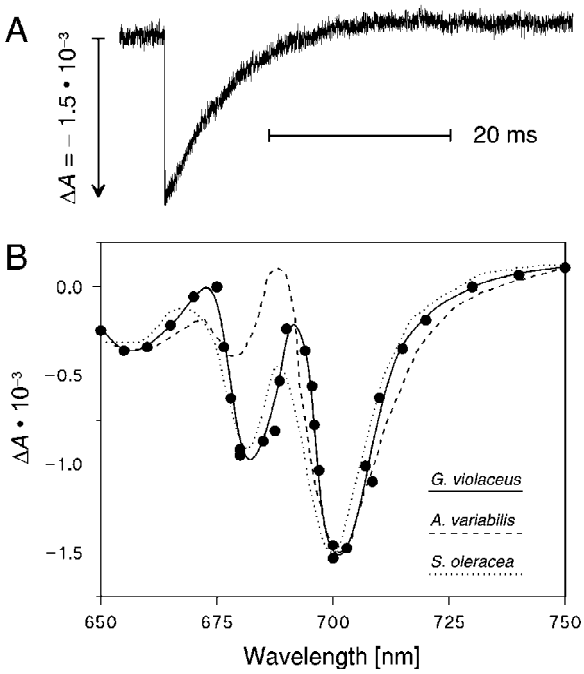

Figure 5. Characterisation of purified PS I complexes from *Gloeobacter violaceus* by flash-induced absorption changes. (A) Time course at 700 nm, (B) difference spectrum.

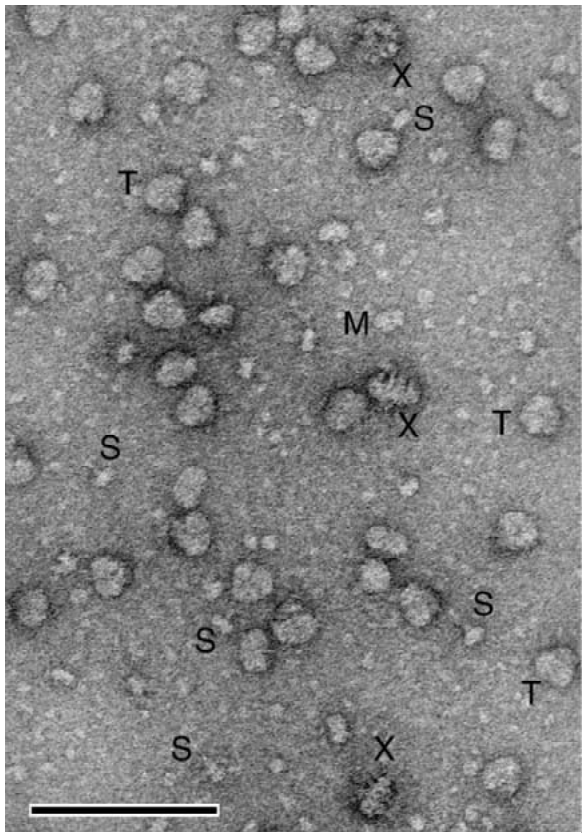

Figure 6. Electron micrograph of purified PS I complexes. The particles were negatively stained with 2% uranyl acetate. Projections of trimeric (T) and monomeric (M) PS I complexes have been marked, as well as a the T-shaped protein complex (S) and an unknown type of aggregate (X). The scale bar represents 100 nm.

red-shift of its 730 nm emission band under oxidising conditions to 765 nm under reducing conditions. In contrast, addition of dithionite (reducing conditions) induces only small but distinct changes in the PS I emission spectrum from *Gloeobacter*: The broad and heterogeneous emission band (693 nm) observed under oxidising conditions becomes focused, slightly blue-shifted (689 nm) and more homogeneous, resembling a Gauss distribution under reducing conditions (data not shown). The smaller emission band at 750 nm is not influenced by the redox conditions.

Flash-light induced absorption changes of isolated PS I

Purified PS I complexes were analysed further by difference spectroscopy after excitation by a flash of blue light (Figure 5A). The half time for signal relaxation at 700 nm is 5 ± 1 ms (average of 4 measurements). The corresponding difference spectrum for the red spectral part is plotted in Figure 5B. For comparison, two P700 difference spectra from the literature (Hiyama and Ke 1972) are included, normalised to the maximum amplitude of the *Gloeobacter violaceus* spectrum. The overall shape of the *Gloeobacter* P700 spectrum (solid line) is very similar to the spectrum from spinach (dotted line), while the spectrum for the cyanobacterium *Anabaena variabilis* (dashed line) shows slight differences in the 680 nm region. The choice of the $\Delta\varepsilon$ value was made on the basis of the great similarity of the *Gloeobacter* and spinach spectra. From the amplitude of the *Gloeobacter* spectrum at 700 nm a Chl/P700 ratio of 147 is obtained, when this extinction coefficient of 64 mM^{-1} cm^{-1} (Hiyama and Ke 1972) is used.

Electron microscopy and image analysis

Isolated PS I trimer fractions after IEC were subjected to size exclusion chromatography. The peak fraction of trimeric PS I (Figure 2) was characterised by transmission electron microscopy of negatively stained particles. The preparation contains monodispersely solubilised trimeric PS I particles, besides some unexpected components (Figure 6). For a more precise

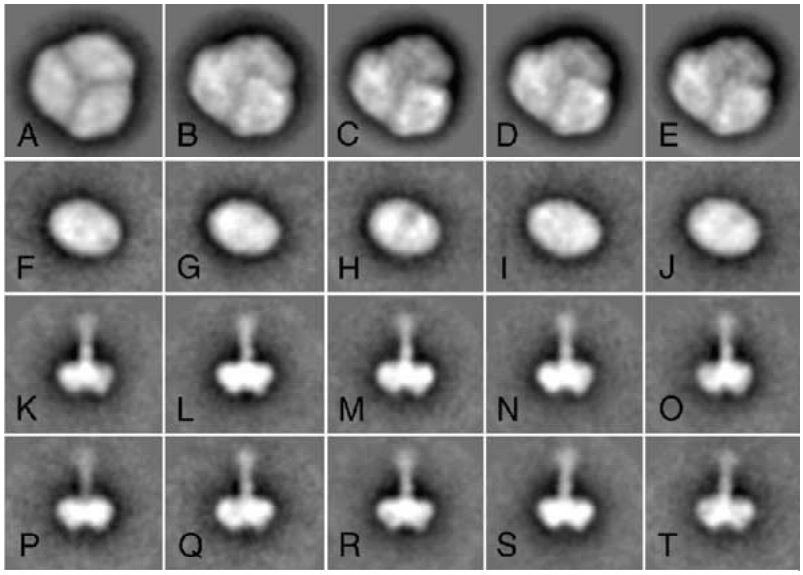

Figure 7. Results of multivariate statistical analysis. Classification of projections from trimeric and monomeric PS I and from a novel T-shaped complex are shown. (A–E) Best 5 classes (out of 6) from a classification of 2517 PS I trimers with 478, 352, 362, 390 and 251 class-members, respectively. For the images A–D, the best 300 images were summed with the cross-correlation coefficient as a quality parameter. (F–J) Best 5 classes (out of 8) from a classification of 512 monomeric PS I complexes, each consisting of between 61–91 projections, (K–T) best 10 classes (out of 14) from a classification of 1139 T-shaped complexes with 82–134 projections for each of the classes.

characterisation by image analysis, a large data set was extracted from 34 images. Apart from PS I trimers (marked T), three types of components can be observed in significant numbers: Firstly an oval-shaped particle with features of monomeric PS I (marked M), which was present in a ratio of about 1:10–20 (in numbers of particles to PS I trimers), secondly a T-shaped complex (marked S) with an overall length of almost 20 nm and thirdly a complex (marked X) about as large as the PS I trimers, variable in size and shape and present in a ratio of about 1:50–100 to PS I trimers. Figure 6 shows three projections of particles marked X, which show peculiar protrusions, reminiscent to the spikes of certain viruses, protruding from protein-dense regions.

The T-shaped complex consists of a bilobed scaffold from which a long and narrow stem extends (see various particles marked S in Figure 6). In many of these complexes the stem is easily recognisable and the particle projections are present in a ratio of about 1:3–5 relative to PS I trimers. The larger aggregates marked X might be small clusters of this protein in side or top view projection. These aggregates are, however, quite rare and variable in shape, thus excluding further image analysis. Otherwise, the samples were free of contaminants, such as dimers of PS II, tetramers of RubisCO or phycobilisome fragments, which are frequently present in PS I preparations.

A set of about 2500 top-view projections of the PS I trimers was analysed by a combination of multivariate statistical analysis and classification. A decomposition into six classes shows that only a minority of the projections had well-preserved three-fold rotational symmetry (Figure 7A). Deviation from the symmetry is quite strong in the other classes (Figures 7B–E), due to a thicker stain-embedding of the smaller-appearing monomer in the upper position. A similar deviation of the three-fold symmetry was found earlier for PS I trimers and could be attributed to local variations in the roughness of the carbon support film causing a different degree of tilting of the molecules (Lelong et al. 1996).

Image analysis of a set of oval particles indicated that they were rather homogeneous in overall shape (Figures 7F–J), but quite featureless in internal structure (Figure 8C). They are very similar to previously analysed PS I monomers (Rögner et al. 1990).

Analysis of 1139 T-shaped complexes showed only one predominant type of projection (Figures 7K–T). The complex has an overall length of 19 nm, consisting of a bilobed scaffold with a width of 13 nm and a height of 6.5 nm, from which a long (12 nm)

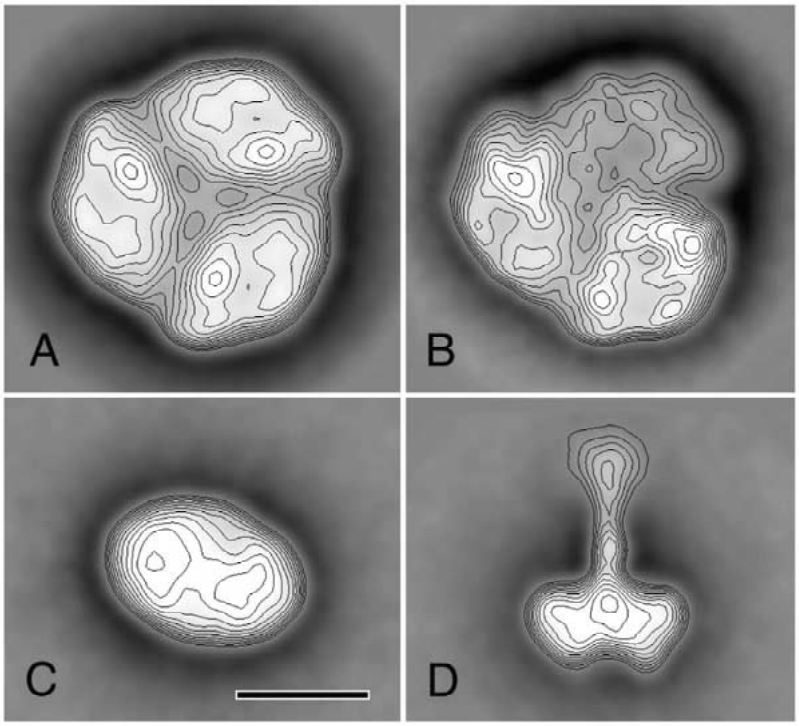

Figure 8. Final sums of the single particle analysis. (A) Sum of 300 images from Figure 7A with three-fold symmetry imposed after analysis and contoured with equidistant contour lines; (B) sum of Figure 7C; (C) sum of 406 T-shaped complexes; (D) sum of 471 PS I monomers. The scale bar is 10 nm.

and narrow (2.5 nm) stem extends. The narrow stem protrudes almost vertically from the scaffold in most of the projections. A small tilt of the stem is visible in some of the classes (Figures 7Q–T), possibly indicating some flexibility. On the other hand the tilt could also be explained by variations in the position on the carbon support film, similar as found for the PS I trimers.

Discussion

Purification of PS I

The purification method published here is a modified version of a powerful perfusion chromatographic approach published earlier (Kruip et al. 1999; Wenk and Kruip 2000). Major changes were introduced in the salt content regarding solubilisation and elution buffers. The purification scheme yields pure PS I, devoid of typical contaminants like PS II or RubisCO, which exhibits an absorption spectrum that fits classical PS I spectra. The elution profile of the ion exchange perfusion chromatography column is typical for trimeric PS I. Its oligomeric state was confirmed by size exclusion chromatography and electron microscopy. The absence of any 77 K fluorescence emission peaks due to phycobiliproteins shows the purity of the preparation. The isolated PS I particles are very stable: even over a time span of 36 h at room temperature, no decay in flash-induced signal intensities could be found.

Biochemical properties

The polypeptide pattern is a typical cyanobacterial PS I pattern (cf. Kruip et al. 1993), except for additional bands which could not be attributed to known proteins. The assignment of PS I subunits was done by immunoblotting, which unambiguously confirmed the presence of PsaC, PsaD, PsaF and PsaL, by mass spectrometry (data not shown) and by comparison to PS I preparations from other cyanobacteria, e.g. *Synechocystis* (Kruip et al. 1993). The apparent molecular weight of all PS I subunits was similar to subunits from *Synechocystis*. As can be concluded from the results of size exclusion chromatography the PS I preparation from *Gloeobacter* contains trimeric PS I although the molecular mass of around 1100 kDa is significantly

higher than the 800 kDa for trimeric PS I from other cyanobacteria. An increase in the ionic strength of the buffer did not change the apparent molecular weight and therefore the unusual size may not be attributed to interactions of the particles with the column material. The most plausible explanation for the higher molecular weight is that PS I forms complexes with other proteins present in the preparation leading to a larger size in gel filtration.

As mentioned before, additional subunits could be found in the PS I preparation. Attempts to identify these proteins by N-terminal sequencing failed. One protein could be sequenced, but database searches of the resulting sequence information failed to find homologues. Determination of the total genome sequence of *Gloeobacter violaceus* is under way at the Kazusa Institute (Chiba, Japan) and is expected to be available soon (S. Tabata, personal communication). These data will be indispensable in order to gain more information on the proteins present in the PS I preparation. Nevertheless, some of the additional proteins cross-react with antibodies raised against the major LHC protein from the diatom *Cyclotella cryptica* (Rhiel et al. 1997), which also cross-react with light-harvesting complexes of the rhodophyte *Galdieria sulphuraria* (Marquardt and Rhiel 1997). This is an unexpected and interesting finding since it had generally been assumed that LHC proteins are absent in cyanobacteria. Indeed, it is not possible to immunodecorate subunits from a *Synechocystis* PS I preparation with the diatom LHC antibody. It could already be shown that *Prochlorococcus marinus*, a marine prochlorophyte phylogenetically affiliated with the cyanobacteria, does contain a chlorophyll *a/b* antenna besides genes encoding phycobiliproteins (Hess et al. 1996). Furthermore, a group of genes encoding polypeptides related to the early light-inducible proteins (ELIP) and LHCs of higher plants, the one-helix high-light-inducible proteins [HLIP, for reviews see Green and Durnford (1996); Montané and Kloppstech (2000)] has been found in cyanobacteria (Funk and Vermaas 1999). The results presented here, however, constitute the first evidence on protein level for membrane intrinsic light harvesting proteins in cyanobacteria besides the most recently found IsiA ring (Boekema et al. 2001).

LHCs from *Cyclotella cryptica* generally have molecular weights in the range of 18 to 22 kDa, correlating nicely to the largest of the immunodecorated protein bands in the *Gloeobacter* preparation (23 kDa). In contrast to the diatom *Cyclotella cryptica*, *Gloeobacter* contains neither fucoxanthin (G. Sandmann, personal communication) nor chlorophyll *c* (P. Jöstingmeyer, unpublished data). This observation raises questions concerning a possible variability of the pigment content of different light harvesting complexes. The new findings support the hypothesis discussed recently that light-harvesting proteins were already invented in the prokaryotic ancestor of chloroplasts rather than upon evolution of the eukaryotic cell (Green 2001).

Fluorescence spectroscopy

It has already been shown that fluorescence emission spectra of both whole *Gloeobacter* cells and isolated plasma membranes obtained upon excitation with blue light are dominated by the 690 nm fluorescence besides minor peaks due to phycobiliproteins (Koenig and Schmidt 1995). Similarly, fluorescence emission spectra of the purified PS I complexes at 77 K show a single peak with a maximum around 690 nm and lack an intense long wavelength emission peak. Shape and maximum of the peak are influenced by the redox conditions. Usually, 77 K fluorescence emission peaks between 685 and 695 nm are attributed to cyanobacterial PS II. However, as the preparation presented here does not contain any PS II, the 690 nm emission must be attributed to PS I. In conclusion, the PS I 77 K fluorescence emission, which was or has so far been believed to be missing in *Gloeobacter*, is in fact present but shows an extreme blue-shift.

A similar blue-shift of the long wavelength fluorescence emission has been observed for the prochlorophyte *Prochlorococcus*, which due to its unusual pigment pattern has only recently been attributed to the cyanobacteria (Garczarek et al. 1998). Also, a spectral blue shift of the 77 K emission could be shown for the rhodophyte *Rhodella violacea* under iron deprivation (Marquardt et al. 1999). Interestingly, the *Gloeobacter* spectrum resembles the PS I emission from other cyanobacteria grown under iron limitation (J. Kruip and E. Pistorius, unpublished observation). As *Gloeobacter* was grown in Allen's medium iron limitation is unlikely unless it has an inefficient iron-uptake system which cannot be ruled out presently. Energy transfer measurements on a femto-second time scale will be necessary to probe energy distribution in the *Gloeobacter* antenna.

Difference spectroscopy

The re-reduction kinetics of the isolated PS I complex after flash-induced turnover has a half-time of 5 ms (Figure 5). This confirms the functional intactness of the purified PS I complex, since this time constant is within the range of values obtained under identical conditions (30 μM PMS, 5 mM ascorbate, pH 6.5) for other cyanobacterial PS I preparations: we have observed relaxation times of 2.8±0.2 ms for *Synechocystis* sp. PCC 6803 and 6.2±2.0 ms for *Synechococcus* sp. PCC 7942 (S. Berry, J. Kruip, K.-P. Michel, M. Rögner and E. Pistorius, unpublished data).

The difference spectrum of *Gloeobacter* PS I in the red spectral region has the shape of a typical P700 spectrum. With respect to the relative minimum around 680 nm it resembles more the literature spectrum for spinach rather than the one for the cyanobacterium *A. variabilis* (Hiyama and Ke 1972). In general, the amplitude of this absorbance change relative to the major peak at 700 nm, i.e. the $\Delta A_{680}/\Delta A_{700}$ ratio, can be quite variable as it depends on both the organism and the protocol used for isolation of PS I (Hiyama and Ke 1972; Sonoike and Katoh 1988, 1989). The variation of the peak at 680 nm is assumed to be mainly caused by slight differences in the electrochromic response of antenna chlorophylls upon formation of the $P700^+$ cation. Different $\Delta A_{680}/\Delta A_{700}$ ratios, therefore, do not imply specific differences in the electronic structure of the special pair. Although the shape of the *Gloeobacter* PS I spectrum is normal, it has a surprisingly low amplitude, indicating a Chl:P700 ratio of 147. This is large in comparison with the antenna size for PS I preparations from higher plants and other cyanobacteria, which is about 90 Chl per P700 (Schubert et al. 1997). An even larger value of 175 chlorophyll molecules per P700 was observed earlier for isolated plasma membranes from *Gloeobacter* under illumination with continuous blue light (Koenig and Schmidt 1995). This difference to the isolated PS I is probably due to chlorophyll associated with PS II. Assuming a similar PS I structure, the different antenna size of *Gloeobacter* PS I in comparison with PS I from other cyanobacteria is most probably due to Chl bound by polypeptides which do not belong to the PS I core.

Electron microscopy

PS I monomers and trimers from *Gloeobacter violaceus* closely resemble previously analysed PS I complexes from other cyanobacteria like *Synechocystis* PCC 6803 and *Synechococcus elongatus* regarding size and shape (Rögner et al. 1990; Kruip et al. 1997). However, the T-shaped complex in the preparation was never observed in any of the previously studied PS I samples (Kruip et al. 1997). Its shape is distantly reminiscent of a recently described TolC protein from gram-negative bacteria spanning both the outer membrane and the periplasmic space (Koronakis et al. 2000). This protein has an overall length of 14 nm, of which nearly 10 nm consists of a uniform cylinder of about 35 Å internal diameter. Because of its longer overall length and its smaller diameter, the complex described here is unlikely to be a TolC homologue.

It is somewhat surprising that the size of the trimers as deduced from electron microscopy is comparable to PS I trimers from *Synechococcus* or *Synechocystis* (Rögner et al. 1990b; Kruip et al. 1997) considering that difference spectroscopy implies that *Gloeobacter* PS I contains substantially more Chl molecules, and gel filtration shows the trimers to have a higher molecular weight. It is unlikely that the PsaA/B heterodimer can have additional binding sites for significantly more Chl molecules than the 90 found in other cyanobacterial strains, since high resolution X-ray crystallography has shown the PS I complex to be almost filled with chlorophyll molecules which are spaced and tilted in an optimal fashion for efficient energy transfer (Jordan et al. 2001). This would imply the necessity for one or more additional chlorophyll bearing polypeptides on which the superfluous Chl molecules could be organised. One attractive hypothesis would be that *Gloeobacter* contains additional membrane-integral light-harvesting proteins resembling the LHC proteins found in chloroplasts or the recently found CP43′ (Riethman and Sherman 1988; Park et al. 1999) or IsiA (Boekema et al. 2001; Bibby et al. 2001) in cyanobacteria. Such a hypothesis would support the assumption that *Gloeobacter* is the predecessor of chloroplasts. Indeed, novel PS I associated proteins are present in the preparation described here in high amounts. If it turns out that *Gloeobacter* indeed contains membrane-bound antenna proteins it will become the most likely predecessor of chloroplasts. The interesting question remains whether the lack of an internal membrane system is an old feature reflecting the position of *Gloeobacter* in phylogenetic trees or whether it is a secondary loss.

Taken together, a purification protocol which yields monomeric as well as trimeric PS I from *Gloeo-*

bacter violaceus has been established. The trimeric complex has the same subunit composition and dimensions as PS I from other cyanobacteria. However, the preparation shows some unexpected properties: an unusually high Chl/P700 ratio, an extraordinarily high molecular weight, an atypical fluorescence spectrum and some co-purifying polypeptides three of which cross-react with an antibody against LHC proteins. Moreover, the preparation contains T-shaped complexes, as can be seen in electron micrographs, which up to now have not been observed.

Gloeobacter violaceus is a key-stone organism for understanding the evolution of complex membrane systems and the evolution of membrane based energy generating systems. The unusual features of PS I from this organism presented here form the basis for a further molecular understanding of membrane organisation and energy metabolism.

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