

University of Groningen

## Unraveling the structure of the photosynthetic membrane using electron microscopy approach

Arteni, Ana Andreea

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2007

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Arteni, A. A. (2007). *Unraveling the structure of the photosynthetic membrane using electron microscopy approach*. s.n.

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

## **CHAPTER 2**

### **Single particle electron microscopy in combination with mass spectrometry to investigate novel complexes of membrane proteins**

Ana A. Arteni, Marc Nowaczyk, Julia Lax, Roman Kouřil, Matthias Rögner  
and Egbert J. Boekema

**Published in:**

J. Struct. Biology (2005) 149(3), 325 - 331.

**Abstract**

Large data sets of molecular projections of the membrane proteins Photosystem I and Photosystem II from cyanobacteria were analyzed by single particle electron microscopy (EM). Analysis resulted in the averaging of 2D projections from the purified complexes but also in the simultaneous detection and averaging of 2D projections from large contaminating complexes, which were present in frequencies as low as 0.1%. Among them T-shaped and L-shaped contaminants were found. The L-shaped particles could be assigned to Complex I just from the shape, although no Complex I from a cyanobacterium has been structurally characterized. A systematic comparison by single particle EM and mass spectrometry of two differently purified Photosystem II complexes resulted in the assignment of PsbZ, a small peripheral subunit of 6.8 kDa, within the structure.

Together these data suggest that screening for membrane protein structures by single particle EM and mass spectrometry may be a new approach in order to find novel structures of such proteins. We propose here a scheme for searching for novel membrane protein structures in specific types of membranes. In this approach single particle EM and mass spectrometry, after pre-fractionation using 1- or multidimensional protein separation techniques are applied to characterize all its larger components.

---

---

## Introduction

A proteomics analysis of transmembrane proteins is often focussed on the discovery of novel protein components or on the study of modifications by phosphorylation and so on. Such a systematic analysis is based on one- or multidimensional protein/peptide separation depending on the complexity of the sample followed by extensive (tandem) mass spectrometric analysis (Stauber et al. 2003). In comparison with sequence tags and genomic databases, the presence of fully novel or multiple, closely related proteins can be demonstrated. Studies to test this approach have been carried out on several types of membranes, such as mitochondrial membranes from the yeast *Saccharomyces cerevisiae* (Everberg et al. 2004). In this paper we will focus on photosynthetic membrane proteins from oxygen evolving organisms. As an illustrative example for a proteomics analysis, Hippler and coworkers were able to reveal nine Lhca antenna proteins associated to Photosystem I (PS1) from the green alga *Chlamydomonas reinhardtii*. Three of them were identified for the first time (Stauber et al. 2003). These proteins have a mass around 25 kDa and are functionally related to the light-harvesting complex II (LHCII) antenna proteins of Photosystem II (PS2), which will be discussed below.

Because of the small dimensions of proteins with a mass below 50 kDa and the low signal-to-noise ratio of transmission EM images of biomacromolecules, single particle EM analysis is not (yet) able to reveal much detail in such proteins, although it is our experience that individual trimers of LHCII (75 kDa) can already be recognized, even without averaging, by their typical shape. But the potential of single particle EM is certainly on the larger complexes and their association into supercomplexes.

Over the last 20 years, single particle analysis of electron microscopy (EM) projections has become a well-established technique to obtain structural information about large biomacromolecules at a resolution of 10–20 Å (Van Heel et al. 2000; Frank 2002). In some favourable cases a much higher resolution has been achieved on ice-embedded virus particles or other complexes with a high symmetry (Van Heel et al. 2000; Ludtke et al. 2004). Single particle averaging is simple and attractive if applied to negatively stained specimens with a mass between about 300 kDa and 2000 kDa, since one can process several thousands of projections within a few days, yielding 2D projection maps of at least 20 Å resolutions. The statistical analysis and classification procedures used in single particle analysis are often effective in the sorting of different projection views originating from different conformations or subunit compositions.

During the analysis of a large set of 16,600 projections of PS2 complexes, purified from chloroplast grana membranes from spinach, we noticed a strong heterogeneity of particles due to a variable binding of peripheral subunits around the PS2 core complex (Boekema et al. 1999). As a result, 10 protein complexes with a different subunit composition or arrangement were resolved. However, in this analysis which consisted of several cycles of alignments, multivariate statistical analysis and classification about 25% of the projections could not be assigned to any of these PS2 particles (Boekema et al. 1999). One triangular

---

shaped protein contaminant with a diameter of about 200 Å was present in about 200 copies. By averaging these projections, the contaminant could be designated as a multimer of 7 copies of trimeric LHCII (Dekker et al. 1999). This assignment was possible for two reasons. First, a high-resolution structure of LHCII trimers was solved by electron microscopy (Kühlbrandt et al. 1994). Even at the 20 Å level the strong similarity between the contaminant and the LHCII structure was apparent. Second, it is known that single LHCII trimers which do not bind to PS2 are present in variable, but appreciable numbers within the thylakoid membrane, from which PS2 complexes were purified. However, the discovery that these trimers would be present as supercomplexes with seven LHCII trimers was a surprise.

These results on a rather heterogeneous set of EM projections indicate that single particle EM analysis has the potential to detect specific unknown large complexes in large data sets and to obtain reasonably well defined 2D maps, even if such contaminants are present in small numbers. Usually we will not be able to assign the structures of the contaminants to specific proteins. Nevertheless, in several studies we could detect remarkably large contaminants, such as a T-shaped particle that co-purified with trimeric PS1 from the cyanobacterium *Gloeobacter violaceus* (Mangels et al. 2002). These studies also indicated that it would be likely that by checking “rather pure” membrane protein complexes for contaminants more of such novel structures could be found if averaging of several dozens to hundreds of projections is possible.

In a next step of reasoning we consider here that if it were possible to assign such novel structures to polypeptides in a rather systematic way the technique of single particle analysis could get a novel useful application in the expanding field of proteomics. Here we would like to propose a method which combines single particle EM and protein mass spectrometry. Its strategy is specially meant for membrane proteins, which are harder to characterize by a proteomics analysis than water-soluble proteins and aims to discover novel and larger complexes rather than the single smaller proteins.

To test the possibilities of combining single particle EM with protein mass spectrometry we describe the comparison of two batches of PS2 from the cyanobacterium *Thermosynechococcus elongatus*. PS2 is a multisubunit membrane protein complex which catalyzes one of the key reactions on our planet – the light-driven oxidation of water (Hankamer et al. 1997). It is a dimer with over 25 subunits (PsbA-PsbZ and others) per monomer depending on the species. About half of them are small intrinsic membrane proteins with a molecular weight below 10 kDa, which extremely complicates the mass spectrometry analysis. However, in a detailed characterization of our His-tagged PS2 preparation almost all of the small subunits could recently be assigned in a rapid and gel-free MALDI-TOF approach (Nowaczyk et al., submitted). In contrast to WT PS2 which was purified by a standard HPLC procedure (Kuhl et al. 1999), the His-tagged PS2 complex shows the presence of an additional mass which can be assigned to PsbZ. Single particle EM analysis can reveal the presence of PsbZ in the His-tag preparation and localize it at the periphery of the PS2 complex.

These and other results on proteins contaminating the photosynthetic membrane proteins PS1 and PS2 will be discussed in the framework of a proposal

---

for a combination of the single particle EM technique with a mass spectrometry analysis.

## Materials and methods

### *Purification of wt and his-tagged photosystem II*

PS2 complexes from *Thermosynechococcus elongatus* were prepared by a two-step HPLC procedure as described in Kuhl et al. (1999), with the following modification: after solubilization of the thylakoid membranes by dodecyl- $\beta$ -D-maltoside ( $\beta$ -DM) and centrifugation, the supernatant was loaded onto a 27 ml sucrose gradient consisting of 14 % sucrose (w/w) in buffer B (20 mM MES pH 6.5, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 0.5 M mannitol and 0.03 %  $\beta$ -DM); this gradient was layered on top of an 80 % sucrose cushion in the same buffer. After overnight centrifugation (18 h, 4°C, 83.000  $\times$ g), the core centers – visible as a green band in the upper layer – were collected with a syringe and prepared for the first chromatographic step.

For the preparation of His-tagged PS2 complexes, thylakoid membranes were solubilized in 20 mM MES pH 6.5, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.2%  $\beta$ -DM, 0.5% Na-cholate at a Chl concentration of 1 mg/ml. After centrifugation at 45.000  $\times$  g for 90 min at 4°C, the supernatant was loaded onto a chelating sepharose fast flow column (Pharmacia) which had been equilibrated with starting buffer (50 mM MES pH 6.5, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 300 mM NaCl, 10% glycerol, 0.02%  $\beta$ -DM, 1 mM imidazole). The column was then flushed with 4 volumes of equilibration buffer at a flow rate of 2 ml min<sup>-1</sup>. PS2 core complexes were eluted by a linear gradient from 1 mM to 200 mM imidazole and dialysed over night against buffer B. In order to separate PS2 monomers and dimers, the resulting protein solution was loaded onto a second column (IEC) as in (Kuhl et al. 2000).

### *Mass spectrometry*

For the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis, PS2 preparations (adjusted 1 mg/ml Chl) were mixed with a saturated solution of alpha-cyano-4-hydroxy cinnamic acid at a ratio of 2:5 and spread on a MALDI-target. After calibration with insulin, protein masses between 6000 and 7000 (m/z) were determined in linear mode with a MALDI-TOF mass spectrometer (Autoflex, Bruker Daltonics).

### *Electron microscopy and image analysis*

Negatively stained specimens were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. From both types of purified PS2 particles, images were recorded with a Gatan 4K slow-scan CCD camera on a Philips CM20FEG electron microscope. 2000  $\times$  2000 pixel images were recorded at 66,850  $\times$  magnification with a pixel size of 15  $\mu$ m and a binning factor of 2, corresponding to a size of 4.49 Å at the specimen level. The data sets were

---

analysed with Groningen image processing (Grip) software on a PC cluster. Resolution in averaged single particle 2D projections was measured according to van Heel (1987).

## **Results**

### ***PS2 EM analysis***

Two sets of about 15,000 projections of dimeric PS2 complexes from the cyanobacterium *Thermosynechococcus elongatus* were analyzed. After several cycles of multi-reference alignments, statistical analysis and classification the best top-view projections from each set were averaged. The non-symmetrized 2D projections were obtained with a resolution of 14 Å (Fig. 1A, B). By comparing the two dimers, it appears that the monomers of each are highly similar, even without imposing any 2-fold symmetry, except for the outer tips (Figure 2C). This tip is larger in the particles with a His-tag.

### ***PS2 analysis by MALDI-TOF***

The 1D SDS-PAGE pattern showed no difference between WT- and His-tagged PS2 (data not shown); however, the resolution in the region below 10 kDa is very weak, so that different protein bands could be shielded by overlay. For this reason we developed a technique to apply PS2 complexes directly to MALDI-TOF analysis in order to determine the entire mass of the small subunits (Nowaczyk et al., submitted). With this method we could assign a single mass in the range of 7000 to 8000 m/z to subunit PsbZ in the sample of the His-tagged PS2 complex (Fig. 2a); this mass is not present in the WT PS2 preparation (Fig. 2b). Studies with different preparations showed, that the amount of PsbZ varies between different batches and with more sensitive techniques traces of PsbZ could also be detected in the WT preparation (data not shown).

### ***L-shaped contaminant of PS2***

In the PS2 preparation studied above one contaminant appeared. L-shaped particles were present with an abundance of 2% and 10% in the batches of PS2 with the His-tag and the ones without, respectively. Image analysis indicated that this L-shaped particle appeared in two dominant views (Fig. 3A, B). The longer horizontal “leg” of the particle does not show much internal detail, which is in general a typical feature for negatively stained membrane proteins in side-view position. Therefore this part is probably a membrane-embedded protein part. Because projections in both views have the same overall dimensions, they likely come from the same particle and thus only have a different handedness in respect to the carbon support film.

---

### *T-shaped contaminant of PS1*

Another large contaminant of photosynthetic membrane proteins seen in other samples is a T-shaped particle which copurifies with PS1 trimers purified from *Gloeobacter violaceus* (Mangels et al. 2002). It has an overall length of 190 Å, consisting of a bilobed scaffold with a width of 130 Å and a height of 65 Å, from which a 120 Å long stem extends (Fig. 4B). In comparison to trimeric PS1 (Fig. 4A) it appeared with a frequency of about 20-30% in the batch which was used for EM analysis. A search in data sets from other PS1 particles showed that it was also present with 70 copies among projections from the cyanobacterium *Synechococcus* PCC7942 (Boekema et al. 2001), corresponding with a frequency of only 0.1%. After inspection of original micrographs from which these projections were picked the same frequency was found. Another more spherical contaminant is present in different views in PS1 data sets from *Synechococcus* PCC7942 (Fig. 4D-E) (Boekema et al. 2001). It is also found in preparations of another cyanobacterium (*Synechocystis* PCC 6803, Yeremenko et al. 2004).

### **Discussion**

Before discussing the role which single particle EM could play in combination with proteomics, we will first discuss our non-systematic observations on contaminants, presented above. For a number of reasons it is possible to assign the L-shaped projection views to molecules of Complex I in side-view position. First, to our knowledge Complex I is the only known major membrane complex which is L-shaped. Second, just as in our data set it appears in all negatively stained single particle EM specimens only in side-views. Third, Complex I sometimes copurifies with PS2 (Berger et al. 1991). However, the assignment is tentative because no cyanobacterial Complex I has been analyzed by single particle EM. Interestingly, the upper leg of the complex is appreciably shorter (Fig. 3A, B) than found for Complex I from other species (Yagi and Matsuno-Yagi 2003). This makes sense because three hydrophilic subunits which are responsible for the NADH dehydrogenase activity and which are considered to form the upper unit of the vertical leg (red mass in Fig. 3C) are lacking in cyanobacteria (Friedrich et al. 1995). These subunits have a mass of about 150 kDa in total and the lack of this amount of mass seems in accordance with the much smaller vertical leg observed in our projections (Fig. 3C).

The T-shaped particle is more difficult to assign in the absence of a specific biochemical characterization. Most remarkably, however, it is about 300 times more abundant in *Gloeobacter violaceus* than in other cyanobacteria. In contrast to *Synechocystis* and *Synechococcus* species this cyanobacterium lacks a special thylakoid membrane in which PS1, PS2 and other photosynthetic membrane proteins are located (Rippka et al. 1974). Therefore PS1 is a component of the cytoplasmic membrane, which is the only intracellular membrane in *Gloeobacter*. If the T-shaped particle is always restricted to the cytoplasmic membrane this

---



---

could give a possible explanation for its low frequency in the PS1 batch of *Synechococcus* PCC7942. Due to its long stem, it could be that this particle is involved in protein secretion. The rather spherical contaminant of unknown origin (Fig. 4D-F) is likely a water-soluble protein, given its excellent staining profile.

### **Assignment of a PS2 subunit**

Comparison of our EM data with the 3.7 Å X-ray structure from PS2 (Ferreira et al. 2004) indicates that two membrane-spanning  $\alpha$ -helices at the tip of the projection are responsible for this difference (Fig. 1). The mass spectroscopy results show that the difference observed between top views projections can be attributed to the PsbZ subunit (Fig. 2). This observation encourages us to propose a scheme for a more systemic search for protein complexes by single particle EM and proteomics tools, especially mass spectrometry.

### **Proposed scheme for EM plus proteomics analysis**

The following strategy specially focuses on a characterization of membrane proteins by proteomics. The first step is to detect novel 2D structures present in a specific protein fraction of a detergent-solubilized membrane by single particle EM. It could be a specialized type of membrane, like the thylakoid membrane from photosynthetic organisms, but also a rather non-specific cytoplasmic membrane. The idea is to study all the larger proteins and the easiest way to obtain them would be a one-step size-exclusion chromatography purification or dialysis with a membrane with a cut-off of 100 kDa to remove the abundant smaller proteins and fragments. The second step would be to obtain a batch from this membrane in which single particle EM indicates that the complex of interest is more abundant or largely absent. This could be achieved by applying further different partial purification procedures, like sucrose gradient centrifugation or dialysis with membranes with a different cut-off. Possibility would be to change the detergent used for solubilization. We also like to emphasize here that partial solubilization often yield larger complexes (Boekema et al. 1999). Third, if differences in the frequencies of observed novel structures complexes are detected by single particle EM, SDS-PAGE patterns from the various purified membrane protein batches should be inspected for major differences. Thereafter, mass spectrometry (MALDI-TOF and other techniques should be used to assign protein bands with a variable intensity. Since the genomes of many organisms have already been sequenced an unambiguous assignment could be achieved in most cases. If necessary additional de novo synthesis could be of help.

Further studies could also be undertaken by subsequent purification of the novel structures to homogeneity. However, this is not the prime objective of our proposal. Moreover, purification to homogeneity of large membrane proteins can be tedious and at the risk of the loss of protein components. Actually, the first indication for the heterogeneity of PS2 due to loosely attached peripheral membrane-bound subunits was obtained by the analysis of single particles from disrupted thylakoid membranes without any purification at all (Boekema et al.

---

---

1998). The advantage of EM over many other biophysical methods is certainly the detection of single molecules. Single particle EM should take full advantage out of this. If so, we can expect that by application of our proposed scheme interesting complexes or transient supercomplexes in many membranes will be discovered.

### Conclusions

1. Membrane protein contaminants can still be detected and averaged from mixtures of membrane proteins by single particle analysis EM if present in frequencies of 0.1%. To get statistical meaningful projections this requires data sets of at least about 20,000 projections. Analysis of such numbers can be performed by a state-of-the-art PC cluster with about 16 processors. This is necessary for performing the most time consuming step in single particle EM, which is the statistical analysis.

2. Mass differences between membrane proteins in the order of 3% are still detectible, as shown for the PsbZ subunit of PS2.

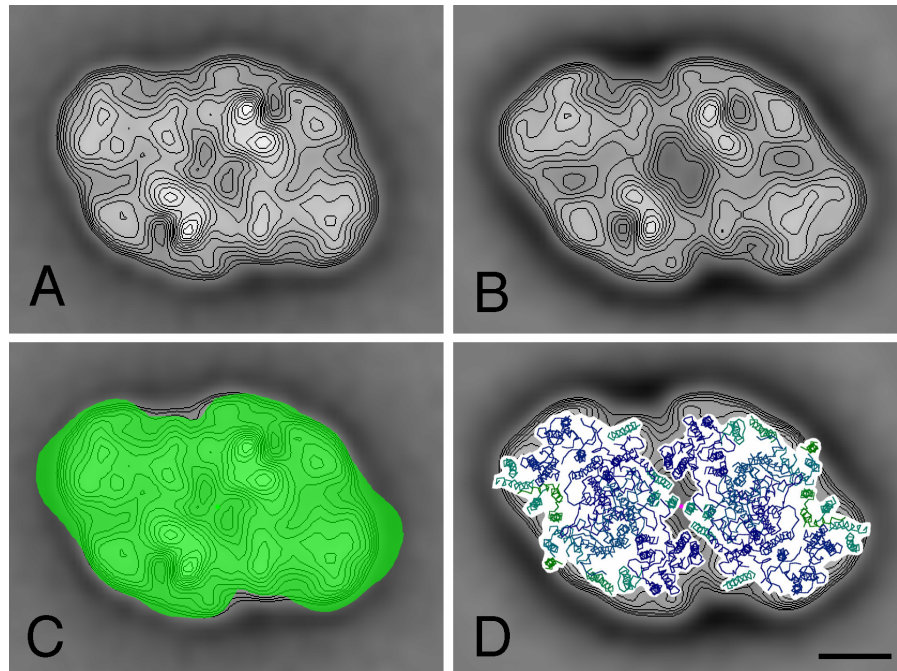
3. Clear differences in frequencies between structures seen by single particle EM can be correlated to clear differences in intensities of protein bands on SDS-PAGE. Since basically a protein band can be assigned by mass spectrometry as long as it is visible with Coomassie staining, we suggest that assignment by mass spectrometry of novel structures seen by single particle EM should be possible.

4. We propose the analysis of the complete set of larger integral membrane proteins from any kind of a specific membrane after detergent disruption in a systematic way by combining single particle EM and proteomics analysis, in particular mass spectrometry.

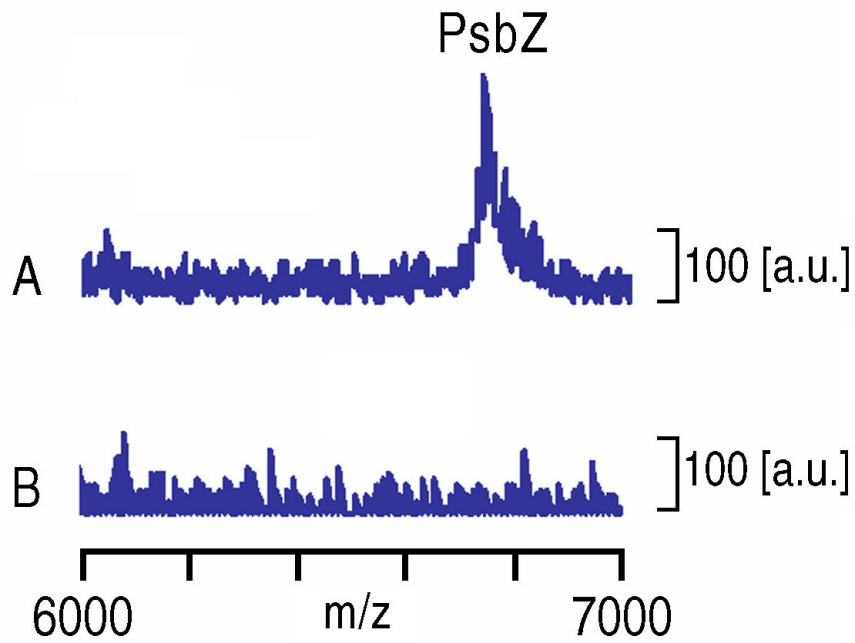
### Acknowledgements

We acknowledge a grant of the the Dutch science foundation NWO-ALW (Programme From Molecule to Cell) awarded to E.J.B. and support from the German Research Council DFG (SFB480 and European Graduate College 795) awarded to M.R.

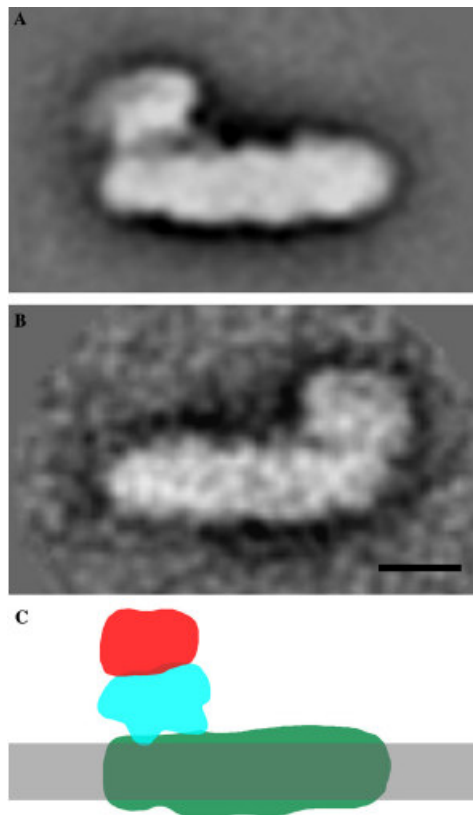
---

**Figure 1**

**Fig. 1. Single particle analysis of top-view projections of PS2 complexes.** (A) averaged top view projection of PS2 complexes purified with a standard HPLC procedure. (B) averaged top view projection of His-tagged PS2 complexes after modified HPLC. (C) map of image of B (in green) superimposed on image A. (D) Fitting of the X-ray structure (Ferreira et al 2004) on image B suggests that the additional mass belongs to two membrane-bound  $\alpha$ -helices (in blue-green). Space bar is 50 Å.

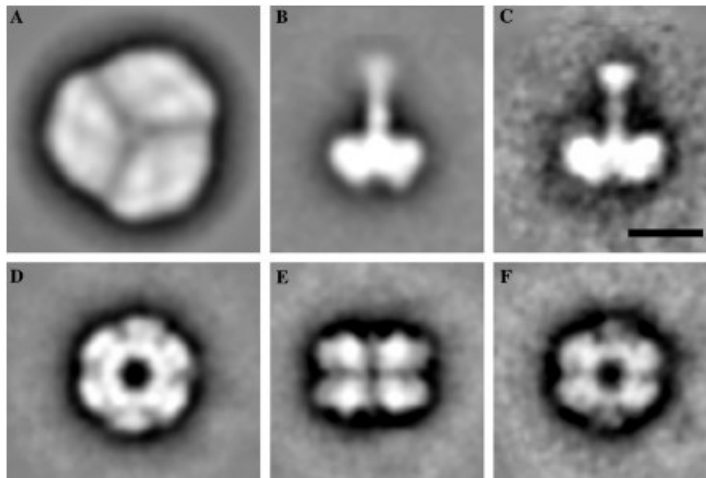
**Figure 2**

**Fig. 2. Determination of PsbZ in His-tagged PS2 complexes by MALDI-TOF MS.** (A) His-tagged PS2 complexes after modified HPLC and (B) PS2 complexes purified with a standard HPLC procedure; both preparations were used directly to determine the entire protein masses between 6,000 and 7,000 Da.

**Figure 3**

**Fig. 3.** Analysis of L-shaped particles from *Thermosynechococcus elongatus*, tentatively assigned to Complex I. (A) sum of 1962 projections. (B) Sum of 259 projections. (C) Scheme for Complex I and its three units in bacteria and mitochondria with the NADH-oxidizing unit (red), the membrane-embedded hydrophobic module (green) and the connecting domain (blue) (modified from Dupuis et al. 2001). The space bar in the frames A and B is equal to 100 Å.

Figure 4



**Fig. 4.** *Analysis of contaminants of trimeric Photosystem I complexes.* (A) average of 300 projections of trimeric Photosystem I from *Gloeobacter violaceus* (reproduced from Mangels et al. 2002). (B) average of 406 projections of an unknown T-shaped contaminating PS1 from *Gloeobacter violaceus* (Mangels et al. 2002). (C) average of 70 projections from a similar T-shaped contaminant in a data set of PS1 projections from *Synechococcus PCC7942*. (D-E) three different views of probably the same type of another contaminant in PS1 preparations from *Synechococcus PCC7942*, with 200, 94 and 53 projections, respectively. Space bar is 100 Å.

