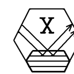

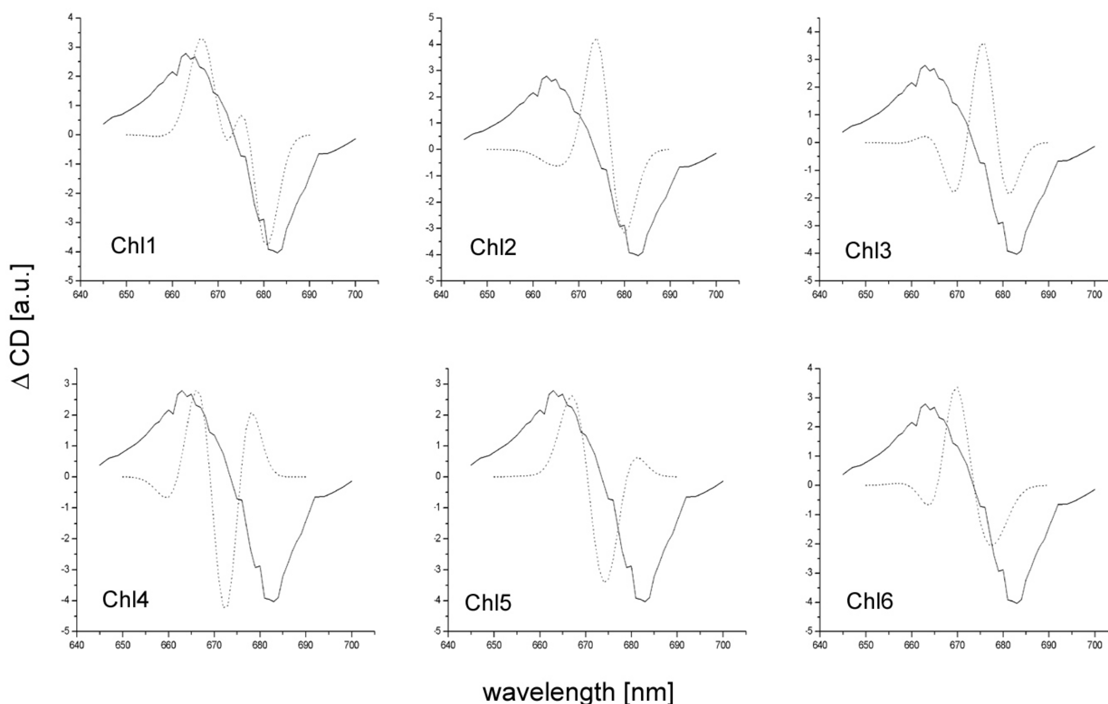

Fig. 3. Light induced (light minus dark) circular dichroism difference spectra of reaction centre of photosystem II measured at 273 K (solid lines) compared with calculated circular dichroism difference spectra (dotted lines). The calculated spectra were obtained by subtracting the original full pigment spectra from spectra where one particular chlorophyll pigment (numbered in the individual figures as Chl1-6) was omitted from the calculation.

FUNCTIONAL RECONSTITUTION OF PHOTOSYSTEM 2 INTO LIPOSOMES

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Abstract
Most recent structural data on photosystem 2 (PS2), the first membrane protein complex in the photosynthetic electron transport chain, confirm that this complex exists as a functional dimer in the thylakoid membrane of cyanobacteria [1, 2]. Besides the membrane embedded part of this dimer with dimensions of 190 Å x 100 Å x 40 Å, this complex also extends about 10 Å out of the membrane in the stromal region and 55 Å in the lumen; the latter is referred to as the oxygen evolving complex, harbouring the water-splitting site. Developing an approriate method to reconstitute dimeric PS2 into liposomes should finally help to answer the fundamental question concerning its structure-based function: Is a dimeric structure a prerequisite for optimal water-splitting activity (monomeric complexes, solubilized by detergent, are active, too, although at a lower level) and which is the impact of the lipid-phase-composition on the water-splitting activity? Here we present data on the orientation of reconstituted dimeric PS2 from the cyanobacterium *Thermosynechococcus elongatus* and also give indications for its activity within the liposomes and monomer-dimer distribution (by EM analysis).

Material and Methods

Liposomes were prepared according to [3]. PS2 core complexes, prepared essentially as in [4], were reconstituted

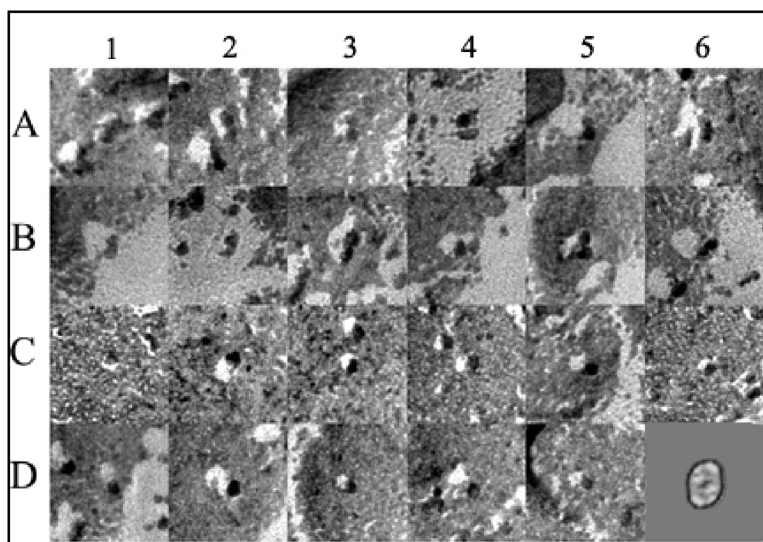

Fig. 1. Selected parts of electron micrographs of incorporated PS2 complexes with a size of 493 A x 493 A. Frame D6 represents an averaged projection image of dimeric PS2.

into these liposomes according to [5]. In detail, 100 g Chl of PS2 was added to 180 L of the liposom suspension; this mixture was diluted by buffer (20 mM MES, pH 6,5; 30 mM $CaCl_2$; 10 mM $MgCl_2$; 1 M glycin-betain) to reach a final volume of 900 L. A stock solution of 1 M OGP dissolved in the same buffer was added in small aliquots to the suspension under stirring at 20°C until the suspension turned from cloudy to clear (final concentration about 30 mM). After addition of 80 mg Biobeads [6], the suspension was incubated under gentle stirring for 1 h at 20°C; this step was repeated once, and in the third step 160 mg Biobeads were added, followed by 1 h incubation.

Orientation of the incorporated PS2 complexes in the membrane was checked by trypsinization of the oxygen evolving complex (OEC). The enzym/protein ratio was 1:5 (w/w) at 20°C and aliquots were taken after 0, 2, 5, 15 and 60 min. After 15 min the sample was divided into two parts of which one was incubated with OGP (final concentration 100 mM). After the indicated incubation times, aliquots were taken from both samples and treated with a protease inhibitor cocktail (final concentration 70 g/mL) and EDTA (final concentration 0,1 M) to stop the protease reaction. From this, an aliquot of 0,05 mg chlorophyll was used for analysis by SDS-PAGE electrophoresis.

For freeze-fracturing and electron microscopy, small droplets of reconstituted PS2 monomers and dimers were quickly frozen in nitrogen slush and fractured. Replicas were examined with a Philips CM10 transmission electron microscope and images were recorded at 52,000x magnification. Images were scanned by a densitometer with a 25 micron scannstep. Representative parts of well-shadowed vesicles were selected and all protein profiles were selected.

Results

Reconstituton experiments have always been performed with highly purified PS2 dimers. In order to determine the amount of incorporated PS2 and estimate roughly the ratio of monomers to dimers, reconstituted liposomes were broken by a freeze fracture technique and observed by transmission electron microscopy (Figure 1). The electron micrographs show a heterogeneous mixture of monomers and dimers, indicating the partial dissociation of the dimeric PS2 particles within the lipid phase.

Incorporated proteins show up as dark spots (because of deposited heavy metal) with a bright shadow where no metal was deposited. Frames B6, D2 and A2 show incorporated dimers, while monomers can be seen in frames C3, C4 and D3. Dimers and monomers could be identified by comparison with averaged images of isolated, negatively stained dimeric PS2 molecules (frame D6). PS2 dimers in freeze-fracturing are expected to be slightly smaller than the negatively stained dimers, because the latter are prepared in the presence of detergent, which forms a boundary layer around the complexes. Another complication is the position of the dimers towards the shadow direction of the metal deposition used in freeze-fracturing. Dimeric particles which have been shadowed with their long axis paralllel to the shadow direction will tend to appear somewhat smaller than particles which are 90° rotated.

Although the amount of particles per liposome varies considerably, the ratio between monomers and dimers is approximately 1:1.

As a compromise between PS2 stability and optimal condition for trypsin activity pH 7,5 was chosen. The extrinsic subunit PsbO served as an indicator for the orientation of the PS2 complex: When oriented towards the outside of the vesicles, it should be gradually digested by trypsin. In contrast, an orientation towards the inside would prevent an attack of PsbO by trypsin, which is only possible after destruction of the vesicle by detergent.

The time-dependent immunoblot (Figure 2) of the external 33 kDa subunit clearly shows an increase in degraded protein within the first 15 minutes. These bands - including the degradation products at about 25 kDa and 14 kDa, focused even more between 15 and 60 minutes. The most significant PsbO trypsination products are at 30 kDa, 25 kDa, 14 kDa and 3 kDa. For comparison, trypsination of an OGP treated sample is shown, where no intact subunits are to be found.

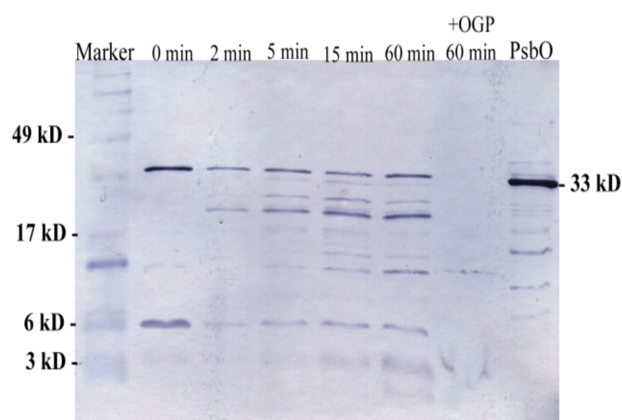

Fig. 2. Immunoblot analysis of the PsbO subunit after trypsinization at pH 7,5. As a reference 0.5 mg in E. coli heterologously overexpressed PsbO was used.

Additionally, the effect of trypsination was also tested by polarographic measurements of oxygen evolution. With ferricyanid (2 mM) and DCBQ (0.4 mM) as electron acceptors, reconstituted PS2 complexes which had been treated by trypsin yielded an activity of 240 mol O_2/mg Chl x h corresponding to about 30% of the activity determined with untreated complexes (780 mol O_2/mg Chl x h).

Discussion

Although each 33 kDa protein contains 23 lysine and 6 arginin residues [7], i.e. sufficient restriction sites for trypsin digestion, the degradation of this subunit resulted in only two main bands at 25 kDa and 14 kDa. In contrast, OGP treated samples showed two signals at 10 kDa and 3 kDa, but none at 25 and 33 kDa. This apparently is due to the action of the OGP detergent, which may induce dissociation of the 33 kDa subunit from the complex and by this may prevent the determination of the PS2-orientation in the lipid bilayer after 60 min. (although the digestion of all trypsin accessible proteins was completed after that time). Comparison of the band intensity after 60 min between OGP-untreated and the control sample at 0 min. shows, that about 55% of the 33 kDa band remains after trypsin action, i.e. about 55 % of PS2 is oriented with the water-splitting site inside.

The oxygen evolution measurements show, that trypsin treated samples lose about 70% of their water-splitting activity in comparison with the untreated reference sample. This may, however, not indicate that 70% of the PS2-donor site is exposed to the outside: According to Honk et al. [7], tryptic digestion also effects the acceptor site of PS2 and impairs the function of ferricyanid and DCBQ as electron acceptors. This in turn would reduce the activity of PS2 particles oriented with their acceptor site towards the outside of the liposomes. As a partial damage of the QB-site by tryptic digestion cannot be excluded under our experimental conditions, polarography of trypsin-treated samples is not ideal to quantify the orientation of the PS2 complex and may be the major reason for the missmatch of the 70% with watersplitting outside vs. the 45% gained by the immunoblot analysis.

Reconstitution of PS2 from *Thermosynechococcus elongatus* was already reported with synthetic lipids (phytanyl-chained glycolipids) and natural sulphoquinovosyldiacylglycerol [9]. Depending on the type of lipid used, oxygen-evolving activities of 5 - 30% with respect to the activity of non reconstituted PS2 were obtained, indicating a significant role of the surrounding lipid for PS2 activity. However, this report yielded no direct information on the orientation of PS2 in the liposome membrane and whether the activity loss was due to a "wrong" orientation of the PS2 particles or the long exposure in OGP during the reconstitution procedure.

Summary

In summary, we have developed an efficient method for the functional reconstitution of PS2 into liposomes. Considering the fact that approximately half of the PS2 particles is apparently oriented "wrong side out", the reconstituted PS2 shows a reasonable oxygen-evolving activity. The establishment of this procedure now enables studies on the reversible oligomerization of PS2 and its impact on oxygen-evolving activity, as well as the impact of "external" factors like lipid and salt environment.

Acknowledgements

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