The Role of Phosphatidylglycerol as a Functional Effector and Membrane Anchor of the D1-Core Peptide from Photosystem II-Particles of the Cyanobacterium *Oscillatoria chalybea*

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D1-Peptide, Phosphatidylglycerol, Lipid-Protein Binding, Cyanobacterium, PSII-Complex

The intrinsic polypeptide D1, isolated from photosystem (PS) II-particles of the cyanobacterium *Oscillatoria chalybea*, was obtained by electroelution and fractionated extraction with organic solvents. Purification was demonstrated by Western blotting and amino acid sequencing. By carrying out D1-immunization in rabbits a polyclonal monospecific D1-antiserum was obtained.

For the qualitative characterization of D1 as a lipid-binding peptide, the effect of the lipids phosphatidylglycerol (PG), monogalactosyldiacylglyceride (MGDG) and phosphatidylcholine (PC) on PSII-oxygen evolution was analysed in reconstitution experiments. In these experiments purified photosystem II (PSII)-particle preparations were treated with the enzyme phospholipase A_2 and supplemented with lipid emulsions. We were able to show that the inhibition of electron transport, as the consequence of this lipase treatment, was only relieved, if phosphatidylglycerol was added to the preparation. A model was proposed, in which phosphatidylglycerol is a functional effector for the optimal conformation of D1 in the PSII core complex. Phosphatidylglycerol molecules are unusually tightly bound to the D1 peptide by hydrophobic interactions. A covalent binding seems not probable. The localisation of phosphatidylglycerol binding sites was found by trypsin treatment of D1 and analysis of the obtained oligopeptides with HPLC and immunoblotting. The binding sites could be confined to the hydrophobic amino acid section between arginine 27 and arginine 225, which is known to be the membrane anchor of D1. This has led us to the conclusion that the phospholipid phosphatidylglycerol plays an important role for anchoring the D1-peptide and for its orientation in the thylakoid membrane. Phosphatidylglycerol with its high amount of palmitic acid has in prokaryotic cyanobacteria apparently a role in stabilization and orientation.

The high turn-over of D1 and the spatial separation of the synthesis- and incorporation-site in the membrane, developed during evolution in eukaryotic organisms, might have changed the requirement on the mobility and the orientation of D1 in photosynthetic membranes.

Introduction

During investigations on possible lipid-protein interactions in isolated oxygen-evolving photosystem (PS) II-membrane preparations from the cyanobacterium *Oscillatoria chalybea* we were able to show that the anionic phospholipid phosphatidylglycerol is specifically and tightly bound to the intrinsic D1 core peptide (Kruse *et al.*, 1994). This binding was demonstrated by means of serology by monospecific antisera as well as chromatographically by HPLC and gas chromatography.

Due to the fact that the structure of the photosynthetic apparatus is part of the whole membrane system, interactions between lipids and photosystem (PS) II-peptides gain in importance. Biomembranes are always in a liquid-cristalline phase condition, in which the maintenance of fluidity is the decisive factor. The most important clues for this view were presented by investigations of different organisms during thermal stress situations. Many species adapt to lower temperatures by changing their membrane lipid composition and, simultaneously, by increasing the concentration of un-

Abbreviations: MSP, manganese stabilizing protein; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglyceride; DGDG, digalactosyldiacylglyceride; PG, phosphatidylglycerol; SQL, sulfoquinovosyldiglyceride, PSII, photosystem II.

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saturated fatty acids (Thompson, 1980). By this reaction the fluidity of the membrane will be preserved at lower temperatures, which is of vital necessity for the organism. According to the function of such a fluid mosaic model, the ability of membrane components for lateral diffusion is essential. This kind of passive mobility is not only limited to the lipids but occurs also with proteins and entire protein complexes, although the mobility of the latter is usually restricted by their size.

Interactions between membrane lipids and proteins seemed to be important for anchoring the complexes as well as for their functional orientation in the biomembrane. At any time lipids are in contact with the proteins, that are integrated in membranes. Here, certain proteins are selectively associated with certain lipids in a condition, in which the binding type may vary from electrostatic and ionic interactions to covalent binding. Many membrane proteins have nonpolar domains, which interact with the hydrophobic centre of the lipid double-layer. Experimental results from studies on the bacterial photosynthetic reaction centre have shown that the nonpolar element consists of a membrane-spanning α-helix of the protein. The protein complexes are surrounded by uncharged galactolipids like monogalactosyldiacylglyceride, digalactosyldiacylglyceride and, in case of *Oscillatoria chalybea*, by trigalactosyldiacylglyceride (Zepke *et al.*, 1978). They may make up for 80% of the total lipid content (Kruse *et al.*, 1990). The lipid composition of the cell is completed by the polar lipids sulfoquinovosyldiacylglyceride and phosphatidylglycerol. To date, in contrast to higher plants, no other phospholipids could be identified in cyanobacteria (Nichols *et al.*, 1965). Furthermore, in these organisms, no lipids were found outside the membrane systems. On the other hand, investigations during the last few years have shown, that the role of lipids in biomembranes lies beyond the simple structural function. In this context, lipids are seen as an active part in many membrane associated processes, for example in processes, in which lipids are responsible for optimal enzyme activities. For example it is known that the Ca^{2+}-ATPase of the sacroplasmatic reticulum and the $Na^{+}K^{+}$-ATPase are unspecifically activated by most of the phospholipids (Bennett *et al.*, 1973). On the other hand, lipids play an important role as regulatory agents, such as phosphatidylinositol in plasmatic membranes of eukaryotes (Parthasarathy and Eisenberg, 1986). Other lipids are active components of biochemical pathways, like phosphatidylglycerol, which participates in the biosynthesis of periplasmatic oligosaccharides in *Escherichia coli* (Jackson *et al.*, 1986) and which is also involved in the protein phosphorylation process of the spinach chloroplast-membrane (Siegenthaler and Bovet, 1993).

Based on this knowledge the role of lipids for photosynthetic protein complexes becomes evident. Despite former assumptions, suggesting that some highly concentrated lipids increase oxygen evolution in photosystem (PS) II-complexes (digalactosyldiacylglyceride (DGDG), phosphatidylcholine (PC)), whereas other lipids, such as phosphatidylglycerol (PG), sulfoquinovosyldiacylglyceride (SQDG)) (Gounaris *et al.*, 1983) would decrease the activity and the finding that photosystem II, if prepared with Triton X-100, is a lipoprotein complex (Gounaris and Barber, 1985), no direct evidence for a specific binding of membrane lipids within photosystem (PS) II components was known. More recent investigations on higher plants led us to the conclusion that an association might exist between the lipids monogalactosyldiacylglyceride (MGDG), digalactosyldiglyceride (DGDG) and sulfoquinovosyldiglyceride (SQDG) on the one and the PSII complex on the other hand (Voß *et al.*, 1992). Nevertheless, it is important to note that this kind of association is not synonymous with a functional aspect of these lipids in photosynthetic electron transport. For example, Benning *et al.*, 1993, were able to show that the association of sulfoquinovosyldiacylglyceride with photosynthetic membranes is of no importance for photosynthetic electron transport.

In contrast to this, it has been observed, that the D1 subunit is more resistant to glutaraldehyde crosslinking than the D2 subunit and it has been suggested that the D1 conformation might be stabilized by bound lipids, which would imply that lipids may have an important role in the centre of the reaction core (Adir and Ohad, 1988). In support of this conclusion are observations that phospholipids are essential for the charge separation in PSII (Jordan *et al.*, 1983) Based on this knowledge the evidence of a specific binding of phosphatidylglycerol onto the D1-peptide gains importance. In addition, the fact that the enzymatic cleavage of

the lipid disturbs photosynthetic electron transport, brings the role of phosphatidylglycerol to the fore.

In this publication we report on the role of phosphatidylglycerol as a functional effector for charge separation at the D1/D2-heterodimer and as a membrane anchor for the D1-core peptide.

Material and Methods

Isolation of PSII-particle preparations

Isolated PSII-particles were obtained according to a method described earlier (Kruse *et al.*, 1994) by solubilization of PSII complexes with the non-ionic detergents *n*-dodecyl-β-D-maltoside and octyl-β-glucopyranoside and a following purification by sucrose density gradient-centrifugation.

Analysis of polypeptide composition

Polypeptides of different samples were analysed, as described earlier (Kruse *et al.*, 1994), by polyacrylamide gel electrophoresis according to Laemmli (1970).

Immunoblotting

Peptides were separated by SDS polyacrylamide gel electrophoresis and analysed immunologically via the Western blot procedure described earlier by Rennart *et al.* (1979), and described by Kruse *et al.* (1994).

Dot blotting

Using the Dot blot procedure the antigen (1 μg/1–3 μl suspension) was applied directly to nitrocellulose membranes (Schleicher a. Schuell No. B85) or to methanol-stable PVDF-membranes (Millipore). After a short period of drying the experiment was continued with the washing procedures and the treatment with different antibodies according to Rennart *et al.* (1979).

Isolation of the D1-peptide

Purified D1-peptides were obtained by electroelution as described earlier (Kruse *et al.*, 1994).

In a second step the separation of D1 from the coeluated hydrophilic manganese-stabilizing enzyme was achieved by extraction with the organic solvents chloroform/methanol (2:1; v/v). For this purpose, the D1-suspension, isolated by electroelution, was diluted with water to 10 ml and extracted with 30 ml of the organic mixture.

After a period of phase-separation (30 min), chloroform was removed by vacuum evaporation and the peptide was diluted in the electroelution buffer. Its purity was tested by immunoblotting with antibodies raised against the PSII-polypeptides.

Generation of a polyclonal antiserum against D1

To obtain a monospecific antiserum against D1 the method used, was that described by Schmid *et al.*, 1975. The immunization of the rabbit was obtained by injection of 180 μg of the D1-peptide in 0.8 ml 20 mM TRIS (hydroxymethyl) aminomethane (TRIS HCl-buffer) (pH 9.0) containing 15 mM glycine and 0.01% SDS. 24 days after the first immunization the rabbit was boostered by injecting intraveneously 130 μg D1-peptide, diluted in 0.4 ml buffer. The first blood withdrawal was carried out 9 days later with further blood withdrawals in one week intervals. To obtain the serum, the blood was coagulated for 3–5 h at 20 °C and then over night at 4 °C. After centrifugation at 3000×*g* (Heraeus Christ, Minifuge 2) the supernatant was filled in ampoules and stored at −20 °C.

Amino acid-sequencing of the D1-polypeptide

Sequencing of D1 was carried out by the Edman-procedure in a Knauer sequencer (Modell 810). The method used was described by Reinke *et al.* (1991) in which in a step by step procedure the N-terminal amino acid was transformed into a phenylthiohydantoin derivative and then hydrolyzed.

Trypsin-treatment of the D1-peptide

D1-peptide suspensions were concentrated by vacuum-centrifugation (Bachofer) and then diluted in 0.1 M TRIS HCl-buffer (pH 8.5) containing 0.1% SDS to give a final concentration of 0.5 mg/ml. 25 μg Trypsin (HPLC-grade, Boehringer) were diluted in 100 μl 1 mM HCl. 5 μl of this solution were mixed with 50 μl of the D1-sample (enzyme/peptide ratio 1:20; v/v). Incubation was carried out in a water bath at 37 °C for 10 h.

HPLC-analysis of the D1-oligopeptides

D1-oligopeptides produced by trypsin treatment were separated by reversed HPLC. The following solutions were used for a gradient elution: solution A: H_3PO_4 (1.7% in water), solution B: acetonitrile (86% in water). The elution was carried out over a period of 55 min, in which the portion of solution B changed as described below: 0–5 min: constant 5%; 5–45 min: from 5 to 90%; 45–48 min: from 90 to 100%; 48–50 min: from 100 to 5%; 50–55 min: constant 5%. The analysis was carried out with a 100-RP-18-column (Merck; 5 μm; 250×4mm), which contained LiChrosphere as the stationary phase and using a HPLC-apparatus from LKB (gradient pump 2249) and a flow rate of 0.8 ml/min. Detection of the eluated peptides was achieved by spectroscopic analysis with a spectral photometer (LKB 2140) at 220 nm. The identification of the D1-oligopeptides was obtained by dot blotting with specific D1-antibodies.

Results

Isolation of the D1-peptide and evidence of purity

The intrinsic D1 polypeptide is together with the D2 peptide the site of photosynthetic charge separation. The D1-peptide was obtained by electroelution after peptide separation by SDS gel electrophoresis. Since purified PSII particles contain only a limited number of peptides, it was easy to cut the individual peptides out of the gel.

In order to achieve maximal separation in the 30 kDa area a separation gel containing 10% acrylamide was used. After the following electroelution the purity of D1 was checked by the method of immunoblotting. As a result, it could be recognized that in addition to the 32 kDa-peptide, a second peptide (manganese stabilizing peptide, MSP) was cut out. The difference in the molecular weight between these peptides proofed to be too small for a total separation from D1.

Complete separation was achieved, however, by a subsequent extraction with organic solvents, in which the more hydrophobic D1 went into the nonpolar chloroform phase, whereas the more hydrophilic MSP remained in the polar water–methanol phase. The purity of D1 was attested by immunoblotting.

Fig. 1 shows the result of this treatment. In lane a the D1-antiserum labelled the 32-kDa peptide of a purified PSII-sample. As seen in lane b–e the purified D1-peptide only reacted with the antibodies raised against D1 (b) and against the phospholipid phosphatidylglycerol (c) but not with antibodies raised against D2 (d) and MSP (e). This result represents the evidence for purity of the intrinsic 32 kDa peptide D1 from the cyanobacterium *Oscillatoria chalybea*.

Amino acid sequencing of the D1-polypeptide from *Oscillatoria chalybea*

Partial N-terminal sequencing of D1 was used to further proof the purity of the D1-preparation and was supposed to reveal potential differences to known D1 sequences from other cyanobacteria. The first 10 N-terminal amino acids, identified by the method described above, are listed in the following chart and compared to those of *Synechococcus* PCC7942 and *Synechocystis* PCC6714 using the usual abbreviations of amino acids (Table I).

Except for the *psb*A-gene product, which encodes for D1, no other sequence could be identified. This result was taken as a further proof for

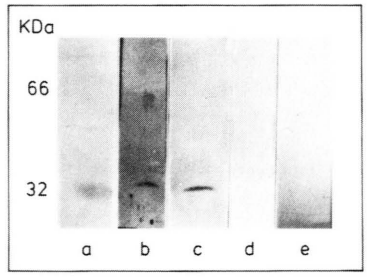

Fig. 1. Reaction of a PSII-particle preparation (lane a) and of the purified D1-peptide (lanes b–e) with different antisera in the Western blot. The figure shows the nitrocellulose membrane after reaction with antisera raised against a) and b) D1, c) phosphatidylglycerol, d) D2 and e) MSP.

Table I. N-terminal amino acid sequences of the D1-polypeptides from different cyanobacteria.

a) *O. chalybea psb*A	H_2N-M-T-L-I-L-E-L-V-V-I
b) *S.* PCC7942 *psb*A-1	H_2N-M-T-S-I-L-E-R-E-Q-R
c) *S.* PCC6714 *psb*A-1	H_2N-M-T-T-I-L-Q-Q-R-E-S

a) *O. chalybea: Oscillatoria chalybea;* b) *S.* PCC7942: *Synechococcus* PCC7942; c) *S.* PCC6714: *Synechocystis* PCC6714.

the purity of the isolated D1-polypeptide. Peculiarities in this sequence compared to those of other cyanobacteria could not be identified. The whole gene product has about 360 amino acids.

Preparation of a polyclonal monospecific antiserum to the D1-polypeptide from Oscillatoria chalybea

The purified D1-peptide was used for the preparation of a polyclonal monospecific antiserum. Avidity and monospecifity was determined by the usual immunological methods. The dot blot test of the D1-antiserum with a purified D1-peptide and a PSII-peptide sample led to a positive reaction. The control serum tested in trace d did not react. Monospecifity of the serum was tested with different PSII-antibodies in the Western blot procedure (Fig. 2). Peptides of a purified PSII-sample were separated by gel electrophoresis and incubated with antibodies raised against MSP and D1 from oat (lanes a and b) as well as against the D1-antiserum obtained from *Oscillatoria chalybea* (lane c) and compared to the corresponding control serum (lane d). We were able to show that the D1-antiserum from *Oscillatoria chalybea* in lane c only reacted with the 32 kDa peptide, which is the evidence for monospecifity.

Qualitative characterization of the binding structure between phosphatidylglycerol and D1

Evidence for the reaction of a human high density lipoprotein (HDL)-antibody with the polypeptide D1 from *Oscillatoria chalybea*

Investigations with the HDL-Serum (Sigma) led us to the result that this serum reacted positively with the D1-peptide from *Oscillatoria chalybea* (Fig. 3). This is an indication for common antigen determinants of D1 and a human lipoprotein. All human lipoproteins consist of a nucleus of hydrophobic lipids which is surrounded by hydrophilic lipids and an envelope of apolipoproteins. They are classified according to their density and, in case of HDL, are synthesized in the liver. Characteristic for these lipoproteins is the high amount of phospholipids, which are linked to the α-helices of the apolipoproteins by hydrophobic interactions. The positive reaction of the HDL-antibodies with the D1-peptide from *Oscillatoria chalybea* is

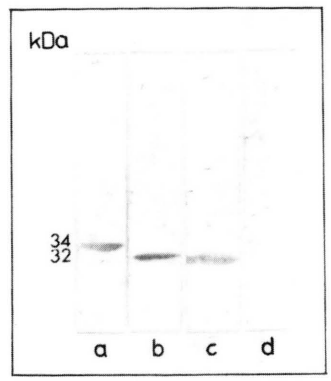

Fig. 2. Western blot reaction of a PSII-particle preparation with different antisera. The figure shows the nitrocellulose membrane after reaction with antisera raised against a) MSP from oat, b) D1 from oat, c) D1 from *Oscillatoria chalybea* and d) the control serum belonging to the D1 immunization.

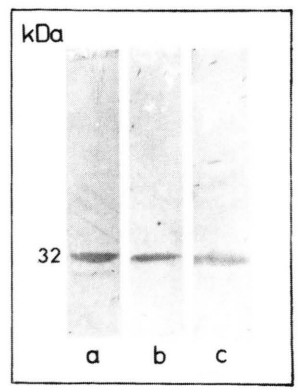

Fig. 3. Reaction of PSII-particle preparations with different antisera in the Western blot. The figure shows the PVDF membrane after reaction with antisera raised against a) human high density lipoprotein (HDL), b) D1 and c) phosphatidylglycerol.

therefore an additional hint for the characterization of D1 as a lipoprotein.

Evidence for the influence of phosphatidylglycerol on oxygen evolution of intact PSII-polypeptide complexes by treatment with phospholipase A_2

The intrinsic 32 kDa peptide is an important component of the PSII-complex. By showing the binding of phosphatidylglycerol onto the D1-Peptide the idea came up, that this lipid-peptide interaction might be important for an optimal electron transport and for the resulting oxygen evolution.

In order to test this aspect, the influence of lipids on electron transport was studied. As described above (Kruse *et al.*, 1994), as a consequence of incubation of photosynthetically active PSII-complexes with phospholipase A_2, oxygen evolution decreases by 20% in comparison to untreated samples (Fig. 4).

This result was clearly due to the reaction of lipase with phosphatidylglycerol. This was demonstrated by the fact that in the immunoblot the PSII-samples reacted differently before and after treatment with phospholipase A_2 (Fig. 4). Antibodies raised against the phospholipid labelled the D1-peptide before the enzyme-treatment (lane a), but not afterwards (lane b). This result was taken as the direct evidence for a relationship between the binding of phosphatidylglycerol onto D1 and the capacity for photosynthetic oxygen evolution of the PSII-complex.

The regeneration effect, as shown earlier (Kruse *et al.*, 1994), was the consequence of reconstitution of the lipid-binding complex, in which we were able to show that other lipids than phosphatidylglycerol, such as MGDG or PC, were not suitable. Thus, it looks, as if phosphatidylglycerol plays a specific role for the functionallity of the PSII-complex and is not replaceable by other lipids.

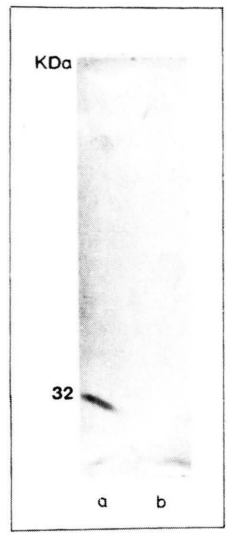

Fig. 4. Western blot reaction of a PSII-particle preparation a) before and b) after treatment with the enzyme phospholipase A_2 with the antiserum raised against phosphatidylglycerol.

Localization of the phosphatidylglycerol-binding site by trypsin treatment of the purified D1-peptide

The aim of these investigations was to recognize further details of the function of the D1-PG binding-system by localization of the binding site. Therefore, a purified D1-sample was treated with the protease trypsin and with the oligopeptides separated by HPLC. Trypsin catalyses only the hydrolysis of peptides, whose carboxy group is provided by the amino acids lysine or arginine. Therefore the maximum number of fragments produced by trypsin treatment can be determined via determination of the number of lysine and arginine residues. To reach an optimal separation a gradient using a LiChrosphere column was chosen with the elution profile shown in Fig. 5. Two maxima are seen with retention times of about 29 min 41 sec and 37 min 51 sec. Both fractions were collected and reacted positively with antibodies raised against D1 and phosphatidylglycerol. Dot blot

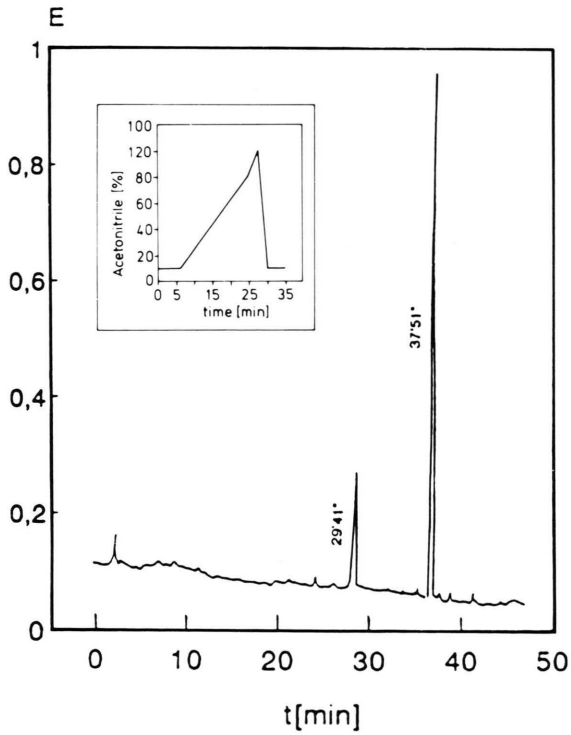

Fig. 5. Oligopeptide analysis of a purified D1-preparation by HPLC after treatment with trypsin. Column: LiChrosphere RP-18 (250mm×4mm, Merck); gradient elution (see methods); E = absorbance.

analysis of the first fraction and a comparison with the HPLC-spectra of a purified D1-sample led to the result that this component was the natural not-cleaved D1-peptide, whereas the second sample was a fragment of D1. This was the first hint for the existence of a specific lipid-binding fragment of D1 after trypsin treatment. As this result gave no information on the size of the fragment nor information on how to insert it into the amino acid sequence, a separation of the D1-oligopeptides by gel electrophoresis after treatment with trypsin was made. To identify smaller components between 2 and 30 kDa a 14%-acrylamide gel was used. The molecular weights of the oligopeptides could be determined by immunoblotting using the monospecific D1-serum (Fig. 6). Besides the non-cleaved D1-peptides in monomeric and dimeric forms (32 kDa and 66 kDa), four fragments with molecular weights of about 9, 12, 20 and 22 kDa could be identified (lane a). On the other hand the antibodies raised against phosphatidylglycerol in lane b only labelled, besides the 32 kDa-peptide, the 20 and 22 kDa fragments. According to Marder *et al.* (1984), these two fragments are nearly identical and only distinguished by a segment of 13 amino acids between arginine 225 and arginine 238. A separation of these nearly identical oligopeptides by HPLC was not possible with the consequence that we could only identify one maximum at 29 min 41 sec, which probably represents a mixture.

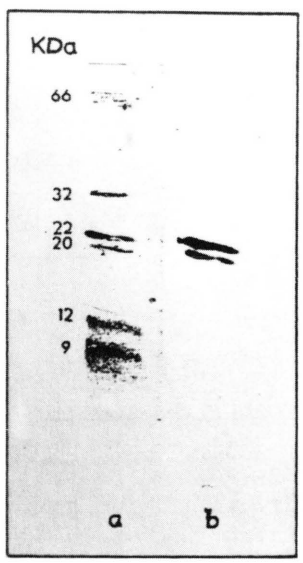

Fig. 6. Reaction of D1-oligopeptides after treatment with trypsin in the Western blot with antisera raised against a) D1 and b) phosphatidylglycerol.

As a result we could recognize that the binding of the anionic phospholipid phosphatidylglycerol could be identified on a hydrophobic area of the intrinsic 32-kDa peptide D1 and that this peptide is a lipoprotein, which binds specifically and tightly one or more phospholipids. The binding has a direct influence on electron transport of photosynthesis and is essential for the function of the PSII-complex.

Discussion

Isolation of the D1-peptide obtained from the cyanobacterium Oscillatoria chalybea

For the isolation of D1 a purification method has been chosen, making use of the high hydrophobicity of the peptide. According to the isolation of the oldest known lipoprotein, which is the Braun lipoprotein from *Escherichia coli* (Braun and Rehn, 1969), we succeeded in separating the D1-peptide from the hydrophilic MSP by extraction with chloroform/methanol. The MSP peptide remained in the water phase. The purification offered the opportunity to produce a specific antiserum against D1 and was the basis for investigations on type and site of the phosphatidylglycerol binding.

Characterization of the D1-corepeptide from PSII particles as a lipoprotein.

Influence of phospholipase A_2 on the PSII-activity and characterization of phosphatidylglycerol as a functional effector

By means of this enzymatic treatment a 20% reduction of the oxygen evolving activity was obtained. This value corresponds approximately to the degradation capacity of phospholipase A_2 towards phosphatidylglycerol as a reaction product. Siegenthaler *et al.* (1989) deduced from similar investigations the existance of interactions between phosphatidylglycerol and other components of the thylakoid membrane in spinach. Furthermore, Siegenthaler and Mayor (1992) discussed a possible change of the D1-conformation after phospholipase A_2-treatment, leading as a consequence to a decreased herbicide-inhibition. With

this suggestion they postulated a relationship between peptide-phospholipid interaction and the conformation of D1 in the membrane.

The experiments led to the result that the consequence of such conformational changes, after enzymatic cleveage of the fatty acid from the C_2-glycerol fragment, is a partial disturbance of the electron transport chain and an inhibition of photosynthetic oxygen evolution. The determinants of the lipid seemed to be no longer accessible to antibodies, raised against phosphatidylglycerol, as after the enzymatic reaction a positive antigen-antibody reaction failed to show up. On the other hand it is improbable that the phospholipase treatment would lead to the cleveage of a covalent binding between PG and D1. Fatty acids of polar lipids are, like alkanes, very inert and have to be ruled out as binding partners.

The observed restoration of the original oxygen evolving capacity by the addition of phosphatidylglycerol is obviously the consequence of a vesicle-formation and incorporation of the PSII complexes into these vesicles. Murata *et al.* (1990) assumed that the PG-molecules are important for the maintenance of the structural organization of the PSII complex.

The orientation of the protein complex in these vesicles might take place by hydrophobic interactions and ionic bindings with one or more tightly associated PG molecules. Since phosphatidylglycerol has been the only lipid, which was able to regenerate the oxygen evolving activity, a specific role of this phospholipid for the enzymatic PSII-reaction has to be assumed. In context with the idea on the importance of PG for orientation and stabilization of D1, the role of PG can be compared with that of a functional effector. Thus, it looks as if many lipids were essential for the catalytic activity of membrane-bound enzymes. They seem to stabilize proteins in the biomembrane against tendencies of denaturation and simplify the interaction between different components by maintaining the conformation via allosteric effects. As a typical example for this function the β-hydroxybutyrate-dehydrogenase should be mentioned, which is an enzyme, which needs specifically phosphatidylcholine for its catalytic activity (Sanderman *et al.*, 1986).

The phospholipid phosphatidylglycerol has to be considered as essential for the maintenance of the D1-peptide and therefore essential for the function of this PSII-component and cannot be replaced by other lipids because of its high specificity.

According to the presented results a model is proposed, which describes the consequence of the enzymatic treatment on the conformation of the D1/D2 heterodimer (Fig. 7). The type of binding between the phospholipid and D1 remains unknown. Fragata *et al.* (1990) were able to show in vesicle experiments a direct dependence of the oxygen evolving activity and the concentration of the added PG. This hints at a cooperative reaction in photosystem II, in which a high charge separation in/at D1 and D2 only occurs, if all lipid binding sites are occupied, leading to an optimal D1-conformation. On the other hand, if these polypeptides are treated with phospholipase A_2, a conformational change seems to occur, caused by a partial decomposition of the stabilizing phospholipids. The consequence of this reaction might be the interruption of the electron transport chain between D1 and D2.

Such a model makes assume that ionic interactions and van der Waal's forces in hydrophobic pockets or clefts might be the principle binding systems between D1 and the phosphatidylglycerol molecules. Under proper conditions these binding systems are very stable and cannot be disrupted by organic solvents.

Since we were not able to exclude that the observed high stability of the lipid-protein is due to covalent binding, we looked for reports on such binding systems. It appeared that covalent binding

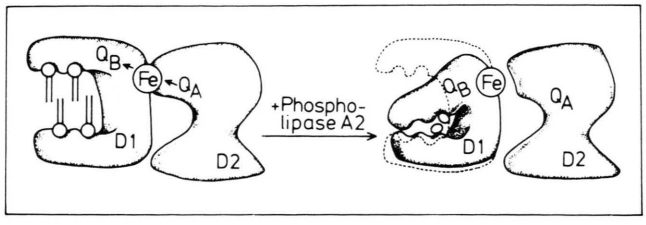

Fig. 7. Model for the influence of phospholipase A_2-treatment on the electron transport chain at the D1/D2 heterodimer. The dashed line represents the initial structure of D1 before enzymatic treatment.

systems are rather the exception, especially in prokaryotic or plant cells. Weyer *et al.* (1987) were able to show that the cytochrome subunit of the photosynthetic reaction center of *Rhodopseudomonas viridis* is a lipoprotein. With the help of masspectroscopic analyses, they found out that the N-terminal amino acid of this protein is a cysteine, connected by a thioester-binding onto a diglyceride. Nevertheless, the N-terminal sequencing of D1 from *Oscillatoria chalybea* did not give any support for such a binding, since no cysteine could be detected.

Since it was not possible to solve the question of binding, that is, wheather binding is covalent or not, the emphasis of the work was put onto the significance of the binding. Therefore, a better localization of the binding site at the D1-peptide would allow a firmer statement, which would go beyond the description of PG as a functional effector.

In order to address this question, a proteolytic cleveage of D1 was made. If the phospholipid could be identified specifically on one oligopeptide, the possiblitiy to precise the binding site might exist.

Localization of the phosphatidylglycerol-binding site and identification of the phospholipid as a membrane anchor in cyanobacteria

Trypsin proved to be the suitable protease for the splitting of D1. This enzyme cleaves specifically at the amino acids lysine and arginine. Its influence onto the D1-peptide has been investigated by others (i.e.: Moskalenko and Kuznetsova, 1990) and the experience of these authors with the identification of the obtained oligopeptides was successful. The separation and analysis of the fragments followed, as described, by HPLC-analysis and by immunological methods as well. Here, a gradient elution instead of an isocratic one was chosen and a LiChrosphere-column was used. The evaluation of the obtained chromatogram did not lead to a final statement on the site of the lipid-binding. Dot blot analyses of different samples led to the result that one main fragment existed, which reacted with antibodies against D1 as well as with those against phosphatidylglycerol. In order to obtain an identification, by comparing the molecular weights of fragments, a separation of the D1-oligopeptides after trypsin-treatment was made by SDS-gelelectrophoresis. Thus, four D1-fragments were identified (Fig. 8). In the immunoblot only the 20 kDa- and 22 kDa-fragments reacted with antibodies against PG, whereas the other fragments (9 and 12 kDa) did not. The classification of these four fragments in comparison to the amino acid sequence of D1 from spinach is shown in Fig. 8.

Marder *et al.* (1984) analysed the cleaving-products by Edman degradation and found, that the 20 kDa and 22 kDa-oligopeptides were nearly identical and differed only in a small segment between arginine 225 and arginine 238, which does not occur in the 20 kDa-fragment. Both fragments represent the so called 'membrane anchor' of the D1-peptide with a high amount of hydrophobic amino acids. Since the binding site of phosphatidylglycerol could be confined to this part of the D1-peptide, the role of the phospholipids for membrane anchoring came to the fore. The HPLC-analysis made before did not result in a dif-

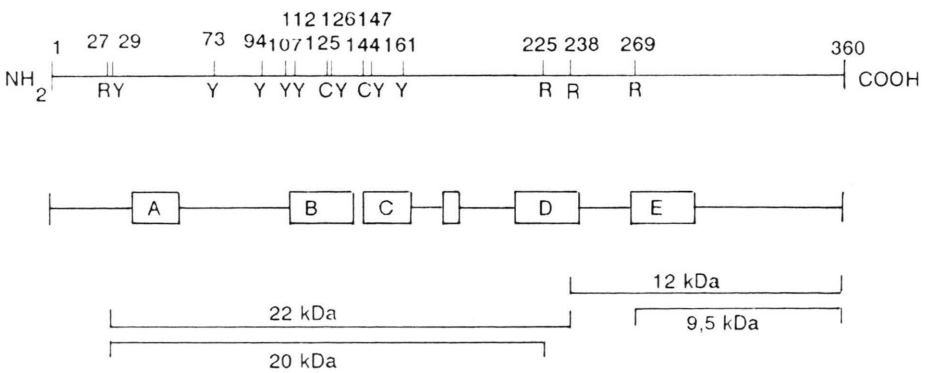

Fig. 8. Illustration of the amino acid sequence of D1 (*Spinacia oleracea*) according to Svensson *et al.* (1991) and classification of the obtained D1-oligopeptides from *Oscillatoria chalybea* after trypsin treatment. The positions of the amino acids cysteine (C), arginine (R), and tyrosine (Y) are specially marked. A–E ≈ α-helices.

ferentiation between the 20- and 22 kDa- fragments, because of their similarity.

This result on the peptid-lipid-interaction, limited to the hydrophobic area of D1, is an important observation with respect to its function and fits in the observations of others.

Adir and Ohad (1988) recognized that hydrophobic interactions are responsible for the stable and tight conformation of D1, stabilized by fatty acids und lipids. They also attached importance to the two 20/22 kDa-fragments obtained by trypsin treatment. Sayre *et al.* (1986) described this fragment between arginine 27 and arginine 225, which contains the first four α-helices of D1 as a highly conserved amino acid-part. The binding of phosphatidylglycerol, shown in the frame of this work, could be assigned to this 20/22 kDa-oligopeptide. This result led to the assumption that the phospholipid molecules, which stabilize D1, are playing an important role as an anchor of the 32 kDa-peptide in the thylakoid membrane. The anchoring may occur on the basis of hydrophobic interactions between membrane lipids and phosphatidylglycerol molecules bound onto D1. Because of the high *turnover* of the D1-peptide *in vivo*, an anchoring must have a high degree of flexibility. Matto and Edelman (1987) described the palmytoylation of the D1-precursor in the stroma lamella of higher plants which would be the prerequisite for the incorporation of the transformed 32 kDa-D1-peptide into the grana regions of the PSII complex. Since this differentiation of the thylakoid membrane does not exist in cyanobacteria, the requirement of lipids and fatty acids for the maintenance of the D1-structure and its function are not necessarily comparable. Besides this, it is important to emphazise that the anionic phospholipid phosphatidylglycerol increases the hydrophobicity of D1 and leads to a negative charge in its surface, which would favor the uptake of cations like calcium and magnesium. This way the direct influence between salt- and phospholipid-concentration would become understandable.

The anionic phospholipid phosphatidylglycerol plays an important role in the PSII complex by binding onto the intrinsic polpeptide D1 as an effector for charge separation as well as a stabilizer in the membrane system. This result has to be particularly emphazised, because these lipid-peptide interactions are important for future *in vitro* reconstitution experminents of PSII. Not taking lipids into account would not lead to the experimental solution of the problem of the water splitting reaction.

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