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The presence of low-molecular-weight polypeptides in spinach Photosystem II core preparations. Isolation of a 5 kDa hydrophilic polypeptide

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Spinach Photosystem II core preparations have been analyzed by sodium dodecyl sulphate urea-polyacrylamide gel electrophoresis, with very high resolution in the low-molecular-weight region. In preparations active in oxygen evolution five low-molecular-weight polypeptides of 7, 6.5, 5.5, 5 and 4 kDa were resolved, in addition to the previously identified subunits of the Photosystem II core. These low-molecular-weight polypeptides were present even if the preparation was performed in the presence of several protease inhibitors. Four of these polypeptides showed a high degree of copurification with the larger Photosystem II core polypeptides during the isolation. At least one of the low-molecular-weight polypeptides is hydrophilic, and has been isolated in a pure form. It has an apparent molecular weight of 5 kDa and contains no prosthetic group or metal. A Photosystem II core preparation, unable to perform oxygen evolution but able to perform electron transport with diphenylcarbazide as donor, lacked two of the low-molecular-weight polypeptides (6.5 and 7 kDa), in addition to the hydrophilic 33 kDa protein. These 6.5 and 7 kDa polypeptides may therefore be necessary for the oxygen-evolving reaction, while the other low-molecular-weight polypeptides may be associated with the primary reactions of Photosystem II.

Higher plant Photosystem II is composed of both peripheral and integral protein subunits (for a review, see Ref. 1), and is surrounded by its light-harvesting antenna, the LHC II. A number of preparations, consisting of membrane fragments derived from the appressed thylakoid region and highly enriched in Photosystem II, has been reported [2–7]. These preparations, which are highly active in Photosystem II electron transport and oxygen evolution, have been extensively characterized and found to contain a relatively large number of polypeptides.

A Photosystem II preparation with a much simpler polypeptide composition, which was unable to perform oxygen evolution but active in the Photosystem-II-dependent catalysis of DCIP reduction by DPC, has also been isolated and characterized [8]. This Photosystem II core preparation was originally reported to consist of only two polypeptides [9], but refined SDS-polyacrylamide gel electrophoresis systems later resolved at least five different polypeptides [10]. These have been identified as the apopolypeptides of the chlorophyll *a*-proteins of Photosystem II (CPa_I and CPa_{II}) of 47 and 43 kDa respectively, the Q_B- or herbicide-binding apoprotein of 32 kDa, a 34 kDa protein which probably is the D2 [11,12] or 32 kDa-like protein [13] and the 9 kDa apopolypeptide of cytochrome *b*-599. A similar polypeptide

Abbreviations: DAN, diazoacetyl nor-leucine methyl ester; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazid; LHC II, light-harvesting chlorophyll *a*/*b* protein of Photosystem II; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulphonyl fluoride; Cyt, cytochrome.

composition has also been found in another DPC-to-DCIP-active Photosystem II core preparation [14].

Only recently have Photosystem II core preparations able to perform the complete Photosystem II reaction at high rates, using the in vivo electron donor water, been reported [15,16]. These preparations contained, in addition to the five proteins listed above, a hydrophilic 33 kDa protein. Additionally, Tang and Satoh [15] found that various amounts of a 22 kDa protein were present, while Ikeuchi et al. [16] detected a polypeptide of approx. 30 kDa. The 33 kDa hydrophilic protein has previously been isolated and extensively characterized [17–19], and implicated to be associated with the catalytic manganese of the photosynthesic oxygen-evolving complex [20–22]. Preparations with similarly simple polypeptide profiles have recently also been isolated from cyanobacteria [23,24].

In the present study, the polypeptide contents of two spinach Photosystem II core preparations, one active in oxygen evolution and the other having only DPC to DCIP activity, were analysed with SDS urea-polyacrylamide gel electrophoresis with a very high resolution in the low-molecular-weight region. In the oxygen-evolving Photosystem II core preparation, in addition to the previously detected polypeptides, five low-molecular-weight polypeptides of 4, 5, 5.5, 6.5 and 7 kDa could be resolved. Two of these (6.5 and 7 kDa) were absent from the Photosystem II core preparations, inactive with respect to oxygen-evolving capacity. The 5 kDa hydrophilic polypeptide was purified to homogeneity and characterized.

Materials and Methods

Spinach thylakoid membranes were prepared as previously described [25]. Stroma lammellae vesicles were prepared by differential centrifugation after Yeda press treatment as in Ref. 26. Photosystem II membranes were prepared according to Berthold et al. [2], as modified in Ref. 27. A Photosystem II core preparation, active in DPC-to-DCIP electron transport, but inactive in oxygen evolution, was prepared as described by Bricker et al. [14], omitting the Tris treatment. An oxygen-evolving Photosystem II core preparation was prepared from the Photosystem II membranes according to Ikeuchi et al. [16]. The only modification was that the *n*-octyl-β-D-glucopyranoside solubilized sample was loaded onto a 5 ml cushion of 30% sucrose instead of a linear gradient. Material pelleted after 5.5 h at $250000 \times g$ was used as the Photosystem II core complex. Oxygen evolution was measured polarographically in the media decribed in Ref. 16. DPC-to-DCIP activity was measured as in Ref. 28. Protease inhibitors, when used, were 2 mM PMSF/1 mM PCMB/5 mM DAN + 5 mM $Cu(Ac)_2$. These were added to the grinding medium before the preparation of oxygen-evolving Photosystem II core complex, while thylakoids were prepared with 2 mM PMSF/1 mM PCMB/5 mM 1,10-phenantroline in the grinding medium and with 1,10-phenantroline present throughout the preparation. Stock solutions were made up and added to the media as described in Ref. 29. Unless indicated all procedures were performed at 4°C.

Triton X-114/water phase partitioning [30] was performed by adding Triton X-114 (20% stock in water) to a final concentration of 5% to the Photosystem II core preparation. After incubation on ice for 30 min, the sample was brought to room temperature where phase separation occurred. The upper aqueous phase and the lower Triton X-114 phase were collected after phase settling at $10000 \times g$ centrifugation for 2 min. Proteins in the lower phase were freed from Triton X-114 by precipitation in 95% ice-cold acetone for 30 min and pelleted by centrifugation at $10000 \times g$ for 5 min. The supernatant was discarded and the pellet dried by evaporation and finally dissolved in SDS urea-polyacrylamide gel electrophoresis solubilization buffer. The aqueous phase was used without further treatment in SDS urea-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed in the buffer system of Laemmli [31] in the presence of 4 M urea in the gel and in the solubilization buffer, with an acrylamide gradient from 12 to 22.5%. After casting, the gels were kept at room temperature overnight to allow for maximal polymerization and then stored at 4°C. Solubilization was carried out on ice for 15 min. Electrophoresis was run at 0°C at a current density of 7 mA/cm^2. The gels were fixed in 50% methanol/

7% acetic acid/43% water for 30 min, prior to staining with Coomassie brilliant blue R-250. The gels were scanned with an LKB 2202 laser densitometer. For calculation of molecular weights from the SDS urea-polyacrylamide gel electrophoresis, the following marker polypeptides were used: bovine serum albumine (68 kDa), ovalbumine (45 kDa), carboanhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), myoglobin from sperm whale (17.8 kDa), aprotinin (6.5 kDa), B chain from insulin (3.6 kDa) and the fragments from myoglobin cleaved by CNBr of 14.6 kDa, 8.3 kDa and 6.3 kDa [32]. The lower limit of resolution of this gel system was found to be approx. 3 kDa.

A 5 kDa hydrophilic polypeptide was prepared from Photosystem II membranes. A suspension of Photosystem II membranes, prepared as in Ref. 27, corresponding to 200 mg Chl at a concentration of 5 mg/ml in 10 mM sodium phosphate buffer (pH 6.5) was made to 3% Zwittergent TM-314 and 2% Triton X-100. After 30 min incubation on ice, unsolubilized material was pelleted at $250000 \times g$ for 45 min. The greyish pellet was discarded and the supernatant was pumped on to a 40×1.6 cm column containing CM-Sepharose^R, equilibrated with 10 mM sodium phosphate buffer, pH 6.5, 0.05% Triton X-100, at a flow rate of 20 ml/h. Proteins were eluted with a 0–250 mM NaCl gradient in 10 mM sodium phosphate buffer (pH 6.5) 0.05% Triton X-100. The 5 kDa polypeptide eluted at approx. 210 mM NaCl and was further purified by repeated runs on the same column using a 0–250 mM NaCl gradient in 10 mM sodium phosphate buffer (pH 6.5)/0.05% Triton X-100. Protein fractions were concentrated and desalted by ultrafiltration.

Metal analysis of protein fractions were performed by particle induced X-ray emission [33].

Amino acid analysis were performed on a Beckman System 6300 High Performance Amino Acid Analyzer according to Beckmans standard program. Tryptophan determination was done after hydrolysis in mercaptoethanesulphonic acid. The content of the other amino acids was determined after Triton X-100 removal as described by Piccioni et al. [34]. Protein and Triton X-100 were precipitated in 10% trichloroacetic acid for 30 min in the hydrolysis tubes. The precipitate was collected by centrifugation at $10000 \times g$ for 10 min, then the Triton X-100 was removed by two extractions in ice cold 100% acetone. The pellet was finally dried under vacuum and then hydrolyzed for amino acid analysis according to Beckmans instructions.

Spectra of the purified 5 kDa polypeptide were obtained from a sample precipitated in 90% cold ethanol, washed in 100% ethanol, dried and then dissolved in 10 mM sodium phosphate buffer, pH 6.5. Immunoblotting [35] was performed as in Ref. 36.

Results

Identification of low-molecular-weight polypeptides in Photosystem II core preparations

A large number of low-molecular-weight poly-

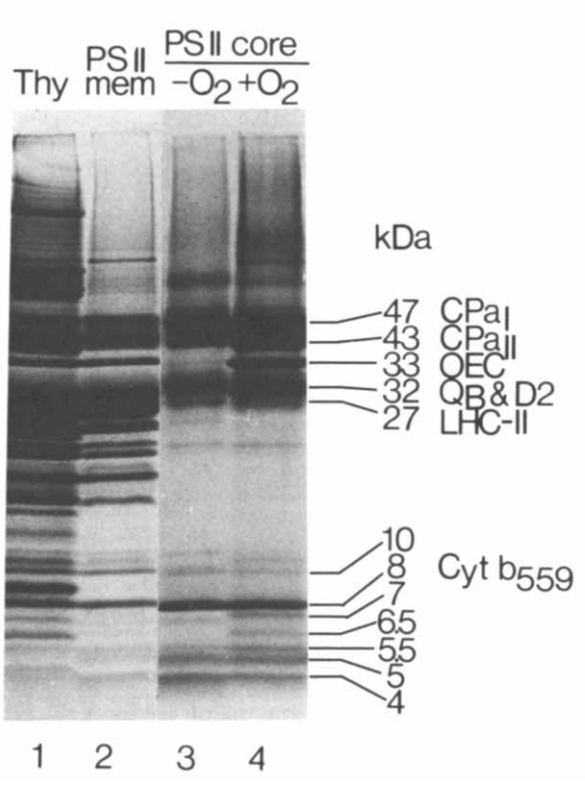

Fig. 1. SDS urea-polyacrylamide gel electrophoresis of: (1) intact thylakoids (30 μg Chl); (2) Photosystem II membranes (30 μg Chl); (3) non-oxygen-evolving Photosystem II core preparation (15 μg Chl); and (4) oxygen-evolving Photosystem II core preparation (15 μg Chl). OEC, oxygen-evolving complex.

peptides from intact thylakoids could be resolved by the SDS urea-polyacrylamide gel electrophoresis procedure employed (Fig. 1). These low-molecular-weight polypeptides had an electrophoretic mobility higher than the apopolypeptide of cytochrome *b*-559 (identified by immunoblotting and in our SDS urea-polyacrylamide gel electrophoresis system running as 8 kDa). Some of these polypeptides were highly enriched in the Photosystem II membranes (Fig. 1), whereas others showed a marked depletion and instead were enriched in a stroma lammellae preparation. In order to investigate whether the low-molecular-weight polypeptides in the Photosystem II membranes were also present in the Photosystem II core complex, two such preparations were made: one active only in DPC-to-DCIP electron transport and the other active also in oxygen evolution. As has previously been demonstrated [14,16], both the Photosystem II core preparations contained the apopolypeptides of CPa_I and CPa_{II} at 47 and 43 kDa, respectively, the 32–34 kDa polypeptides of Q_B and D2 respectively, and the apopolypeptide of cytochrome *b*-559 (Fig. 1). The oxygen evolving Photosystem II core preparation contained, in addition, the 33 kDa hydrophilic polypeptide in accordance with the original study [16]. Strikingly, the two Photosystem II core preparation also contained prominent polypeptide bands in the low-molecular-weight region (Fig. 1). The oxygen-evolving Photosystem II core preparation contained five low-molecular-weight polypeptides of 4, 5, 5.5, 6.5 and 7 kDa. The 6.5 and 7 kDa polypeptides were completely missing and the amount of the 5.5 kDa polypeptide was reduced in the Photosystem II core preparation not active in water oxidation (Fig. 1). The latter preparation contained a faint band at 6.8 kDa. This polypeptide was not detected in the oxygen evolving preparation which makes its presence obscure. The use of urea in the gel was a prerequesite for resolution of the low-molecular-weight polypeptides. If urea was omitted, no bands below the cytochrome *b*-559 could be resolved (Fig. 2). The migration of the five different molecular-weight markers below 10 kDa produced a slowly bending curve with a remarkably good fit when plotted against the log values of their molecular-weights. The estimation of the molecular weights should therefore be fairly accurate, despite the presence of urea and the polyacrylamide gradient in the gel.

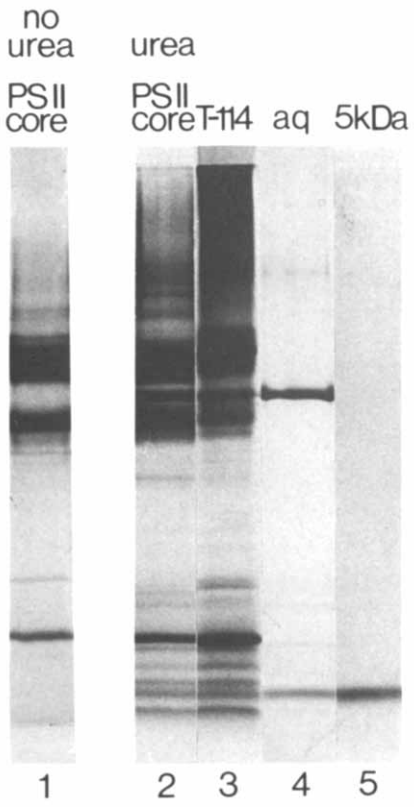

Fig. 2. SDS urea-polyacrylamide gel electrophoresis of the oxygen-evolving Photosystem II core preparation (15 μg Chl) without (1) and with (2) urea in the gel; (3) Triton X-114 phase and (4) aqueous phase after phase partitioning; and (5) pure 5 kDa polypeptide.

If the low-molecular-weight polypeptides are integral components of the Photosystem II core complex they should show the same extent of copurification as the previously identified subunits of this complex. To examine this, the relative content of each polypeptide resolved in the oxygen evolving Photosystem II core preparation was compared to the relative content of the same polypeptide in the Photosystem II membranes, used as starting material. The copurification of each polypeptide was normalized to the enrichment of the sum of the CPa_I, CPa_{II}, Q_B and D2 apoproteins, which was set to 100% (Table I). The 7, 5.5 and 5 kDa polypeptides copurified closely (72–99%) with the previously identified Photosystem II core poly-

TABLE I

ENRICHMENT OF THE LOW-MOLECULAR-WEIGHT POLYPEPTIDES OF THE OXYGEN-EVOLVING PHOTOSYSTEM II CORE PREPARATION

The enrichment was calculated as the copurification with the sum of the apopolypeptides of CPa_I, CPa_{II}, Q_B and D2 from the Photosystem II membranes as estimated from gel scans. A perfect copurification is 100%, i.e., the ratio of the examined polypeptide and the four apopolypeptides mentioned above is the same in the Photosystem II core preparation as in the Photosystem II membranes used as starting material. OEC, oxygen-evolving complex; Cyt, cytochrome; (–), not present in non-oxygen-evolving Photosystem II core preparation.

Molecular weight (kDa)	Copurification (%)
33 (OEC)	96 (–)
27 (LHC II)	3
23 (OEC)	0
22	12
16 (OEC)	0
10 (OEC?)	20
8 (Cyt *b*-559)	69
7	99 (–)
6.5	148 (–)
5.5	72
5	97
4	45

peptides. The extrinsic 33 kDa protein was found to copurify to 96%, and the apopolypeptide of the cytochrome *b*-559 copurified to 69%, which corresponds well to the value reported in the original work [16]. The 4 kDa polypeptide showed 45% copurification, while an unrealistic high value, 148%, was obtained for the 6.5 kDa polypeptide. This high value is probably due to difficulties in determining the amount 6.5 kDa polypeptides in the Photosystem II membranes used as starting material, because of closely comigrating polypeptides. Many of the polypeptides present in the Photosystem II membranes showed quite low copurification in the Photosystem II core preparation, e.g., the Tris-releasable 10 kDa polypeptide [37] (20%) and a 22 kDa polypeptide [38] (12%). Moreover, the copurification of the apo-LHC II subunits was only 3% and there were no traces of the extrinsic 23 and 16 kDa polypeptides [1]. Thus, at least four of the five low-molecular-weight polypeptides showed a high degree of copurification with the four larger and previously characterized polypeptide constituents of the Photosystem II core complex.

The possibility of degradation was examined by performing preparations in the presence of protease inhibitors against the four types of proteases identified in plants [29]. The presence of the substances PMSF, PCMB, DAN + Cu^{2+} and 1,10-phenantroline, inhibitors of serine-, thiol-, carboxyl- and metalloproteases, respectively, did not affect either the presence or the abundance of the low-molecular-weight polypeptides. In addition, immunoblotting with antisera against other Photosystem II proteins such as the hydrophilic 16, 23 and 33 kDa [1], the 10 and 22 kDa polypeptides [37,38], and the cytochrome *b*-559 have not given any crossreaction with the low-molecular-weight polypeptides, indicating that these are not proteolytic fragments formed from the proteins tested (not shown).

To determine whether the low-molecular-weight polypeptides are hydrophobic or hydrophilic the oxygen-evolving Photosystem II core preparation was subjected to Triton X-114/water phase partitioning. Using this technique, hydrophobic proteins, which partition into the Triton X-114 phase, can be separated from hydrophilic proteins, which partition into the aqueous phase [30]. The majority of the Photosystem II core polypeptides, the CPa_I, CPa_{II}, Q_B, D2 and the cytochrome *b*-559 apopolypeptides, partitioned into the Triton X-114 phase (Fig. 2). Moreover, four of the five low-molecular-weight polypeptides, the 4, 5.5, 6.5 and 7 kDa polypeptides, partitioned to the hydrophobic phase (Fig. 2). The 33 kDa protein and the 5 kDa low molecular weight polypeptide were found also in the aqueous phase (Fig. 2). When the same experiment was performed on the Photosystem II core preparation inactive with respect to oxygen evolution only the 5 kDa polypeptide was found in the aqueous phase (not shown).

Isolation of a 5 kDa Photosystem II core polypeptide

The 5 kDa polypeptide was isolated from Photosystem II membranes by ion-exchange chromatography. The polypeptide was purified to homogeneity (Fig. 2) by repeated chromatographic runs. Its behaviour in ion-exchange chromatography indicates a p*I* higher than 6.5 (Fig. 3). The

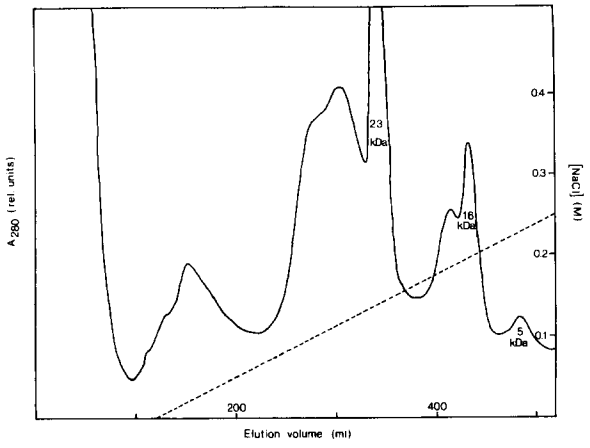

Fig. 3. Elution diagram from CM-Sepharose® chromatography of solubilized Photosystem II membranes. Proteins were eluted with a gradient from 0 to 250 mM NaCl in 10 mM sodium phosphate buffer (pH 6.5)/0.05% Triton X-100.

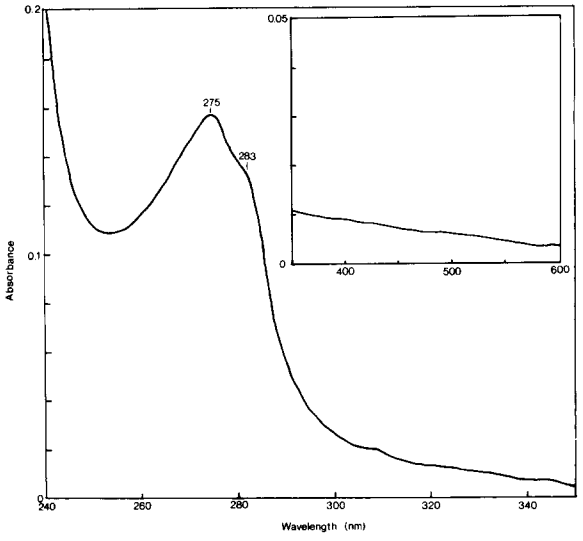

Fig. 4. Spectra of 300 μg pure 5 kDa polypeptide in 1 ml 10 mM sodium phosphate buffer (pH 6.5). The spectra were recorded with an Aminco DW-2 spectrophotometer.

TABLE II

AMINO-ACID COMPOSITION OF THE 5 kDa HYDROPHILIC POLYPEPTIDE OF THE PHOTOSYSTEM II CORE COMPLEX

Figures within parenthese denote the nearest integer. The polarity index was calculated as in Ref. 39, and the extinction coefficient as in Ref. 40. Absorbance maximum, 275 nm; calculated extinction coefficient (275 nm), 1950 $M^{-1} \cdot cm^{-1}$; polarity index, 45%; metal content (particle-induced X-ray emission), none.

Amino acid	Mole%	Residues per protein (mol/mol)
Asp/Asn	2.7	1.1 (1)
Thr	6.8	2.7 (3)
Ser	4.7	1.8 (2)
Glu/Gln	10.9	4.3 (4)
Pro	11.9	4.7 (5)
Gly	6.7	2.7 (3)
Ala	9.5	3.7 (4)
Cys	2.8	1.1 (1)
Val	7.0	2.8 (3)
Met	1.7	0.7 (1)
Ile	3.8	1.3 (1)
Leu	2.8	1.1 (1)
Tyr	4.8	1.9 (2)
Phe	1.7	0.7 (1)
His	0.2	0.1 (0)
Lys	15.9	6.3 (6)
Trp	0.0	0.0 (0)
Arg	6.1	2.4 (2)
Total	100.0	39.4 (40)

isolated polypeptide did not contain any metals as determined by particle-induced X-ray emission [33]. Neither could any cofactor with absorbance in the visible region of the spectrum be detected (Fig. 4). The polarity index, 45%, strongly indicates that it is a hydrophilic polypeptide [39], in agreement with its solubility in aqueous solution without the aid of detergents, and the observation that the pure 5 kDa polypeptide partitioned with a ratio of approx. 10 : 1 to the aqueous phase when subjected to Triton X-114 phase partitioning. The absence of tryptophan is clearly indicated by the ultraviolet spectrum (Fig. 4), where the peak absorbance is at 275 nm, due to tyrosine [40]. One consequence of this is that the 5 kDa polypeptide has a low-molar-extinction coefficient (1950 $M^{-1} \cdot cm^{-1}$ at 275 nm), which makes it difficult to detect with ultraviolet monitoring (Fig. 3). As can be seen in Table II, the most abundant amino acid in the 5 kDa polypeptide is lysine. Furthermore, it contains one cysteine and no histidine. Based on a molecular weight of 5 kDa, it contains a total of 40 amino acids. The presence and pronounced enrichment of the 5 kDa polypeptide in both types of Photosystem II core preparation were confirmed by immunoblotting using an antiserum against this isolated polypeptide (not shown).

Discussion

In the present study we show that the oxygen-evolving Photosystem II core complex contained at least five low-molecular-weight polypeptides in addition to the six previously resolved, i.e., the CPa_I, CPa_{II}, Q_B, D2 and the cytochrome *b*-559 apopolypeptides and the hydrophilic 33 kDa protein. Strikingly, two of these low-molecular-weight polypeptides (6.5 and 7 kDa) together with the extrinsic 33 kDa protein, were absent from a Photosystem II preparation devoid of manganese and oxygen evolution capacity. The 6.5 and 7 kDa polypeptides may therefore be considered, in addition to the 33 kDa extrinsic polypeptide, as being responsible for the retention of the oxygen-evolving capacity and manganese content in the oxygen-evolving Photosystem II core preparation. The other three low-molecular-weight polypeptides, the hydrophilic 5 kDa and the hydrophobic 4 and 5.5 kDa polypeptides, which were present in both preparations, may then rather be involved in the DPC-to-DCIP activity either on the donor or on the acceptor side of Photosystem II.

When dealing with the presence of low-molecular-weight polypeptides, one must be observant of possible proteolytic breakdown, particularly since the work of Ikeuchi et al. [16] indicated possible proteolysis of the 43 kDa apopolypeptide of CPa_{II}. However, the presence of four types of protease inhibitors during the preparation of the oxygen-evolving Photosystem II core did not influence the presence or abundance of the low-molecular-weight polypeptides in the Photosystem II core preparation. Furthermore, the possibility of proteolysis was also tested by immunoblotting with antisera against Photosystem II proteins of larger molecular weight. These did not show any cross-reactivity with the low-molecular-weight polypeptides. Moreover the Photosystem II core preparation active in water oxidation showed a higher abundance of low-molecular-weight polypeptides than the inactive preparation. This provides another indication against the possibility of proteolysis as the source of the low-molecular-weight polypeptides.

The relatively low copurification of the polypeptides in the 10–30 kDa weight region (less than 20%) makes it unlikely that any of these are catalytic subunits in Photosystem II. This would also imply that the 24, 22 and Tris-releasable 10 kDa polypeptides, identified by an immunoprecipitation study [38] to be associated with the hydrophilic 33 and 23 kDa proteins, are regulatory or structural subunits of Photosystem II.

One of the low-molecular-weight polypeptides probably corresponds to the newly discovered 4.3 kDa subunit of cytochrome *b*-559 [41]. However, it is not the hydrophilic 5 kDa polypeptide, since this polypeptide lacks the amino acids histidine and tryptophan (Table II), in contrast to the cytochrome subunit. We were also unable to verify whether any of the other low-molecular-weight polypeptides corresponded to this cytochrome subunit, since immunoblotting with an antiserum against the purified cytochrome *b*-559 did not react with any Photosystem II core polypeptide other than the 8 kDa apopolypeptide.

Low-molecular-weight polypeptides have been detected in many enzyme-complex preparations during the course of development of better resolving SDS-polyacrylamide gel electrophoresis systems, and the preparations have been found to be more complex than originally thought. The case of the mammalian cytochrome *c*-oxidase is strikingly analogous to that of the Photosystem II core complex. Originally, preparations of this enzyme system were reported to consist of only seven polypeptides, ranging in molecular weight from 43 to 10 kDa [42]. However, high resolution SDS urea-polyacrylamide gel electrophoresis resolved six additional subunits, all below 10 kDa [43].Low-molecular-weight Photosystem II polypeptides have also been reported in a study of the green algae *Chlamydomonas*. Mutant studies indicated the presence of a 6 kDa hydrophilic and a 3 kDa hydrophobic polypeptide in the Photosystem II of this algae [44]. Whether these polypeptides correspond to any of those identified in this work remains to be established.

Since a stretch of 18–23 amino acids, corresponding to a molecular weight of 2.5–3 kDa, is normally considered sufficient to span a biological membrane [45], the hydrophobic low-molecular-weight polypeptides reported here can be considered as potential membrane spanning Photosystem II core polypeptides, while the hydrophilic 5 kDa polypeptide probably is an extrinsic membrane polypeptide, located at the inner or outer surface of the thylakoid membrane.

Note added in proof (Received February 20th, 1986)

Since the submission of this paper we have verified that the 4 kDa hydrophobic polypeptide identified in the present study is the 4.3 kDa cytochrome *b*-559, reported in Ref. 41. This was done by coelectrophoresis of the purified cytochrome *b*-559 with the oxygen-evolving Photosystem II core preparation.

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