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Topological studies of spinach 22 kDa protein of Photosystem II

Sunyoung Kim [a], Eran Pichersky [a], Charles F. Yocum [a,b,*]

[a] *Department of Biology, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, USA*

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Abstract

An intrinsic 22 kDa polypeptide is associated with the O_2-evolving Photosystem II core complex in a variety of green plants, although it does not appear to be required for O_2 evolution. Digestion of thylakoid membranes and isolated Photosystem II preparations with trypsin, followed by immunoblotting using spinach anti-22 kDa antibodies, leads to two observations: (1) the domain between the 2nd and 3rd transmembrane helices of the 22 kDa protein is stromally exposed, and (2) only in a reaction center complex preparation, lacking the chlorophyll *a*/*b*-light harvesting complex II, is there extensive proteolytic cleavage of the 22 kDa protein. We also found that after, but not prior to, selective extraction of the 22 and 10 kDa proteins from Photosystem II membranes, the chlorophyll *a*/*b*-light harvesting complex II can be separated from the Photosystem II reaction center core by precipitation with $MgCl_2$. This result suggests that the 22 kDa polypeptide is located between the Photosystem II reaction center polypeptides and light-harvesting complex II; it is possible that the protein serves as a link between the two protein complexes. The presence of the 22 kDa protein in several species was also examined by immunoblotting with polyclonal spinach anti-22 kDa antibodies.

Keywords: Photosynthesis; Membrane protein; Polypeptide, 22 kDa; Western blot; Trypsin

1. Introduction

Photosystem II (PS II) is a multisubunit structure containing the catalytic components necessary for light-driven oxidation of water to O_2 coupled to reduction of plastoquinone, as well as an extensive antenna system comprised of chlorophyll *a*/*b*-binding (CAB) proteins. Biochemical resolution has identified three functional protein groups within PS II: a core complex (47, 42, 34 (D1), 32 (D2) and 9 and 4.5 kDa (cytochrome *b*-559) polypeptides), a light harvesting chlorophyll–protein complex (LHC II), and an ensemble of extrinsic polypeptides of 33, 23, and 17 kDa. An examination of O_2-evolving PS II preparations from spinach has revealed the presence of additional intrinsic protein components (28, 22, 10, 5–3 kDa polypeptides) [1]. The 22 kDa protein appears to reside between PS II polypeptides comprising the reaction center (polypeptides obligately required for charge separation) and the light harvesting antennae proteins LHC II and CP24 [2,3]. The polypeptides of the reaction center include the chlorophyll *a* antennae CP47 and CP43, the chlorophyll *a*/*b* antenna CP26, a 10 kDa polypeptide, and several low molecular weight components. Removal of the 22 and 10 kDa polypeptides has little effect on the kinetics of water oxidation, but the fact that the 22 kDa polypeptide is found in PS II of all plants examined [2] and also is reported to be present in *Synechocystis* 6803 PS II [4] suggests that it must

Abbreviations: Chl, chlorophyll; PS II, Photosystem II; RCC, Photosystem II reaction center complex; swPS II, salt-washed Photosystem II; uswPS II, urea, salt-washed Photosystem II; LHC II, light-harvesting complex II; CAB, chlorophyll *a*/*b*-binding protein; OTG, octyl-β-D-thioglucopyranoside; PEG, polyethylene glycol, M_r = 8000; Tween-20, polyoxyethylene (20) sorbitan monolaurate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Tris, 2-amino-2-hydroxyethylpropane-1,3-diol; AP, alkaline phosphatase; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; BCIP, bromochloroindolyl phosphate; NBT, nitroblue tetrazolium; TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone; DCBQ, 2,6-dichloro-*p*-benzoquinone).

* Corresponding author. (Address a.) E-mail: userLDDG@um.cc.umich.edu. Fax: +1 (313) 7470884.

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have a role in PS II function or assembly. Removal of the 10 kDa polypeptide does not appear to affect PS II activity [5].

The amino acid sequence of the 22 kDa protein, derived from the *psbS* cDNA sequence [6,7], indicates that the protein is highly hydrophobic, with four potential membrane-spanning regions predicted from hydropathy plotting analyses. The N-terminus of the mature polypeptide, defined as the sequence preceding the first predicted transmembrane helix, is extremely basic since it contains 8 Lys residues, but only 2 Glu and 1 Asp residue; the C-terminus is highly acidic, consisting of the sequence Asp-Asp-Glu-Glu-Asp. The two halves of the mature protein show high sequence similarity to each other, suggesting that the *psbS* gene arose from an internal gene duplication. In addition, the 22 kDa protein also has sequence similarity to chlorophyll *a*/*b*-binding (CAB) proteins and a recent report suggests that it might bind chlorophyll [8], although other reports did not observe this [6,7,9–12]. These data suggest that the CAB proteins of LHC II and the 22 kDa polypeptide derived from a common ancestor containing four transmembrane helices [6,7,13].

Although several studies have attempted to define the function of the polypeptide, the role of the 22 kDa PS II protein remains unclear. Bowlby and Yocum [9] demonstrated that removal of the 22 kDa polypeptide modified the ability of PS II to reduce exogenous acceptors (substituted *p*-benzoquinones, $Fe(CN)_6^{3-}$), but the sensitivity of these reactions to the herbicide DCMU was not strongly affected. Ljungberg et al. [10] have examined the associations among PS II polypeptide components after solubilization of PS II membranes in detergent. Using antibodies against the extrinsic 33 and 23 kDa PS II polypeptides, it could be shown that 24, 22, and 10 kDa proteins were co-precipitated by antisera directed against the extrinsic proteins. On the basis of these results, it was proposed that the 24, 22, and 10 kDa polypeptides are closely associated with the PS II complex, and that they might be required for binding of the extrinsic polypeptides. However, Mishra and Ghanotakis [11] demonstrated that selective extraction of the 22 and 10 kDa polypeptides from PS II membranes with OTG does not release the extrinsic polypeptides.

Additional investigations by Ljungberg et al. [12] showed that extraction of the 22 and 10 kDa polypeptides by NaCl/Triton X-100 treatment results in the release of the 23 and 17 kDa extrinsic proteins and loss of O_2 evolution activity, which could be restored with $CaCl_2$ but not by readdition of the purified 23 kDa polypeptide. This suggested that the 23 kDa protein could not rebind to the PS II complex in the absence of the 22 and 10 kDa polypeptides.

In the present study, we have probed the orientation of the 22 kDa polypeptide within the thylakoid membrane by subjecting a variety of O_2-evolving complexes to limited proteolysis. In addition, we sought to define the location of the 22 kDa protein within PS II. Lastly, the presence of the 22 kDa polypeptide was examined in several species using immunoblotting.

2. Materials and methods

2.1. Photosystem II preparations

Thylakoids were prepared from spinach using a method that produces membranes capable of high rates of electron transfer and ATP synthesis [14], and were used for topological studies as well as for the preparation of PS II membranes [1]. Thylakoid samples were resuspended in 50 mM Hepes (pH 7.5)/10 mM NaCl/0.4 M sucrose to 3 mg Chl/ml. Photosystem II preparations were resuspended in 50 mM MES (pH 6.0)/10 mM NaCl/0.4 M sucrose (SMN) to 3 mg Chl/ml. Photosystem II reaction center complexes (RCC) were isolated according to Ref. [15]; samples were resuspended in 50 mM MES (pH 6.0)/15 mM $CaCl_2$/0.4 M sucrose (SMC) to 1.5 mg Chl/ml. All samples were stored at −80°C until use.

Salt-washed PS II preparations (swPS II) were prepared by incubating PS II preparations at 1.5 mg Chl/ml with 2 M NaCl/1 mM EDTA-NaOH (pH 6.0) for 1 h in the dark at 4°C, with stirring [16–18]. Pellets obtained after a 30 min centrifugation at $40000 \times g$ were resuspended in SMN to one-half the salt-wash volume, and used immediately for proteinase digestion studies or used for urea, salt-washed PS II preparations.

Urea, salt-washed PS II preparations (uswPS II) were prepared by incubating five volumes of 3.1 M urea (freshly prepared)/50 mM MES (pH 6.0)/240 mM NaCl with one volume of swPS II for 30 min in the dark at 4°C, with occasional swirling [19]. The mixture was centrifuged for 30 min at $40000 \times g$. Pellets were washed once with SMN in twice the volume of the urea-wash treatment. The solution was centrifuged for 30 min at $40000 \times g$ and pellets were resuspended to 1.5–3 mg Chl/ml in 50 mM MES-NaOH (pH 6.0)/200 mM NaCl/0.4 M sucrose. Samples were pipetted into small volumes, flash-frozen in liquid nitrogen and kept at −80°C until use.

Arabidopsis thaliana (ecotype Columbia) plants (a gift from Mr. Mark Kinkema) were grown from seed at 22°C under 16:8 h light/dark cycles. Leaves from 4–6-week-old plants were harvested immediately after bolting; thylakoids were prepared immediately, in the same manner as spinach thylakoids above. Greenhouse-grown, mature *Nephrolepis exaltata* (Boston fern) thylakoids were prepared as described above for

spinach; after resuspension in 50 mM Hepes (pH 7.5)/10 mM NaCl/0.4 M sucrose, the thylakoids were incubated with cellulase for 12 h at 4°C and then sonicated briefly. Membrane preparations were stored at −80°C until use. *Synechocystis* 6803 and *Chlamydomonas reinhardtii* thylakoids were generous gifts from Professors Bridgette Barry and Richard Sayre, respectively.

2.2. Octyl-β-D-thioglucopyranoside treatment

Treatment of spinach PS II membranes with OTG was carried out as described in Mishra and Ghanotakis [11]. After a first centrifugation (30 min at 40 000 × *g*), the supernatant was divided in half. One half was mixed with 40% PEG and centrifuged for 30 min at 40 000 × *g*. The other half of the supernatant was diluted to twice its volume with SMN to obtain a 0.3% OTG concentration, and $MgCl_2$ was added to a final concentration of 40 mM; this solution was allowed to incubate for 30 min on ice in the dark and then was centrifuged for 30 min at 40 000 × *g* to obtain a pellet (LHC II) and supernatant. The supernatant, which does not retain LHC II, was precipitated with 40% PEG and centrifugation (30 min at 40 000 × *g*). Variations of this protocol are listed in the figures.

2.3. Trypsin treatment

For trypsin treatment, appropriate amounts of TPCK-treated trypsin (Sigma) were added to thylakoid membranes or PS II preparations to obtain a specific trypsin:Chl ratio at a final Chl concentration of 1 mg/ml. The use of TPCK-treated trypsin avoided spurious results from chymotrypsin contamination [20]. To standardize the proteinase treatment among different PS II preparations, trypsin concentrations were determined on the basis of PS II reaction center concentrations. All original thylakoid and PS II samples (≥ 2 mg Chl/ml) were diluted in SMC. Two different ratios of trypsin:Chl (w/w) were used for each preparation; the first ratio was equal to 1/3 of the second. Trypsin:Chl ratios were as follows: RCC, 1:15 and 1:5; uswPS II, 1:58 and 1:19; PS II, 1:58 and 1:19; and thylakoids, 1:93 and 1:31. All samples were exposed to trypsin in the dark on ice for 15 min. At the end of this time, a 20-fold excess (w/w) of soybean trypsin inhibitor (Sigma) was added, samples were pelleted in a cold microcentrifuge (7 min, 12 000 rpm), and resuspended in 50 mM Hepes (pH 7.5)/15 mM $CaCl_2$/0.4 M sucrose (thylakoids) or SMC to a final concentration of 1 mg Chl/ml.

The intactness of the spinach thylakoid membranes was tested by immunoblotting trypsin-treated thylakoids with antibodies against the lumenal 23 kDa polypeptide of PS II, as described below. No tryptic fragments of the 23 kDa protein were detected.

Table 1
Effect of trypsin treatment on percent control activity of different PS II preparations

Preparation	Low trypsin:Chl treatment	High trypsin:Chl treatment
Thylakoid	86.5 ± 5.3	81.8 ± 5.8
PS II	81.9 ± 3.4	73.7 ± 7.4
uswPS II	45.5 ± 8.4	28.7 ± 12.6
RCC	76.8 ± 3.3	71.6 ± 2.1

Control oxygen evolution activity (100%) of thylakoids, PS II, uswPS II, and RCC preparations was 498, 543, 108, and 1264 mmol O_2 h/mg Chl per ml, respectively. Final assay concentration of the artificial acceptor DCBQ was 312.6 μM. The assay buffer for thylakoids was 50 mM Hepes (pH 7.5) and 10 mM NaCl, 50 mM MES (pH 6.0) and 10 mM NaCl for PS II preparations, and 50 mM MES (pH 6.0) and 20 mM $CaCl_2$ for uswPS II and RCC preparations. Trypsin:Chl concentrations are listed in Section 2.

2.4. Analyses

Chlorophyll was determined by the method of Arnon [21] (spinach, *N. exaltata*, *C. reinhardtii* and *A. thaliana* preparations) or Lichtenthaler [22] (*Synechocystis* 6803). Steady-state rates of O_2 evolution were measured in a water-jacketed cuvette (1.6 ml) at 25°C with the buffers, salts, and artificial acceptors listed in Table 1. Saturating actinic illumination was provided by two 125 W microscope illuminators fitted with red acetate filters (cut-on at 600 nm) and focused on the cuvette from opposite sides. The initial rate of activity was derived from the average rate of O_2 evolution between 12 and 30 s after the onset of illumination.

Polyacrylamide gel electrophoresis was performed as in Ref. [23] using the Tris-borate discontinuous buffer system. The 13.5% acrylamide/4 M urea gels were stained with Coomassie Brilliant blue R-250 or electroblotted (BioRad) to nitrocellulose (BioRad) or PVDF membranes (Dupont). Transfer was carried out in a buffer containing 10 mM CAPS (pH 11.0)/10% methanol [24,25].

Immunoblotting reactions were carried out on a rotary shaker at 37°C [26]. Filters were blocked for 2 h with 5% dry, nonfat milk (Saco)/0.2% Tween-20/0.02 M Tris/0.154 M NaCl (blocking solution). Filters were incubated with the primary antibody diluted in the blocking solution at a 1:1000 ratio for 1 h. The filters were washed for three times (5 min each) with the blocking solution. The secondary antibody used was either goat anti-rabbit conjugated with HRP (BioRad) or goat anti-rabbit-AP conjugate (Sigma); both antibodies were incubated with the filters at a 1:30 000 or 1:1000 dilution in blocking solution, respectively, for 1 h. Filters were washed two times with the blocking solution for 5 min intervals and then washed for 15 min

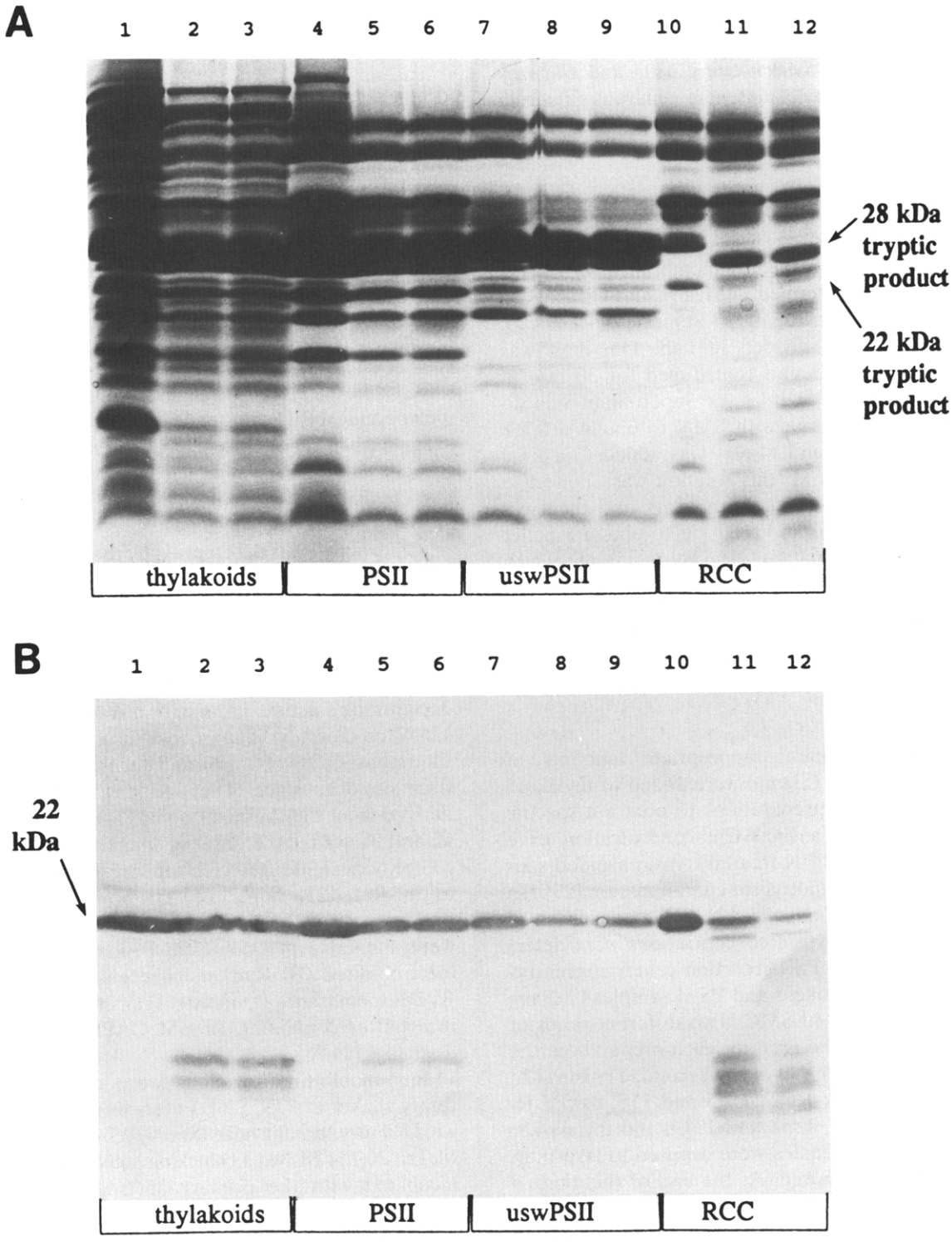

Fig. 1. Susceptibility of the 22 kDa polypeptide to trypsin hydrolysis in spinach thylakoids and PS II preparations. (A) Coomassie-stained 4 M urea/13.5% polyacrylamide gel of trypsin-treated and control photosynthetic preparations. Lanes 1–3 show spinach thylakoid preparations; lanes 4–6 spinach PS II preparations; lanes 7–9 spinach uswPS II preparations; and lanes 10–12 spinach RCC preparations. Ratios of trypsin : Chl were: lane 2, 1 : 93; lane 3, 1 : 31; lane 5, 1 : 58; lane 6, 1 : 19; lane 8, 1 : 58; lane 9, 1 : 19; lane 11, 1 : 15, and lane 12, 1 : 5. Lanes 1, 4, 7, and 10 are control preparations. (B) Immunoblot of trypsin-treated and control photosynthetic preparations using spinach anti-22 kDa antibody. The polyacrylamide gel shown in Fig. 1A was transferred to PVDF membrane in a buffer containing 10 mM CAPS (pH 11.0)/10% methanol. The secondary antibody was protein A-AP conjugate; visualization was by color reaction via NBT and BCIP.

with 0.02 M Tris/0.154 M NaCl. For the HRP conjugate, antigen-antibody complexes were visualized by chemoluminescence reagents (Dupont) according to the manufacturer's specifications [27]. For the alkaline phosphatase conjugate, the color reaction was developed by incubating the filter in 100 mM NaCl/5 mM $MgCl_2$/100 mM Tris (pH 9.5)/4 mM BCIP/40 mM NBT for 5 min. The rabbit anti-22 kDa antiserum was reported in Bowlby and Yocum [9]; SDS-PAGE-purified 22 kDa protein was used as the antigen. Antisera against the 33 and 23 kDa extrinsic proteins were provided by Professor Bridgette Barry.

3. Results

3.1. Orientation of the intrinsic 22 kDa polypeptide in the thylakoid membrane

Since the membrane of intact thylakoids shields the lumenal space from large exogenous agents, proteinase treatment of intact thylakoids will target only stromally-exposed cleavage sites. However, proteinase treatment of isolated PS II preparations in theory should target both stromal and lumenal cleavage sites. After preparations were treated with two different concentrations of trypsin for 15 min and analyzed by SDS-PAGE, minimal changes in polypeptide composition were observed (Fig. 1A). Little reduction in the amount of 22 kDa polypeptide was observable in thylakoids (lanes 1–3), isolated PS II preparations (lanes 4–6), or urea-treated PS II preparations depleted of the 33, 23, and 17 kDa extrinsic polypeptides (lanes 7–9). However, the reaction center complex (RCC), a PS II preparation depleted of the extrinsic 23 and 17 kDa polypeptides and LHC II, showed visible proteolysis of the 22 kDa protein (lanes 10–12).

To examine the effect of trypsin treatment on the 22 kDa polypeptide specifically, a Western blot of an identical gel probed with spinach anti-22 kDa antibody was performed (Fig. 1B). In thylakoids, tryptic fragments of the 22 kDa protein, ranging in molecular mass between 10 and 14 kDa, were generated (lanes 1–3). Similarly, in PS II (lanes 4–6) and urea-treated membranes (lanes 7–9), small amounts of proteolytic products from the 22 kDa protein appeared; the same pattern of tryptic fragments were generated in thylakoids and intact PS II preparations. In the absence of all extrinsic proteins (uswPS II, Fig. 1B), the larger proteolytic fragments are not observed and the yield of the smaller fragments is lower when compared to thylakoid and intact PS II samples. Exposure of the RCC to trypsin generated a new band at about 21 kDa as well as significant amounts of the 10–14 kDa fragments (Fig. 1B, lanes 10–12); the majority of the 22 kDa protein was cleaved. These results indicate that the 22 kDa polypeptide was most accessible to trypsin attack when the LHC II was removed from the PS II complex. Additionally, in thylakoid, PS II, and urea-treated PS II samples, the anti-22 kDa antibody cross-reacted weakly with CP26 (Fig. 1B). This cross-reactivity was not visible in the RCC preparation after trypsin treatment.

Since a 20-fold excess of trypsin inhibitor (M_r = 21.5 kDa) was used to terminate proteolysis in these experiments, it is possible that residual amounts of this protein comigrate with the 22 kDa tryptic product (Fig. 1A); however, immunoblotting definitively identifies a 22 kDa tryptic product at approximately the same apparent molecular mass (Fig. 2B).

Oxygen evolution rates for the trypsin treated samples decreased to approximately 70–80% of untreated control samples of each preparation (thylakoids, PS II, and RCC) (Table 1). Trypsinization of urea-treated samples lowered O_2 evolution rates to 30% of the control, a finding which suggests that the removal of the 33, 23, and 17 kDa extrinsic polypeptides may have increased the susceptibility of the catalytic sites for H_2O oxidation to damage by proteolysis.

3.2. Location of the 22 kDa polypeptide within Photosystem II

The proteolysis results indicate that access of trypsin to the 22 kDa protein is impeded by the presence of LHC II. This finding and previous observations that the 22 kDa protein is located on the periphery of the PS II reaction center [2] suggest that the 22 kDa polypeptide is located between the LHC II antenna complex and the PS II reaction center core. Using the methods in Ref. [11] we examined the effect of OTG on PS II preparations. After incubating PS II preparations with 0.6% OTG for 5 min followed by centrifugation, the resulting pellet contained the 22 and 10 kDa proteins (Fig. 2A, lane 2), in agreement with the observations of Mishra and Ghanotakis [11]. The other proteins of LHC II and the PS II core complex remain in the supernatant and can be precipitated with PEG (Fig. 2A, lane 3), as was performed in the original protocol [11].

To examine whether the PS II reaction center and LHC II in the supernatant remained associated with each other after the 0.6% OTG treatment and removal of the 22 kDa polypeptide, the above supernatant from the OTG treatment was diluted two-fold with SMN to lower the detergent concentration to 0.3% OTG (slightly above the critical micelle concentration) to allow protein sedimentation, 40 mM $MgCl_2$ was added, and the suspension was incubated for 30 min in the dark on ice, followed by centrifugation at 40000 × g. The pellet contained LHC II and some minor chlorophyll-binding proteins (Fig. 2A, lane 4) while the su-

pernatant contained the proteins of the PS II reaction center (Fig. 2A, lane 5). Thus, removal of the 22 and 10 kDa polypeptides by OTG coincides with disruption of interactions between LHC II and the PS II core complex, so that the LHC II subunits can be selectively precipitated with Mg^{2+} [28].

When the protocol described above was performed without first removing the 22 kDa polypeptide by centrifugation, the pellet obtained after $MgCl_2$ incubation and centrifugation contained the LHC II (Fig. 2B, lane 2), while the supernatant contained the PS II reaction center (Fig. 2B, lane 3), again suggesting that the detergent affected the PS II complex so as to dissociate the 22 kDa polypeptide, PS II reaction center, and LHC II from one another. A set of control experiments were performed to examine the effect of salt and/or detergent alone on PS II preparations without the prior removal of the 22 kDa protein. When intact PS II preparations retaining the 22 kDa protein were incubated with 0.3% OTG, rather than 0.6% OTG, in the presence of 40 mM $MgCl_2$, centrifugation produced a pellet containing most of the PS II reaction center and all of the LHC II; a residual amount of the PS II reaction center polypeptides were in the supernatant (Fig. 2C, lanes 1 and 2). Treatment of PS II preparations with 0.3% OTG alone resulted in a pellet containing all of the proteins of the original preparation and a pale green supernatant (Fig. 2C, lanes 3 and 4). Treatment of PS II with 40 mM $MgCl_2$ alone resulted in a pellet with all of the components of the PS II (Lane 5) and a clear supernatant. Thus, incubation of PS II with 0.3% OTG or salt alone is not sufficient to

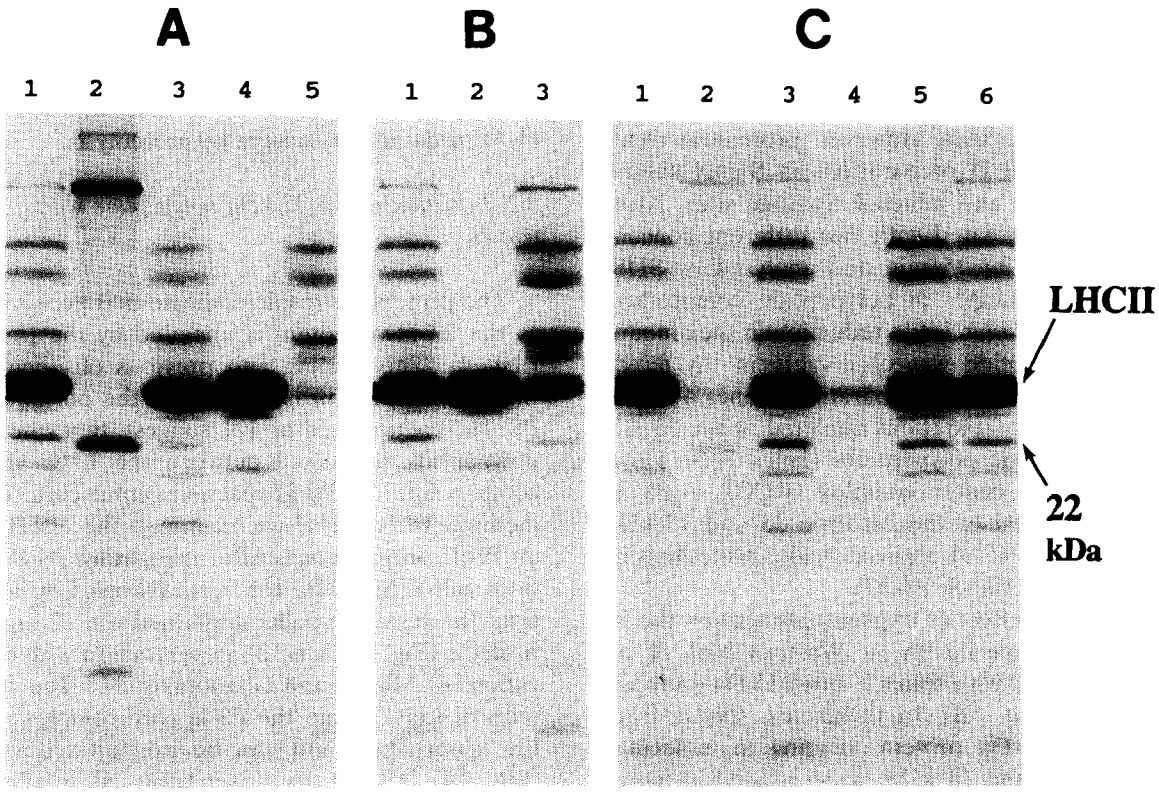

Fig. 2. Location of the 22 kDa polypeptide within Photosystem II. All samples described below were run on a Coomassie-stained 13.5% polyacrylamide gel with 4 M urea. (A) PS II preparations were incubated at 0.5 mg Chl/ml with 0.6% (w/v) OTG in the dark on ice with stirring for 5 min [11]. After centrifugation for 30 min at 40 000 × *g*, a pellet (lane 2) and a supernatant were obtained; the supernatant was divided in two halves. One half was mixed with 40% PEG and centrifuged for 30 min at 40 000 × *g*; the resulting pellet is shown in lane 3. The other half of the supernatant was diluted to twice its volume with SMN to obtain a 0.3% OTG concentration, and $MgCl_2$ was added to a final concentration of 40 mM; this solution was allowed to incubate for 30 min on ice in the dark and then was centrifuged for 30 min at 40 000 × *g* to obtain a pellet (lane 4) and supernatant. The supernatant was precipitated with 40% PEG and a 30 min centrifugation at 40 000 × *g* (lane 5). Lane 1 is a control PS II preparation. (B) A PS II preparation was incubated with OTG for 5 min as described above. After this time period, the solution was diluted to twice its volume with SMN to obtain a 0.3% OTG concentration and $MgCl_2$ was added to obtain a final concentration of 40 mM; this solution was incubated for 30 min on ice in the dark and then centrifuged for 30 min at 40 000 × *g* to obtain a pellet (lane 2) and supernatant, which was precipitated with 40% PEG and centrifugation (lane 3). Lane 1 is a control PS II preparation. (C) Control treatments for OTG and $MgCl_2$. Three different experiments were performed, where PS II preparations were incubated with salt and/or detergent for 30 min in the dark on ice and then centrifuged to obtain a pellet and a supernatant, which was precipitated with 40% PEG. The three treatments were: (a) 0.3% OTG + 40 mM $MgCl_2$: pellet (lane 1) and supernatant (lane 2); (b) 0.3% OTG: pellet (lane 3) and supernatant (lane 4); (c) 40 mM $MgCl_2$: pellet (lane 5). Lane 6 is a control PS II preparation.

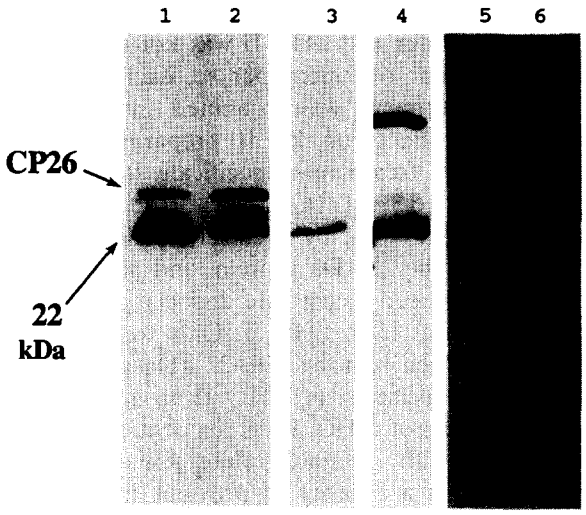

Fig. 3. Presence of the 22 kDa polypeptide in different species. Samples were run on a 13.5%+4 M urea polyacrylamide gel and transferred to PVDF membrane in a buffer containing 10 mM CAPS (pH 11.0)/10% methanol. Immunoblot was performed using spinach anti-22 kDa primary antibody and HRP-conjugated secondary antibody; visualization was via chemoluminescence reaction. Lane 1 shows a spinach PS II preparation, lane 2 an *A. thaliana* thylakoids, lane 3 *N. exaltata* thylakoids, lane 4 *C. reinhardtii* thylakoids, lane 5 spinach PS II preparation, and lane 6 *Synechocystis* 6803 thylakoids.

completely separate the reaction center and LHC II without the prior removal of the 22 kDa polypeptide.

3.3. Presence of the 22 kDa polypeptide in different species

Since the genes encoding for the 22 kDa and CAB polypeptides appear to have a common ancestor, plants which do not have chlorophyll *a*/*b*-binding proteins may not contain the 22 kDa protein. To test for the presence of the 22 kDa polypeptide, *A. thaliana*, *N. exaltata*, *C. reinhardtii* and *Synechocystis* 6803 thylakoids were examined by immunoblot using spinach anti-22 kDa polyclonal antibody (Fig. 3). Two different types of secondary antibody conjugates were used: alkaline phosphatase (AP) and horseradish peroxidase (HRP). For the AP conjugate, a color reaction employing BCIP and NBT was utilized to visualize the antigen-antibody complexes (data not shown). For the HRP conjugate, antigen-antibody complexes were visualized by chemoluminescence reaction; this method of antigen-antibody complex detection was at least 30-fold more sensitive than visualization via the color reaction catalyzed by alkaline phosphatase.

The 22 kDa polypeptide in *Arabidopsis* thylakoids was weakly detected by AP (data not shown) and strongly visible with HRP (Fig. 3, lane 2); however, the reactivity of the spinach anti-22 kDa antibody with the *Arabidopsis* protein was much lower than that of the spinach protein. The *N. exaltata* 22 kDa protein was only weakly visible with chemoluminescence (Fig. 3, lane 3). In *C. reinhardtii*, two bands were detectable by the HRP-chemoluminescence method with the spinach anti-22 kDa antibody; the more strongly-decorated *C. reinhardtii* protein is approximately 40 kDa and the weakly-antigenic *C. reinhardtii* protein is approximately 22 kDa (Fig. 3, lane 4). However, we could not detect any protein in *Synechocystis* that cross-reacted with the spinach anti-22 kDa antibody, even with the highly sensitive HRP-chemoluminescence method of detection (Fig. 3, lane 6). As a positive control, an immunoblot using spinach anti-33 kDa antibody was performed on the same sample; strong cross-reactivity with a protein of the appropriate size was observed (data not shown).

4. Discussion

4.1. Presence of the 22 kDa polypeptide in different species

The presence of the 22 kDa PS II polypeptide has been verified in a variety of angiosperms [4] and the *psbS* gene has been isolated and characterized in spinach [6,7], tomato [29], and tobacco (Kim, S. and Pichersky, E., unpublished data). We have also detected the presence of a cross-reacting polypeptide in *Arabidopsis* thylakoids and the fern *Nephrolepis exaltata* thylakoids using spinach anti-22 kDa antibody in western blots (Fig. 3). These data suggest that the psbS protein is a ubiquitous component of PS II in angiosperms and fern species as well. From the lower intensity of the immunoblot signal, it appears that the *Arabidopsis* and *Nephrolepis* 22 kDa PS II polypeptides have few epitopes similar to those of the spinach protein. We also investigated the presence of the 22 kDa protein in a green alga and a cyanobacterium. There appears to be at least one protein of appropriate size that reacts with the spinach anti-22 kDa antibody in *C. reinhardtii* as well as a second protein, significantly larger than 22 kDa, that also reacts with the spinach antiserum (Fig. 3, lane 4). However, we were unable to detect cross-reacting proteins from the cyanobacterial PS II particles, even when the sensitive HRP-chemoluminescence reaction was used. The cyanobacterial PS II particles were active in O_2-production and the use of spinach anti-33 kDa antibody gave a strong signal. Therefore, it appears that either cyanobacteria do not possess a 22 kDa PS II polypeptide that is homologous to those found in higher plants (contrary to a previously published report of the detection of the protein in *Synechocystis* 6803 by immunoblot [4]), or that the 22 kDa PS II polypeptide in this prokaryote has diverged sufficiently to preclude detection by the spinach antibody. The latter possibility

is supported by the observation that fern and *Chlamydomonas* proteins cross-react weakly with this antibody.

4.2. Location of the 22 kDa polypeptide within Photosystem II

Exposure of PS II preparations to 0.6% OTG appears to disrupt interactions between the 22 kDa polypeptide, light-harvesting complex and the PS II reaction center, after which the 10 kDa and the hydrophobic 22 kDa polypeptides can be pelleted by centrifugation, as observed by Mishra and Ghanotakis [11]. Since removal of the 10 kDa protein does not affect Photosystem II activity [5] and it is a nonintegral component of PS II [30], it is not likely to contribute to the structural effects observed in PS II preparations upon OTG treatment. Removal of the 22 kDa polypeptide, dilution of the supernatant (to 0.3% OTG), and introduction of Mg^{2+} caused the precipitation of LHC II (Fig. 2A, lane 4), while the PS II reaction center remained in solution (Fig. 2A, lane 5). This demonstrates a loss of interactions between the reaction center and LHC II.

To ensure that observed loss of interactions between the reaction center and LHC II was not due to a salt and/or detergent treatment per se, each variable in the OTG experiment was examined individually to determine its effects on PS II preparations. Three different control treatments, specifically 0.3% OTG + 40 mM $MgCl_2$, 0.3% OTG alone, or 40 mM $MgCl_2$ alone, were used that, by themselves, do not cause dissociation of the 22 kDa protein, and none of these treatments produced a complete dissociation of the PS II reaction center from LHC II. Only treatment with 0.6% OTG, which removes the 22 kDa protein, also resulted in dissociation of LHC II from PS II. Although isolation of LHC II by incubation of PS II preparations with nonionic detergents has been demonstrated in a number of previous investigations [31,32], it was not previously established that dissociation of the 22 kDa protein from PS II correlates with the dissociation of LHC II from the PS II core complex.

It was noted in the control experiments on PS II preparations, i.e., without the prior removal of the 22 kDa polypeptide, that the presence of salt and detergent together resulted in some loss of 22 kDa polypep-

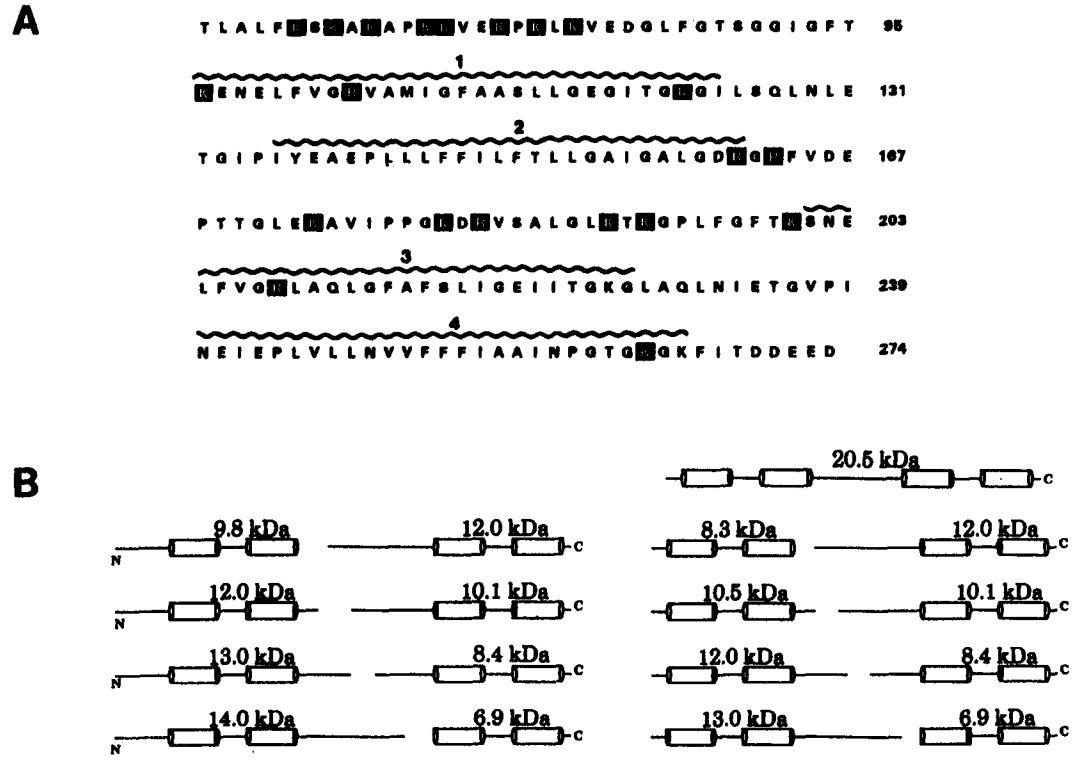

Fig. 4. Orientation of the 22 kDa polypeptide within the thylakoid membrane. (A) Amino acid sequence of the 22 kDa polypeptide. Highlighted in black are the potential sites for trypsin cleavage. Wavy lines designate the transmembrane α-helical domains of the protein. The transit peptide is not shown; the first amino acid shown in the sequence is residue 60. (B) Putative tryptic fragments from various cleavage sites in the 22 kDa polypeptide. Transmembrane α-helical domains are represented by cylinders and there are four transmembrane domains in the 22 kDa polypeptide. The drawings on the left side of the figure represent putative tryptic fragments that could be generated if the N-terminus is not accessible to trypsin and if there is a single cleavage between the second and third transmembrane helices. The drawings on the right side of the figure represent putative tryptic fragments that could be generated if the N-terminus is accessible to trypsin and if there is no cleavage (top) or a single cleavage between the second and third transmembrane helices.

tide precipitation (Fig. 2B lane 2 and 3; Fig. 2C lanes 1 and 2). In addition, the 22 kDa polypeptide did not precipitate when PS II preparations were incubated with 0.6% OTG and 40 mM $MgCl_2$ simultaneously for 5 min followed by centrifugation (data not shown). These observations suggest that, although the 22 kDa polypeptide can be dissociated from the PS II reaction center by 0.6% OTG, the 22 kDa protein is insoluble in the absence of $MgCl_2$ and therefore can precipitate out of solution. However, in the presence of the salt, the protein is soluble and therefore cannot precipitate out of solution easily.

4.3. Orientation of the intrinsic 22 kDa polypeptide in the thylakoid membrane

Spurred by elucidation of the *psbS* gene sequence and the topological structure of the 22 kDa protein predicted from hydropathy plotting routines, we have sought to determine the orientation of this polypeptide in the thylakoid membrane. We selected TPCK-treated trypsin, which specifically cleaves peptide bonds at lysine and arginine residues [33], since the structural model of the protein predicts that only two domains in the 22 kDa polypeptide would be susceptible to trypsin attack: the N-terminal domain and the domain between the predicted 2nd and 3rd transmembrane helices (Fig. 4A). There are no lysine and arginine residues between either the first and second membrane helices or between the third and fourth helices predicted by hydropathy plotting routines. Our results indicate that in thylakoids and PS II membranes, proteolytic fragments of 10–14 kDa were generated (Fig. 1B). Trypsin-treated uswPS II preparations (Fig. 1B, lanes 7–9) show diminished yields of smaller peptide fragments and the larger fragments seen in other preparations are absent. It is possible that urea exposure itself or the removal of the extrinsic polypeptides may change the structural conformation of the protein complex such that normally inaccessible tryptic sites are exposed and therefore generate smaller tryptic fragments which could not resolved on the polyacrylamide gel. Alternatively, failure to observe enhanced proteolysis upon removal of extrinsic proteins from the lumenal side of PS II by urea treatment would suggest that sites of trypsin attack are either rare or entirely lacking on this surface of the enzyme system.

N-terminal sequencing of tryptic fragments was unsuccessful and therefore the specific sites of cleavage cannot be ascertained. However, molecular weight predictions for potential tryptic fragments (Fig. 4B) suggest that the fragments observed in the immunoblots could only arise from proteolytic cleavage between the second and third transmembrane helices of the 22 kDa polypeptide. This domain is accessible in all membrane preparations examined; since these proteolytic products occur upon proteinase treatments of intact thylakoid membranes, the region between the 2nd and 3rd transmembrane helices must be located on the stromal face of the thylakoid membrane. Consequently, it follows that both the N- and C-termini of the protein are stromally exposed (Fig. 5).

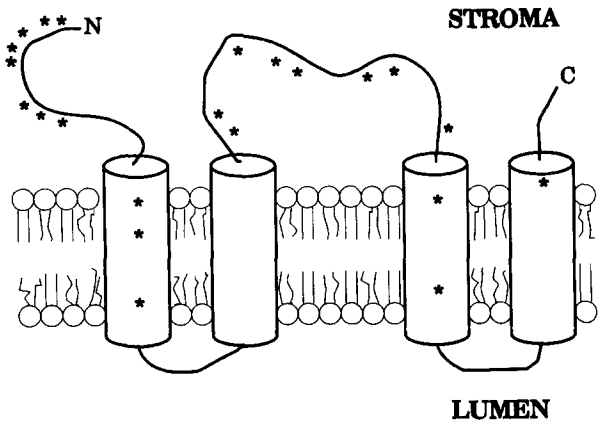

Fig. 5. Schematic representation of the mature 22 kDa polypeptide in the thylakoid membrane. Transmembrane α-helical domains are represented by cylinders. Potential sites of trypsin cleavage are designated by asterisks.

A proteolytic fragment of approximately 21 kDa is observed only in the LHC II-depleted RCC preparation (Fig. 1B, lanes 10–12). This correlates well with the calculated molecular weight of peptides that would be produced by trypsin cleavage at the N-terminus (Fig. 4B). Since the N-terminus of the protein appears to be most accessible to proteolysis after removal of LHC II, it is possible that LHC II is in contact with or shields the N-terminus of the 22 kDa protein on the stromal surface of the thylakoid membrane. The cross-reactivity of the anti-22 kDa antibody with CP26, which had been noted before [3,34], is eliminated when the RCC is exposed to trypsin. Since the greatest sequence similarity between CP26 and the 22 kDa polypeptide is at their N-termini [6], loss of anti-22 kDa antibody reactivity with CP26 suggests that the N-terminus of CP26 is also accessible to trypsin cleavage only after the removal of LHC II.

Trypsin treatment reduces the apparent molecular mass of LHCB1 and LHCB2, the CAB proteins of LHC II, by approximately 2 kDa owing to removal of a short segment from the N-terminus [35,36], similar to the result obtained with the 22 kDa polypeptide. Additionally, this treatment eliminates the ability of Mg^{2+} to precipitate LHCB2/LHCB1 [37]. Magnesium ions are thought to interact with negative charges at the N-termini of the LHC II proteins [38]. The first and third transmembrane helices of the 22 kDa polypeptide have sequence similarity with the first and third transmembrane helices of chlorophyll *a*/*b*-binding proteins [6,7]. Thus, for both the 22 kDa and CAB polypeptides, it appears that there are few domains protruding from

the thylakoid membrane and that most of the protein structure remains embedded in the membrane, as suggested from crystallographic data of pea LHCB1 [39,40].

4.4. Summary

Removal of the 22 and 10 kDa polypeptides has little effect on the kinetics of water oxidation, but the fact that the 22 kDa polypeptide is found in PS II of all plants examined [2], with the possible exception of the PS II of *Synechocystis* 6803, suggests that it must have some role in PS II function or assembly. Its amino acid sequence indicates that the 22 kDa protein is a highly hydrophobic polypeptide, with four potential membrane-spanning regions. The experimental evidence presented here indicates that the orientation of the protein in the thylakoid membrane is one where the domain between the second and third transmembrane helices as well as the N-and C-termini of the polypeptide are stromally exposed. In addition, the 22 kDa polypeptide is positioned between the PS II reaction center and LHC II, and may serve as a linkage between the two complexes. These observations regarding the 22 kDa polypeptide suggest a role in the structure or biogenesis of PS II for this protein. Additional experiments are now in progress to determine the exact function of the 22 kDa protein of Photosystem II and the relative organization of the PS II reaction center, LHC II, and the 22 kDa polypeptide within the thylakoid membrane.

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