Topography of the Protein Complexes of the Chloroplast Thylakoid Membrane[1]

STUDIES OF PHOTOSYSTEM I USING A CHEMICAL PROBE AND PROTEOLYTIC DIGESTION

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ABSTRACT

The transverse heterogeneity of the polypeptides associated with the Photosystem I (PSI) complex in spinach thylakoid membranes and in a highly resolved PSI preparation has been studied using the impermeant chemical modifier, 2,4,6-trinitrobenzenesulfonate (TNBS) and the proteolytic enzyme, Pronase E. The present study has shown that the PSI reaction center polypeptide of ~62 kilodaltons and the 22 and 20 kilodalton polypeptides of the PSI light-harvesting chlorophyll protein (LHCPI) complex are not labeled by [14C]TNBS in unfractionated thylakoids. On the other hand, the 23 kilodalton polypeptide of the PSI LHCP and the 19 and 14 kilodalton polypeptides associated with the PSI primary electron acceptor complex are readily labeled by [14C]TNBS and are exposed to the stromal side of the thylakoid. Differences and similarities in the labeling of polypeptides associated with the PSI complex in thylakoids and in the isolated PSI complex are also noted. Treatment of thylakoids with pronase had no effect on the organization of the polypeptides in the LHCPI or the reaction center core complex, as manifested by the separation of these two subcomplexes from pronase-treated membranes. The 62, 19, and 14 kilodalton polypeptides associated with the reaction center core complex and the 23 and 22 kilodalton polypeptides associated with LHCPI are sensitive to pronase treatment while the 20 kilodalton polypeptide of LHCPI was inaccessible to the protease. The proteolysis of the 62 kilodalton polypeptide generated first a single immunodetectable fragment at about 48 kilodaltons, and further proteolytic digestion generated two other fragments at 30 and 17 kilodaltons respectively. These results are discussed in relation to the organization of the PSI complex in spinach thylakoids. A model for the transmembrane topography of the polypeptide constituents of PSI has been developed.

Thylakoid membranes in higher plants contain four integral protein complexes: PSI, PSII, the Cyt b_6-f complex, and the ATP-synthetase (CF_0-CF_1) (17). These complexes function in a series of reactions that involve the absorption of photons and the conversion of this light energy into the stable chemical products, ATP and NADPH, that are ultimately used for the fixation of CO_2 via the reductive pentose phosphate cycle. Although the function of these individual complexes has been studied in great detail, the nature of the organization of the individual components in the respective complexes and the relationship of these complexes to the overall structure of the chloroplast membrane is less well understood.

It has recently been possible to isolate all the chloroplast membrane electron transfer complexes in highly resolved functional form (7, 16, 25), and subsequently it has been demonstrated that these complexes are reconstitutively active in catalyzing noncyclic electron transport from water to NADP when supplemented with soluble protein cofactors (19). The availability of these resolved complexes allows for detailed examination of structure-function relationships in the individual complexes and examination of the nature of the interactions between complexes. In the present study, we have considered the topographical orientation of the chloroplast PSI complex. The impermeant chemical probe, TNBS,[3] labeled with ^{14}C, has been used to modify stroma-exposed amino acid side chains on proteins in unfractionated thylakoid membranes and reactive groups in the resolved PSI complex of Mullet *et al.* (25). The labeling pattern of PSI polypeptides in the membranes has been compared with that of the isolated PSI complex and subfractions isolated from this complex. Since any one specific technique used in the study of membrane-protein topology has limitations, different techniques should be applied before drawing conclusions on membrane organization. To that effect, we have used proteolytic digestion with pronase E to probe the PSI complex for components accessible to the stroma phase. This approach has been previously employed in studies of the organization of mitochondrial (5) and chromatophore (29) membrane proteins as well as in the characterization of thylakoid pigment-protein complexes (3, 10). Based on these results, a model for the PSI polypeptides that shows a transverse heterogeneity with respect to the membrane is presented and the organization of the thylakoid PSI complex is considered.

MATERIALS AND METHODS

Preparation of Chloroplasts. Chloroplast thylakoid membranes were prepared from freshly harvested greenhouse-grown spinach. Leaves were homogenized in a Waring Blendor in a solution containing 0.3 M sucrose, 10 mM NaCl, and 50 mM K-phosphate (pH 8.0) and the resulting slurry filtered through filtering silk. Intact chloroplasts, collected by centrifugation of the filtrate at 3000g for 1 min, were resuspended in a 1:10 dilution of the blending solution. Thylakoid membranes were isolated by centrifugation at 35,000g for 10 min and were washed once with 0.3 M sucrose and 30 mM K-phosphate (pH 8.0) by centrifugation. The membranes were resuspended in the same

[3] Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonate; CPI, Chl-protein complex of PSI; LHCPI, light-harvesting Chl protein of PSI; DABS, diazonium benzenesulfonate; P_{700}, reaction center Chl of PSI.

solution at a Chl concentration of 1 mg/ml.

Chemical Modification with TNBS. Chloroplast thylakoid membranes (5 mg Chl) were modified by reaction with 10 μCi [14C]TNBS (Research Products International, Inc.; Mt. Prospect, IL; 45–55 mCi/mmol) for 3 h at 4°C. The membranes were washed twice by centrifugation with 20 mM Tricine-KOH buffer (pH 8.0) to remove unreacted [14C]TNBS. The modified membranes were then used for the isolation of the resolved PSI complex according to the procedure of Mullet *et al.* (25).

Preparation and Chemical Modification of the Resolved PSI Complex. The resolved PSI complex (1.5 mg Chl) was treated with 15 μCi [14C]TNBS for 2 h at 4°C. An aliquot of this reaction mixture was diluted with 4 to 5 volumes of cold 20 mM Tricine-KOH buffer (pH 8.0) and then centrifuged at 360,000g for 1 h in a Beckman SW-60 rotor. The PSI complex was recovered as a pellet with very little Chl remaining in the supernatant. Alternatively, an aliquot of the reaction mixture was dialyzed at 4°C overnight against 50 mM sorbitol. The PSI complex was recovered as a pellet following centrifugation at 35,000g for 10 min in a Sorvall SS-34 rotor.

The isolated PSI complex was further fractionated into two Chl-containing fractions: a light-harvesting antenna complex (LHCPI) containing Chl *a* and *b*, and an antenna-depleted reaction center complex containing Chl *a*. The procedure involved treatment with Zwittergent-16 and dodecyl-β-D-maltoside, according to the procedure of Haworth *et al.* (15), followed by centrifugation at 360,000g for 3 h in a Beckman 60Ti rotor. When necessary, the isolated Chl-protein complexes were further labeled with [14C]TNBS at 4°C for 1 to 2 h. The samples were dialyzed overnight against 20 mM Tricine-KOH buffer (pH 8.0) to remove unreacted [14C]TNBS.

In some experiments, the resolved PSI complex was isolated from protease-treated thylakoid membranes. Thylakoids were resuspended in a solution containing 0.3 M sucrose and 30 mM K-phosphate (pH 8.0) at a Chl concentration of 1 mg/ml. The suspension was incubated for 20 min at 25°C in the presence of Protease type XIV (Sigma Chemical Co.) at 300 μg pronase/ml. The treated membranes were washed extensively with ice-cold incubation medium and finally with 5 mM EDTA (pH 8.0). The resolved PSI complex was isolated from these membranes by the procedure of Mullet *et al.* (25) and, when necessary, the preparation was further fractionated into the two Chl-protein complexes described above by the procedure described by Haworth *et al.* (15).

Solubilization of Plastocyanin from Thylakoid Membranes. The labeling of plastocyanin by [14C]TNBS was used to monitor the ability of this reagent to cross the thylakoid membranes. Two procedures were used: (a) thylakoids were labeled with [14C]TNBS, as described above, and then sonicated to release plastocyanin, or (b) thylakoids were sonicated in the presence of [14C]TNBS. Sonication was done for 1 min at 4°C with a Branson Sonifier (power setting 1—microtip). The soluble protein fraction was isolated by centrifugation for 1 h at 144,000g and the supernatant solution, which contained plastocyanin, was concentrated by precipitation with 10% TCA. This fraction was analyzed without further purification. Pure spinach plastocyanin, a gift from R. K. Chain, was prepared by modification of the procedure of Katoh (18). The pure protein (~0.5 mg) was labeled with [14C]TNBS (0.02 μCi) by reaction for 2 h at 4°C.

Preparation of Pronase-Digested Thylakoids and Pronase-Digested PSI. Thylakoids were incubated at 25 to 27°C with various concentrations of pronase (Sigma Protease type XIV) for 20 min. The final concentration of Chl was approximately 1 mg/ml during the treatment. The pronase-treated thylakoids were then washed with ice-cold blending solution at least three times to remove the protease prior to the isolation of the PSI complex. Pronase treatment of the isolated PSI complex was as follows:

The isolated PSI complex (0.1 mg Chl/ml) was incubated at 27°C with various concentrations of pronase for 30 min in a medium containing 50 mM K-phosphate (pH 7.8). After the incubation, the samples were diluted with about 8 to 9 volumes of distilled H$_2$O before centrifugation at 360,000g for 45 min in a Beckman 60 Ti rotor. The pellets were then resuspended in 0.1% Triton, 50 mM Tris-HCl buffer (pH 7.8).

SDS-PAGE and Fluorography. The resolved PSI complex and fractions derived from this complex were analyzed by SDS-PAGE on slab gels (1.5 mm thickness) using the method described by Chua (11) for 10 to 15% gradient gels. Approximately 10 to 15 μg of Chl were loaded for each sample. During the analysis of the PSI complex by SDS-PAGE, we have included 2 M urea in the stacking and resolving gels because of an increased resolution of polypeptides, particularly in the mol wt range below ~25 kD. We have observed, however, that higher mol wt peptides displayed smearings and distortions after [14C]TNBS treatment on such urea gels, while lower mol wt peptides showed no such effects. At this time, we cannot offer an explanation for the behavior of the high mol wt peptides, since distortions did not occur when such treated samples were analyzed on gels from which urea had been omitted. As far as the TNBS modification work is concerned, we present data relevant to the high mol wt peptides (60–70 kD) using SDS-PAGE in the absence of urea and data relevant to the lower mol wt peptides (below 25 kD) using SDS-PAGE in the presence of 2 M urea because of improved resolution. All samples were solubilized prior to electrophoresis in a sample buffer that contained urea in order to obtain fully denaturing conditions.

Gels were prepared for fluorography according to the method of Laskey and Mills (22) as modified by Burkhardt *et al.* (9).

The immunoblotting procedure has been described previously (21).

Spectroscopy. Light-induced changes of P$_{700}$ were measured in an Aminco DW-2 spectrophotometer. Samples were illuminated with saturating red light passing through two Corning 2-64 filters and the photomultiplier was protected by a Corning 4-96 filter. A differential extinction coefficient of 44 mM^{-1} cm^{-1} was used in the calculation of P$_{700}$ concentration. Absorbance spectra were recorded at 25°C in a Cary 219 spectrophotometer with automatic baseline correction. Total Chl concentrations and amounts of Chl *a* and *b* were determined by the method of Arnon (4). Fluorescence emission spectra (77 K) were recorded in a Perkin-Elmer spectrofluorometer (courtesy of C. Krasnow, AGS, Berkeley).

Materials. Triton X-100 and pronase (Sigma Protease type XIV) were purchased from Sigma. Dodecyl-β-D-maltoside and Zwittergent-16 were purchased from Calbiochem. The antibody to CPI was obtained from Dr. Stephen Mayfield (Dept. of Genetics). The antibody to LHCPIb was obtained by injection of the isolated protein (20, 21) into white rabbits in complete Freund's adjuvant and serum samples collected by standard procedures.

RESULTS

The strategy for studying the topography of membrane complexes that has been used is the following. (a) Label or modify surface-exposed groups. (b) Isolate specific membrane protein complexes after modification. (c) Identify modified polypeptides. This strategy has been applied using a radioactive chemical modifying reagent, trinitrobenzenesulfonate ([14C]TNBS). This reagent is specific for nucleophilic side chains which, under the present experimental conditions, will be mainly lysine residues. Thus, if the portions of the polypeptide which are extruded out toward the stroma do not contain lysines, there may not be labeling by TNBS. To augment these limitations, we have used a nonspecific protease, pronase E, as a surface probe since this

large enzyme would not be expected to cross the thylakoid membrane.

TNBS and Pronase E as Chemical Probes. TNBS has previously been reported not to penetrate erythrocyte membranes and other membranes (8, 12) and therefore might not be expected to cross the thylakoid membrane. The characteristics of the reaction of TNBS with thylakoids are shown in Figures 1 and 2. At a fixed concentration of TNBS, the time course of incorporation of TNBS into thylakoids is relatively slow at 4°C, taking ~3.5 h to go to completion. Shown in Figure 2 is the concentration dependence of incorporation, and a saturating level of 5 mM was observed. The results of these two studies were used to establish optimal conditions for TNBS labeling of thylakoids in subsequent experiments. In order to confirm the impermeability of thylakoids to TNBS under our experimental conditions, we have done experiments to show that the probe does not traverse the thylakoid membrane and gain access to the lumenal space. The
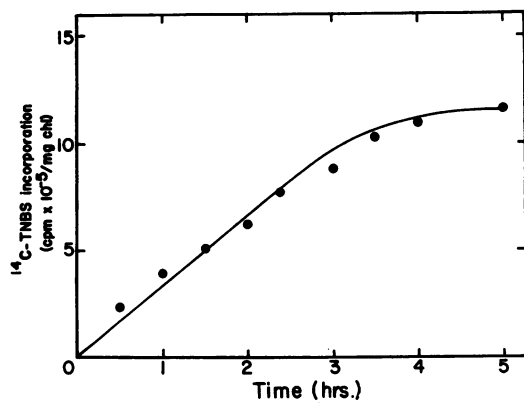

FIG. 3. [^{14}C]TNBS labeling of peripheral and lumenal proteins and isolated plastocyanin. Thylakoids or sonicated thylakoids were incubated with [^{14}C]TNBS for 3 h at 4°C and plastocyanin extracted as described in "Materials and Methods." TCA precipitates from both extracts corresponding to equal amounts of Chl were analyzed by SDS-PAGE. The gels were stained (lanes 1–5) with Coomassie Blue, then fluorographed (lanes 6–9) for about 2 weeks. Lanes 2, 3, 7, 8 correspond to a partially purified plastocyanin preparation obtained from [^{14}C]TNBS labeled thylakoids. Lanes 4 and 9 correspond to a partially purified plastocyanin preparation obtained from thylakoids sonicated in the presence of [^{14}C] TNBS. Lanes 1 and 6 correspond to pure plastocyanin modified with [^{14}C]TNBS. Lane 5 corresponds to unmodified pure plastocyanin and the corresponding fluorography is shown in lane 10.

FIG. 1. Time course of [^{14}C]TNBS incorporation into spinach thylakoids. Thylakoids (0.2 mg/ml) were suspended in ice-cold 300 mM sucrose + 30 mM K-phosphate (pH 8), and incubated in the presence of 5 mM TNBS (~2 μCi/μmol [^{14}C]TNBS) at 4°C in the dark. At the times indicated, samples of 10 μg Chl were placed on filter paper discs and immediately soaked in 5% TCA. The discs were prepared for scintillation counting by washing twice with 5% TCA, once with hot 5% TCA, twice with ethanol:ether (1:1), and once with ether and allowed to air dry before adding scintillation liquid.
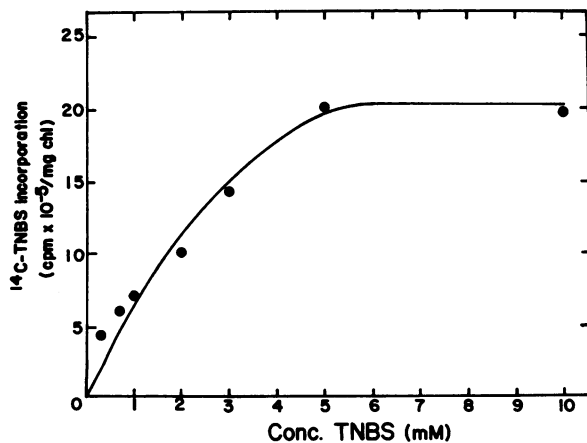

FIG. 2. Concentration-dependence curve for the reaction of TNBS with spinach thylakoids. Thylakoids were resuspended in ice-cold 300 mM sucrose + 30 mM K-phosphate (pH 8) and incubated in the presence of various concentrations of TNBS (~4 μCi/μmol) for 4 h at 4°C in the dark. At the end of the incubation period, samples containing 20 μg Chl were placed on filter paper discs and soaked in 5% TCA. Discs were prepared for scintillation counting described in the legend to Figure 1.
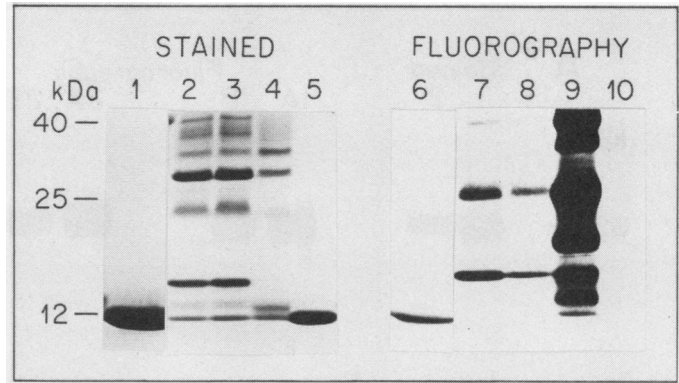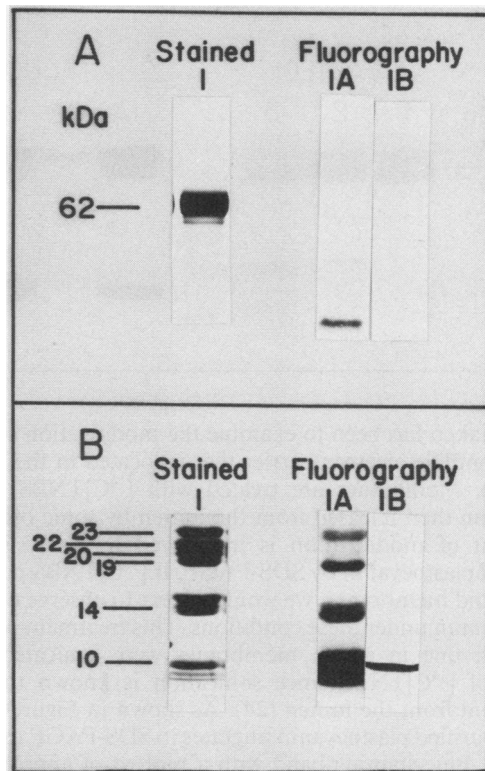

FIG. 4. [^{14}C]TNBS labeling of the reaction center polypeptide (A) and the low mol wt polypeptides (B) of PSI. The resolved PSI complex was prepared by the method of Mullet et al. (28) from [^{14}C]TNBS-modified thylakoids. The preparation was analyzed by SDS-PAGE in the presence (B) or absence (A) of urea (for explanation, see "Materials and Methods"), then stained with Coomassie Blue (lane 1), and fluorographed at −80°C for 1 week (lanes 1A) or at room temperature for 3 d (lanes 1B).
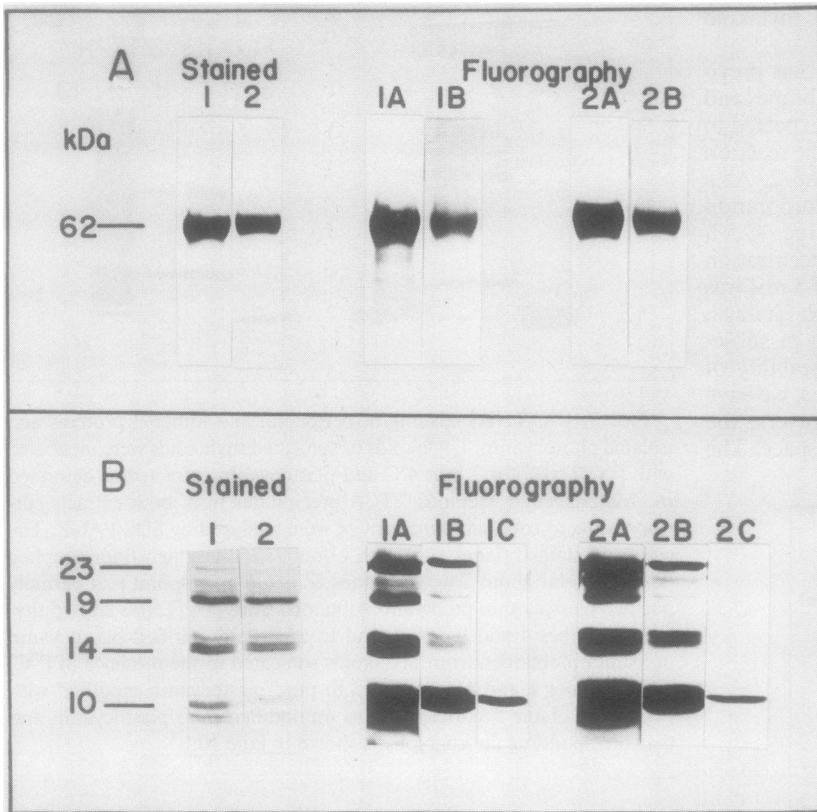
FIG. 5. Distribution of radioactivity in an antenna-depleted PSI complex obtained from a resolved PSI complex directly modified with [^{14}C]TNBS: Reaction center polypeptide (A), small mol wt components (B). A resolved PSI complex was modified with [^{14}C]TNBS, as described in "Materials and Methods," and then fractionated into an antenna-depleted PSI complex and LHCPI (Fig. 6), according to (13, 20). An aliquot of the antenna-depleted PSI complex was also relabeled with [^{14}C]TNBS. The fractions were analyzed by SDS-PAGE with (B) or without (A) urea in the gel. The gels were stained with Coomassie Blue (lanes 1 and 2) and fluorographed at −80°C for 1 week (lanes 1A, 2A), at room temperature for 3 d (lanes 1C, 2C) or at room temperature for 7 to 10 d (lanes 1B, 2B). Lanes 1, 1A, and 1B contain the antenna-depleted PSI reaction center complex obtained from a [^{14}C]TNBS-modified resolved PSI complex. Lanes 2, 2A, and 2B contain an aliquot of the same antenna-depleted complex relabeled with [^{14}C] TNBS.
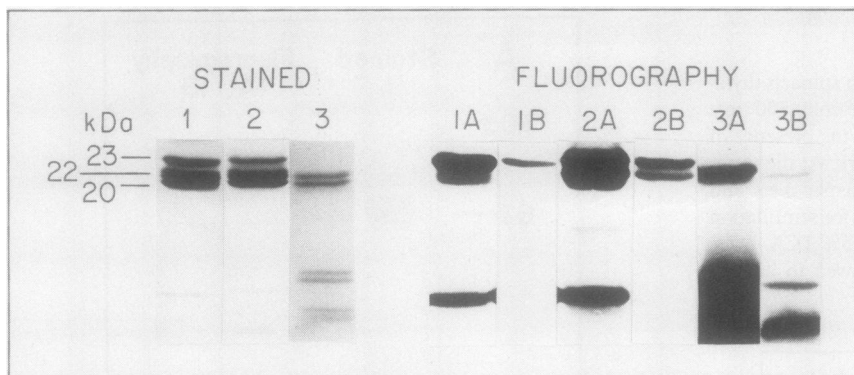

FIG. 6. Distribution of radioactivity in LHCPI. LHCPI was obtained as described in Figure 5. An aliquot of LHCPI was also relabeled with [^{14}C] TNBS. The fractions were analyzed by SDS-PAGE with urea in the gels. The gels were stained with Coomassie Blue (lanes, 1, 2, and 3) and fluorographed for 1 week at −80°C (lanes 1A, 2A, and 3A) or for 3 d at room temperature (lanes 1B, 2B, and 3B). Lanes 1, 1A, and 1B contain LHCPI isolated from a [^{14}C]TNBS-labeled resolved PSI complex. Lanes 2, 2A, and 2B contain an aliquot LHCPI which has been relabeled with [^{14}C]TNBS. Lanes 3, 3A, and 3B contain LHCPI isolated from pronase-treated membranes (300 μg pronase E/ml of thylakoid suspension) and then labeled with [^{14}C]TNBS.

approach taken has been to examine the modification of plastocyanin, a mobile electron carrier that is located in the lumenal space (14). Membranes are treated with [^{14}C]TNBS and the plastocyanin then released from the lumen by sonic oscillation. The extent of modification is monitored by analysis of the solubilized plastocyanin by SDS-PAGE. If [^{14}C]TNBS penetrates the thylakoid membranes, we would expect to observe a labeling of plastocyanin under these conditions. This treatment was compared with one in which membranes were sonicated in the presence of [^{14}C]TNBS since sonication is known to release plastocyanin from the lumen (24). As shown in Figure 3 (lanes 1 and 5), purified plastocyanin migrates in SDS-PAGE as a single Coomassie Blue-staining band with a mol wt of approximately 11 kD. The corresponding fluorograph of a TNBS-labeled sample is shown in lane 6. This sample was underlabeled with [^{14}C] TNBS to avoid extreme darkening on gels. Lane 2 shows the Coomassie-Blue staining pattern of thylakoid membranes modified with [^{14}C]TNBS in the absence of osmoticum (0.3 M sucrose) and sonicated to release lumenal proteins. Several protein bands are present, including a prominent one at 11 kD that comigrates

with pure plastocyanin. The corresponding fluorograph (lane 7) shows no labeling of the 11 kD band. An experiment using thylakoid membranes modified in the presence of osmoticum (lanes 3 and 8) shows no labeling of the 11 kD polypeptide. A labeled band at 11 kD is present, however, in lumenal extracts from thylakoids that are sonicated in the presence of [^{14}C]TNBS (lanes 4 and 9). This indicates that plastocyanin has become accessible to the modifying reagent after disruption of the membrane structure.

As was found with TNBS based on SDS-PAGE analysis, pronase did not modify plastocyanin (data not shown). In addition, electron paramagnetic resonance analysis of control and pronase-treated membranes for plastocyanin (24) indicated no degradation of this protein.

The effect of pronase on PSI activities has also been investigated. The PSI complex has been extracted from control and pronase-treated thylakoids by the method of Mullet *et al.* (25), and the PSI-catalyzed photoreduction of Fd and methyl viologen studied. No significant difference in rates was observed even after treatment of thylakoids with the highest pronase concentration
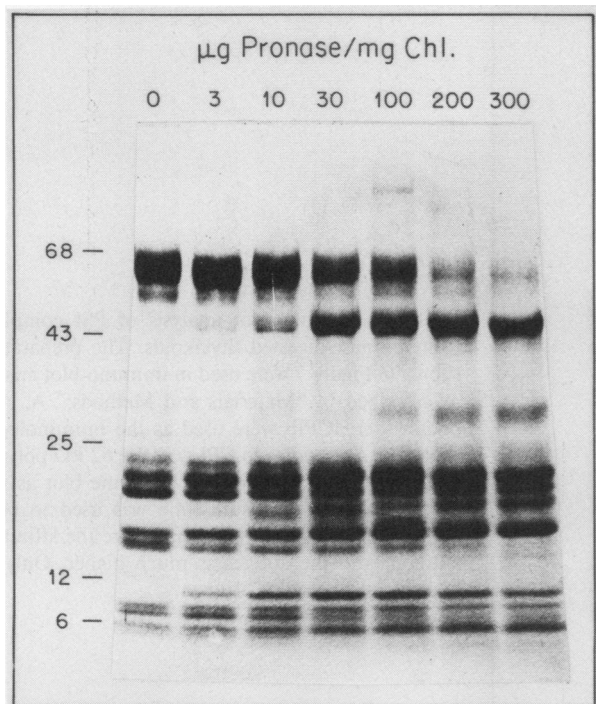
FIG. 7. Effects of pronase digestion of thylakoids on the PSI polypeptide composition. SDS-PAGE in the presence of 2 M urea was carried out as described in "Materials and Methods." PSI preparations from thylakoids treated with various amounts of pronase are shown.
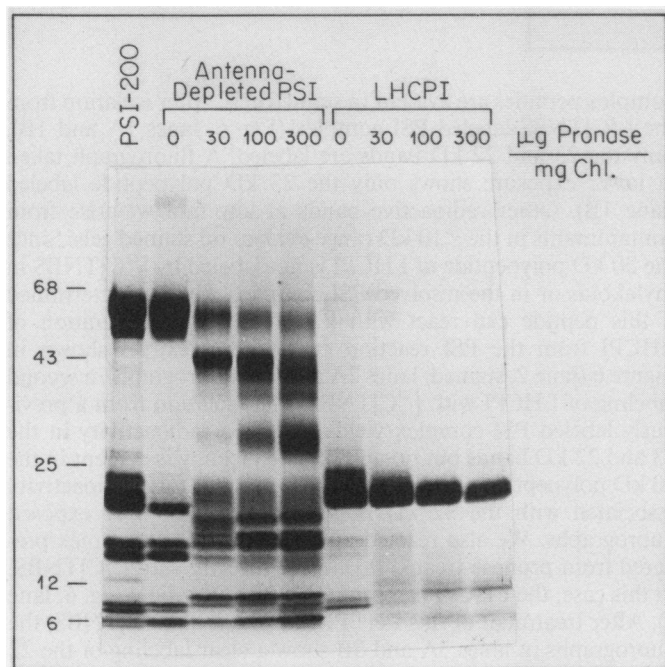

FIG. 8. Fractionation of the PSI complex from pronase-digested thylakoids. The various PSI complexes were fractionated into the LHCPI and reaction center-containing subcomplexes. The polypeptide composition of these two Chl-containing complexes were then analyzed by SDS-PAGE.

used (*i.e.* 300 μg pronase/mg Chl). All PSI complexes showed a Chl/P$_{700}$ ratio of 230 ± 30 and a Chl *a/b* ratio of 6. Fluorescence emission spectra for these preparations (77 K) show a 735 nm maximum while their absorption spectra at room temperature are identical. Thus, functionally and spectrally, the PSI com-

plexes isolated from control and pronase-treated thylakoids were indistinguishable. Pronase treatment also did not affect P$_{700}$ photooxidation and the subsequent reduction in the presence of added plastocyanin (data not shown).

On the basis of the results on modification of plastocyanin and on effects on PSI activities, we conclude that TNBS or pronase E do not cross the thylakoid membrane to any significant extent under our experimental conditions and can therefore be used to modify surface-exposed (stroma-exposed) protein side chains.

Peptide Composition of the Resolved PSI Complex. The resolved PSI complex prepared by mild detergent treatment of thylakoid membranes contains a number of major Coomassie Blue-staining polypeptides when analyzed by SDS-PAGE (Fig. 4, A and B, lane 1): (a) the 60 to 70 kD polypeptide(s) that contain the reaction center Chl, P$_{700}$ (6). Under our SDS-PAGE conditions, this polypeptide migrates as a broad band centered at ~62 kD; (b) the 23, 22, and 20 kD polypeptides associated with the LCHPI (15, 20, 21); (3) polypeptides of 19, 16, and 14 kD whose function may be related to the PSI primary electron acceptor complex (23, 27); and (d) several low mol wt peptides (<10 kD) of unknown function.

Modification of the PSI Complex in Unfractionated Thylakoids. When unfractionated thylakoids are modified with [^{14}C] TNBS and the PSI complex isolated and then analyzed by SDS-PAGE, we find that a number of the major polypeptides are labeled. The Coomassie Blue-stained bands of this material is shown in Figure 4, A and B (lane 1). The corresponding fluorography is also shown in Figure 4, A and B (lanes 1A and 1B) at two different exposure conditions (see legend for details). The most significant labeling occurs on polypeptides of mol wt of 23, 19, 16, and 14 kD, and the bulk of the labeling occurs in the low mol wt polypeptides. (For the sake of simplicity, we will consider these polypeptides of <10 kD as a group since they are not well resolved from each other in our gel system.) We often observe a faintly labeled band at about 30 kD (lane 1A), although the presence of this polypeptide is not apparent in stained gels (lane 1). Although the above-described bands are clearly labeled, no labeling of the reaction center polypeptide of 62 kD is observed, and two of the LHCPI peptides (22 and 20 kD) are also unmodified by [^{14}C]TNBS. The fluorographs in Figure 4, A and B (lane 1A) are considerably overexposed and, at lower exposure, the only low mol wt polypeptide showing significant labeling is that at 10 kD (lane 1B).

Modification of the Isolated PSI Complex. The isolated PSI complex has been modified directly with [^{14}C]TNBS and then immediately fractionated into the antenna-depleted PSI reaction center core complex and the light-harvesting PSI antenna complex (LHCPI). These two fractions were used for subsequent SDS-PAGE analysis of labeling patterns. The Coomassie-Blue stained gel for the PSI reaction center core complex is shown in Figure 5, A and B (lane 1). This preparation contains the high mol wt (~62 kD) reaction center polypeptide, the 19, 16, and 14 kD polypeptides and two low mol wt peptides. The bands visible in the 20 to 23 kD region arise from small amounts of the antenna complex still present in the preparation. The corresponding fluorography is shown in lanes 1A and 1B at two (Fig. 5A) or three (Fig. 5B) different exposure conditions (see legend for details). In contrast to the results with unfractionated thylakoids, the 62 kD polypeptide of PSI is now heavily labeled, as are all the major peptides of the PSI reaction center core complex (Figure 5, A and B, lane 1A). A fluorograph at lower exposure (lane 1B) still indicates the heavy labeling of the 62 kD peptide relative to other polypeptides of the complex. Labeling in the 20 to 23 kD region originates from the contaminating antenna peptides (Fig. 5B, lanes 1A and 1B).

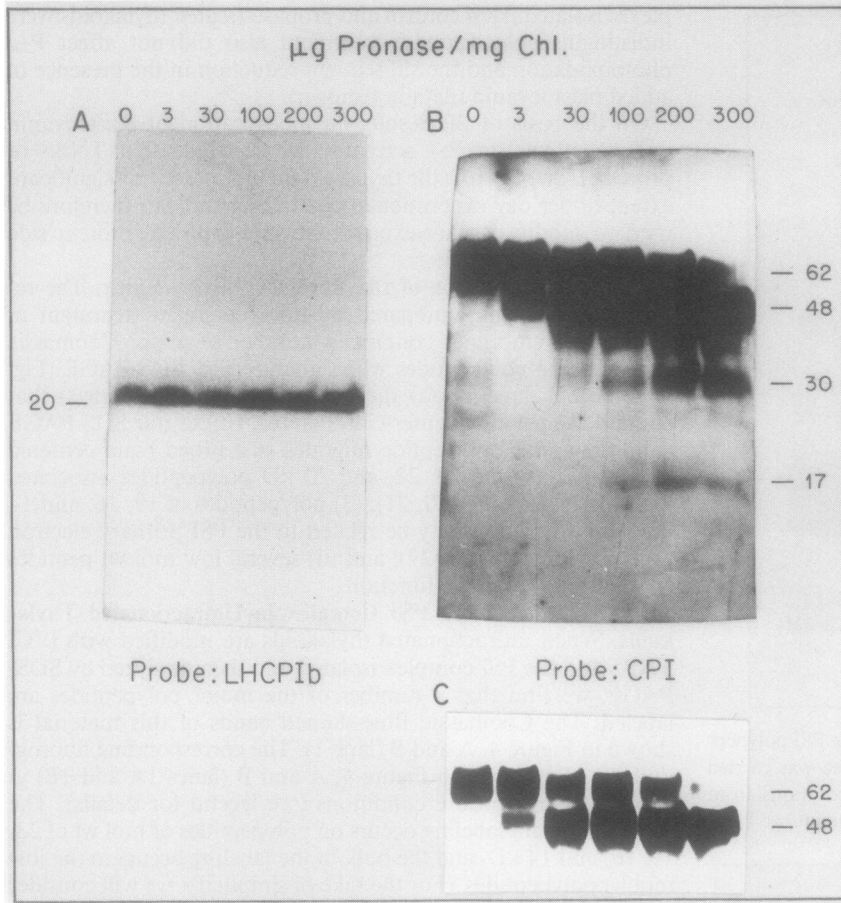Also shown in Figure 5, A and B (lane 2) is the Coomassie-

FIG. 9. Immuno-blot analysis of PSI complexes from pronase-digested thylakoids. The preparations shown in Figure 7 were used in immuno-blot analysis as described in "Materials and Methods." A, Antibodies to LHCPIb were used as the immunological probe. B, Antibodies to CPI (*i.e.* the 62 kD polypeptide) were used as the probe. C, Same blot as in B except a shorter exposure time was used to better define the higher mol wt region where the affinity of antibodies to the antigen is much higher. Only the higher mol wt region is shown.

Table I. *Topography of the Chloroplast PSI Complex*

	PSI Peptides	TNBS Thylakoids	TNBS PSI	Pronase Thylakoids		
	kD					
1)	62.0	–	++	++		
2)	23.0	++	+++	+++		
3)	22.0	–	++	+		
4)	20.0	–	–	–		
5)	19.0	+++	+	+++		
6)	16.0[a]	++	+++	–	PSI	LHCPI
7)	14.5	++	++	++	Core	
8)	14.0	++	+	+++		
9)	8.7	( )[b]	( )	–		
10)	7.8	( )	( )	+++		
11)	6.8	( )	( )	–		
12)	5.6	( )	( )	+++		

[a] Often lost during separation of antenna complex from PSI core complex.

[b] ( ), Unsure due to overlap of strong labeling with [14C]TNBS which restricts resolution during fluorography.

Blue staining pattern of a PSI reaction center complex labeled directly with [14C]TNBS. Lanes 2A, 2B, and 2C are fluorographs taken at different exposure conditions that indicate that all of the major bands of this fraction, including some contaminating bands from the LHCPI, are heavily labeled with [14C]TNBS. Increased labeling of the 62 kD polypeptide is also observed (compare in Fig. 5A, lanes 1B *versus* 2B).

The results of a similar study using LHCPI are shown in Figure 6. As shown in lanes 1 and 2, LHCPI contains three major polypeptides (23, 22 and 20 kD), and none of the reaction center complex peptides are evident in stained gels. After isolation from the [14C]TNBS-labeled PSI complex (Fig. 6, lanes 1A and 1B), only the 23 and 22 kD bands are labeled. A fluorograph taken at lower exposure shows only the 23 kD polypeptide labeled (lane 1B). Other radioactive bands at low mol wt arise from contaminants in the <10 kD range evident on stained gels. Since the 20 kD polypeptide of LHCPI is not labeled by [14C]TNBS in thylakoids or in the resolved PSI complex, we have determined if this peptide can react with [14C]TNBS after separation of LHCPI from the PSI reaction center complex. As shown in Figure 6 (lane 2, stained; lanes 2A and 2B, fluorograph), a second labeling of LHCPI with [14C]TNBS after isolation from a previously labeled PSI complex yields increased radioactivity in the 23 and 22 kD bands but no appreciable labeling is evident in the 20 kD polypeptide other than that originating from radioactivity associated with the 22 kD polypeptide, *e.g.* in over exposed fluorographs. We also reacted an isolated LHCPI complex prepared from pronase-treated thylakoids directly with [14C]TNBS. In this case, the LHCPI lacks the 23 kD polypeptide (Fig. 6, lane 3). After treatment of this LHCPI complex with [14C]TNBS, the fluorographs in lanes 3A and 3B show a clear labeling of the 22 kD polypeptide but no appreciable label of the 20 kD polypeptide.

Polypeptides in PSI Accessible to Pronase. The polypeptide constituents of the PSI complexes isolated from pronase-treated membranes have been compared in Figure 7. Many polypeptides are found to be digested by the protease treatment, and new fragments are observed as the pronase concentration was increased. The polypeptides most sensitive to pronase were the ones with relative mol wt of 62, 14, and 7.8 kD. The new fragments at 48 and 30 kD have been presumed to arise from the 62 kD peptide since the appearance of these peptides paral-
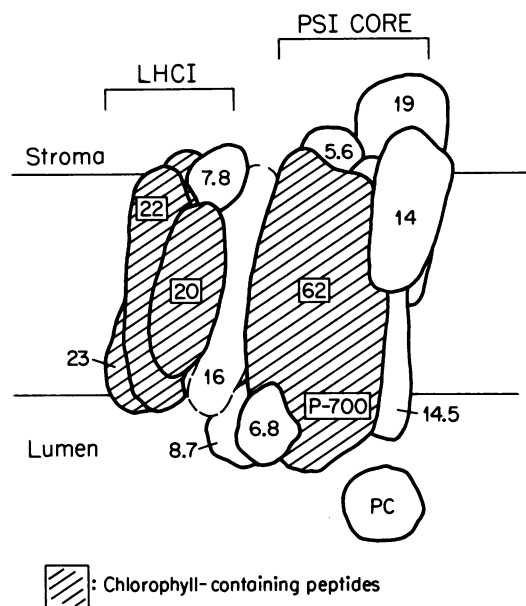
FIG. 10. Model of the transmembrane organization of the PSI complex. The qualitative data of Table I are represented in this model. The locations of the lower mol wt peptides (≤10 kD) are at present questionable although an organization based on pronase treatment is shown in this figure. The exact location of each polypeptide in relationship to each other and their stoichiometry remain to be determined. The model, however, attempts to emphasize the transmembrane organization of each peptide component in the PSI complex. The grouping of peptides into the LHCPI and PSI reaction center core complexes is based on the composition of peptides in the fractionation studies.

leled the loss of the 62 kD peptide, and the control PSI complex did not contain any appreciable amount of polypeptides in this mol wt range.

Analysis of pronase-treated membranes in relation to PSI has been aided by the ability to fractionate the resolved PSI complex into the two Chl-containing complexes described above. The purpose of this is 2-fold. (a) In the presence of urea during SDS-PAGE, which is included to insure solubilization of hydrophobic polypeptides, the 20 kD polypeptide in the PSI light-harvesting Chl complex (LHCPI) at times overlaps the 19 kD polypeptide of the reaction center core complex. By analyzing the two PSI Chl complexes separately, it should be possible to obtain a clearer picture since the number of polypeptides in these two complexes is better defined. This is especially important when many new peptide fragments are generated by the protease treatment. (b) It is possible to test whether the hydrophobic interactions among the transmembrane fragments of the constituent polypeptides are strong enough to hold the subcomplexes together after the pronase digestion of the stromal-exposed portions. Thus, during solubilization of the PSI complexes by high concentrations of dodecyl-β-D-maltoside and Zwittergent 16, one might expect fragments of the subcomplexes to separate from each other in the absence of strong hydrophobic interactions.

It has been found that the yield of the two Chl subcomplexes following solubilization of the PSI complex was not significantly altered by prior pronase treatment. In each case, about half of the Chl in the PSI preparation is recovered in LHCPI and half in the reaction center core complex. No significant amount of free pigment was observed.

The polypeptide composition of the Chl-protein subcomplexes of PSI are shown in Figure 8 from control and pronase-treated thylakoids. In the reaction center core complexes (antenna-depleted PSI in the figure), the 62 kD polypeptide is sensitive to pronase, and new fragments of 48 and 30 kD and several distinct

new fragments in the mol wt range between 19 and 16 kD are observed. The prominent polypeptides at 19 and 14 kD have been found to be most sensitive to pronase treatment. The other polypeptides associated with the reaction center core complex that were sensitive to pronase were low mol wt peptides of 7.8 and 5.6 kD. Of these, the 7.8 kD was found associated with the LHCPI complex. The low mol wt peptides at 8.7 and 6.8 kD of the reaction center core complex appear to be insensitive to protease treatment. The LHCPI complex contained four main polypeptides with relative mol wt of 23, 22, 20, and 7.8 kD. Of these, the 7.8 kD was extremely sensitive to pronase treatment while the 23 kD polypeptide also disappeared at about 100 µg pronase/mg Chl. The 22 kD peptide was also sensitive to pronase digestion, although to a lesser extent than the other two polypeptides. The 20 kD polypeptide, on the other hand, appeared to be unaffected by the highest protease concentration used in this series of studies.

Immunoidentification of Pronase PSI. A major problem in the use of proteases to probe topography is the positive identification of digestion products seen after treatment. In a gel system containing many different polypeptides, indications that a specific peptide fragment originates from a specific polypeptide is rarely without ambiguity as new fragments overlap with existing polypeptides of similar mol wt. This problem is especially severe in the lower mol wt regions since more polypeptides can potentially give rise to low mol wt fragments. One way to overcome these problems is through the use of antibodies for the polypeptide(s) in question to probe the various digested complexes after separation of the peptides. Antibodies for the 62 and 20 kD polypeptides of PSI have been prepared. In Figure 9, these antibodies have been used in a Western Blot procedure to probe specifically the effects of pronase treatment on these two polypeptides. The antibodies to the 20 kD polypeptide (Fig. 9A), also known as LHCPIb (20), demonstrate that no significant digestion of this peptide occurred, even at the highest pronase concentration used. The antibody against the apoprotein of CPI (62 kD), on the other hand, cross-reacted with the 48, 30 (Fig. 9C), and a 17 kD fragment (Fig. 9B) in the PSI preparations from pronase-treated thylakoids. The 48 kD fragment appeared significantly earlier than the other two and would appear to be a precursor of these lower mol wt fragments.

Pronase Digestion of Isolated PSI. To address the question as to whether the pronase-insensitive polypeptides in PSI complexes isolated from treated membranes were exposed on the lumenal side of the membrane, studies with the isolated PSI complex have been done. Since this complex has been demonstrated to be functional in NADP photoreduction with plastocyanin as electron donor, it is reasonable to assume that both surfaces are exposed. The PSI complex was treated with varying amounts of pronase at 27°C as described in "Materials and Methods" and the digested complex reisolated by centrifugation at 360,000g. Interestingly, no free Chl resulted and the pelleted complex was spectrally indistinguishable from a control PSI complex. Functionally, however, it was found that plastocyanin was unable to donate electrons to P_{700}^+ effectively after pronase treatment of the isolated PSI complex. It is interesting that the P_{700}/Chl ratio was not altered in the digested PSI. This indicates that the reaction center Chl(s) were not accessible to pronase although the plastocyanin binding site has been altered.

The polypeptide patterns for the digested PSI complexes show that all of the polypeptides in the isolated complex were subject to proteolytic attack (data not shown). This observation suggests that either the detergent isolation procedure makes normally membrane-imbedded proteins accessible to the protease, or that the availability of the lumen-exposed portions enabled the pronase to digest both stromal and lumenal portions of the PSI complex. These two possibilities are not mutually exclusive.

However, the first case is unlikely since we would have expected a loss of Chl due to the attack of the membrane-imbedded regions by the protease. No such loss has been observed. Thus, the loss of plastocyanin donation site and the sensitivity of all the polypeptides in the isolated PSI complex supports the view that pronase does not penetrate the thylakoid membranes under our conditions and that the lumenal portions of PSI become exposed in the isolated complex.

DISCUSSION

The availability of a resolved, reconstitutively active PSI complex (25) with a well-defined polypeptide composition allowed a study of the organization of specific polypeptides associated with this complex. Little is known concerning the membrane organization of this integral membrane complex, although a recent study of a cyanobacterial PSI complex has proposed a transmembrane orientation (13). In the case of the spinach thylakoid PSI complex, we are in a position to relate specific polypeptides to specific functions and consider structure-function relationships in detail.

A number of polypeptides appear labeled when whole thylakoids are reacted with [14C]TNBS, among them the 23 kD polypeptide of the LHCPI, polypeptides of 19, 16, and 14 kD, and a number of small mol wt polypeptides (<10 kD). Other polypeptides, like the reaction center polypeptide (62 kD) and the 22 and 20 kD polypeptides of the LHCPI are not labeled. We interpret these results to indicate that the labeled polypeptides are exposed to the stromal side of the thylakoid, while the unlabeled polypeptides are either buried in the lipid matrix and/ or are preferentially exposed to the lumenal portion of the thylakoid, or have no reactive amino groups that are accessible to [14C]TNBS. The fact that we observe no labeling of the 62 kD reaction center polypeptide by TNBS agrees with reports in the literature which indicate that this polypeptide is resistant to proteolytic digestion by trypsin (3, 10).

We have shown that the 23 kD polypeptide of the LHCPI is accessible to [14C]TNBS. These results parallel observations that the LHCP of PSII is surface-exposed, since a 2,000-D segment could be removed by proteolysis (10, 26). On the other hand, the 20 kD polypeptide, which we have recently shown to be a Chl *a/b*-containing polypeptide of PSI (20), is not accessible to [14C]TNBS, and appears to be buried in the hydrophobic portions of the membrane. Studies using proteolytic digestion are in agreement with this conclusion.

Although the specific function of the 19 and 14 kD polypeptides in PSI is not known, evidence is accumulating that indicates an association of these peptides with the primary electron acceptor complex (23, 27). From a functional point of view, we would expect at least one of these polypeptides to be involved in a reaction with soluble chloroplast Fd as electrons are transferred to NADP. This would argue for a stroma-exposed location for some of the acceptor peptides and our finding that both the 19 and 14 kD polypeptides are labeled with [14C]TNBS in unfractionated thylakoids is consistent with this role.

Our work on the chemical modification of the isolated PSI complex of Mullet *et al.* (25) revealed interesting parallels as well as differences in the pattern of polypeptide labeling when compared with results obtained for thylakoids. One significant difference is that the reaction center polypeptide of 62 kD and the 22 kD polypeptide of LHCPI are labeled when the isolated PSI complex is modified with [14C]TNBS but not when whole thylakoids are modified with the reagent. We conclude that as the PSI complex is removed from its native environment in the thylakoid, reactive amino groups in the 62 kD polypeptide and in the 22 kD polypeptide become acessible to TNBS. This could be due to a rearrangement of the polypeptides in the complex during the isolation procedure as well as that the lumen-exposed

portions of these peptides become accessible to TNBS. The fact that we observe increased labeling of these peptides when isolated antenna-depleted PSI or isolated LHCPI are chemically modified with [14C]TNBS suggests that even in the isolated complex of Mullet *et al.* (25) not all of the reactive amino groups are accessible to the reagent.

A most interesting observation is the failure to chemically modify the 20 kD polypeptide of LHCPI when either thylakoids or the isolated PSI complex reacts with [14C]TNBS. This indicates that the 20 kD polypeptide is buried in the membrane and sequestered in the innermost (and least accessible) portions of the isolated complex. We believe that the absence of labeling of the 20 kD polypeptide is not due to the lack of reactive amino groups since preliminary amino acid analysis shows that the 20 kD polypeptide contains lysine residues (Lam and Malkin, unpublished observations).

In the present study, we have also investigated the topography of PSI constituent polypeptides by pronase treatment of intact thylakoids followed by isolation of a modified PSI complex (25). This procedure differs from that previously used by Carter and Staehelin (10) in a study where thylakoids were treated with pronase in that the isolation of the resolved PSI complex after treatment allows for a specific localization of the effects of proteolysis on components of this membrane complex. Of the 12 distinguishable polypeptides in PSI, about nine are accessible to pronase in varying degrees. Surprisingly, many of the functional and spectral characteristics of the PSI complex have not been altered significantly by this treatment. In addition, LHCPI and the PSI reaction center core complex are stable enough to be separated in an intact form followed by detergent solubilization of the isolated PSI complexes. These observations suggest the following: (a) The binding site for Fd on the stromal side of the thylakoids must be buried to some extent in the hydrophobic region of the membrane phase and inaccessible to the protease since Fd reduction is not inhibited by pronase. (b) The binding sites for Chl in the antenna (LHCPI) and the PSI reaction center core complex must be in the intramembrane regions of their respective Chl proteins, since the P_{700}/Chl ratio was not altered by degradation of surface-exposed segments of the polypeptides. (c) The hydrophobic interactions among the intramembrane segments of the various constituent polypeptides appear to be the major factors in the stabilization of the structure of the functional PSI complex since it is possible to isolate a complex from pronase-treated membranes. The surface exposed, hydrophilic portions are not required for either a functional complex or the association between polypeptides in the complex. This last point is similar to that observed in the widely studied membrane protein, bacteriorhodopsin (1), since it was shown that proteolytic digestion does not alter the spectral or functional characteristics of this protein.

The study on the topography of membrane protein complexes by proteases is not without deficiencies. Being a protein itself, the size of the protease is a limiting factor in its ability to react with surface exposed protein segments which either do not protrude far enough from the membrane surface or might be surrounded (shielded) by other members of the complex from the protease. A small molecule, such as a hydrophilic reagent, will probably be affected by these factors to a much lesser extent. Thus, the use of chemical modifiers in conjunction with protease treatment will be complementary in elucidating membrane protein topography. Table I summarizes results from our studies with TNBS modification as well as those with pronase digestion of thylakoids. Most apparent is the lack of modification of the 62 kD polypeptide by TNBS while pronase was found to degrade this polypeptide. The most obvious conclusion is that the stroma-exposed portions of the 62 kD polypeptide do not contain lysine residues. The 19 and 14 kD polypeptides found in the PSI

reaction center core are relatively sensitive to pronase treatment and also are readily labeled by TNBS. In the LHCPI complex, the 23 kD polypeptide is most readily digested by pronase and also readily labeled by TNBS. In contrast, the 20 kD polypeptide of LHCPI is neither affected by pronase nor labeled by TNBS.

The qualitative data summarized in Table I are presented in a model of the transmembrane topography of the PSI complex (Fig. 10). In this model, the surface or non-surface exposed nature of the constituent polypeptides is emphasized. However, several points of interest in this model are relevant to our current knowledge on the structure-function relationship in the PSI complex. The high mol wt polypeptides of the PSI complex, which have recently been demonstrated to be heterogeneous (28), are known to contain the reaction center Chl, P_{700}, and all the Chl a of the PSI reaction center core complex ($\sim$130 Chl a/P_{700}) (2, 6). Our results indicate that all these polypeptides have a transmembrane organization and that the Chl associated with these proteins is located in the intermembrane section since none is released by pronase digestion of an isolated PSI complex even though substantial digestion to low mol wt fragments has occurred. The observation that the 62 kD polypeptide is converted to a lower mol wt polypeptide of 48 kD and subsequently to polypeptides of 30 and 17 kD during pronase digestion suggests a minimum of three transmembrane segments for the original polypeptide. Recent work from several laboratories has characterized a Chl a/b-containing antenna complex associated with PSI (15, 20, 21, 30) and these polypeptides have different organizational characteristics with the 23 kD peptide being the most stromal exposed and the 22 kD peptide less exposed. The 20 kD polypeptide is inaccessible to the probes we have used and this polypeptide would appear to be totally buried in the thylakoid membrane. At present the function of the other polypeptides in the PSI complex is not known, but the organization of these peptides is also shown in Figure 10.

Acknowledgment—We thank Dr. S. Mayfield for CPI antibody.

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