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P_{700} Chlorophyll *a*-Protein[1]

PURIFICATION, CHARACTERIZATION, AND ANTIBODY PREPARATION

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ABSTRACT

The P_{700} chlorophyll *a*-protein was purified by preparative sodium dodecyl sulfate (SDS) gel electrophoresis from SDS-solubilized barley (*Hordeum vulgare* L., cv Himalaya) chloroplast membranes. After elution from the gel in the presence of 0.05 to 0.1% Triton X-100, the recovered protein had a chlorophyll/P_{700} ratio of 50 to 60/1 and contained no chlorophyll *b* or cytochromes. Analysis of the polypeptide composition of the chlorophyll-protein revealed a 58 to 62 kilodalton (kD) polypeptide component but no lower molecular weight polypeptides. The 58 to 62 kD component was further resolved into two distinct polypeptide bands which were subsequently mapped by partial cyanogen bromide digestion and *Staphylococcus aureus* proteolysis. Based on results from the mapping experiments and other data, we suggest that the two components are conformational variants of a single polypeptide. Measurement of the chlorophyll to protein ratio by quantitative amino acid analysis and consideration of the yield of P_{700} in the protein isolate suggest that, contrary to previous models (Bengis and Nelson, 1975, 1977), P_{700} *in vivo* is associated with a minimum of four subunits of approximately 60 kD.

Antibodies raised against the photochemically active chlorophyll-protein complex from barley reacted specifically with the 58 to 62 kD apoprotein. The same preparative electrophoresis procedure was used to isolate photochemically active P_{700} chlorophyll *a*-protein from soybean (*Glycine max* L.), tobacco (*Nicotiana tabacum* L.), petunia (*Petunia* × *hybrida*), tomato (*Lycopersicum esculentum*), and *Chlamydomonas reinhardti*. The isolated complex from all species exhibited identical polypeptide compositions and chlorophyll/P_{700} ratios. Antibodies to the barley protein cross reacted with all species tested demonstrating the highly conserved structure of the apoprotein.

The P_{700} Chl *a*-protein is defined as the Chl-protein which binds the reaction center Chl of PSI (P_{700}) and approximately 40 molecules of light-harvesting Chl *a* (32). Current knowledge of the structure and function of this important protein has been derived from the analyses of P_{700}-enriched chloroplast membrane fractions prepared by several methods (5, 6, 12, 25, 30). Although there is general agreement that the P_{700} Chl *a*-protein is the structural and functional center of PSI, there is conflicting evidence regarding the number and mol wt of the polypeptides which comprise the apoprotein component of this complex. Several workers have concluded that the P_{700} Chl *a*-protein is composed of a single 60 to 70 kD polypeptide species (5, 10, 19, 26). However, a broad protein band or two distinct protein bands in the 60 to 70 kD range have also been observed upon denaturation of the Chl-protein complex (2, 10, 20, 23, 27–29). One suggested explanation for these observations is that the two protein bands are the result of proteolytic degradation of a single component (10, 19).

The greatest P_{700} enrichment (low Chl/P_{700} ratios) is routinely observed in PSI preparations containing the 60 to 70 kD polypeptide(s) and additional polypeptide components of less than 25 kD (22). Further purification of the high mol wt polypeptide(s) often results in loss of P_{700} activity. This could be explained as resulting from denaturation of the Chl-protein complex by the harsh treatments required to remove additional polypeptides. Alternatively, the low mol wt components may be required to preserve P_{700} photochemical activity. Low Chl/P_{700} (40–60/1) ratios have been reported recently for higher plant isolates which appear to contain only the 60 to 70 kD polypeptide(s) (18).

As a first step toward studying the biosynthesis of the P_{700} Chl *a*-protein, we chose to reexamine the polypeptide composition of the photochemically active protein, characterize the component polypeptide(s) biochemically, and develop monospecific antibodies to the major protein component.

MATERIALS AND METHODS

Chloroplast Membrane Preparation. Membranes were prepared from 10- to 14-d-old greenhouse-grown *Hordeum vulgare* L. (cv Himalaya) seedlings. Leaves (10 g fresh weight) were chilled and homogenized at 4°C using a Polytron (Brinkman) with two, 5-s high speed bursts in 250 ml 0.5 M sucrose, 0.1 M NaCl, 1 mM DTT, 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged 5 min at 12,000*g*. The membrane pellet was washed twice by resuspension in 100 ml 0.1 M NaCl, 1 mM EDTA, 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0), and centrifugation for 10 min at 20,000*g*, twice.

The same procedure was used to prepare membranes from soybean (*Glycine max* L.), tobacco (*Nicotiana tabacum* L.), petunia (*Petunia* × *hybrida*), and tomato (*Lycopersicum esculentum*). *Chlamydomonas reinhardti* cells were harvested at 10,000*g* for 10 min, resuspended in the homogenization medium, and broken by one passage through a cooled French pressure cell (Aminco) at 8,000 p.s.i. Remaining steps were performed as for leaf homogenates.

Preparative Electrophoresis. Membranes were homogenized directly after isolation using 1 ml of 1% SDS (w/v), 10 mM Tris-glycine (pH 8.3) per mg Chl. The membrane detergent mixture was incubated no more than 10 to 15 min at room temperature and then insoluble material was removed by centrifugation at 15,000*g* for 1 min. Protein separation was performed on modified Laemmli SDS-gels (17) in which 25 mM Tris-glycine (pH 8.3) was used to buffer the 7.5% acrylamide separating gel. Electrophoresis of membranes (500–700 μl) on 0.3- × 11- × 14-cm slabs was carried out at 15°C for 4 h at 200 v. The slowest migrating Chl-

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containing band was excised and macerated. Protein was passively eluted overnight at 4°C with an equal volume (ml/ml gel macerate) of 10 mM Tris-glycine (pH 8.3) containing 0.05 to 0.10% Triton X-100. Eluted protein was separated from the gel by centrifugation through glass wool.

Spectroscopy. Absorption spectra and P_{700} measurements were recorded at room temperature using an Aminco DW-2 spectrophotometer. Corrected fluorescence excitation and emission spectra were recorded at room temperature on an Aminco SPF-500 spectrofluorometer. Chl content of whole membranes, detergent solubilized preparations, and isolated P_{700} Chl *a*-protein was determined in 80% acetone extracts according to Arnon (3). P_{700} content was determined from the reversible light-induced absorbance change at 697 nm according to Thornber (31) or by chemical difference spectroscopy (21).

Amino Acid and Amino Terminal Analyses. All solutions used for protein isolation were buffered with 50 mM Tris-HCl (pH 8.0) rather than Tris-glycine to avoid glycine contamination of the amino acid analyses. Protein prepared by preparative electrophoresis was dialyzed against double distilled H_2O and lyophilized. Samples were hydrolyzed in 5.7 N HCl for 24, 48, or 72 h at 100°C under vacuum. Samples were analyzed on a Dionex D-502 amino acid analyzer. Cysteine was determined after performic acid oxidation. Quantitative analyses were performed by addition of norleucine to the sample which was then used to estimate percentage recovery of Ala, Val, His, and Pro.

Amino terminal analysis was performed by combined Dansyl-Edman microsequencing (9).

Analytical Electrophoresis. Analytical electrophoresis was performed according to Laemmli (17). Gels were stained using Coomassie Brilliant Blue R, or silver stain using the procedure of Oakley *et al.* (24).

Peptide Mapping. The 58 and 62 kD polypeptides of the P_{700} Chl *a*-protein complex were isolated for CNBr[4] digestion or proteolysis by electrophoresis on 7.5% acrylamide gels. For CNBr digestion, gel slices were placed in 80% HCOOH which contained 10 or 100 mg/ml CNBr and then incubated overnight in the dark. After digestion, the HCOOH which contained the cleavage products was removed and evaporated to dryness under N_2. Dried peptides were solubilized in 2% SDS, 6 M urea, 50 mM DTT, 0.125 M Tris-HCl (pH 6.8), and analyzed on 15% acrylamide gels. BSA was cleaved as a control.

Partial proteolysis using *S. aureus* V-8 protease (Miles) was performed essentially according to Cleveland *et al.* (11). Gel slices containing the polypeptide to be analyzed were placed in sample wells of a second analytical gel composed of a 4-cm stacking gel (3% acrylamide) and a 10-cm separating gel (15% acrylamide). Protease (20 μl) at 5, 17, or 50 μg/ml in 0.1% SDS, 1 mM EDTA, 0.125 M Tris-HCl (pH 6.8) was overlayed and electrophoresis performed at 150 v for 7 h.

Antibody Preparation. Antibodies to the P_{700} Chl *a*-protein were raised in five New Zealand white female rabbits following standard procedures (35). Before inoculation, all rabbits were bled to obtain 30 to 50 ml preimmune serum. P_{700} Chl *a*-protein, isolated by preparative electrophoresis, was adsorbed to $AlNH_4(SO_4)_2 \cdot 12H_2O$ (alum) following the procedures of Jockusch *et al.* (15). No attempt was made to denature the protein before alum coupling. P_{700} photooxidation and dark reduction were observed in the alum-coupled protein, indicating that a portion of the protein retained native conformation.

Primary injections were made using 200 to 250 μg protein in 1.0 ml of 0.15 M NaCl, 10 mM Na phosphate (pH 7.0) emulsified with an equal volume of Freund's complete adjuvant (Miles). Subsequent injections of 100 to 200 μg protein were made in emulsions using Freund's incomplete adjuvant (Miles). A total of four injections, each a minimum of 2 weeks apart, were administered and animals bled for antiserum 8 to 10 d following the final injection.

Nitrocellulose Protein Blots. Proteins separated by analytical electrophoresis were transferred to nitrocellulose paper (0.45 μm, Schleicher and Schull) by passive capillary blotting. Gels were equilibrated for 10 to 15 min in 100 to 200 ml of 0.22 M Na phosphate, 0.02% Na_2N_3 (pH 7.0) and then placed between Whatman No. 1 paper (which served as a wick to a supply of phosphate buffer) and the nitrocellulose paper. Several inches of absorbent toweling and a 2-L weight were placed on top of the sandwich. After 12 to 18 h, 20% of the total gel protein was transferred.

The nitrocellulose-bound proteins were reacted with antiserum and [^{125}I]-protein A (NEN) according to Towbin *et al.* (33). Additional nitrocellulose binding sites were blocked by incubating with 1% gelatin, 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4) for 2 h at room temperature. Blots were incubated with antiserum or control serum (3–30 μl/ml, 1 ml/5 cm^2 nitrocellulose) in 0.25% gelatin, 0.05% NP-40, 0.005 M EDTA, 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4) for 1 to 2 h. Blots were rinsed for 1 to 2 h in the same buffer without serum and then incubated with 1 μl/ml of [^{125}I]-protein A (0.1 μCi, 89 μCi/μg protein A) as for the antiserum step. Blots were rinsed again and allowed to air dry before exposure to Kodak X-Omat AR 5 film with an intensifying screen (Cronex). Using this technique, it was possible to detect an original gel load of 16 ng of P_{700} Chl *a*-protein.

RESULTS

Yield and Spectral Characteristics. Figure 1 shows segments of a typical preparative gel used for P_{700} Chl *a*-protein isolation from barley chloroplast membranes. In the unstained segment (lane A), three Chl-containing bands are visible, the P_{700} Chl *a*-protein (or CPI), the Chl *a*/*b*-protein (or CPII), and free Chl which is not complexed to protein. When the gel is stained for protein (lane B), the P_{700} Chl *a*-protein is seen to be well separated from all other chloroplast membrane proteins. The Chl-protein complex has an apparent mol wt of approximately 120 kD. There is no evidence of protein banding at the position of the P_{700} Chl *a*-apoprotein (60 kD), indicating no significant dissociation of the Chl-protein complex occurred before or during the electrophoresis. There was also little or no staining material on top of the stacking (not shown) or separating gel. It is therefore reasonable to assume that all of the P_{700} Chl *a*-apoprotein present in the starting extract was recovered in the high mol wt Chl-protein complex.

All Chl-containing gel bands were excised, the protein and Chl eluted, and the Chl and P_{700} content quantified. Resulting estimates of the yield of Chl and P_{700} indicate that approximately 6 to 8% of the total Chl and 43 to 56% of the total P_{700} activity applied to the gel were recovered in the P_{700} Chl *a*-protein containing gel eluate (Table I). There is no indication that the unrecovered P_{700} activity is associated with an additional distinct Chl-binding protein. We hypothesize that incomplete recovery of activity results from inactivation of 50% of the P_{700} reaction centers during electrophoresis or gel elution. Consistent recovery of only 50 to 60% of the starting Chl is presumably due to Chl degradation and loss of Chl in low concentration in the interband regions.

The room temperature absorption characteristics of the isolated P_{700} Chl *a*-protein are presented in Figure 2. The red absorbance maximum, 674 to 676 nm, is shifted to a longer wavelength compared to the starting membrane extract (669 nm) as is expected for this complex (32). The complex has essentially no Chl *b* which is demonstrated by the absence of both a Soret band at 470 nm and a shoulder at 650 nm. Absorbance at 490 nm is attributed to β-carotene. There is no evidence of pheaophytin (545 nm) in the preparation. The complex has a corrected fluorescence emission

[4] Abbreviations: CNBr, cyanogen bromide; PAGE, polyacrylamide gel electrophoresis; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride.

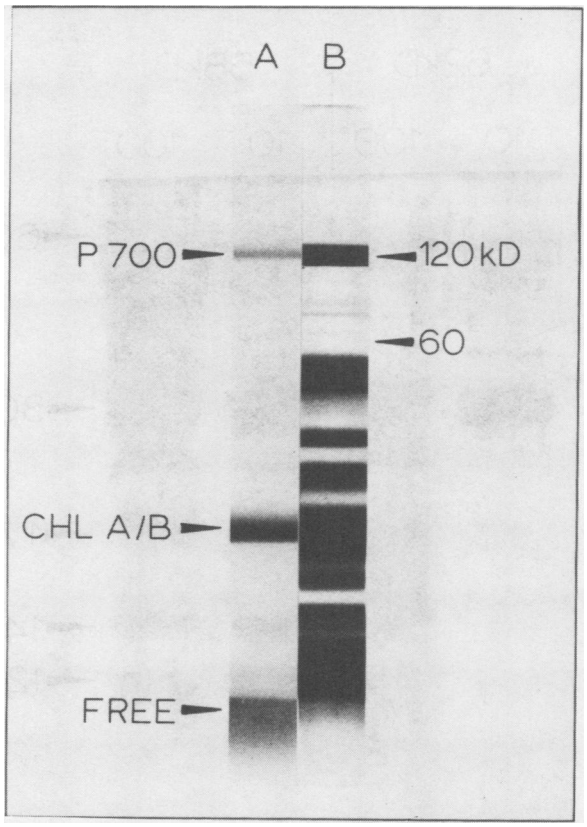

FIG. 1. Separation of barley chloroplast membrane proteins by preparative PAGE. Lane A, Slice of gel before staining. Lane B, Same gel slice stained with Coomassie blue to reveal other major membrane protein bands. Chl-proteins and free Chl as indicated. The mol wt of the Chl-binding form of the P_{700} Chl *a*-protein is 120 kD as indicated. There is no staining material at the position of the apoprotein (60 kD).

Table I. *Chl Content and P_{700} Activity of Whole Membranes and Gel Fractions*

Fraction	Chl	Chl *a/b*	Chl/P_{700}	Chl	P_{700}
	μg	*ratio*		*%*	
Whole membranes[a]	475	3.2	370	100	100
P_{700} Chl *a*-protein	36	>7.0	65	7.5	43
Chl *a/b*-protein	123	0.87	>9,000[b]	26	0
Free Chl	90	5.0	>9,000	18	0

[a] Fraction applied to the gel.
[b] >9,000—not detectable.

peak at 678 nm, and the fluorescence excitation spectrum lacks a band at 470 nm substantiating the absence of Chl *b* in the complex (data not shown; see Ref. 34).

The isolated P_{700} Chl *a*-protein exhibited photo-induced bleaching characteristic of the reaction center of PSI (Fig. 2, inset). In the presence of added methyl viologen and sodium ascorbate, the reaction center is oxidized in the light with a half-time of <2 s and re-reduced in the dark with a half-time of 10 s. No such signal is detected in the gel isolates of either the Chl *a/b*-protein or free Chl.

The wavelength dependence of the light-induced absorbance change shows a maximum at 697 nm (Fig. 3). The chemical difference spectrum has the same spectral properties and, in addition, confirms the absence of other redox components, such as Cyt *f* (λ max = 554 nm) and b_6 (λ max = 563 nm), which are associated with PSI in the thylakoid membrane (34). The ratio of Chl to P_{700} (mol/mol) in the gel isolates ranged from 50 to 60 Chl

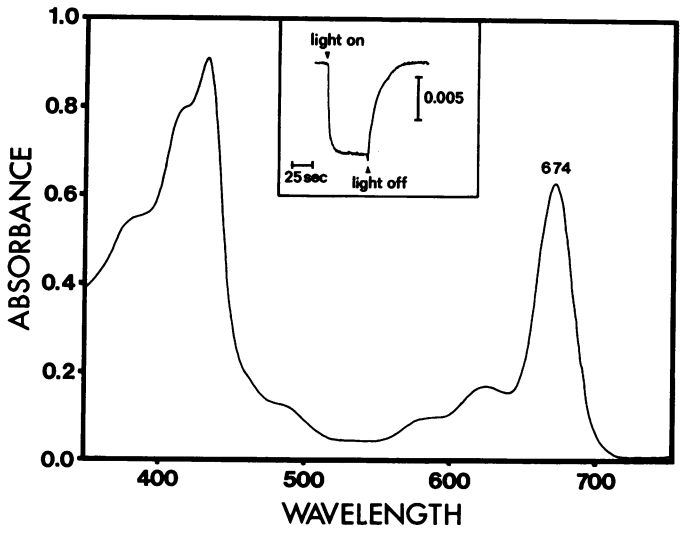

FIG. 2. Spectral characteristics of the isolated P_{700} Chl *a*-protein. Gel isolates were diluted 4- to 10-fold with H_2O or 10 mM Tris-glycine (pH 8.3) for spectroscopy. Inset shows the light-induced difference signal of the same preparation measured at 697 nm.

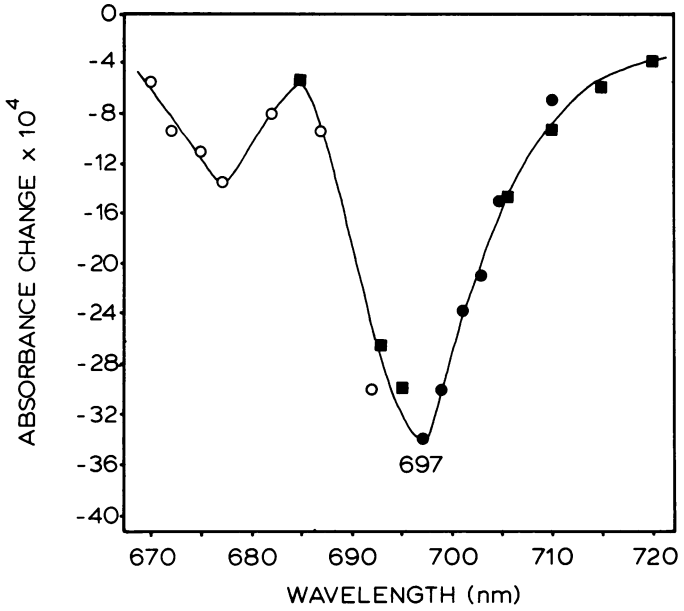

FIG. 3. Wavelength dependence of the magnitude of the light minus dark difference signal produced by the isolated P_{700} Chl *a*-protein. The absorbance change was measured at all wavelengths using a reference beam held constant at 720 nm. Different symbols indicate separate samples. Chl concentration was approximately 2.9 μM in all samples.

per P_{700} reaction center. This result was consistent when P_{700} was measured either by light-induced or chemical difference spectroscopy. The presence of 0.05 to 0.10% Triton X-100 during elution of the protein from the gel was required to obtain this level of P_{700} enrichment. In the absence of Triton X-100, measurable P_{700} activity was decreased by 50 to 70% (Chl/P_{700} = 120:1).

The same preparative procedure was used successfully for the isolation of the P_{700} Chl *a*-protein from petunia, tobacco, tomato, soybean, and *Chlamydomonas*. The isolated protein from all species had similar absorption spectra with red maxima of 676 nm. The Chl/P_{700} ratio of the complex was 50 to 60:1 for all species.

Analysis of Polypeptide Composition. The polypeptide composition of the photochemically active P_{700} Chl *a*-protein preparation was examined using analytical SDS-PAGE. When the Chl-protein

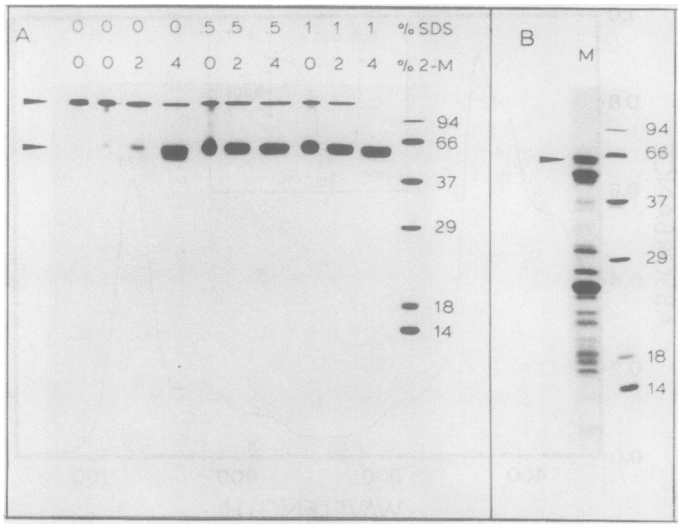

FIG. 4. Polypeptide composition of the P_{700} Chl *a*-protein. Panel A, Samples of the P_{700} Chl *a*-protein (approximately 5 μg) were made to the indicated final concentrations of SDS and 2-mercaptoethanol (% 2-M). All samples, except the sample to the far left (0% SDS, 0% 2-M). were heated to 40°C for 15 min before electrophoresis. Gel acrylamide concentration was 12.5%. Upper arrow indicates the 120 kD Chl-binding form; lower arrow indicates the apoprotein of the P_{700} Chl *a*-protein. Panel B, Sample of chloroplast membranes (M) solubilized in 2% SDS, 2% 2-mercaptoethanol, 10 mM Tris-glycine (pH 8.3). The P_{700} Chl *a*-apoprotein is indicated (arrow).

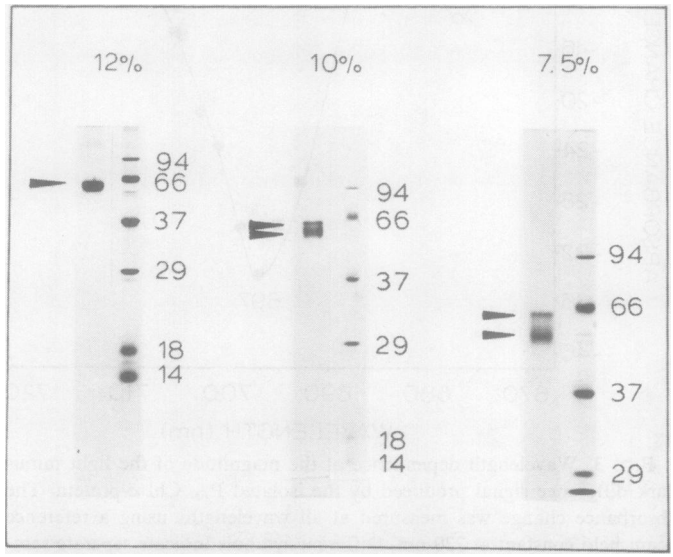

FIG. 5. Separation of the 58 and 62 kD components of the P_{700} Chl *a*-protein. Five μg of protein were denatured by heating at 50°C for 15 min in 1% SDS, 50 mM DTT, 10 mM Tris-glycine (pH 8.3) and separated on 12, 10, or 7.5% acrylamide gels.

was re-electrophoresed without the addition of SDS and 2-mercaptoethanol (or DTT), the P_{700} Chl *a*-protein migrates as a Chl-binding complex with a mol wt of approximately 120,000 (Fig. 4) demonstrating the stability of the complex as isolated. Addition of SDS and sulfhydryl reagent dissociates the 120 kD form to what appears to be a single polypeptide species of 62 kD, when analyzed on 12.5% acrylamide gels. No lower mol wt components are observed, and >95% of the total protein is accounted for by the 62 kD band as determined from densitometric scans. The absence of protein bands between the 120 and 62 kD forms of the protein, despite the obvious incomplete denaturation at lower SDS and 2-

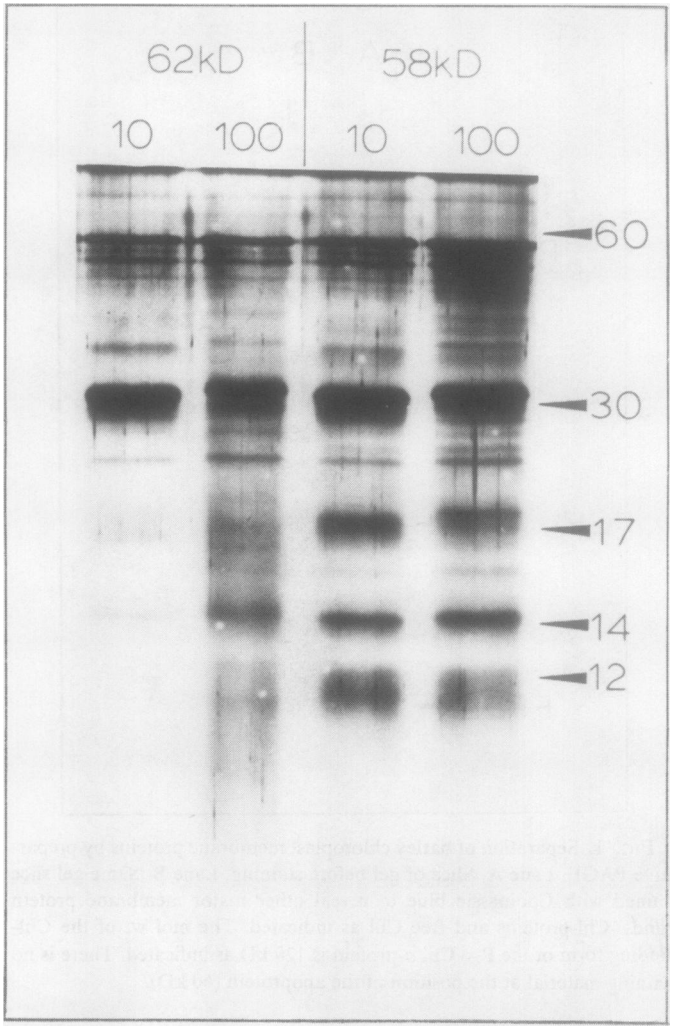

FIG. 6. Peptide fragments from CNBr digestion of the isolated 58 and 62 kD polypeptides. The CNBr concentration (mg/ml) used is indicated above each gel lane. Peptides were separated on 15% acrylamide gels and stained with silver.

mercaptoethanol concentrations, suggests that the 120 kD band is comprised of a maximum of two subunits.

Observations of a doublet at 58 to 62 kD in some experiments prompted further analysis of the polypeptide composition. Electrophoresis on 10% and 7.5% acrylamide gels clearly resolved two distinct polypeptide components, one at 62 kD and one at 58 kD (Fig. 5). The 58 kD component was always observed as a poorly resolved, diffuse component. The stoichiometry of the two polypeptides is 1:2, 62:58 kD, as estimated by integration of densitometric scans of the Coomassie blue-stained gels. The same polypeptide composition has been observed in all species examined (data not shown).

The possibility that the double-banded appearance was produced by electrophoresis was tested in two ways. After denaturation and electrophoresis in 7.5% acrylamide tube gels, the protein was subjected to a second dimension SDS-PAGE. The two components were observed to retain their integrity. Similarly, the 62 and 58 kD bands could be excised, eluted from the gel, and re-electrophoresed. In this experiment, they also migrated at their original mol wt.

Diffuse banding patterns are often exhibited by glycoproteins (14). We found (34) that neither component contains carbohydrate stainable by the periodic acid Schiff reaction (14). Recent work examining lectin binding to thylakoid membrane proteins con-

firms this result (16).

It has been suggested previously that the double-banded appearance of the P_{700} Chl *a*-apoprotein results from proteolytic degradation during isolation (10). However, two protein bands were also observed when PMSF (2 mM) was used throughout the membrane isolation and solubilization procedures. Furthermore, despite variation between isolations in the length of the different procedural steps during which proteolysis might be proposed to occur, in no case was only the 62 or 58 kD band observed, nor did the band stoichiometry appear to vary significantly between isolations.

Partial Pepide Mapping. In order to test critically the possibility that the 58 and 62 kD polypeptides represented distinct, unrelated protein components, CNBr digestions and partial proteolysis using *S. aureus* protease were performed (Figs. 6 and 7). CNBr analysis proved difficult, presumably due to the insolubility of the protein and peptide fragments. Cleavage performed and analyzed as described resulted in only partial digestion as judged from the predicted number of fragments based on Met content. The BSA control was similarly incompletely cleaved. However, comparison of the partial cleavage products of the 58 and 62 kD polypeptides reveal strikingly similar major fragment sizes of approximately 30, 17, 14, and 12 kD (Fig. 6). A number of the minor high mol wt bands present across the gel most probably represent gel or buffer contaminants detected by the sensitive silver staining procedure.

The homology, or identity, of the 58 and 62 kD polypeptides is further demonstrated by *S. aureus* partial digests (Fig. 7). Although variations in the band stoichiometries are evident, all fragments produced are detectable in digests of both polypeptides. It is particularly striking that the same major initial cleavage product

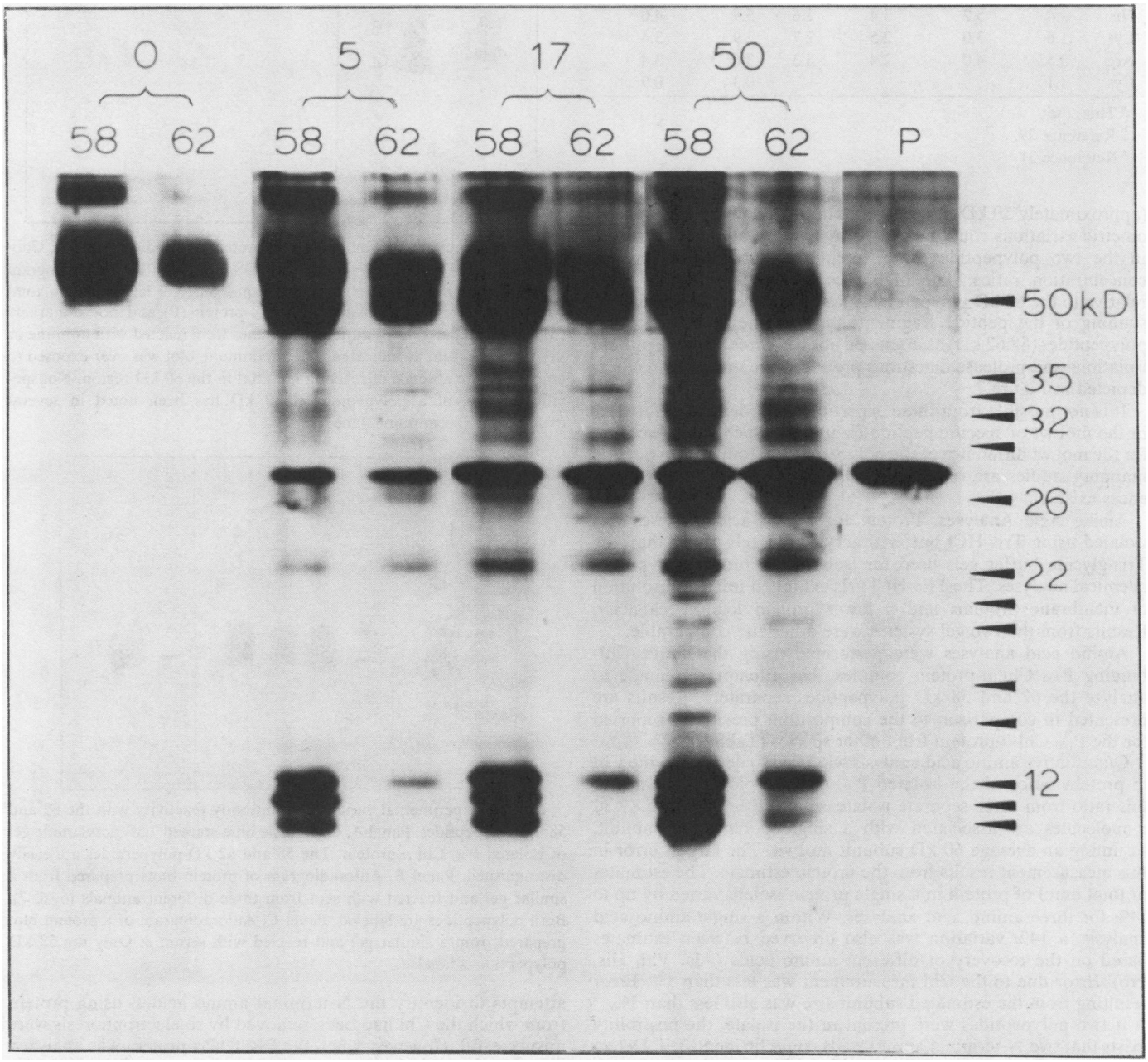

FIG. 7. Peptide fragments produced by *S. aureus* protease digestion of the 58 and 62 kD polypeptides. The polypeptide digested (58 or 62 kD) is indicated above each gel lane. Polypeptides were digested with 20 μl of 0, 5, 17, or 50 μg/ml protease as indicated. Lane P is 20 μl of 50 μg/ml protease. Arrows indicate fragments which are common in digests of both subunits. Gel was stained with silver.

Table II. *Amino Acid Composition of the P_{700} Chl a-Protein*

	Barley[a]	Spinach[b]	Tobacco[c]	Oat[c]	Beet[c]	Phormidium[c]
			mol %			
Asx	7.8	7.7		7.6	8.4	8.8
Thr	6.1	5.6	4.2	4.5	5.2	5.9
Ser	6.1	6.5	4.4	3.5	5.5	5.9
Glx	6.8	6.9	6.6	6.6	7.8	6.5
Pro	3.6	4.9	4.9	3.6	4.2	4.7
Gly	10.6	12.6	10.1	9.9	11.0	10.0
Ala	10.5	9.8	9.0	9.2	9.8	10.0
Val	4.5	4.7	5.6	4.8	6.3	6.2
Met	1.4	3.3			1.4	1.9
Ile	8.6	5.4	6.2	6.6	6.5	6.2
Leu	14.6	11.3	10.6	10.6	11.4	11.2
Tyr	2.6	3.0	1.8	2.2	2.7	3.1
Phe	7.2	6.1	6.8	6.2	6.8	6.2
His	3.4	5.2	3.4	3.6	5.9	4.0
Lys	1.6	3.0	2.5	2.7	2.9	3.4
Arg	3.5	4.0	2.4	3.3	3.6	3.4
Cys	1.1				0.3	0.9

[a] This study.
[b] Reference 29.
[c] Reference 31

(approximately 50 kD) is seen for both polypeptides. The stoichiometric variations could be caused by conformational differences in the two polypeptides or differences in protease to protein concentration ratios. The higher concentration of the 58 kD relative to the 62 kD polypeptide, as evidenced by the intensity of staining of the peptide fragments, reflects the 2:1 ratio of the polypeptides (58:62 kD) as discussed above. Three separate protein isolations and protease digestions produced the same fragments as depicted in Figure 7.

It is not possible from these experiments to identify differences in the mol wt of specific peptide fragments which would account for the mol wt difference of the polypeptides. More precise peptide mapping studies are required to determine whether such differences exist.

Amino Acid Analyses. Protein for amino acid analyses was isolated using Tris-HCl buffered acrylamide gels rather than the Tris-glycine buffer gels used for isolation of protein for photochemical analyses. The Tris-HCl gels exhibited inferior resolution of membrane proteins and a lower protein loading capacity. Results from the two gel systems were otherwise comparable.

Amino acid analyses were performed using the native Chl-binding P_{700} Chl *a*-protein complex. No attempt was made to analyze the 62 and 58 kD polypeptides separately. Results are presented in comparison to the composition previously reported for the P_{700} Chl *a*-protein from other species (Table II).

Quantitative amino acid analysis was used to determine the Chl to protein ratios of the isolated P_{700} Chl *a*-protein. Estimates of this ratio from three separate isolates showed that 4.5 to 5.8 Chl *a* molecules are associated with a single polypeptide subunit, assuming an average 60 kD subunit mol wt. The largest error in this measurement results from the protein estimate. The estimates of total nmol of protein in a single protein isolate varied by up to 14% for three amino acid analyses. Within a single amino acid analysis, a 14% variation was also observed between estimates based on the recovery of different amino acids (Ala, Val, His, Pro). Error due to the Chl measurement was less than 1%. Error resulting from the estimated subunit size was also less than 1%.

If two polypeptides were present in the isolate, the possibility exists that two N-terminal amino acids could be identified. Detection of a single N-terminal amino acid would be consistent, though not unequivocal, with the interpretation that the isolated P_{700} Chl *a*-protein is composed of a single polypeptide species. Initial attempts to identify the N-terminal amino acid(s) using protein from which the Chl had been removed by re-electrophoresis were unsuccessful. However, when the P_{700} Chl *a*-protein was analyzed directly after gel elution, a single N-terminal residue, Phe, was detected.

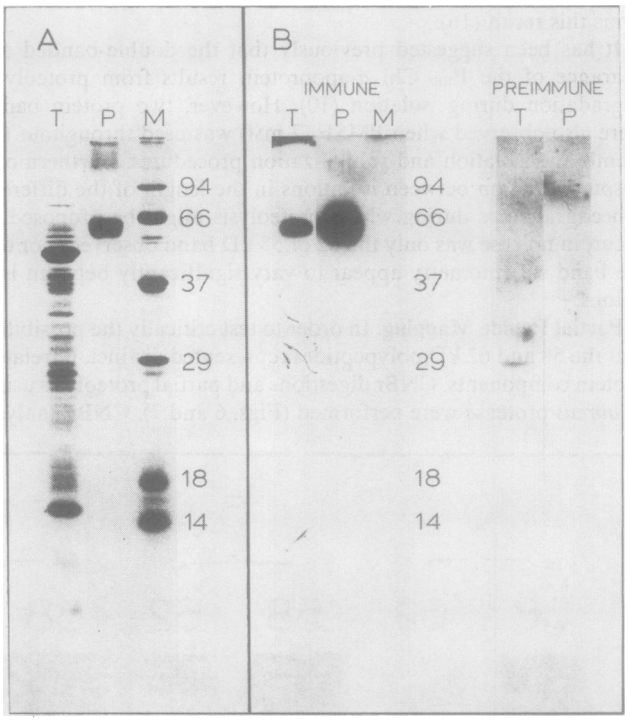

FIG. 8. Specificity of the P_{700} Chl *a*-protein antibodies. Panel A, Coomassie blue-stained 12.5% acrylamide SDS gel. Panel B, Autoradiogram of a protein blots prepared from the same gel. Gel lanes contain total barley leaf proteins (T), isolated P_{700} Chl *a*-protein (P), and mol wt markers (M). Blots prepared from duplicate gel lanes were reacted with immune or preimmune serum as indicated. The preimmune blot was over-exposed to emphasize the absence of reacting material in the 60 kD region. Nonspecific labeling of a polypeptide at 30 kD has been noted in several experiments (preimmune, lane T).

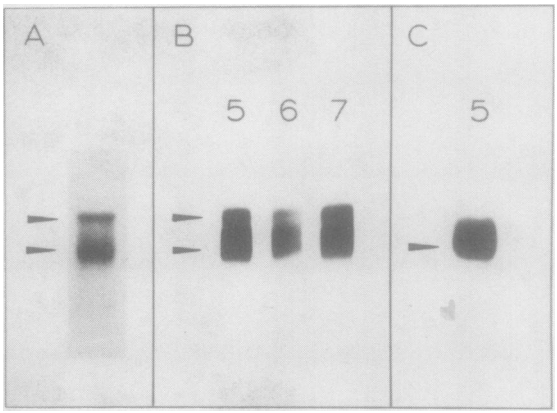

FIG. 9. Experimental variation in antibody reactivity with the 62 and 58 kD polypeptides. Panel A, Coomassie blue-stained 7.5% acrylamide gel of isolated P_{700} Chl *a*-protein. The 58 and 62 kD polypeptides are easily distinguished. Panel B, Autoradiogram of protein blots prepared from a similar gel and reacted with sera from three different animals (5, 6, 7). Both polypeptides are labeled. Panel C, Autoradiogram of a protein blot prepared from a similar gel and reacted with serum 5. Only the 58 kD polypeptide is labeled.

Antibodies. To demonstrate precisely the specificity of the anti-

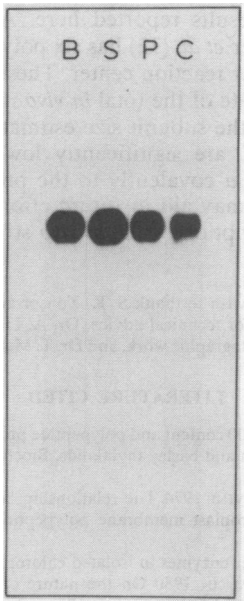

FIG. 10. Antibody recognition of the P_{700} Chl *a*-protein from other species. Autoradiogram of a protein blot comparing the P_{700} Chl *a*-protein from barley (B), soybean (S), petunia (P), and *Chlamydomonas* (C).

bodies generated to the P_{700} Chl *a*-protein, protein blotting experiments were performed. Isolated P_{700} Chl *a*-protein and total barley leaf proteins (from 10- to 14-d-old seedlings) were separated on 12.5% acrylamide gels and blotted to nitrocellulose. In experiments of this type, sera from all five animals inoculated reacted with a polypeptide component of identical mol wt as the denatured P_{700} Chl *a*-protein, and preimmune sera exhibited no reactivity with this polypetide (Fig. 8). When the 58 and 62 kD polypeptides of the P_{700} Chl *a*-protein were resolved on 7.5% acrylamide gels, in some experiments, both polypeptides were observed to react, while in other experiments preferential reaction with 58 kD polypeptide was observed (Fig. 9). The cause of this variation remains undetermined. The antibodies produced also showed greater reactivity with the denatured apoprotein than with the Chl-protein complex, although the photochemically active protein complex was used as an antigen. No evidence for the production of antibodies to Chl was obtained, and no cross-reactivity with the Chl *a*/*b* protein was observed.

The barley antibodies were shown to cross-react with the P_{700} Chl *a*-protein from soybean, tobacco, petunia, tomato, corn, and *Chlamydomonas*. Equivalent amounts (5 μg) of isolated P_{700} Chl *a*-protein from barley, soybean, petunia, and *Chlamydomonas* were denatured, electrophoresed, blotted to nitrocellulose, and processed as described (Fig. 10). Cross-reactivity was observed for corn and tomato on protein blots of whole leaf extracts (data not shown). No significant differences in intensity of the reaction were observed between species using this type of assay, except in the case of *Chlamydomonas* which exhibited somewhat less intense labeling.

DISCUSSION

A current model of P_{700} Chl *a*-protein structure *in vivo* proposes that two identical polypeptides of approximately 60 kD bind one PSI reaction center and 40 molecules of light harvesting Chl *a* (5, 6). However, numerous researchers have observed two poorly resolved high mol wt polypeptides, rather than a single polypeptide, upon dissociation of this Chl-protein (2, 19, 22, 23, 27, 29). Results of these workers were obtained using various isolation procedures involving different detergents such as SDS, Triton X-100, and digitonin, and different separation methods including gel electrophoresis, sucrose gradients, and isoelectric focusing. None of these studies have reported the reproducibility of these results, the stoichiometry of the two polypeptides, or the successful separation and characterization of the two components.

Results of the present study demonstrate that the P_{700} Chl *a*-protein can be routinely and reproducibly dissociated into two electrophoretically distinct polypeptide species which migrate at 58 and 62 kD in the electrophoretic system employed. Although on 12.5% acrylamide SDS-gels these components migrate as a single protein band, the two polypeptides have been readily resolved using 7.5 to 10% acrylamide gels. These two polypeptide species have also been observed in P_{700} Chl *a*-protein preparations from all species examined to date. These data suggest that observations by other workers identifying a single polypeptide component (5, 10, 20, 26) may have in some cases resulted from the use of lower resolution gel electrophoresis. Furthermore, the 58 kD polypeptide is difficult to detect in total thylakoid membrane protein profiles because of its diffuse banding pattern. This fact could also lead to the failure in previous studies to recognize the 58 kD polypeptide as a component of the P_{700} Chl *a*-protein.

Although the 58 and 62 kD polypeptides have been isolated electrophoretically as distinct components, peptide data demonstrate that the two polypeptides are largely homologous in primary structure. One-dimensional, partial peptide maps generated by two different methods, CNBr cleavage and *S. aureus* proteolysis, show that the same peptide fragments are produced from the 58 and 62 kD polypeptides. These results also demonstrate that the 58 kD polypeptide is not a contaminant polypeptide unrelated to the P_{700} Chl *a*-protein. Nechustai and Nelson (23) have examined *S. aureus* proteolytic digests of the high mol wt polypeptides from a *Chlamydomonas* PSI preparation. Although the two polypeptides were poorly resolved in their preparation, their results support our conclusion that the polypeptides are similar.

It is not possible from these studies to determine definitively the molecular nature of the observed difference in electrophoretic mobility of these homologous polypeptides. The apparent mol wt difference could result from differences in primary protein structure, or from stable variations in secondary structure or differential post-translational protein modifications of polypeptides with identical primary structure. In at least two reports, the observation of two polypeptides has been attributed to proteolysis during isolation (10, 19). Although it is difficult to unambiguously rule out this possibility, our data indicate that proteolysis is not involved. First, isolation in the presence of PMSF did not alter the polypeptide composition. Second, reproducibility of the results and failure to observe only the 58 kD polypeptide (the presumed product of proteolysis), indirectly argue against a proteolytic origin of the two polypeptides. Based on the assumption that the protein is encoded by the chloroplast genome (13), there is only a single copy of the P_{700} Chl *a*-protein gene. This excludes the possibility that the two polypeptides are the products of a gene family as is the case for the nuclear encoded Chl *a*/*b*-protein (18). Therefore, differences in primary structure, if not generated artifactually duing isolation, would have to result from differential post-translational protein processing or differential post-transcriptional processing of the apo-protein mRNA. No data are available addressing these latter possibilities. Thus, it is reasonable to propose that the 58 and 62 kD polypeptides represent altered forms of a single component.

Inability to interconvert the 58 and 62 kD polypeptides, as judged from re-electrophoresis experiments, suggests that a stable difference exists between the two polypeptide forms. Nechustai and Nelson (23) briefly speculate that the mol wt heterogeneity of the polypeptides from *Chlamydomonas* arises from post-translational modification. There is no evidence that the P_{700} Chl *a*-protein from any plant species is modified by glycosylation (16, 34) or phosphorylation (7). Other protein modifications have not

been examined to date. We suggest that the mol wt heterogeneity arises from irreversible denaturation of identical polypeptides into two different denatured conformations which exhibit different mobilities in SDS gels. The equilibium between these conformations could be determined by membrane isolation, denaturation, and/or electrophoresis conditions. Recent data of Schmidt *et al.* (28) indicate that ionic conditions may regulate the apparent mobility of both the holo- and apoprotein forms of the P_{700} Chl *a*-protein. Using the same membrane preparation, they observed two polypeptides in the absence of Mg^{2+} during electrophoresis, while 5 to 10 mM Mg^{2+} produced a single protein band. Ionic effects which could alter secondary structure or SDS binding were not examined in our study and deserve further consideration. The equilibrium between the 58 and 62 kD forms may also reflect *in vivo* heterogeneity in orientation of the polypeptides within the thylakoid membrane.

We have used the P_{700} Chl *a*-protein to raise successfully rabbit antibodies which specifically react with the apoprotein. These antibodies recognize the apoprotein from a wide variety of plant species. The observed preferential antibody reactivity with the 58 kD polypeptide may also be explained by conformational differences between the 58 and 62 kD polypeptides; such differences could restrict the available antigenic sites. Experiments demonstrating that the antibodies recognize the same CNBr peptide fragments from both polypeptides further support this conclusion (34). These antibodies should prove useful in studies of P_{700} Chl *a*-protein structure and biosynthesis.

Other SDS-PAGE preparations of the P_{700} Chl *a*-protein which exhibit P_{700} activity have been described recently (1, 4, 18). However, limited photochemical (18) or biochemical analyses (1, 4) of these preparations have left the relationship of polypeptide composition to activity ambiguous. Combined with the protein analysis, the spectral and activity measurements presented here clearly demonstrate that the P_{700} reaction center is associated with the same polypeptide subunits responsible for binding PSI associated Chl *a*. We have found that addition of 0.05 to 0.10% Triton X-100 during elution of the protein from the SDS-gel acts to preserve P_{700} activity and to prevent pheaophytinization of Chl *a* which is frequently observed in other SDS-treated PSI isolates. These results indicate that the lower mol wt polypeptides (less than 25 kD) generally present in other PSI preparations are not required for P_{700} activity *in vivo* although they may act to stabilize the complex *in vivo* and *in vitro*.

Measurements of the Chl to protein ratio and the Chl *a* to P_{700} ratio of the isolated P_{700} Chl *a* protein result in a calculated ratio of one P_{700} reaction center per 8 to 12, 60 kD subunits. However, while our data indicate essentially all of the P_{700} Chl *a*-apoprotein present in the starting extract was recovered, only 50% of the P_{700} activity was recovered. Also, assuming that 50 to 60 Chl *a* molecules are associated with each functional P_{700} Chl *a*-protein, based on the ratio of Chl *a* to P_{700} in the starting membrane extract (400:1), 12 to 15% of the total membrane Chl should be associated with the P_{700} containing fraction. Approximately half this amount was obtained. Therefore, it would appear that one-half of the Chl *a* associated with the protein *in vivo* has been lost during the isolation procedure. This loss of Chl *a* could also explain the 50% recovery of P_{700} activity. Thus, from our data it is reasonable to propose, accounting for the unrecovered Chl *a* and P_{700} activity, that four to six polypeptide subunits are associated with each P_{700} reaction center *in vivo*.

Bengis and Nelson (5, 6) have derived a two-subunit model for the P_{700} Chl *a*-protein. This model assumes one reaction center is specifically associated with 140 kD of protein, as opposed to 240 to 360 kD of protein as is suggested by our data. In their procedure, 10% of the total P_{700} activity was recovered; however, no estimate of apoprotein recovery was made. Therefore, differential yield of protein and activity may explain the discrepancies between this earlier model and results reported here. An alternative model proposed by Thornber *et al.* (32) has six polypeptides, with a total mol wt of 300 kD, per reaction center. These values more closely agree with our estimate of the total *in vivo* mol wt of the P_{700} Chl *a*-protein. However, the subunit size estimates of Thornber *et al.* (32), 45 and 48 kD, are significantly lower than we observe. Methods to link Chl *a* covalently to the protein and alternative isolation procedures may aid in future efforts to provide a complete molecular description of the *in vivo* strucure of the P_{700} Chl *a*-protein complex.

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