Identification of a Chloroplast-encoded 9-kDa Polypeptide as a 2[4Fe-4S] Protein Carrying Centers A and B of Photosystem I*

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The photochemical transfer of electrons from reduced plastocyanin to ferredoxin is catalyzed by PS-I. The complex is known to contain P700, the reaction center chlorophyll of PS-I, and several electron acceptors which become reduced upon illumination. The signals obtained by a large number of spectrophotophysical techniques (e.g. ESR and chemically induced dynamic electron polarization) have revealed the involvement of at least five different electron acceptors denoted A0, A1, X, B, and A (1–3). Whereas spectrophotophysical techniques have been very useful in detecting these acceptors in complex preparations, the same techniques are of limited value in determining the identity of the chemical structures giving rise to the signals detected. As a result, the chemical identity of P700 and centers A0 and A1 still remains unresolved (1–3). Mössbauer and ESR spectrometry indicated that centers B and A are iron-sulfur centers (4–7). Based on microwave power saturation studies, these two centers were further assigned as [4Fe-4S] clusters (8). The ESR spectrum of center X also had some resemblance to those of iron-sulfur centers (9), and Mössbauer spectroscopy suggested that center X could be a [4Fe-4S] center (10). However, microwave power saturation studies indicated that center X was not a typical [4Fe-4S] or [2Fe-2S] center and that the spectrum possibly represented a chlorophyll anion magnetically interacting with iron (8).

An alternative approach to study PS-I is to characterize the structural and functional role of each of the PS-I polypeptides. Using this strategy, Høj and Müller (11) and Golbeck (2) provided biochemical evidence demonstrating that the 82-kDa polypeptides of P700-chlorophyll a-protein 1 bind an iron-sulfur center, most likely center X. PS-I preparations contain additional polypeptides of lower molecular mass (2, 3, 11). Although the number reported varies, four polypeptides of approximate molecular masses 18, 16, 14, and 9 kDa appear to belong to the PS-I core (2, 3, 11). The 18-kDa polypeptide has been claimed to carry the iron-sulfur centers A and B (12, 13). However, this identification was based solely on correlation between the gradual depletion of the 18-kDa polypeptide and the disappearance of centers A and B as monitored by ESR spectroscopy. Malkin et al. (14) isolated an 8-kDa polypeptide from chloroplasts of spinach (Spinacia oleracea). The

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The abbreviations used are: PS-I, photosystem I; DTT, 1,4-dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-N-(morpholino)ethanesulfonic acid; ORF, open reading frame; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
preparation showed an ESR spectrum similar to that of a ferredoxin but different from those of centers, A, B, and X. It remains to be established whether the reported spectra could have been derived from an artificial complex of similar molecular mass and generated from cysteine sulfide. As demonstrated by Høj and Møller (11), the coincidental co-migration of such artificially formed oligomeric complexes with the 18- and 16-kDa polypeptides was previously interpreted to indicate that these two polypeptides were binding the vesicles to chaotropic agents. From its ability to bind zero-valence sulfur (20) and for their polypeptide content. Fractions rich in the 9-kDa polypeptide were combined, lyophilized, dissolved in a minimal volume of AcA buffer, and finally applied to a column (1.6 x 95 cm) of AcA 34 equilibrated in AcA buffer and eluted with the equilibration buffer at a flow rate of 2 cm/h. Fractions judged by SDS-PAGE to contain pure 9-kDa polypeptide were combined, lyophilized, and stored at -80 °C.

18- and 16-kDa Polypeptides—PS-I vesicles (146 ml) obtained from the AcA column were concentrated to a volume of 10 ml, diluted with 120 ml of 20 mM imidazole HCl (pH 7.8), and concentrated to 17 ml by ultrafiltration. Solid urea (7 g) was added to the concentrated PS-I vesicles (16 ml, 1.20 mg of chlorophyll/ml), and the material was applied (25 ml/h) to a column (0.9 x 46 cm) of Polybuffer Exchanger 94 (Pharmacia Biotechnology AB) equilibrated in 20 mM imidazole (pH 7.6), 5 mM urea. Washing of the column with 160 ml of equilibration buffer served to elute the major part of the 18-kDa polypeptide originally bound to PS-I. The column was subsequently washed with 75 ml of 25 mM imidazole (pH 7.6) and eluted with 60 ml of 25 mM imidazole HCl (pH 7.6), 0.1% (w/v) Triton X-100, 0.05% Empigen BB (Albright & Wilson Ltd., Marchon Works, Whitehaven, Cumbria, United Kingdom) which eluted some of the 16-kDa polypeptide in a homogenous form. P700-chlorophyll a-protein 1, the 9- and 14-kDa polypeptides, and some of the 16-kDa polypeptides were eluted by applying 7-fold diluted Polybuffer 74/HC (pH 4.5), 0.1% (w/v) Triton X-100 to the column. The fractions containing P700-chlorophyll a-protein 1 were concentrated by ultrafiltration and treated with NaSCN as described above for the 9-kDa polypeptide. These manipulations allowed the isolation of a particle which contained only P700-chlorophyll a-protein 1 and the 14- and 9-kDa polypeptides.

Polycrylamide Gel Electrophoresis

Analytical SDS-PAGE was carried out in slab gels at 6 °C according to Fling and Gregerson (21) with a 5% stacking gel (1.5 cm) and an 8-25 or 18% resolving gel (18 cm), both containing 0.1% SDS. Acrylamide was present in the gel and in the stacking gel. The SDS concentration was lowered to 0.033% and the length of the stacking and resolving gel was 1 and 6 cm, respectively. When stated, the cysteine residues of the PS-I vesicle and isolated polypeptides were labeled by S-carbamoylmethylation using iodo[14C]acetamide in the presence of 2-mercaptoethanol and 8 M urea (22) before analyses by SDS-PAGE and autoradiography. Apparent molecular masses were deduced from the electrophoretic mobility of the following standards: catalase, aldolase, bovine serum albumin, ovalbumin, and cytochrome c. Determination of the amount of Coomassie Brilliant Blue R-250 bound to individual polypeptides after staining and destaining of the polycrylamide gels was carried out according to Ball (23).

Amino Acid Analysis and Sequencing

Samples were hydrolyzed with 6 N HCl at 110 °C in sealed, evacuated tubes for 24 h, and the hydrolysates were analyzed using a Durrum D500 amino acid analyzer. Half-cystine was determined after performic acid oxidation (24) or by S-carbamoylmethylation. S-Carbamoylmethylation of isolated polypeptides was carried out by dialyzing the purified proteins against several hundredfold excess of 20 mM ammonium acetate (pH 6.3), 0.05% (w/v) Triton X-100, followed by lyophilization. The lyophilized protein sample was dissolved in 7 M guanidine chloride, 0.2 M Tris/HCl (pH 8.0), 5 mM EDTA, and 1% Empigen BB and autoclaved. Apparent molecular masses were deduced from the electrophoretic mobility of the following standards: catalase, aldolase, bovine serum albumin, ovalbumin, and cytochrome c. Determination of the amount of Coomassie Brilliant Blue R-250 bound to individual polypeptides after staining and destaining of the polycrylamide gels was carried out according to Ball (23).
Lyophilized polypeptide material was easily dissolved in 30% (v/v) H2O. Amino acid sequences were determined with both a Beckman 890C spinning cup sequenator (25) and an Applied Biosystems gas-phase sequenator Model 479A using the program provided by the company. Phenylthiodyantoins were identified by reverse-phase high-pressure liquid chromatography as described by Svendsen et al. (26). C-terminal amino acid analysis (27) was carried out by carboxypeptidase Y (649 ng) digestion of the S-carbamoylmethylated 9-kDa polypeptide (13 nmol) in a reaction mixture (150 μl) containing 40 mM Mes (pH 6.5), 0.5% SDS, and 14 nmol of norleucine as an internal standard. Aliquots withdrawn at different times were acidified (pH 2.2) to stop the enzymatic reaction and used directly for amino acid analysis.

Acid-labile Sulfide and Zero-valence Sulfur

Acid-labile sulfide and zero-valence sulfur were determined as described by Haj and Müller (20) using the methylene blue procedure with EtOAc extraction steps included to avoid interference from chlorophyll. To secure accurate spectrophotometric determination of methylene blue, the absorption spectrum (500–750 nm) of each sample was recorded using an Amino DW-2c spectrophotometer with a typical full-scale setting of 0.05. The absorbance at 660 nm was determined from the spectra. Barley ferredoxin was isolated essentially as described (28) and used as a reference.

Additional Analytical Procedures

Thylakoids 35S-labeled in vivo were obtained as previously described (11). Chlorophyll was determined according to Arnon (29). P700 was quantitated from its ferricyanide-oxidized minus ascorbate-reduced spectrum using an Amino DW-2c spectrophotometer and an extinction coefficient of 64 mmol cm−1 (30).

RESULTS

Compared to our previous study (11), an ion-exchange chromatography step has been introduced in the preparation of the PS-I vesicles from barley. This allows processing of large amounts of starting material and eliminates contamination with chloroplast coupling factor (Fig. 1, lane 1). The isolation procedure here reported has routinely been used to isolate PS-I vesicles from thylakoids containing several hundred milligrams of chlorophyll. The yield of P700 is approximately 1 nmol/mg of chlorophyll of the thylakoids. This corresponds to a yield of 30%. The PS-I preparation used in Ref. 11 had a chlorophyll to P700 ratio of 110, whereas the ratio is 60 in the present preparation. This difference is explained by the prolonged exposure time to Triton X-100 caused by inclusion of the ion-exchange step.

In the PAGE systems previously used, the major PS-I polypeptides were assigned molecular masses of 70 (doublet), 18, 15, 10, and 8 kDa (11). In this study, the polypeptide composition of the isolated PS-I vesicles was analyzed in the high Tris gel system of Fling and Gregerson (21). Although devoid of urea, this system proved superior in focusing the low molecular mass polypeptides of PS-I. Based on the electrophoretic mobility of known standards in the high Tris system, the calculated apparent molecular masses of the major PS-I polypeptides were 82 (doublet), 18, 16, 14, and 9 kDa, respectively. Apparent molecular masses of 82 kDa for the two apoproteins of P700-chlorophyll a-protein 1 are in close agreement with the molecular masses predicted from the nucleotide sequence of their genes (31). The band at 105 kDa represents P700-chlorophyll a-protein 1 which has not been converted into the apoprotein. The band at 195 kDa is probably a dimer of P700-chlorophyll a-protein 1. A minor component migrating just above the 9-kDa polypeptide was observed. In some preparations, an additional minor component was observed in the 23-kDa region. This component is thought to represent residual amounts of light-harvesting chlorophyll-protein I (32) and was not detectable in most of the PS-I preparations.

Fig. 1. Analysis of the polypeptide composition of purified PS-I vesicles and isolated polypeptides by SDS-PAGE. Electrophoresis was carried out overnight at 6 °C using an 8–25% high Tris gradient gel. Unless otherwise indicated, the gel was stained with Coomassie Brilliant Blue R-250 (CBB) and visualized by alkaline silver staining; lane 1, purified PS-I vesicles; lane 2, isolated 18-kDa polypeptide; lane 3, isolated 16-kDa polypeptide; lane 4, isolated 9-kDa polypeptide after extensive handling; lane 5, isolated 9-kDa polypeptide; lane 6, thylakoids; lane 7, PS-I vesicles devoid of the 18- and 16-kDa polypeptides as obtained after NaSCN treatment of PS-I vesicles originally treated with urea (the bands were visualized by alkaline silver staining); lane 8, purified PS-I vesicles obtained from plants 35S-labeled in vivo; lane 9, autoradiography of sample in lane 8.

The Coomassie Brilliant Blue R-250 bound to the individual polypeptide bands of the PS-I preparation was eluted from the gels and quantified spectrophotometrically (23). Normalization based on their apparent molecular masses and a uniform binding of Coomassie Brilliant Blue R-250 gave the following stoichiometry for the five main components: 2:0:1:2:1.2:0:5:1:1, indicating a stoichiometry in the native PS-I complex of 4:2:2:1:2 for the apoproteins of P700-chlorophyll a-protein 1 and the 18-, 16-, 14-, and 9-kDa polypeptides, respectively.

The relative distribution of sulfur amino acids among the PS-I polypeptides was assessed by electrophoresis and autoradiography of a 35S-labeled PS-I preparation obtained from barley seedlings grown in the presence of [35S]sulfate (Fig. 1, lanes 8 and 9). In agreement with earlier observations (11, 17, 33), the 18- and 14-kDa polypeptides were found to incorporate small amounts of 35S label, whereas the 16-kDa polypeptide was not labeled at all. P700-chlorophyll a-protein 1 and the 9-kDa polypeptide were both strongly labeled. The superimposition of the labeled band at 9 kDa with that obtained by Coomassie Brilliant Blue R-250 staining (Fig. 1, lanes 8 and 9) shows that the reported (17, 34, 35) inability to visualize the 9-kDa polypeptide by Coomassie Brilliant Blue R-250 staining was not due to an intrinsic property of the protein, but merely reflected the poor characteristics of the SDS-PAGE systems earlier used. Specific assessment of the content of cysteine residues in the individual PS-I polypeptides was achieved by [35S]-carbamoylmethylation of the PS-
I preparation from spinach (17), but is in agreement with the published nucleotide sequences of the genes encoding the two apoproteins (36).

An isolated intact iron-sulfur protein can be detected by its property to release acid-labile sulfide, whereas a denatured iron-sulfur protein may retain acid-labile sulfide in the form of zero-valence sulfur (37). Incubation of the PS-I preparation with 3.4 M NaSCN and subsequent gel filtration on AcA 34 allowed collection of fractions containing varying amounts of the 18-, 16-, and 9-kDa polypeptides as monitored by SDS-PAGE and Coomassie Brilliant Blue R-250 staining of each individual fraction. The fractions also contained zero-valence sulfur, whereas no acid-labile sulfide was detectable. The elution of zero-valence sulfur correlated with that of the cysteine-rich 9-kDa polypeptide, but not with that of the 18- and 16-kDa polypeptides. Fractions rich in zero-valence sulfur were combined and dialyzed. Subsequent chromatography on CM-Sepharose CL-6B followed by a final gel filtration step on AcA 34 resulted in a homogeneous preparation of the 9-kDa polypeptide. From both columns, the elution of the 9-kDa polypeptide and zero-valence sulfur coincided. The homogeneity of the isolated 9-kDa polypeptide was assessed by SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining (Fig. 1, lane 5) and autoradiography after 14C-S-carbamoylmethylation (Fig. 2, lane 6). The zero-valence sulfur contained in the isolated 9-kDa polypeptide was stable to dialysis at pH 5 and to lyophilization and was therefore bound covalently to the polypeptide backbone, most likely as a trisulfide (37). The isolated polypeptide lacked chromophores absorbing around 420 nm. Such chromophores are present in native ferredoxins (37). Extensive handling of the isolated polypeptide, e.g., by repeated lyophilizations, tended to generate traces of a component of slightly faster electrophoretic mobility (Fig. 1, lane 4).

The procedure developed to purify the 9-kDa polypeptide also provided homogeneous preparations of the 18- and 16-kDa polypeptides. However, large amounts of these two polypeptides were more easily obtained after treatment of the PS-I preparation with 6 M urea. When such an extract was applied to a column of Polybuffer Exchanger 94 equilibrated in 20 mM imidazole HCl (pH 7.6), 5 M urea, the 18-kDa polypeptide did not bind to the column and could be collected in a homogeneous form (Fig. 1, lane 2). Upon subsequent washing of the column with 25 mM imidazole HCl (pH 7.6), 0.1% Triton X-100, 0.05% Empigen BB, a proportion of the 16-kDa polypeptide was released in a homogeneous form (Fig. 1, lane 3). A preparation containing P700-chlorophyll a-protein 1 and low molecular mass polypeptides was obtained by elution with Polybuffer 74 (pH 4.5). Subsequent treatment of this preparation with NaSCN followed by gel filtration resulted in a preparation containing P700-chlorophyll a-protein 1 and the 14- and 9-kDa polypeptides (Fig. 1, lane 7).

A specific association of acid-labile sulfide or zero-valence sulfur with the 18- or 16-kDa polypeptides was not observed under any of the isolation procedures tested. Even when purified, the 16-kDa polypeptide was not reactive toward iodo-1-14C] acetamide (Fig. 2, lane 5), whereas a weak labeling was obtained with the 18-kDa polypeptide (Fig. 2, lane 4). A condition for the successful application of the purification procedures reported here to obtain homogeneous preparations of the 18-, 16-, and 9-kDa polypeptides of PS-I is the use of a highly purified preparation of PS-I vesicles as the starting material.

The amount of zero-valence sulfur bound to the isolated 9-kDa polypeptide was quantitated with native PS-I as a standard (Fig. 3). Increasing amounts of PS-I were subjected to
SDS-PAGE. After electrophoresis, the Coomassie Brilliant Blue R-250 specifically bound to the 9-kDa polypeptide band of each gel lane was quantitated spectrophotometrically (Fig. 3B) (23). Identical amounts of the PS-I preparation were assayed for acid-labile sulfide and zero-valence sulfur (Fig. 3A), and two standard curves were constructed. In an analogous manner, the Coomassie Brilliant Blue R-250 bound to the purified 9-kDa polypeptide after SDS-PAGE and the corresponding content of zero-valence sulfur were determined. The yield of zero-valence sulfur obtained by assaying the 9-kDa polypeptide from two different preparations was 32 and 37% of that obtained when assaying an amount of native PS-I containing exactly the same amount of 9-kDa PS-I. However, when the native PS-I vesicle was subjected to a NaSCN treatment analogous to that used to isolate the 9-kDa PS-I, the recovery of acid-labile sulfide was less than 60% even after incubation with DTT. It is therefore evident that the native 9-kDa polypeptide must bind a major part of the acid-labile sulfide of the native PS-I particle.

To determine the relative distribution of zero-valence sulfur between P700-chlorophyll a-protein 1 and the 9-kDa polypeptide under identical experimental conditions, the purified PS-I vesicles were subjected to preparative SDS-PAGE. After electrophoresis, the gel was cut into narrow horizontal segments. The content of acid-labile sulfide and of zero-valence sulfur was determined after DTT incubation as described (20). Small, equally sized parts of each segment were used for re-electrophoresis to establish the polypeptide composition in the segments and to spectrophotometrically quantify the polypeptides present (23). When an 8–25% gradient gel was used, between 30 and 35% of the recovered acid-labile sulfide was associated with P700-chlorophyll a-protein 1, whereas 65–70% was associated with polypeptides in the low molecular mass region (Fig. 4). To separate the 9-kDa polypeptide more efficiently from those at 18, 16, and 14 kDa, a similar experiment using an 18% resolving gel was performed (Fig. 5). This experiment demonstrated that the acid-labile sulfide recovered after incubation with DTT was derived from the 9-kDa polypeptide. Thus, after separation of the polypeptides of the PS-I vesicle by SDS-PAGE, 65–70% of the acid-labile sulfide recovered after DTT treatment is associated with the 9-kDa polypeptide, whereas 30–35% resides in the large polypeptides of P700-chlorophyll a-protein 1. The apoproteins of P700-chlorophyll a-protein 1 barely enter the 18% resolving gel, which is therefore not suitable for determination of the relative distribution of zero-valence sulfur between P700-chlorophyll a-protein 1 and the low molecular weight polypeptides.

It was of interest to obtain sequence information on the isolated 9-kDa polypeptide. The S-carbamoylmethylated 9-kDa polypeptide was therefore subjected to Edman degradation in a liquid-phase spinning cup sequenator (24) as well as a gas-phase sequenator (25). The sequence for the 29 N-terminal residues of the isolated 9-kDa polypeptide is shown on Fig. 6. The spacing of the 4 identified cysteine residues is strongly indicative of a [4Fe-4S] protein (38). Amino acid analysis of the 9-kDa polypeptide after treatment with performic acid (24) revealed the presence of 8 cysteine residues/78 amino acids (Table I), indicating that the protein might be a [2[4Fe-4S]] protein. Very recently, the complete nucleotide sequences of chloroplast DNA from the liverwort Marchantia polymorpha (39) and from tobacco (Nicotiana tabacum) (40) have been determined. Ohyama et al. (39) pointed out that the M. polymorpha sequence contained two ORFs denoted frxA and frxB in which the periodic appearance of cysteine residues resembled that of [4Fe-4S] ferredoxins. When the N-terminal sequences predicted from these two ORFs were compared with the N-terminal amino acid sequence of the 9-kDa polypeptide isolated from PS-I vesicles of barley, the homology with frxA, but not with frxB, was striking and leaves no doubt that the isolated PS-I polypeptide is coded for by the corresponding ORF on the barley chloroplast genome (Fig. 6). To accord with the nomenclature used by Ohyama et al. (39) and Gray et al. (41) for the previously identified chloroplast genes encoding membrane proteins catalyzing light reactions of photosynthesis, we designate this gene as psaC. The psaC of the M. polymorpha chloroplast genome is located between the ndhI and ndh4 genes (39). An initial search on the chloroplast genome of tobacco (40) for a similar ORF coding for an N-terminal...
of the cysteines bound to the two \([4Fe-4S]\) centers (42). One \([4Fe-4S]\) cluster is bound to Cys-X-Cys-X-Cys (where X represents amino acid) in the N-terminal half of the protein and to Cys-Pro in the C-terminal half. The second cluster is bound to Cys-X-Cys-X-Cys in the C-terminal half and to Cys-Pro in the N-terminal protein of the partial amino acid sequence of the isolated 9-kDa polypeptide described in tobacco and \(M. polymorpha\).

The C-terminal amino acid of the 9-kDa polypeptide was determined by carboxypeptidase Y digestion (27) and was found to be tyrosine. Thus, apart from the removal of the N-terminal methionine residue, the primary translation product of the 9-kDa polypeptide is not proteolytically processed. X-ray crystallographic analyses of the soluble 2\([4Fe-4S]\) ferredoxin from \(P. aerogenes\) established the identity of the cysteines bound to the two \([4Fe-4S]\) clusters (42). One \([4Fe-4S]\) cluster is bound to Cys-X-Cys-X-Cys (where X represents amino acid) in the N-terminal half of the protein and to Cys-Pro in the C-terminal half. The second cluster is bound to Cys-X-Cys-X-Cys in the C-terminal half and to Cys-Pro in the N-terminal protein of the partial amino acid sequence of the isolated 9-kDa polypeptide and the deduced amino acid sequence for the corresponding proteins in tobacco and \(M. polymorpha\) reveal identical cysteine-containing segments, thereby identifying these proteins as 2\([4Fe-4S]\) proteins.

A hydrophathy plot according to Hopp and Woods (43) is shown in Fig. 7. It is interesting to note that the 4 cysteine residues at positions 10, 13, 16, and 57 anchoring one of the \([4Fe-4S]\) centers (42) are positioned in relatively hydrophobic regions, whereas the cysteine residues at positions 20, 47, 50, and 53 coordinating the second center are located in more hydrophilic stretches of the molecule. The positioning of the cysteine residues in the bacterial 2\([4Fe-4S]\) ferredoxin (38) suggests that the cysteine residue at position 33 is not involved in anchoring an iron-sulfur center.

The amino acid composition of the isolated 16- and 18-kDa

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Total 78.0 79 81

* Deduced from the nucleotide sequence identified on the genome of \(N. tabacum\) (40) after insertion of a missing nucleotide (see "Results").

* Deduced from the nucleotide sequence identified on the genome of \(M. polymorpha\) (39).

* Determined as cysteic acid after performic acid oxidation (24).

* ND, not determined.

part of the polypeptide homologous to the barley 9-kDa polypeptide was unsuccessful. Search at the nucleotide level, however, revealed extensive homology between the psaC region of \(M. polymorpha\) and the corresponding region in tobacco. It turned out that the ORF found in \(M. polymorpha\) was not listed in the analysis of the tobacco chloroplast genome, most probably because a single base pair had been missed in the sequencing of the tobacco chloroplast genome. Thus, insertion of a thymine residue in the noncoding strand of the tobacco sequence at a position corresponding to the wobble position of codon 20 in the psaC ORF of \(M. polymorpha\) restored a nucleotide sequence which coded for a protein identical to the N-terminal part of the barley 9-kDa protein (Fig. 6). The amino acid composition of the isolated 9-kDa PS-I polypeptide of barley resembles that predicted from the psaC genes of \(M. polymorpha\) and tobacco (Table I). The identity between the partial sequence of the 9-kDa PS-I of barley and the sequence deduced from the psaC gene of tobacco strongly indicates that the sequence of the remaining part of the barley protein will be very homologous to the corresponding sequences in tobacco and \(M. polymorpha\).

The C-terminal amino acid of the 9-kDa polypeptide was determined by carboxypeptidase Y digestion (27) and was found to be tyrosine. Thus, apart from the removal of the N-terminal methionine residue, the primary translation product of the 9-kDa polypeptide is not proteolytically processed. X-ray crystallographic analyses of the soluble 2\([4Fe-4S]\) ferredoxin from \(P. aerogenes\) established the identity of the cysteines bound to the two \([4Fe-4S]\) clusters (42). One \([4Fe-4S]\) cluster is bound to Cys-X-Cys-X-Cys (where X represents amino acid) in the N-terminal half of the protein and to Cys-Pro in the C-terminal half. The second cluster is bound to Cys-X-Cys-X-Cys in the C-terminal half and to Cys-Pro in the N-terminal protein of the partial amino acid sequence of the isolated 9-kDa polypeptide and the deduced amino acid sequence for the corresponding proteins in tobacco and \(M. polymorpha\) reveal identical cysteine-containing segments, thereby identifying these proteins as 2\([4Fe-4S]\) proteins.

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The amino acid composition of the isolated 16- and 18-kDa
polypeptides was also determined. The 16-kDa polypeptide contained neither methionine nor cysteine, in accordance with the earlier reported absence of label in this protein after carbamoylmethylation and after labeling with 35S in vivo (11). This polypeptide also lacks histidine. The 18-kDa polypeptide contained small but significant amounts of cysteine (~1 residue/molecule). Thus, the 16- and 18-kDa polypeptides are not iron-sulfur apoproteins. Both the 18- and 16-kDa polypeptides contained very high levels of alanine and proline. This was reflected in the partial amino acid sequences which we have obtained for these two proteins.

**DISCUSSION**

Partial amino acid sequencing of the 9-kDa polypeptide isolated from PS-I vesicles of barley permitted identification of its corresponding gene psaC on the chloroplast genomes of tobacco (40) and *M. polymorpha* (39) and identification of the protein as a carrier of two [4Fe-4S] clusters (Fig. 6). The spacing of the cysteine residues does not fit that of soluble [2Fe-2S] proteins which also lack the Cys-Pro segment (38, 44-46). The soluble [2Fe-2S] ferredoxins reveal a strong internal homology presumably due to gene duplication (47). The deduced sequence for the 9-kDa polypeptide of tobacco displays a similar internal homology between residues 3-25 and 40-62 (Fig. 8).

The availability of analytical procedures (20) permitting fast and reliable determination of acid-labile sulfide and zero-valence sulfur was essential in the development of the procedure which resulted in isolation of the 9-kDa polypeptide from barley as a partially denatured [4Fe-4S] protein. Partial denaturation was evidenced by the lack of absorption around 420 nm and by the inability of the polypeptide to release acid-labile sulfide without prior reduction. The amount of zero-valence sulfur bound to the isolated 9-kDa polypeptide was quantified by two different procedures. After SDS-PAGE of the purified PS-I vesicle, the amount of zero-valence sulfur associated with the 9-kDa polypeptide was twice the amount found to be associated with P700-chlorophyll a-protein 1 (Fig. 4). In its purified state, the amount of zero-valence sulfur associated with the 9-kDa polypeptide was one-third of the amount of acid-labile sulfide present in a native PS-I preparation containing an identical amount of 9-kDa polypeptide (Fig. 3). This is explained by the less than 60% yield of acid-labile sulfide obtained from denatured PS-I vesicles after DTT reduction. Incomplete conversion of zero-valence sulfur into acid-labile sulfide upon reduction may be one reason for the lower recovery of acid-labile sulfide from the isolated 9-kDa polypeptide. Thus, the yield obtained with cysteine trisulfide as a standard was 77% (37). In addition, Petering et al. (37) observed that the zero-valence sulfurs bound in the oxidatively denatured bacterial [2[4Fe-4S]] ferredoxins of *M. lactuca*, *Clostridium pasteurianum*, and *P. elsdenii* were only 42, 48, and 63% of the acid-labile sulfide found in the native proteins. Of these soluble ferredoxins, that of *P. elsdenii* shows the highest degree of structural homology with the psaC gene product (Fig. 8) (38). The low recoveries led Petering et al. to conclude that the zero-valence sulfur of these proteins is bound mainly in a cysteine trisulfide structure (37). Quantitative retention of the acid-labile sulfide originally present in native [4Fe-4S] proteins would require the formation of a cysteine tetrasulfide structure (37). However, the recovery of zero-valence sulfur from oxidatively denatured [2Fe-2S] proteins was also low (37). In contrast to the results of Petering et al. (37) and to the results obtained in this study, Golbeck and Kok (48) have reported a 100% recovery of acid-labile sulfide following denaturation of PS-I particles and regeneration of acid-labile sulfide from zero-valence sulfur by DTT treatment. Using recoveries of 63 and 77% for the formation of zero-valence sulfur and the regeneration of acid-labile sulfide, respectively, the amount of acid-labile sulfur calculated to correspond to the amount of zero-valence sulfur detected on the isolated 9-kDa PS-I polypeptide corresponds to 70% of the acid-labile sulfide in the native PS-I particle. This value is in close agreement with the relative distribution of zero-valence sulfur between P700-chlorophyll a-protein 1 and the 9-kDa polypeptide as determined after the denaturing conditions of SDS-PAGE (Fig. 4) by which acid-labile sulfide is converted into zero-valence sulfur (11, 29, 34, 35).

Native PS-I vesicles are generally found to contain 12 molecules of acid-labile sulfide for each molecule of P700 (1, 2, 3, 11). From the results presented here, we can assign 8 of the molecules of acid-labile sulfide to the 9-kDa polypeptide and the remaining 4 to the apoproteins of P700-chlorophyll a-protein 1. Heij and Møller (11) and Golbeck and Cornelius (49) have recently demonstrated that P700-chlorophyll a-protein 1 carries center X. Centers A and B are therefore identified as the two [4Fe-4S] clusters of the chloroplast-encoded 9-kDa [4Fe-4S] polypeptide. Experiments based on ESR spectroscopy have revealed a differential sensitivity of centers A and B toward oxidative denaturation (48) and toward reactivity with mercurials (50), with center B as the most sensitive center. Similarly, center B has been demonstrated to be sensitive to the membrane-impermeant probe p-diazonium benzene sulfonate (51). A hydrophobic plot of the 9-kDa polypeptide reveals that the [4Fe-4S] cluster coordinated by cysteine residues 10, 13, 16, and 57 is buried in the membrane, whereas the cluster coordinated by cysteine residues 20, 47, 50, and 53 is more external (Fig. 7). We therefore conclude that these two [4Fe-4S] clusters represent centers A and B, respectively. Selective destruction of one [4Fe-4S] center had also been reported in the soluble [2Fe-2S] ferredoxin 1 of *Azotobacter vinelandii* (52). The localization of centers A and B on the same polypeptide chain is in agreement with the strong interaction observed between these two centers by ESR spectroscopy (5, 7, 54).

One important aspect regarding the biosynthesis of the [2Fe-2S] holoprotein is the formation of the iron-sulfur cluster. Denatured soluble [2Fe-2S] ferredoxins are easily reconstituted (55). Although N- and C-terminal analyses of the isolated 9-kDa polypeptide established that the primary gene product of psaC is not post-translationally cleaved except for the loss of the N-terminal methionine residue, it has not yet been possible by reconstitution experiments to regenerate the ESR signals of the iron-sulfur centers from denatured PS-

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I vesicles (56). Takahashi et al. (57) have recently demonstrated that sulfur atoms of the iron-sulfur cluster of chloroplast ferredoxin are derived from cysteine and that a soluble stroma enzyme is involved in the cluster formation. It will be interesting to test the activity of this enzyme toward the isolated 9-kDa apoprotein.

P700-chlorophyll a-protein 1 was shown in the present study to carry 4 of the 12 molecules of acid-labile sulfide associated with the native PS-I vesicle per molecule of P700. We have previously reported that P700-chlorophyll a-protein 1 binds 4.3 iron atoms/molecule of P700 (11). Using experimental conditions where P700-chlorophyll a-protein 1 had been functionally detached from the lower molecular mass polypeptides, Golbeck and Cornelius (49) obtained absorbance transients at 698 nm, indicating that the iron-sulfur center is present. Mossbauer spectroscopy identified center X as a \([4Fe-4S]\) center. With 4 iron and 4 sulfur atoms present on P700-chlorophyll a-protein 1 per molecule P700, this would permit four large polypeptides to constitute the 8 cysteine residues necessary to form a [4Fe-4S] center. With 4 iron and 4 sulfur atoms present on P700-chlorophyll a-protein 1, this would require the availability of 8 cysteine residues. P700-chlorophyll a-protein 1 is composed of two apoproteins with approximate molecular masses of 83 kDa and which are completely conserved stretch of 12 amino acids: Phe-Pro-Cys-

In all these species, both genes specified the following amino acid sequence indicated that the two polypeptides were also purified to homogeneity. The function of these two polypeptides remains unknown. However, partial amino acid sequencing indicated that the two polypeptides are related.

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