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The 110-kDa Reaction Center Protein of Photosystem I, P700-Chlorophyll *a*-Protein 1, Is an Iron-Sulfur Protein*

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Germination and growth of barley (*Hordeum vulgare* L.) in the presence of $^{59}Fe^{2+}$ or $^{35}SO_4^{2-}$ allows heavy incorporation of both isotopes into the thylakoid membranes and into isolated photosystem I particles. Analysis of ^{59}Fe-labeled preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under mild conditions demonstrates that a minimum of four iron atoms/P700 is carried on P700-chlorophyll *a*-protein 1. When isolated from ^{35}S-labeled preparations, P700-chlorophyll *a*-protein 1 binds zero valence ^{35}S, which is converted into acid-labile [^{35}S]sulfide by dithiothreitol reduction. Isolated photosystem I particles contain 14 acid-labile sulfide atoms and 10 iron atoms for each molecule of P700 and are composed of polypeptides of 110, 18, 15, 10, and 8 kDa of which the 10-kDa component is loosely bound. Under the electrophoretic conditions used, none of the low molecular weight polypeptides could be shown to be specifically associated with iron or acid-labile sulfide. Carboxymethylation of cysteine residues shows a high cysteine content in the 8-kDa polypeptide and an intermediate content in the 110- and 18-kDa polypeptides, whereas the 15-kDa polypeptide is devoid of sulfur amino acids. The experiments with the ^{59}Fe-labeled thylakoids reveal other labeled polypeptides not associated with photosystem I, namely cytochrome *f* and possibly cytochromes b_6 and b_{559}.

In higher plants, photochemical transfer of electrons from reduced plastocyanin to ferredoxin is catalyzed by PS I.[1] PS I is a membrane-bound protein complex containing several photoreducible electron acceptors (A_0, A_1, X, A, B) (1). Centers A_0 and A_1 may be specialized chlorophyll molecules (2–5). Centers X, A, and B have been detected and identified as Fe-S centers using low temperature ESR spectroscopy (6, 7). A comparative study with clostridial ferredoxin indicated that both centers A and B are 4 Fe-4 S centers (8), and their magnetic interaction suggests a close spatial arrangement possibly on the same polypeptide (9, 10). However, it has also been shown that chemical modification of center B may take place without any effect on center A (11) and that center B is much more susceptible to oxidative destruction than center A (12). PS I complexes have been prepared from a variety of sources. They all contain the reaction center protein P700-chlorophyll *a*-protein 1 and a varying number of low molecular weight polypeptides (<25,000). Thus, Møller *et al.* (13) prepared a PS I core particle from barley which by CBB staining was judged to contain only three polypeptides (110, 18, and 15 kDa) but all the ESR signals characteristic of PS I (14). PS I core complexes prepared from other sources contain additional polypeptides (15–22), and these preparations most likely also contain the full complement of PS I acceptors. Efforts to determine directly which of the PS I polypeptides carries Fe-S centers have been unsuccessful. All preparations of P700-chlorophyll *a*-protein 1 have been devoid of Fe-S centers (16, 21, 23–26). Bengis and Nelson (15) prepared PS I complexes from Swiss chard which were subsequently depleted for the small polypeptides by SDS treatment (16). This depletion correlated well with the disappearance of the Fe-S centers as monitored by ESR spectroscopy, and it was suggested that the Fe-S centers were associated with only the low molecular weight polypeptides (16). Similar conclusions were reached by Takahashi and Katoh (25) in experiments with *Synechococcus*. Malkin *et al.* (27) have reported the isolation of an 8-kDa thylakoid bound Fe-S protein, but this protein is probably not carrying any of the Fe-S centers of PS I (28). In the present study we use the isotopes ^{59}Fe and ^{35}S to directly demonstrate that P700-chlorophyll *a*-protein 1 is a major iron-containing protein of the thylakoids and that zero valence sulfur is specifically associated with this purified protein. Taken together these results show that P700-chlorophyll *a*-protein 1 carries at least one of the Fe-S centers of PS I.

MATERIALS AND METHODS

Plant Material

For tracer experiments, 250-g seeds of barley (*Hordeum vulgare* L., cv. Svaløfs Bonus) were surface sterilized by shaking in 400 ml of 0.2% HCHO for 20 min at 20 °C. Following a wash with sterile H_2O, the seeds were transferred to sterile Petri dishes (20 g/dish) containing two layers of Whatman No. 3MM chromatography paper. To each dish was added 3 ml of sterile H_2O containing the isotope material (^{59}Fe: 0.2 mCi of ferrous citrate/dish, 18 mCi/mg, New England Nuclear Chemicals GmbH, 6072 Dreieich, Federal Republic of Germany; ^{35}S: 3.0 mCi of H_2SO_4/dish, 1144 Ci/mmol, Amersham International, Buckinghamshire, England HP7 9LL). The Petri dishes were placed in large transparent plastic boxes with covers and the seeds allowed to germinate and grow for 7 days at 20 °C in continuous white light provided by fluorescent tubes (Osram Lumilux Daylight, 60 $\mu eq/m^2/s$). Each dish received 3 ml of sterile H_2O every day under sterile conditions.For the large-scale isolation of thylakoids and PS I preparations, seeds of barley were germinated and grown in

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[1] The abbreviations used are: PS I, photosystem I; CBB, Coomassie Brilliant Blue R-250; DTT, 1,4-dithiothreitol; MES, 2-*N*-(morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethyl benzidine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

vermiculite moistened with tap water using the light conditions indicated above. The seedlings were harvested after 7 days.

Preparation of PS I Particles

All procedures are carried out at 4 °C. Chloroplasts were isolated as described in Ref. 29 and osmotically lysed by two washings in 5 mM $MgCl_2$, 15 mM NaCl, 20 mM Hepes, pH 6.3. The stacked lamellar systems were pelleted by centrifugation at 10,000 × *g* for 10 min. PS I particles were then prepared by two different methods based on the use of either Triton X-100/Empigen BB or digitonin/Empigen BB.

Triton/Empigen PS I—The lamellar systems were resuspended in the lysis medium above at 2 mg of chlorophyll/ml and incubated with Triton X-100 (25 mg/mg of chlorophyll) with stirring in the dark for 10 min. Centrifugation at 48,000 × *g* for 30 min provided a P700-enriched supernatant (Triton X-100 supernatant). This was incubated with 1% (v/v) of the zwitterionic detergent Empigen BB (CH_3-$(CH_2)_n$-$N^+(CH_2)_2$-CH_2COO^-, n = 9–15) (Albright & Wilson Ltd., Marchon Works, Whitehaven, Cumbria CA28 9QQ, United Kingdom) for 10 min in the dark and applied to a column (2.6 × 95 cm) of AcA 34 (LKB-Produkter AB, S-16126 Bromma, Sweden) equilibrated in 25 mM MES, pH 6.5, 250 mM NaCl, 0.1% Triton X-100. Elution was carried out using the same buffer and a flow rate of 3 cm/h.

Digitonin/Empigen PS I—The lamellar systems were suspended in 5 mM $MgCl_2$, 15 mM NaCl, 20 mM Hepes, pH 6.3 (0.5 mg of chlorophyll/ml) and stirred with 10 mg of digitonin/mg of chlorophyll in the dark for 30 min. The supernatant obtained after centrifugation at 40,000 × *g* for 30 min was subjected to further centrifugation at 144,000 × *g* for 2 h. The resulting pellet was resuspended in a minimal volume of column buffer and incubated with 1% (v/v) Empigen BB for 10 min followed by fractionation on an AcA 34 column (1.6 × 95 cm) as above. The two PS I preparations were used interchangeably in the present study. When desired, the PS I material was pelleted by centrifugation at 240,000 × *g* for 3 h. This step served to concentrate the PS I material, to lower detergent concentration, and to remove traces of chloroplast coupling factor or chlorophyll *a/b*-protein 2.

Polyacrylamide Gel Electrophoresis

Analytical SDS-PAGE was carried out at 6 °C using either the buffer system of Neville (Tris/borate, system II in Ref. 30) or Laemmli (Tris/glycine, system IV in Ref. 30) in slab gels with a 6% stacking gel and a 12% separation gel. Stacking as well as separation gel contained 0.1% SDS. Unless otherwise indicated, samples for analytical electrophoresis (20 μg of chlorophyll) were prepared by addition of 15 μl of 100 mM Na_2CO_3, 100 mM DTT and 5% SDS, 30% sucrose to give a final SDS/chlorophyll ratio of 12 (w/w). After electrophoretic fractionation at 10 mA/cm², gels were stained with CBB, alkaline silver (31), or TMBZ (32). Autoradiography at −80 °C was carried out in cassettes fitted with intensifying screens by exposing stained or unstained dried gels to Kodak XRP-1 or XAR-5 X-Omat films. The x-ray films were scanned at 500 nm using a Carl Zeiss Chromatogramm-Spektralphotometer connected to a Servogor recorder. Preparative SDS-PAGE at 4 °C was performed in 1-mm thick and 300-mm wide slab gels with a 1-cm stacking gel (3.6% acrylamide and 0.25% bisacrylamide) and a 6-cm separation gel (9.6% acrylamide and 0.4% bisacrylamide) and the Tris/glycine buffer system described above. Samples were solubilized with octyl glucoside (detergent/chlorophyll = 8 (w/w)) by gentle mixing for 10 min at 4 °C and were loaded onto gels that had been prerun for 30 min at 6 mA. Electrophoresis was performed for 1 h at 6 mA followed by 1.5 h at 30 mA. The preparative gels were prepared without the use of SDS which was only included in the upper buffer (0.1%). Bands of interest were excised from the gel and analyzed immediately.

Analytical Procedures

Chlorophylls were determined according to Arnon (33). Determination of acid-labile sulfide in the presence of SDS was carried out using a modification of the methods reported in Refs. 34 and 35, whereby the tedious fractionation of each sample by column chromatography was substituted by an EtOAc extraction step. Methylene blue recovered in the final hexanol phase (34, 35) was either quantitated by spectrophotometry or, when [^{35}S]thylakoids were used as starting material, subjected to thin-layer chromatography on Merck Kieselgel 60 F_{254} plates with $CHCl_3$:MeOH:H_2O (78:21:1, v/v) as a developing solvent and subsequently analyzed by autoradiography. Barley ferredoxin was used as a standard in the sulfide assays on the basis of an extinction coefficient of 9.7 mM^{-1} cm^{-1} at 420 nm (36). Iron determinations were carried out by atomic absorption spectrometry at 248.3 nm using a Perkin-Elmer 2380 atomic absorption spectrometer fitted with an HGA 74 graphite tube and autosampler ASE. The cysteine residues of the PS I polypeptides were carboxymethylated by iodo[1-^{14}C]acetamide treatment for 8 h at pH 8.0 in the presence of β-mercaptoethanol and 8 M urea (37). P700 was quantitated from the ferricyanide-oxidized minus ascorbate-reduced difference spectrum in an Aminco DW-2c spectrophotometer using an extinction coefficient of 64 mM^{-1} cm^{-1} (38). The P700 content in an excised gel slice was measured by incubating one-half of the gel slice in 0.1% potassium ferricyanide and the other half in 0.1% ascorbate for 10 min. The two slices were then mounted in the light beams of the spectrophotometer using the surfaces of the masked cuvettes as a support while the difference spectrum was recorded. PS I activity was measured in an oxygen electrode with ascorbate/2,6-dichlorophenol indophenol as electron donor and methyl viologen as electron acceptor (39). Photoreduction of $NADP^+$ was measured with the Aminco DW-2c spectrophotometer operated in the dual wavelength mode (340/375 nm) using reaction mixtures (550 μl total volume) containing: membrane preparation (5 μg of chlorophyll), 0.5 mM $NADP^+$, 80 mM Tricine, pH 8.0, 80 mM NaCl, and 8 mM $MgCl_2$. For analysis of PS I preparations, 2 mM ascorbate and 0.02 mM 2,6-dichlorophenol indophenol were included. Barley plastocyanin, ferredoxin, and ferredoxin-$NADP^+$ oxidoreductase were purified to homogeneity according to published procedures (36, 40, 41) and were added in excess to each reaction mixture. Side illumination was obtained using a Schott KL 1500 light source fitted with red filters (Corning 2-58 and 2-64) and fiber optics. The photomultiplier tube was protected from the actinic light by a blue-green filter (Corning 7-60).

RESULTS

Germination and growth of barley under sterile conditions in solutions containing [^{59}Fe]ferrous citrate or [^{35}S]sulfate allowed the isolation of heavily labeled thylakoids from 7-day-old seedlings (4.4×10^5 dpm/mg of chlorophyll and 1.8×10^7 dpm/mg of chlorophyll, respectively). The labeling observed with ^{59}Fe was specifically bound as washing with lysis buffer containing 5 mM EDTA did not reduce the amount of label recovered in pelleted thylakoids. Furthermore, dialysis against 0.1% Triton X-100 and 2 mM EDTA caused loss of only 20% of the label from a PS I-enriched Triton extract obtained from labeled thylakoids whereas a 99% loss of label was observed upon dialysis of [^{59}Fe]ferrous citrate under identical conditions. When [^{59}Fe]ferrous citrate was added to an unlabeled PS I extract, more than 80% of the label was lost after dialysis.

To demonstrate the specific association of the incorporated ^{59}Fe with individual proteins, thylakoids were subjected to electrophoretic separation on SDS-polyacrylamide gels. Different time periods of electrophoresis were used as it was anticipated that the Fe-S centers would gradually be lost from the holoproteins due to increasing denaturation. P700-chlorophyll *a*-protein 1 and chlorophyll *a/b*-protein 2 were the major chlorophyll-containing components observed at all time periods tested (Fig. 1*A*). Autoradiography of dried gels (Fig. 1*B*) revealed several ^{59}Fe-labeled bands. One of the strong bands observed after 4 h of electrophoresis diminished in intensity upon prolonged electrophoresis and could not be observed after 8 h. The labeled band on the autoradiogram was superimposable with the green band corresponding to P700-chlorophyll *a*-protein 1 and staining of the gel with CBB revealed no other polypeptides in this region (Fig. 1*C*). Autoradiography after staining of the gel in 45% MeOH, 8.5% HOAc, H_2O for 2 h showed a drastic reduction of the label in P700-chlorophyll *a*-protein 1 (data not shown), and boiling of the sample before electrophoresis resulted in complete elimination of the label (Fig. 2). Sample solubilization and the resolution of the electrophoretic system was improved by addition of Na_2CO_3/DTT to the samples (Fig. 2). Addition of Na_2CO_3 without DTT resulted in loss of labeling in P700-

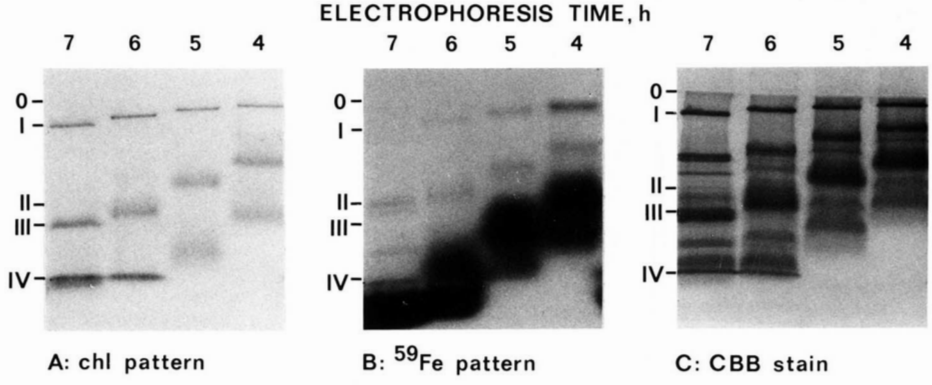

FIG. 1. **The effect of electrophoresis time on SDS-polyacrylamide gel profiles of ^{59}Fe-labeled thylakoids.** *A*, nonstained gel showing chlorophyll-containing components; *B*, autoradiogram showing ^{59}Fe-labeled components; *C*, CBB-stained gel. *0*, border between stacking and separation gel; *I*, P700-chlorophyll *a*-protein 1; *II*, cytochrome *f*; *III*, chlorophyll *a*/*b*-protein 2; *IV*, free pigment. ^{59}Fe-Labeled thylakoids (20 μg) were electrophoresed for the time periods indicated in the analytical Laemmli system.

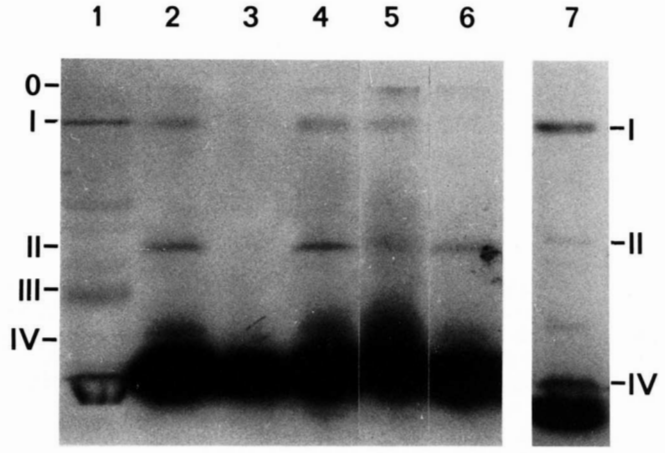

FIG. 2. **The effect of sample preparation on the recovery of ^{59}Fe label in P700-chlorophyll *a*-protein 1.** *1*, ^{35}S-labeled thylakoids; *2*, ^{59}Fe-labeled thylakoids + Na_2CO_3/DTT; *3*, [^{59}Fe]ferrous citrate only + Na_2CO_3/DTT; *4*, ^{59}Fe-labeled thylakoids + DTT; *5*, ^{59}Fe-labeled thylakoids, no additions; *6*, boiled ^{59}Fe-labeled thylakoids + Na_2CO_3/DTT; *7*, as in *2*. *Roman numerals*, see legend to Fig. 1. Electrophoresis was carried out in the analytical Laemmli system for 4 h at 6 °C. The same amount of radioactivity was applied to each slot, and the gel was dried immediately after electrophoresis.

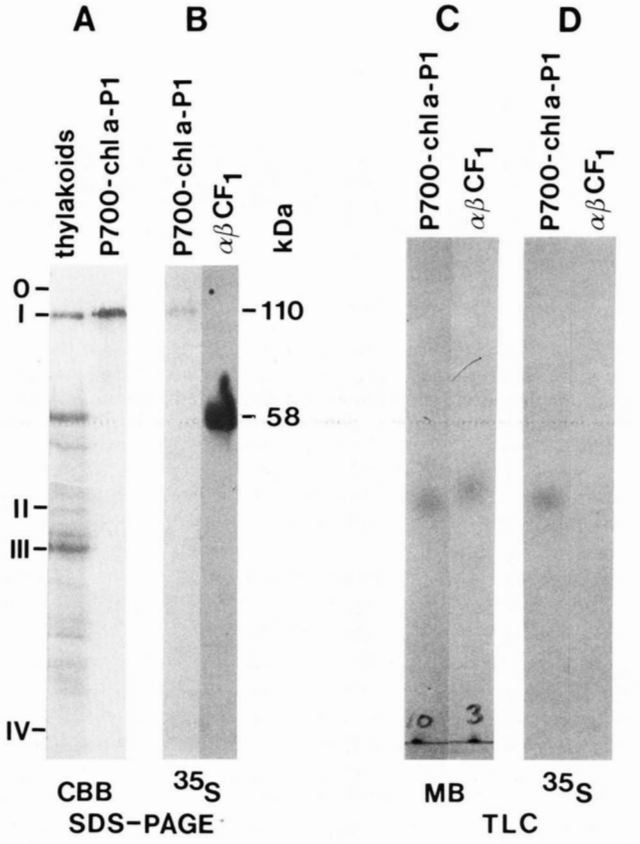

FIG. 3. **The presence of zero valence sulfur on ^{35}S-labeled P700-chlorophyll *a*-protein 1.** A Triton X-100 supernatant was obtained from ^{35}S-labeled thylakoids (10 mg of chlorophyll) and diluted 10-fold with 50 mM Tricine (pH = 7.9). The PS I particles were pelleted and resuspended in 400 μl of 50 mM Tricine (pH = 7.9) and subjected to preparative electrophoresis. Gel segments corresponding to P700-chlorophyll *a*-protein 1 (*P700-chl a-P1*) and the α,β-subunits of CF1 (*α,β CF1*) were excised and macerated. The purity of the polypeptide components of the two excised gel slices was examined after re-electrophoresis of a small part of the gel material under denaturing conditions (Neville system) by CBB staining (*A*) and autoradiography (*B*). After addition of 1 ml of 200 mM Tricine (pH = 8.0), 20 μl of 100 mM DTT,and 2 μl of 100 mM EDTA (pH = 8.0) to each of the macerated gel samples, these were incubated for 2 h and their content of acid-labile sulfide determined as ^{35}S-labeled methylene blue. Identical amounts of unlabeled methylene blue (*MB*) were added as a carrier to the two samples prior to TLC to allow visual detection of the methylene blue spot on the TLC plate (*C*). Subsequent autoradiography of the TLC plate indicated the superimposition of the ^{35}S label of the extract and the methylene blue spot (*D*). *Roman numerals*, see legend to Fig. 1.

chlorophyll *a*-protein 1. In several species of higher plants, careful electrophoretic conditions produce a chlorophyll-protein band containing chlorophyll *a*-protein 1 still bound to low molecular weight polypeptides (42–44). If this was the case here, the ^{59}Fe labeling observed could be dependent on the presence of such low molecular weight polypeptides. The ^{59}Fe-labeled band was, therefore, cut out from the dried gel and re-electrophoresed in the presence of 5 M urea for 16 h. Subsequent CBB staining revealed the presence of two protein bands of 110 and 70 kDa only, corresponding to P700-chlorophyll *a*-protein 1 and its apoprotein, respectively (data not shown). P700-chlorophyll *a*-protein 1 was also labeled in plants grown on [^{35}S]sulfate. Even after electrophoretic separation of the ^{35}S-labeled thylakoids in the mild preparative Laemmli system, re-electrophoresis of P700-chlorophyll *a*-protein 1 followed by autoradiography and CBB staining showed the presence of the 110-kDa component only (Fig. 3). This experiment precluded that the ^{59}Fe labeling of P700-chlorophyll *a*-protein 1 was carried by a sulfur-containing component not staining with CBB or by a low molecular weight component not resolved by the electrophoresis system. During attempts to perform analytical electrophoresis under milder conditions, it was discovered that lowering of the SDS concentration from 0.1 to 0.033% permitted the recovery of ^{59}Fe label in P700-chlorophyll *a*-protein 1 after overnight electrophoresis at 6 °C (Fig. 4). More important, with the high resolution thus achieved, it became apparent that ^{59}Fe label was also present in the 70-kDa region corresponding to the proportion of P700-chlorophyll *a*-protein 1 which had been converted into its apoprotein (Fig. 4). This result demon-

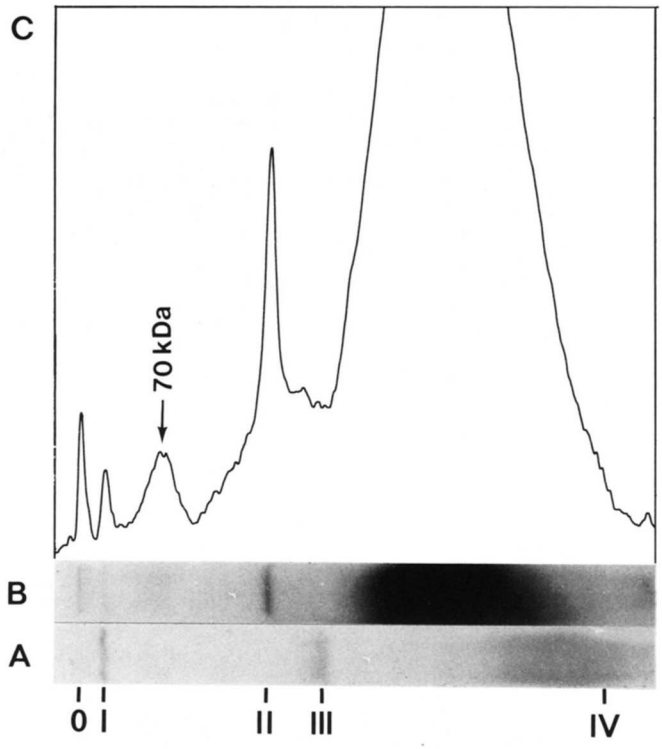

FIG. 4. **Incorporation of ^{59}Fe into P700-chlorophyll *a*-protein 1 and into its 70-kDa apoprotein.** *A*, nonstained gel showing chlorophyll-containing components; *B*, autoradiogram of *A* showing ^{59}Fe-labeled components; *C*, densitometric scan of *B*. *Roman numerals*, see legend to Fig. 1. Electrophoresis of ^{59}Fe-labeled thylakoids was carried out in the Neville system with the SDS concentration lowered to 0.033%. After drying, the distribution of radioactivity was determined by autoradiography and scanning of the x-ray film.

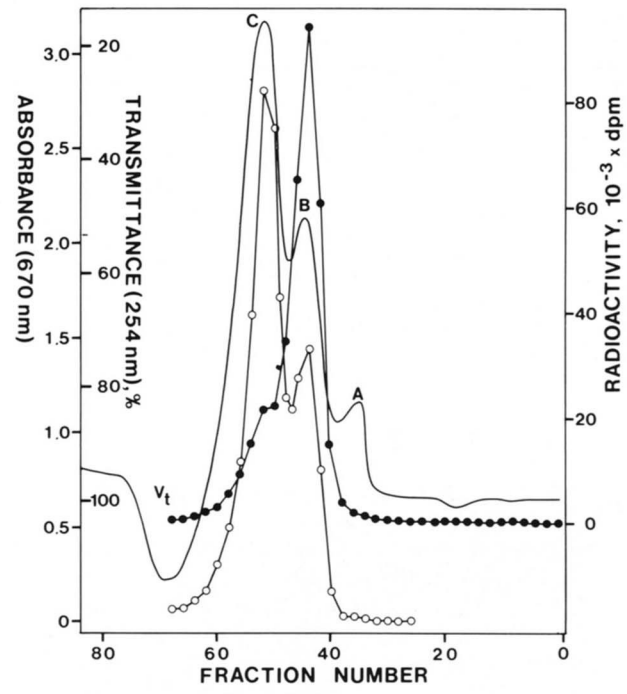

FIG. 5. **Isolation of ^{59}Fe-labeled PS I particles by gel filtration.** A PS I-enriched digitonin/Empigen BB extract was prepared and fractionated on an AcA 34 column. Each 1.8-ml fraction was monitored for absorbance at 670 nm (○——○), transmission at 254 nm (——), and content of ^{59}Fe by scintillation counting (●——●).

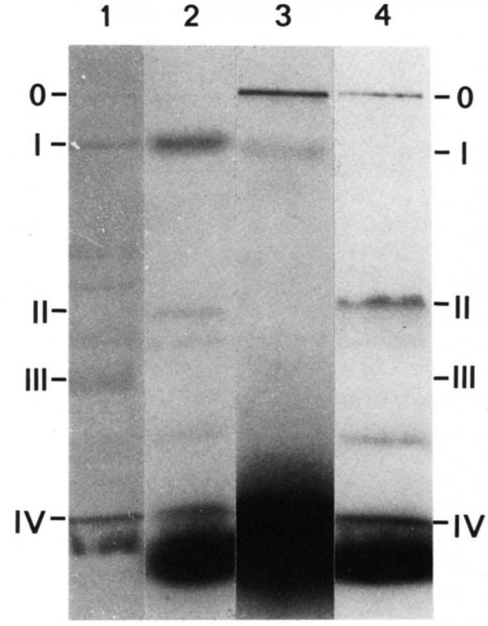

FIG. 6. **The ^{59}Fe content of purified PS I particles.** *1*, ^{35}S-labeled thylakoids; *2*, ^{59}Fe-labeled thylakoids; *3*, pelleted ^{59}Fe-labeled PS I particles (fraction 43, Fig. 5); *4*, pelleted ^{59}Fe-labeled cytochrome f/b_6 complex (fraction 50, Fig. 5). *Roman numerals*, see legend to Fig. 1. Purified PS I particles and the cytochrome f/b_6 complex were obtained by gel filtration on AcA 34 using a digitonin/Empigen BB extract obtained from ^{59}Fe-labeled thylakoids. Immediately after electrophoresis in the analytical buffer system of Laemmli, the gel was dried and subjected to autoradiography.

strates that, at least under some conditions, the antennae chlorophylls and the iron of chlorophyll *a*-protein 1 can be lost independently. The yield of ^{59}Fe label in P700-chlorophyll *a*-protein 1 varied from one experiment to the other (compare Fig. 2, *lanes 2* and *7*) although the experiments were performed under apparently identical conditions. In all experiments, however, SDS-PAGE revealed P700-chlorophyll *a*-protein 1 as a major iron-containing protein of the thylakoids.

The identification of P700-chlorophyll *a*-protein as a labile iron-bearing polypeptide was also possible with purified PS I preparations obtained by digitonin and Empigen fractionation as described under "Materials and Methods." The profile of the gel filtration step performed using AcA 34 showed three main peaks when monitored by transmission at 254 nm (Fig. 5). Peak A mainly contained coupling factor, peak B mainly PS I (chlorophyll *a*/chlorophyll *b* = 15) and peak C chlorophyll *a*/*b*-protein 2 (chlorophyll *a*/chlorophyll *b* = 4.5) in addition to free chlorophyll. Essentially all ^{59}Fe was contained in peak B with tailing into peak C reflecting the elution of the cytochrome $f \cdot b_6$ complex. No iron was found in the salt fraction of the eluate (V_t). This indicated a specific association of the iron with the PS I material contained in peak B. The same distribution of iron was observed by atomic absorption spectrometry (data not shown) and is analogous to that reported by Golbeck (45, 46). On a chlorophyll basis, the iron content of the PS I material was 5 times that found in the thylakoids. SDS-PAGE and subsequent autoradiography of the PS I preparation again revealed strong labeling of P700-chlorophyll *a*-protein 1 (Fig. 6) strengthening the results obtained with thylakoids. The label in P700-chlorophyll *a*-protein 1 of the PS I preparation was even more labile than observed with fresh thylakoids, most likely because of the prolonged exposure to Triton X-100 or digitonin and Empigen.

Due to the instability of the label on the 110-kDa polypeptide under electrophoretic conditions, it was not possible directly to quantitate the number of iron atoms bound per

P700. For each molecule of P700, the PS I preparations used in this study contained 110 molecules of chlorophyll *a*, 14 molecules of acid-labile sulfide, and 10 iron atoms, *i.e.* the generally observed full complement of iron and acid-labile sulfide found in photoreactive PS I preparations carrying the acceptors X, A, and B (45). No cytochrome was detected by difference spectroscopy. The long wavelength absorption maximum of the preparation was 678 nm. Assays of PS I activity by measurement of light-dependent oxygen uptake in the presence of ascorbate/2,6-dichlorophenol indophenol and methylviologen gave rates of 220 μmol of oxygen/mg of chlorophyll/h in the presence of methylviologen compared to only 20 in its absence. Photoreduction of $NADP^+$ with ascorbate/2,6-dichlorophenol indophenol as electron donor progressed at much slower rates, 3 μmol/mg of chlorophyll/h, whereas the rate with thylakoids was 15. SDS-PAGE followed by staining with CBB revealed components at 110, 18, 15, 10, and 8 kDa. The 110-kDa component was identified as P700-chlorophyll *a*-protein 1 based on the P700 content in the excised green gel pieces as demonstrated by difference spectroscopy. The components at 18 and 15 kDa were easily resolved upon electrophoresis in the Tris/borate system of Neville as shown in Fig. 7, but were poorly resolved in the Tris/glycine system of Laemmli. The two components at 8 and 10 kDa proved easier to observe after silver staining than after CBB staining (Fig. 7) and were either absent from or not detected in the PS I particles prepared earlier (13, 39). Some PS I preparations lacked the 10-kDa component. Since it was not possible to demonstrate a quantitative association of all the iron with P700-chlorophyll *a*-protein 1, it was interesting to examine whether any of the low molecular weight polypeptides of the PS I preparation were also labeled. This proved difficult due to the presence of an extensive labeling at this region of the gel which surprisingly was also observed when [^{59}Fe]ferrous citrate was subjected to electrophoresis. Thus, the ferrous ions must somehow be complexed to produce an overall negatively charged complex. The exact location of the label derived directly from this ^{59}Fe-iron complex was strongly dependent on the amount of DTT added to the sample prior to electrophoresis (Fig. 8*A*). This shift in mobility was particularly pronounced in the Tris/borate electrophoresis system probably due to formation of borate-iron complexes. With no DTT in the sample essentially no labeling was observed below 10 kDa, and strong labeling was observed in the 16-kDa region. The latter situation is shown on Fig. 8, which depicts elution of ^{59}Fe-labeled PS I material from the AcA 34 column as monitored by staining the gel with CBB (Fig. 8*B*) and by autoradiography of an unstained gel after overnight electrophoresis (Fig. 8*C*). Based on the presence of a radioactive spot and its incident comigration with the two polypeptides at 18 and 15 kDa, it was speculated (13) that these bands were carrying Fe-S centers. This hypothesis was further supported when the PS I mutant viridis-n[34], which is strongly depleted in these two polypeptides and P700-chlorophyll *a*-protein 1 (39), was found to show only weak labeling at this position.[2] The diffuse nature of the radioactively labeled band compared to the polypeptide bands was thought to reflect postelectrophoretic dissociation and diffusion of radioactivity, *e.g.* during gel handling and drying. From the results presented here, it is apparent that the basis for this suggestion is no longer valid; the PS I mutant only contains neglible amounts of the PS I iron-sulfur centers and thus cannot give rise to the generation of the artificial iron complex during electrophoresis. Thus, the use of the mutant did not disclose the true nature of the labeling in the 16-kDa region. The shift in electrophoretic mobility in dependence of DTT could also indicate the presence of a proteinaceous iron component stable only in the absence of DTT. This possibility was ruled out by proteinase K digestion of the sample in the absence of DTT prior to electrophoresis. The CBB pattern revealed extensive breakdown of the constituent polypeptides whereas the ^{59}Fe-labeling pattern remained unaltered (data not shown). Thus, proteinase treatments or other controls have to be performed before DTT-sensitive iron-labeled bands observed on polyacrylamide gels are related to comigrating polypeptides. The presence of iron-labeled thylakoid proteins destroyable by reductants has been reported in *Anacystis* (47). In the present study, [^{59}Fe]ferrous citrate alone did not produce a band at 110 kDa under any of the electrophoretic conditions tested (Fig. 2). The inclusion of proteinase K in the sample buffer eliminated all the labeled bands observed in the untreated thylakoids except that with the highest electrophoretic mobility which coelectrophoresed with the artificial ^{59}Fe-iron complex (data not shown).

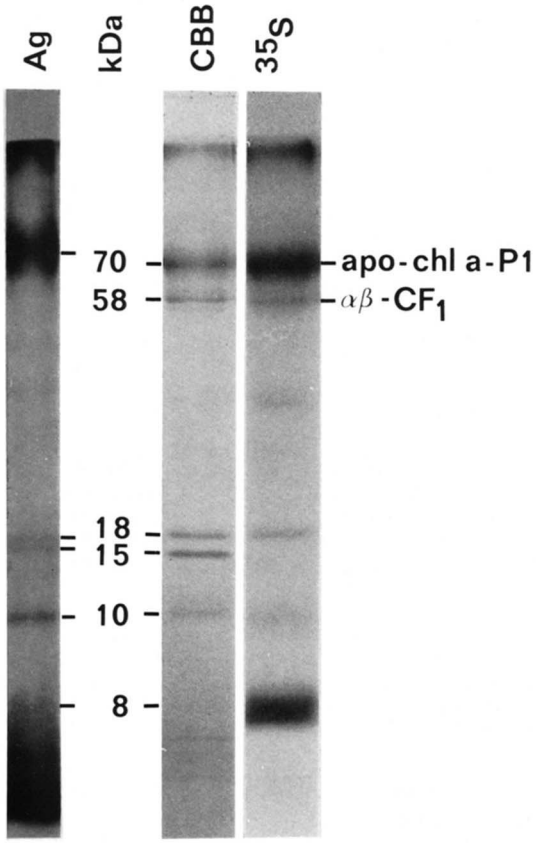

FIG. 7. **Polypeptide composition of purified PS I particles as analyzed by silver staining (*Ag*), CBB staining (*CBB*), and by ^{35}S labeling (^{35}*S*).** Electrophoresis was carried out overnight at 6 °C using the buffer system of Neville. *Apo-chl a-P1*, apoprotein of P700-chlorophyll *a*-protein 1.

Some of the ^{59}Fe labeling at the low molecular weight region of the SDS-polyacrylamide gels results from dissociation of the bound iron from the thylakoid polypeptides during SDS solubilization and electrophoresis and could be removed by washing the gels in aqueous HOAc/MeOH (data not shown). Under these conditions no retainment of iron was observed at positions superimposable with those of the 18-, 15-, and 10-kDa polypeptides of the PS I preparation. The labeling retained in the 8-kDa region of Fig. 1 is situated at a moving boundary but could still reflect the presence of an 8-kDa ^{59}Fe-carrying polypeptide since this labeling is preferentially retained upon washing the gel in aqueous HOAc/MeOH (data

[2] B. L. Møller, unpublished data.

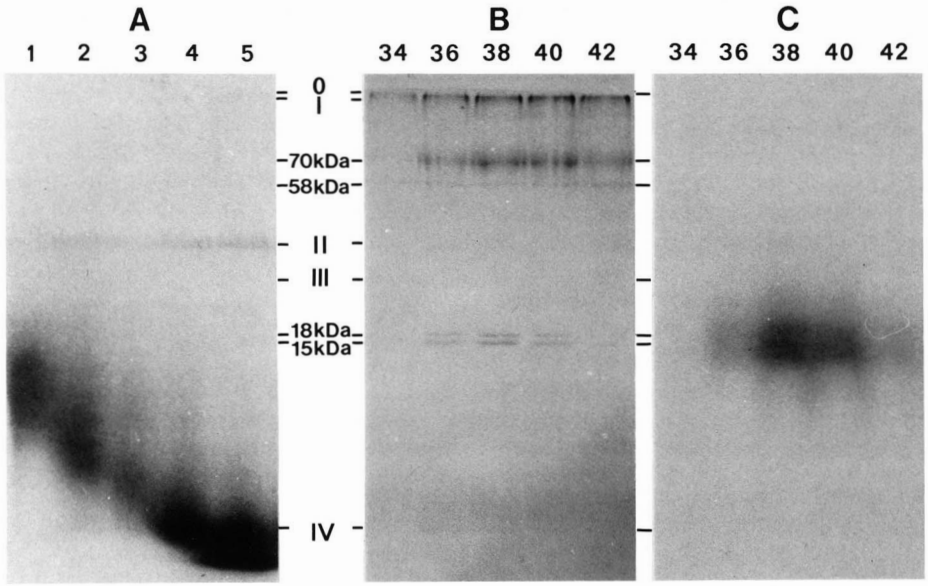

FIG. 8. **The effect of DTT on the electrophoretic mobility of iron complexes formed during sample preparation and SDS-PAGE.** *A*, samples of ^{59}Fe-labeled thylakoids (20 μg of chlorophyll) were solubilized with SDS and made 0 mM (*lane 1*), 14 mM (*lane 2*), 24 mM (*lane 3*), 32 mM (*lane 4*), and 37 mM (*lane 5*) with respect to Na_2CO_3 and DTT before overnight electrophoresis. The gel (Neville system) was dried and the radioactivity distribution analyzed by autoradiography. *B*, PS I fractions (100 μl) obtained after gel filtration on AcA 34 of a Triton/Empigen BB extract. Samples were solubilized with SDS in the absence of Na_2CO_3 and DTT. After overnight electrophoresis (Neville system), the gel was stained with CBB. The appropriate fraction number is indicated on *top of each lane*. *C*, as in *B* except that the gel was dried immediately after electrophoresis and exposed to an x-ray film. *Roman numerals*, see legend to Fig. 1.

not shown). Thus, no conclusion with respect to the existence of iron-carrying polypeptides among the low molecular weight components of PS I could be made.

Denatured Fe-S proteins may retain their acid-labile sulfide as zero valence sulfur (48), and this property was exploited in an attempt to further identify Fe-S-carrying polypeptides of ^{35}S-labeled thylakoids after their separation by preparative gel electrophoresis. Upon DTT treatment, zero valence sulfur is converted into acid-labile sulfide (48) which can then be quantitated by spectrophotometric analysis based on the sulfide-dependent formation of methylene blue in the presence of *p*-aminodimethylanilin (34). Analysis of gel slices containing the various PS I polypeptides following preparative gel electrophoresis of a purified PS I preparation was carried out either before or after reduction with DTT (Fig. 9). Significant methylene blue production was observed from the gel slice containing P700-chlorophyll *a*-protein 1 but only after reduction with DTT (Fig. 9, *B minus C*). Identical amounts of P700-chlorophyll *a*-protein 1 not reduced by DTT were assayed to eliminate interference from chlorophyll degradation products absorbing around 670 nm (Fig. 9, *C minus A*). Incubation of control gel pieces with DTT gave no methylene blue spectrum (Fig. 9*A*). The methylene blue spectrum was thus completely dependent upon the presence of P700-chlorophyll *a*-protein 1. As in the studies with ^{59}Fe, the homogeneity of the preparation was assessed by re-electrophoresis of the ^{35}S-labeled P700-chlorophyll *a*-protein 1 (Fig. 3). Staining with CBB and autoradiography revealed the presence of P700-chlorophyll *a*-protein 1 only. The gel strips cut from the front of the preparative gel containing the 10- and 8-kDa polypeptides gave a smaller but significant methylene blue production after DTT treatment, but the origin of the detected sulfide is as yet uncertain. P700-chlorophyll *a*-protein 1 purified by column chromatography at pH 4.5 in the absence of SDS[3]

[3] P. B. Høj and B. L. Møller, manuscript in preparation.

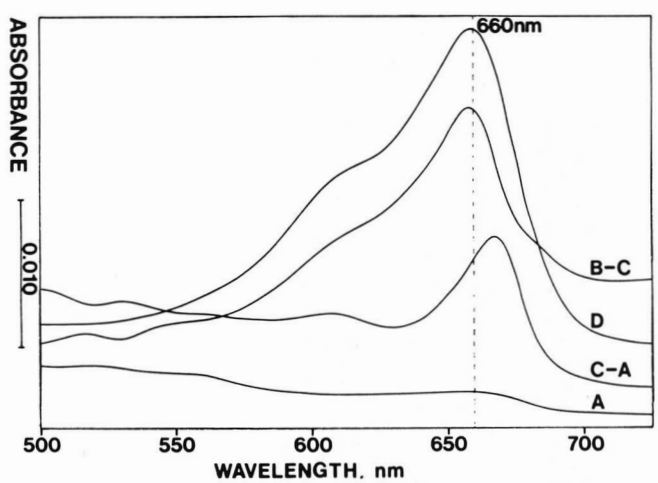

FIG. 9. **Detection of zero-valence sulfur on P700-chlorophyll *a*-protein 1.** P700-chlorophyll *a*-protein 1 was isolated by preparative SDS-PAGE of a purified PS I particle (520 μg of chlorophyll). The green band containing P700-chlorophyll *a*-protein 1 was cut out and divided into equal batches which were then homogenized and assayed for the presence of acid-labile sulfide. Assays using difference spectroscopy were performed on the following material: *A*, control: polyacrylamide gel (0.48 g of gel) recovered in front of the free pigment zone. *B*, P700-chlorophyll *a*-protein 1 (0.48 g of gel) assayed following reduction with 2 mM DTT. *C*, P700-chlorophyll *a*-protein 1 (0.48 g of gel) assayed without prior DTT reduction. *D*, standard: Na_2S in H_2O.

also showed significant amounts of methylene blue production although only after reduction with DTT.

Iron has been shown to catalyze sulfide formation from DTT (35). The sulfide formed might, therefore, originate from DTT mediated by a factor present on P700-chlorophyll *a*-protein 1. In this study, this argument was circumvented by the use of ^{35}S-labeled P700-chlorophyll *a*-protein 1 isolated

from plants grown on [^{35}S]sulfate. Thin layer chromatographic fractionation of the methylene blue containing hexanol phase showed that the radioactivity of the extract resided in the methylene blue spot (R_F = 0.50) (Fig. 3). When *p*-aminodimethylanilin was omitted from the reaction mixture, this radioactive spot was absent. The ^{35}S label found in the methylene blue recovered from P700-chlorophyll *a*-protein 1 did not originate from [^{35}S]cysteine or [^{35}S]methionine since insignificant amounts of ^{35}S-labeled methylene blue (Fig. 3) were produced when the α and β subunits of CF1 (49–51) were assayed. We thus conclude that the isolated P700-chlorophyll *a*-protein 1 carries zero valence sulfur. This result combined with the presence of iron on the same polypeptide strongly suggests that P700-chlorophyll *a*-protein 1 is an Fe-S protein. It is possible that the iron and zero valence sulfur recovered on P700-chlorophyll *a*-protein 1 in this study does not originate from the same Fe-S center. More detailed investigations will be needed to answer this question.

The incorporation of [^{35}S]sulfate into the sulfur amino acids of the various polypeptides was determined by autoradiography after prior fixation or staining of the gels in MeOH-HOAc-H_2O. P700-chlorophyll *a*-protein 1 as well as the 8-kDa polypeptide were strongly labeled (Figs. 7 and 10*A*). The 18- and 10-kDa polypeptide carried a moderate amount of label whereas the 15-kDa component was not labeled (Fig. 7). These results were confirmed when the cysteine residues of an unlabeled PS I preparation were carboxymethylated with [^{14}C]iodoacetamide at pH 8.0 after denaturation with urea. All polypeptides except that at 15 kDa were labeled (data not shown). Thus the 15-kDa polypeptide cannot be a traditional Fe-S protein since it lacks cysteine residues required to coordinate the iron atoms.

It was of interest to quantitate the number of sulfur and iron atoms present on each molecule of P700-chlorophyll *a*-protein 1. The amount of acid-labile sulfide could not be quantitated reliably, because the efficiency of its conversion into S^0-sulfur during the denaturing electrophoretic conditions is unknown as is the yield of S^{2-} formation upon chemical reduction of the electrophoretically purified denatured protein. Calculation of the number of iron atoms for each molecule of P700 was also complicated due to the progressive loss of iron during electrophoresis. Difference spectroscopy revealed that the ratio between P700 and cytochrome *f* in the thylakoids was 1.2. Thus, measurement of the iron content of the stable cytochrome *f* (Fig. 10) compared to that of the labile P700-chlorophyll *a*-protein 1 allowed determination of the minimum number of iron atoms in P700-chlorophyll *a*-protein 1. Cytochrome *f* has a molecular mass of 33–36 kDa, and several iron-labeled bands were present in this region of the SDS gel. The doublet at 35–36 kDa (Fig. 10) was repeatedly seen. The intensity of the doublet neither declined upon prolonged electrophoresis (Fig. 1) nor after boiling (Fig. 2) or staining/destaining in 7% HOAc-containing solutions (data not shown). The 35-kDa component was always more labeled and located at the position of the most intense TMBZ staining band of barley thylakoids (13). This pattern is analogous to that obtained by Hurt and Hauska (52, 53) with a purified cytochrome f/b_6 complex which contained TMBZ bands at 34, 33, and 23.5 kDa. The thylakoids produced a sharp ^{59}Fe band at 30 kDa whenever the electrophoretic sample buffer contained DTT. In the absence of DTT, this band disappeared and the labeling in the 35-kDa region became more diffuse (data not shown). This could indicate that the 30-kDa component was also related to cytochrome *f*. Similar experimental observations have been reported by Guikema and Sherman (47) for ^{59}Fe-labeled thylakoids of *Anacystis nidulans* (R2). In the present study, the intensities of ^{59}Fe label at 35 and 30 kDa were comparable, but the 35-kDa component was far more intense after TMBZ staining than that at 30 kDa. Only the latter component disappeared upon boiling of the sample prior to electrophoresis, and no 30-kDa band was found in a purified cytochrome f/b_6 preparation (52). To investigate the relationship between the two components, the labeled bands at 35 and 30 kDa were cut out from the dried gel using the exposed x-ray film as a template. Re-electrophoresis in the presence of DTT followed by CBB and silver staining showed no interconversion between the two components (data not shown). Only the ^{59}Fe-labeled bands at 36 and 35 kDa were, therefore, assigned to cytochrome *f*. The identity of the labeled 30-kDa component is unknown, but it is of interest that a 31-kDa TMBZ staining component is present in a maize mutant devoid of cytochromes *f* and b_6 (54) and that an iron-quinone protein complex is located on the acceptor side of photosystem II (55). The 20-kDa ^{59}Fe component was tentatively assigned to cytochrome b_6 although it might also represent the Rieske Fe-S protein.

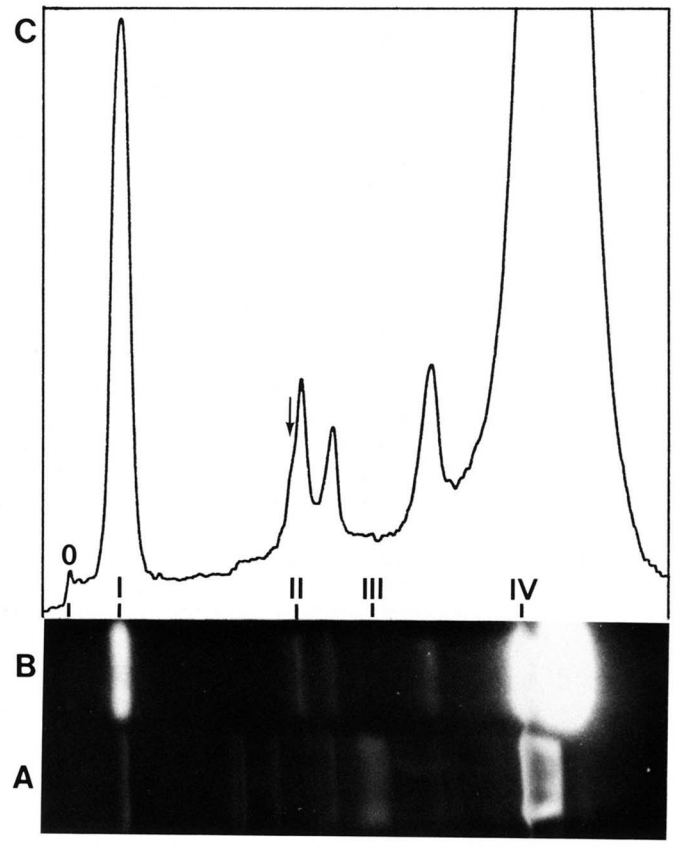

FIG. 10. **Quantitation of the ^{59}Fe label associated with P700-chlorophyll *a*-protein 1.** *A*, ^{35}S-labeled thylakoids and *B*, ^{59}Fe-labeled thylakoids (20 μg of chlorophyll) were electrophoresed for 4 h in the analytical Laemmli system after which the gel was immediately dried and subjected to autoradiography. The x-ray film was used as a template to localize and excise the individual bands of the dried gel. For quantitation, the excised bands were counted by liquid scintillation counting. *C*, densitometric scan of the autoradiogram shown in *B*. *Roman numerals*, see legend to Fig. 1.

Scintillation counting of the gel pieces corresponding to P700-chlorophyll *a*-protein 1, cytochrome *f*, the 30-kDa protein, and to cytochrome b_6 gave average values of 172, 33, 22, and 51 dpm, respectively. Thus, the amount of iron associated with P700-chlorophyll *a*-protein 1 following electrophoretic separation was 5.2 times that in cytochrome *f* whereas the

spectrophotometrically determined ratio between P700 and cytochrome *f* was 1.2. P700-chlorophyll *a*-protein 1, therefore, binds a minimum of 4.3 iron atoms for each P700. Based on scintillation counting, the ratio between cytochrome b_6 and cytochrome *f* was 1.6. The deviation from the expected ratio of 2 probably reflects a partial loss of the heme group of cytochrome b_6 during electrophoresis.

DISCUSSION

The incorporation of ^{59}Fe into thylakoids of barley was first studied by Møller *et al.* (13). Those preliminary studies showed strong labeling of cytochrome *f*. Additional labeling in the 18-kDa region was tentatively associated with the low molecular mass polypeptides of PS I (13), but the present work invalidates this assignment. Subsequent studies with *Vicia faba* also demonstrated the incorporation of ^{59}Fe into cytochrome *f* (56). Guikema and Sherman (47) incorporated ^{59}Fe in the photosynthetic membranes of the algae *Anacystis nidulans*. In contrast, Lagoutte *et al.* (17) reported that they were unsuccessful in detecting any thylakoid-associated labeling when detached green spinach leaves were fed ^{59}Fe. The high incorporation obtained in the present study was achieved by germination of the barley seeds as well as growth of the seedlings in the presence of the radioisotope whereas Lagoutte *et al.* (17) added the radioactive isotope well after the process of thylakoid synthesis and assembly was initiated.

The present study identifies P700-chlorophyll *a*-protein 1 as an Fe-S protein containing at least 4 iron atoms for each molecule of P700. In this context it is interesting to note that studies using iron-deficient sugar beets have shown that P700-chlorophyll *a*-protein 1 was the most depleted chlorophyll-protein and most rapidly recovered upon the resupply of iron (57). Previously obtained pure preparations of P700-chlorophyll *a*-protein 1 (16, 21, 23–26) have all been devoid of Fe-S centers A and B and even of iron (26). The starting material for P700-chlorophyll *a*-protein 1 preparations has normally been a highly resolved PS I particle containing 4–7 polypeptides and all the known acceptors A_0, A_1, X, A, and B. SDS treatment has been used to deplete these preparations of the small molecular weight polypeptides. Since the loss of these small polypeptides correlated well with the disappearance of the Fe-S centers, it was inferred that these centers were carried by low molecular weight polypeptides rather than by P700-chlorophyll *a*-protein 1. However, both from chemical (35) and spectroscopic (24) studies SDS is known to destroy the Fe-S centers. From the results presented in this study, it must be concluded that P700-chlorophyll *a*-protein 1 carries at least one Fe-S center in its functional state, most likely center X (A_2). Very recently, Sakurai and San Pietro (58) reported that a considerable amount of zero-valence sulfur was bound to the large molecular weight polypeptides of a spinach PS I preparation and tentatively concluded that chlorophyll *a*-protein 1 is an Fe-S protein. Bonnerjea *et al.* (28) depleted a spinach PS I preparation significantly of polypeptides of 8, 10, 14, and 16 kDa under conditions where the amplitude of the ESR signals of center A and B only decreased 10%. The remaining 19-kDa polypeptide of their preparation was therefore concluded either to contain centers A and B or to stabilize these centers on P700-chlorophyll *a*-protein 1. Taken together, these lines of evidence strongly argue that at least one but possibly more of the Fe-S acceptors of PS I are associated with P700-chlorophyll *a*-protein 1. In accordance with this, carboxymethylation showed the presence of cysteine residues on this polypeptide. Recently, Fish *et al.* (50) and Lembeck *et al.* (59) determined the nucleotide sequence of the gene (PSIa) which supposedly codes for P700-chlorophyll *a*-protein 1 of maize and pea. These sequences were extremely homologous with four cysteine residues in conserved positions. Thus, if PS I contains traditional Fe-S centers with cysteine residues as ligands for the iron atoms, each monomer can at most carry one 4 Fe-4 S center or one 2 Fe- 2 S center. However, it must be recalled, that the iron in two 2 Fe-2 S centers (60) has recently been shown to be coordinated by a nitrogen atom. It is not unlikely that a similar situation exists in PS I. In this context it may be important to notice that attempts to reconstitute the denatured membrane-bound Fe-S clusters of PS I have been unsuccessful (18, 61) whereas the Fe-S centers of denatured ferredoxin are easily reconstituted (62).

The results obtained with the ^{59}Fe-labeled PS I core particle here studied only give conclusive evidence for the existence of one labeled polypeptide, namely P700-chlorophyll *a*-protein 1. This does not mean that other polypeptides contained in this particle do not carry iron *in vivo*. It is likely that some iron-containing polypeptides may be undetected because of an even more labile association of the iron than that seen with P700-chlorophyll *a*-protein 1. The PS I particle here studied contained polypeptides of 110, 18, 15, 10, and 8 kDa. The 15-kDa polypeptide has been purified to homogeneity in our laboratory and has been shown to contain neither methionine nor cysteine.[3] The 10-kDa polypeptide seems relatively loosely associated with the particle and was absent in the full complement particle of Hiller *et al.* (39). In agreement with the results presented in this study, Lagoutte *et al.* (17) and Bengis and Nelson (16) found that the most heavily ^{35}S-labeled polypeptide chains were P700-chlorophyll *a*-protein 1 and an 8-kDa polypeptide. Carboxymethylation further proved the latter to be the most cysteine-rich component and, therefore, a good candidate for a ferredoxin-like Fe-S protein. Malkin *et al.* (27) reported the isolation of such a low molecular weight Fe-S protein from thylakoid membranes and Sakurai and San Pietro (58) found zero-valence sulfur in the less than 10-kDa region upon SDS-PAGE of a spinach PS I preparation. However, if the 8-kDa polypeptides studied in these reports represent the same polypeptide, then the study of Bonnerjea *et al.* (28) rules out this polypeptide as a candidate for Fe-S centers A, B, and X, and one is left with the 18-kDa polypeptide as the only alternative to chlorophyll *a*-protein 1 as a carrier of the Fe-S centers. In this case, the 8-kDa polypeptide could be a metallothionine involved in the stabilization or activation of PS I (63). If the active PS I involves a dimer (16), a tetramer (18), or a hexamer (64) of chlorophyll *a*-protein 1, it is possible for more than one traditional 4 Fe-4 S center to be carried by this protein alone. If the cysteine residues of the Fe-S cluster are contributed by several identical or different polypeptides, the cluster would be expected to be exceedingly prone to loss upon fractionation. This type of association of both chlorophyll and iron has recently been demonstrated in the reaction center of *Rhodopseudomonas viridis* (65) and for the Fe-S cluster of a bacterial nitrogenase (66). It also applies for the heme group of cytochrome b_{559} which has been shown to be coordinated by two histidine residues (67), and since the gene for spinach cytochrome b_{559} codes for only one histidine (68), the *in vivo* organization of cytochrome b_{559} must be that of a homo- or heteromultimer, possibly with an apparent molecular weight of 30 kDa (69). A similar result has been reported for aspartate transcarbamoylase where the active sites of the enzyme are at the interphases between adjacent polypeptide chains (70). If a similar situation applies for some of the Fe-S clusters of PS I, SDS-PAGE would reveal no attachment to the separated polypeptides. To look into this possibility, limited proteolytic

cleavage of the PS I preparations was attempted in order to permit the isolation of a structure still carrying the Fe-S centers. These studies have not yet been successful. The most direct way to answer the questions posed will apparently be to purify P700-chlorophyll *a*-protein 1 with retention of the bound iron in order to measure the ESR characteristics of this single polypeptide. After the completion of this manuscript, Golbeck and Cornelius (71) interpreted measurements on flash-induced absorbance transients observed in lithium dodecyl sulfate-treated PS I particles to indicate that the Fe-S center X (A_2) is closely associated with and perhaps integral with the P700-containing protein. This interpretation would be in agreement with our results.

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