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The Isolation and Characterization of a New Iron-Sulfur Protein from Photosynthetic Membranes

(photosynthesis/chloroplasts/ferredoxins/electron carriers)

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ABSTRACT **A new iron-sulfur protein, distinct from the soluble chloroplast ferredoxin, was isolated from chloroplast membranes. The isolated protein, purified to homogeneity, had a molecular weight of about 8000 and 4 atoms of iron and 4 inorganic sulfides per mole. Its absorption spectrum had a broad absorbance band in the 400 nm region, a shoulder at approximately 310 nm, and a peak around 280 nm. The absorbance ratio A_{400} to A_{280} was 0.55. The electron paramagnetic resonance spectrum (measured at 12°K) of the reduced protein was similar to that of other reduced iron-sulfur proteins, showing a major resonance line at $g = 1.94$.**

The isolated protein, when photoreduced by spinach chloroplasts, can in turn transfer electrons to mammalian cytochrome *c*. However, the photoreduced protein cannot replace soluble ferredoxin in $NADP^+$ reduction because of its apparent inability to interact with the chloroplast enzyme, ferredoxin-$NADP^+$ reductase.

The relation of the isolated iron-sulfur protein to the bound ferredoxin that acts as the primary electron acceptor in Photosystem I is discussed.

The discovery and characterization of the iron-sulfur protein, chloroplast ferredoxin, led to extensive investigations that elucidated its key role in photosynthetic electron transport (1–3). This article reports the isolation, purification, and characterization of a new iron-sulfur protein from chloroplasts that differs in several respects from ferredoxin.

The presence in chloroplasts of one or more bound iron-sulfur proteins, distinct from soluble ferredoxin, was initially detected by low-temperature electron paramagnetic resonance (EPR) studies (4–6). The low-temperature EPR technique was also used to show that one of these bound iron-sulfur proteins (referred to as bound ferredoxin) serves as the primary electron acceptor of Photosystem I in chloroplasts (7, 8, 5, 9). However, no such membrane-bound iron-sulfur proteins have heretofore been isolated from chloroplasts, and their chemical nature has until now remained unknown.

METHODS

Chloroplast Preparation. Two procedures were used to prepare spinach chloroplast material from which the bound iron-sulfur protein was extracted. Initially, whole chloroplasts were isolated from spinach leaves and totally freed of soluble ferredoxin before the extraction of the bound iron-sulfur protein was begun. After it was demonstrated by this procedure how the bound iron-sulfur protein could be distinguished from soluble ferredoxin, a second, more rapid procedure was used in which broken chloroplasts already free of soluble ferredoxin were isolated from spinach leaves and used directly for the extraction of the bound iron-sulfur protein.

A typical preparation involved the use of about 3 kg of spinach leaves. All preparative steps were carried out at 4°C. In the first procedure, whole chloroplasts were isolated by blending 150-g batches of spinach leaves in a Waring blendor for 30 sec in 250 ml of a solution containing 0.3 M sucrose, 50 mM Tris·HCl buffer (pH 7.8), and 20 mM NaCl. The slurry was filtered through six layers of cheesecloth. Centrifugation of the filtrate for 7 min at 1000 × *g* yielded a pellet containing whole chloroplasts. Broken chloroplasts were made by resuspending the whole chloroplasts in cold distilled water (to give a chlorophyll concentration of approximately 0.5 mg/ml) and centrifuging the suspension for 10 min at 35,000 × *g*. The pellet containing broken chloroplasts was resuspended in cold distilled water (final chlorophyll concentration, 1 mg/ml) and the suspension was sonicated for 2 min at full power with a Branson sonifier. The sonicated suspension was passed over a 3 × 5-cm DEAE-cellulose column equilibrated with water. All of the green eluate was collected and lyophilized for 24 hr.

In the second procedure, broken spinach chloroplasts were prepared directly by blending spinach leaves in 50 mM Tris·HCl buffer (pH 8.0), passing the slurry through six layers of cheesecloth, and centrifuging the filtrate at 35,000 × *g* for 10 min. The pellet, containing the chloroplast fragments, was washed once with distilled water and then lyophilized.

Extraction and Solubilization of the Bound Iron-sulfur Protein. The lyophilized chloroplast fragments (approximately 1.5 g of chlorophyll) were suspended in 1 liter of absolute methanol (precooled to −20°C) containing 1 ml of 2-mercaptoethanol. This suspension was quickly mixed with 15 liters of acetone (precooled to −20°C) containing 10 ml of 2-mercaptoethanol. The resulting suspension was stirred for approximately 1 min and rapidly filtered through a coarse-sintered glass funnel. The residue remaining on the funnel was dried with N_2 gas and was resuspended and stirred for 2 hr in a solution of 500 ml 50 mM Tris·HCl buffer (pH 7.6) containing 2 mM cysteine-hydrochloride (hereafter referred to as buffer). The suspension was centrifuged at 35,000 × *g* for 10 min and the pellet was discarded. The pale yellow-brown supernate constituted the soluble fraction used for further purification of the iron-sulfur protein. The soluble fraction contained approximately half of the total inorganic sulfide, assayed as described below, in the chloroplast fragments prior to extraction.

Abbreviation: EPR, electron paramagnetic resonance.

Purification of the Solubilized Bound Iron-sulfur Protein. The supernate solution was applied to a 3 × 25-cm DEAE-cellulose column (equilibrated with buffer) and the column was developed with a linear salt gradient (0.1–0.6 M NaCl). Fractions (8 ml) were collected and assayed for inorganic sulfide. The sulfide-containing fractions were combined and diluted 5-fold with buffer free of cysteine. The dilute solution was applied to a 2 × 8-cm DEAE-cellulose column (equilibrated with buffer) and the adsorbed brown material was eluted with buffer containing 0.5 M NaCl. This concentrated fraction was applied to a 2.5 × 90-cm Sephadex G-75 column (equilibrated with buffer containing 50 mM NaCl) and the column was developed with the same buffer. Fractions (5 ml) were collected and the absorbancies at 280 and 400 nm were determined. The inorganic sulfide content of the fractions was also determined, and the sulfide-containing fractions were concentrated in an Amicon ultrafiltration apparatus (UM-10 membrane). The recovery of sulfide was 10–20% of the amount present in the crude soluble fraction. The concentrated protein was stored at 4°C under anaerobic conditions; exposure to oxygen gave a loss of absorbance in the visible region.

Assay Procedures. Non-heme iron was determined by the procedure of Miller and Massey (10), inorganic sulfide by the procedure of Brumby *et al.* (11). Soluble chloroplast ferredoxin (prepared by R. Chain) was used as the standard in both of these assays, and the concentration of this protein was calculated on the basis of a millimolar extinction coefficient of 9.68 at 420 nm (12). Protein was estimated by a modified Lowry-phenol reagent procedure (13).

Polyacrylamide gel electrophoresis with 20% acrylamide gels (Tris·glycine buffer, pH 8.3) was carried out as described by Davis (14).

Photoreduction of $NADP^+$ and cytochrome *c* at 340 nm and 550 nm, respectively, was measured in a Gilford spectrophotometer modified for actinic illumination (15) using washed, broken spinach chloroplasts prepared as previously described (16).

X-band EPR spectra were recorded in a modified JEOL spectrometer at 12°K as described elsewhere (8).

The molecular weight of the iron-sulfur protein was estimated by gel filtration (17) on a Sephadex G-75 column (1.5 × 90-cm) equilibrated with buffer containing 50 mM NaCl. The proteins of known molecular weight used as standards were cytochrome *c*, myoglobin, chymotrypsinogen A, and ovalbumin.

RESULTS

Properties of the Purified Iron-Sulfur Protein. After Sephadex gel filtration, the iron-sulfur protein gave a single band in polyacrylamide gel electrophoresis and a single peak in sedimentation velocity ultracentrifugation. Determinations of the non-heme iron and inorganic sulfide content of the purified protein yielded values of 0.5 μmoles each of iron and sulfide per mg of protein. The molecular weight of the protein, estimated by Sephadex gel filtration, was approximately 8000. A molecular weight of 7500 was obtained by sedimentation velocity ultracentrifugation. On the basis of a molecular weight of 8000, the protein contains 4 atoms of non-heme iron and 4 inorganic sulfides per mole. However, because of uncertainty in the determination of protein concentrations of iron-sulfur proteins by the Lowry-phenol procedure used here, these values for iron and sulfide require confirmation by a dry-weight analysis of the protein.

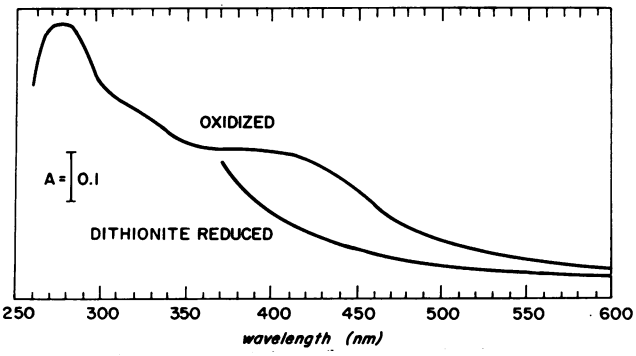

FIG. 1. Absorption spectra of bound chloroplast iron-sulfur protein. Protein concentration was 0.3 mg/ml (in the buffer solution). The spectrum of the reduced protein was obtained by the addition of a small amount of solid sodium dithionite.

The absorption spectrum of the bound iron-sulfur protein in the oxidized form shown in Fig. 1 is clearly different from that of soluble chloroplast ferredoxin, which has peaks in the visible region at 420 and 463 nm and a peak in the near-ultraviolet region at 330 nm (1, 3, 18). The spectrum of the bound iron-sulfur protein shows a broad absorbance band in the 400-nm region, a shoulder at approximately 310 nm, and a peak in the ultraviolet region at approximately 280 nm. This absorption spectrum is similar to the spectra of "bacterial-type" ferredoxins which contain more than two iron atoms per mole of protein (18). The ratio of the absorbance at 400 nm to that at 280 nm for the purified iron-sulfur protein was 0.55. This ratio is similar to the corresponding ratio reported for ferredoxin (type II) isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (19) and for some of the ferredoxins from the nonphotosynthetic bacteria *Azotobacter vinelandii* (20) and *Bacillus polymyxa* (21–23). The computed extinction coefficient per mole of acid-labile sulfide for the bound chloroplast iron-sulfur protein was around 4000—similar to that of other iron-sulfur proteins (18).

Reduction of the iron-sulfur protein with sodium dithionite resulted in a decrease in the absorbance in the visible region (Fig. 1). The difference spectrum of the reduced *minus* oxidized spectra showed a bleaching with a maximum at approximately 430 nm. After reduction, the protein was reoxidizable by oxygen, as evidenced by the restoration of the absorbance in the visible region.

The iron-sulfur protein was reduced by light but the spectrum of the photoreduced protein could not be recorded because, as shown in Fig. 2, the photoreduced protein underwent a rapid reoxidation after the cessation of illumination. This reoxidation did not appear to be related to the presence of oxygen in the reaction mixture since, under comparable conditions, photoreduced soluble ferredoxin, which is also oxidized by oxygen (see Fig. 2 in ref. 24) remained in the reduced state after the cessation of illumination (Fig. 2).

The EPR spectra of the photoreduced and the dithionite-reduced iron-sulfur protein at 12°K are shown in Fig. 3. The dithionite-reduced spectrum is similar to those of other reduced iron-sulfur proteins, showing a marked *g*-anisotropy with a maximum line at $g = 1.94$ (25). A 3-fold increase in signal intensity was obtained when the protein was reduced

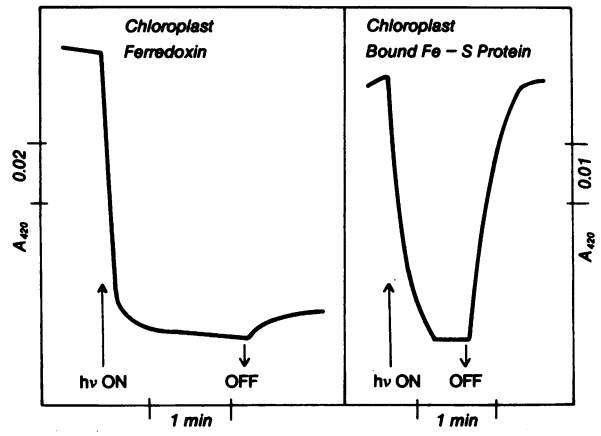

FIG. 2. Photoreduction of soluble chloroplast ferredoxin and bound chloroplast iron-sulfur protein. The reaction mixture (1.0 ml) contained heated broken chloroplast fragments (50 μg of chlorophyll) and (in μmoles): *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 25; sodium ascorbate, 10; 2,6-dichlorophenol indophenol, 0.04; and 3-(3'-4'-dichlorophenyl)-1,1'-dimethyl urea (DCMU), 0.001. Where indicated, 2 mg of soluble chloroplast ferredoxin or 0.6 mg of bound iron-sulfur protein was added.

in the presence of a small amount of methyl viologen (10^{-5} M) in addition to the sodium dithionite. A similar EPR spectrum, except for a small free-radical signal from the chloroplasts (g = 2.00), was obtained when the protein was photoreduced by chloroplasts at room temperature and frozen in the light prior to the recording of its EPR spectrum at 12°K.

The temperature sensitivity of the EPR signal was similar to that of other iron-sulfur proteins in that its intensity markedly decreased as the temperature was raised; no signal could be observed at temperatures above 25°K.

Biological Activity of the Purified Iron-sulfur Protein. As shown in Fig. 4, the iron-sulfur protein was unable to replace soluble chloroplast ferredoxin as an electron carrier in the photoreduction of $NADP^+$ by chloroplasts. The protein was ineffective as an electron carrier in this reaction with either water or reduced 2,6-dichlorophenol indophenol as the electron donor. This result was unexpected because most iron-sulfur proteins of the ferredoxin type isolated from photosynthetic or nonphotosynthetic bacteria can substitute for soluble chloroplast ferredoxin in $NADP^+$ photoreduction (see refs. 1, 19, 20–22, 26). The inability of the bound iron-sulfur protein to replace soluble ferredoxin in $NADP^+$ photoreduction was not the result of the protein not being photoreduced by chloroplasts. As shown in Fig. 5, the iron-sulfur protein was as effective as soluble ferredoxin in the light-dependent transfer of electrons from water to mammalian cytochrome *c*. To avoid any possible differences between chloroplast preparations, the same chloroplasts were used to measure both $NADP^+$ and cytochrome *c* photoreduction.

Another difference between the bound iron-sulfur protein and soluble chloroplast ferredoxin was in their ability to reduce cytochrome *c* with NADPH, in the presence of ferredoxin-$NADP^+$ reductase (EC 1.6.7.1). Cytochrome *c* reduction was totally dependent on the addition of soluble ferredoxin, whereas the bound iron-sulfur protein was ineffective in this reaction.

DISCUSSION

Recent work in this laboratory has led to the isolation and chemical characterization of two different and biologically

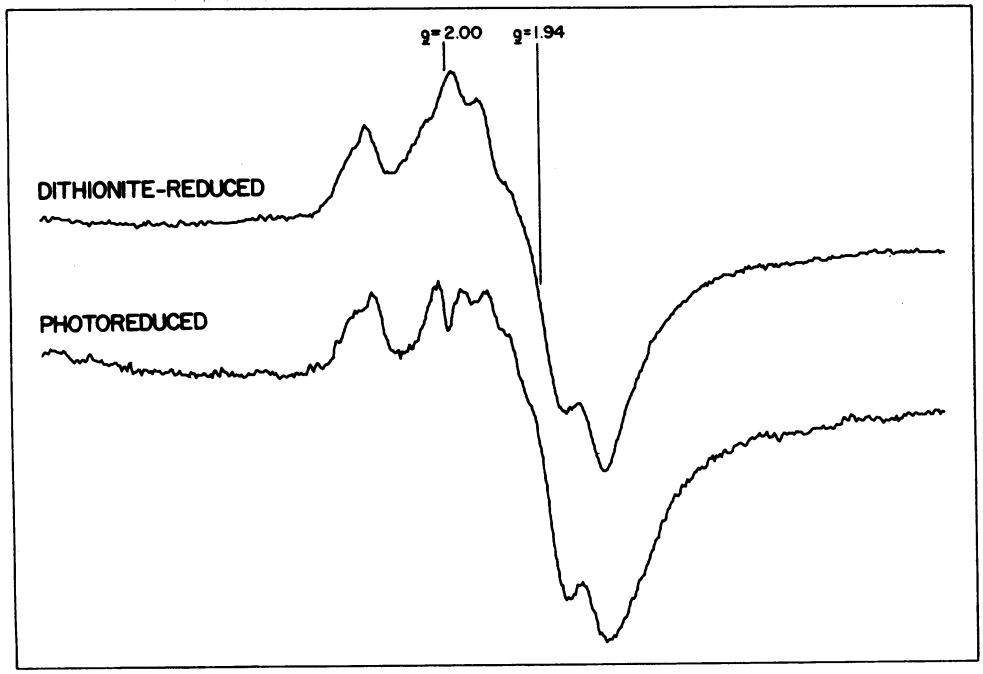

FIG. 3. EPR spectra of bound chloroplast iron-sulfur protein. Protein concentration was 0.6 mg/ml. The chemically reduced spectrum was obtained by the addition of a small amount of solid sodium dithionite. For photoreduction, the reaction mixture (0.5 ml) contained heated broken chloroplast fragments (20 μg of chlorophyll) and (in μmoles): Tricine buffer (pH 7.8), 20; sodium ascorbate, 0.5; 2,6-dichlorophenol indophenol, 0.04; and DCMU, 0.005. The reaction mixture was made anaerobic by evacuation followed by gassing with N_2 in a Thunberg-type EPR tube, illuminated with wide-band red light, and frozen in the light. Conditions of EPR spectroscopy: microwave frequency, 9.1 GHz; modulation frequency, 100 kHz; modulation amplitude, 10 G; microwave power, 4 mW; sample temperature, 12°K.

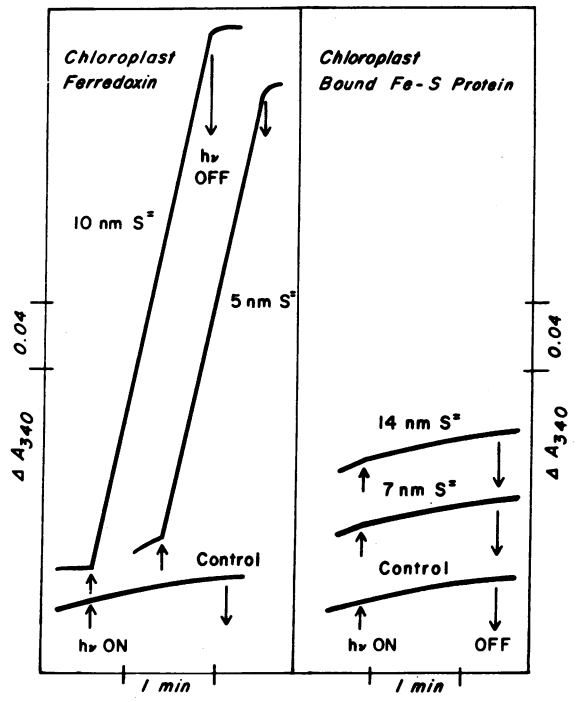

FIG. 4. Effect of soluble chloroplast ferredoxin and bound iron-sulfur protein on NADP+ photoreduction. The reaction mixture (1.0 ml) contained washed broken chloroplast fragments (50 μg of chlorophyll) and (in μmoles): HEPES buffer (pH 7.5), 50; NH_4Cl, 5; and NADP+, 5. Where indicated, soluble ferredoxin or bound iron-sulfur protein containing the specified amount of sulfide was added. NADP+ reduction was measured at 340 nm.

active ferredoxins from the same bacterial species. Two such distinct ferredoxins were isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (19) and also from the nonphotosynthetic nitrogen-fixing bacteria *Azotobacter vinelandii* (20) and *Bacillus polymyxa* (22). The present investigation led to the isolation from the photosynthetic apparatus of plants of a new kind of an iron-sulfur protein, different from soluble ferredoxin.

The physical and chemical differences between the membrane-bound iron-sulfur protein of chloroplasts and the soluble ferredoxin include molecular weight, iron and inorganic sulfide content, EPR spectrum, and absorption spectrum in the visible range. Of special interest are the findings

TABLE 1. *Some properties of soluble ferredoxin and bound iron-sulfur protein from spinach chloroplasts*

	Soluble ferredoxin	Bound Fe-S protein
Molecular weight	12,000	8,000
Fe (per mole)	2	4
$S^=$ (per mole)	2	4
Major spectral bands (nm)	280, 330, 420, 463	280, 400
Electron transport capacity		
Photoreduction of cytochrome *c*	+	+
Photoreduction of NADP+	+	−
Reduction of cytochrome *c* by NADPH	+	−

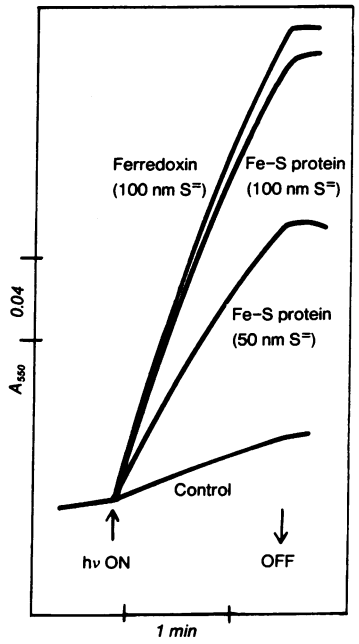

FIG. 5. Effect of soluble chloroplast ferredoxin and bound iron-sulfur protein on cytochrome *c* photoreduction. Reaction mixture as in Fig. 4 except that horse heart cytochrome *c* (4 mg/ml) replaced NADP+. Cytochrome *c* reduction was measured at 550 nm.

that the physical and chemical properties (summarized in Table 1) of the chloroplast iron-sulfur protein resemble more those of the bacterial-type than the plant-type ferredoxin. The absorption spectrum of the iron-sulfur protein is similar to one of the ferredoxins from *Rhodospirillum rubrum* (19) and *Bacillus polymyxa* (21–23). Like the *Bacillus polymyxa* ferredoxin, the isolated chloroplast protein has four iron atoms and four inorganic sulfides and a molecular weight of approximately 8000.

In its biological activity, the bound iron-sulfur protein appears to differ from both plant and bacterial ferredoxins by being unable to interact with ferredoxin-NADP+ reductase, the chloroplast enzyme that reversibly mediates electron transfer from ferredoxin to NADP+ (27). Thus, the photoreduced iron-sulfur protein can reduce cytochrome *c*—a reaction for which ferredoxin-NADP+ reductase is not required—but cannot reduce NADP+, a reaction for which the enzyme is required. Moreover, the iron-sulfur protein cannot reduce cytochrome *c* in the dark with NADPH as the reductant since electron transfer from NADPH to the iron-sulfur protein is also dependent on ferredoxin-NADP+ reductase.

Of greatest interest is the question whether the isolated iron-sulfur protein is identical with the bound ferredoxin that functions as the primary electron acceptor of Photosystem I in chloroplasts or whether it is only one of several bound iron-sulfur proteins present in chloroplasts (4–6). This question cannot as yet be answered with assurance. On the one hand, the difference absorption spectrum (reduced *minus* oxidized) of the isolated protein is similar (in the visible region) to that reported for *P*-430, a chloroplast component thought to be related to the bound ferredoxin (6, 9, 28). On the other hand, the EPR properties of the reduced iron-sulfur protein differ significantly from those of the photoreduced bound ferredoxin *in situ*. It remains to be determined

whether the EPR differences may have resulted from the rather harsh procedure required for the isolation of the protein.

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