Formation of the iron–sulfur cluster of ferredoxin in isolated chloroplasts

cysteine/sulfide/iron–sulfur protein/light/ATP

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Communicated by Emil L. Smith, November 26, 1985

ABSTRACT The formation of the iron–sulfur cluster of ferredoxin was examined in vitro by incubating isolated chloroplasts with [35S]cysteine. The ferredoxin molecule was radioactively labeled in chloroplasts without synthesis of its polypeptide and comigrated with holoferreredoxin during polyacrylamide gel electrophoresis under nondenaturing conditions. When the labeled ferredoxin was denatured by the addition of trichloroacetic acid, radioactive acid-labile sulfide in the cluster was released from the polypeptide as a gas and trapped in a 0.1 M NaOH solution. These results indicate that the sulfur atom derived from cysteine was incorporated into ferredoxin through formation of the iron–sulfur cluster. This process was stimulated by light and inhibited by the electron transport inhibitor, dichlorophenyldimethyleurea, and the uncouplers, atebrin and gramicidin, but not by the protein synthesis inhibitor, chloramphenicol. These inhibitory effects were reversed by the addition of ATP to the incubation mixture. Formation of the iron–sulfur cluster of ferredoxin in chloroplasts is thus dependent on ATP.

Ferredoxin is the name given to the small, acidic, and nonenzymatic protein containing iron–sulfur cluster, and it mediates electron transfer in various important physiological processes such as photosynthesis and nitrite reduction (1). Higher plant ferredoxins contain a 2 Fe + 2 S cluster as an active center and are localized in chloroplasts, whereas their polypeptides are coded by nuclear DNA (2, 3).

The larger precursor of ferredoxin was identified by a cell-free translation system primed with poly(A) RNA from tobacco, bean, and Chlamydomonas (4). The nucleotide sequence of a cDNA clone derived from Silen pratensis ferredoxin has recently been reported (5), and the ferredoxin precursor may possibly have a leader peptide containing 48 amino acid residues at the amino-terminal end of a mature ferredoxin. In vitro studies have indicated that the ferredoxin precursor is incorporated into isolated chloroplasts with a concomitant processing to mature size (6), as is the case for a large number of chloroplast proteins encoded by nuclear DNA (7, 8).

One important aspect regarding the mechanism of ferredoxin biosynthesis is the formation of the iron–sulfur cluster. Apoferreredoxin, the iron- and sulfide-free protein, was reconstituted to native holoferreredoxin by a chemical reaction with iron and sulfide in the presence of 2-mercaptoethanol (9, 10). However, it is not known whether holoferreredoxin is formed in vivo through an analogous reaction. It seems likely that enzymic reactions participate in the formation of the iron–sulfur cluster in apoferreredoxin. In 1971, Finazzi-Agro et al. (11) reported rhodanese (thiosulfate/cyanide sulfurtransferase, EC 2.8.1.1) prepared from beef kidney to contribute to the formation of labile sulfur in the 2 Fe + 2 S cluster of ferredoxin in the in vitro experiment. Although kinetic studies suggest that rhodanese is directly involved in incorporating sulfide into ferredoxin (12, 13), it is possible that rhodanese serves only to provide the sulfide later incorporated into apoferredoxin in a nonenzymatic manner, as in the chemical reconstitution of ferredoxin (9, 10).

In this report, we describe a physiological pathway by which the iron–sulfur cluster is introduced into ferredoxin using cysteine as a sulfide donor in intact chloroplasts.

MATERIALS AND METHODS

Reactions of Spinach Chloroplasts with Labeled Amino Acids. Intact chloroplasts were isolated from spinach leaves by Percoll gradient centrifugation according to Morgenharter et al. (15) with modifications as described (16). Isolated chloroplasts were >90% intact, as estimated by ferricyanide reduction (17). Chlorophyll was determined according to the method of Arnon (18). Isolated chloroplasts were washed with sorbitol N-tris (hydroxymethyl)methylglycine (Tricine) buffer (pH 8.4), consisting of 0.33 M sorbitol and 50 mM Tricine KOH, and resuspended in this buffer to make a concentration of 0.2–0.3 mg of chlorophyll per ml. The chloroplast suspension was incubated with [35S]cysteine (>600 Ci/mmol; 1 Ci = 37 GBq; Amersham) at a concentration of 300 μCi per mg of chlorophyll in a glass test tube in a water bath maintained at 20°C, and illuminated by filtered red light at 8000 lx for 1 hr. Where indicated, [35S]methionine (>800 Ci/mmol; Amersham) was also incubated with chloroplasts at the same concentration as [35S]cysteine.

Polyacrylamide Gel Electrophoresis Under Nondenaturing Conditions. After incubation, chloroplasts were resedimented by centrifugation at 2000 x g for 1 min and the plastid pellet was washed twice with sorbitol Tricine (pH 8.4). The chloroplasts were lysed by suspending them in 50 mM Tris/HCl (pH 7.8), and the lysate was centrifuged at 15,000 x g for 5 min. The supernatant fraction containing the stromal proteins was mixed with glycerol at a final concentration of 5% (wt/vol) with a small amount of bromophenol blue. Then, aliquots of this sample were subjected to conventional polyacrylamide gel (15%) electrophoresis under nondenaturing conditions as described by Williams and Reisfeld (19) using vertical gel slabs. In the gel system used, holoferreredoxin could be separated from other components in the chloroplasts. After the electrophoresis, the gel was fixed with 50% methanol at 4°C to prevent denaturation of ferredoxin and was subjected to autoradiography against x-ray film (Kodak X-Omat AR).

NaDodSO4 Gel Electrophoresis and Fluorography. Aliquots of the stromal fraction of labeled chloroplasts were denatured and analyzed on a 14% polyacrylamide resolving gel, using the buffer system of Laemmli (20). The gel was then treated with EN3HANCE (New England Nuclear) and subjected to fluorography.

Preparation of Sepharose 4B Coupled with Ferredoxin–NADP+ Reductase. Spinach ferredoxin–NADP+ reductase
(ferredoxin-NADP⁺ oxidoreductase, EC 1.18.1.2) was purified essentially by the method described in the preparation from Spirulina (21). About 22 mg of the purified enzyme was obtained from 3 kg of spinach leaves. CNBr-activated Sepharose 4B was prepared by the method of Cuatrecasas (22). About 10 mg of ferredoxin-NADP⁺ reductase was coupled to the activated gel (10 ml in a packed volume) under the conditions suggested by the manufacturer (Pharmacia).

The stromal fraction from labeled chloroplasts was incubated with ferredoxin-NADP⁺ reductase Sepharose 4B for 2 hr at 4°C in a solution containing 25 mM Tris-HCl (pH 7.8). This gel was washed in this buffer, and the ferredoxin adsorbed onto the gel was eluted with 0.1 M Tris-HCl buffer (pH 7.8) containing 0.5 M NaCl.

**Analysis of the Acid-Labile [35S]Sulfide Released from Ferredoxin.** After the electrophoresis under non-denaturing conditions, the area containing ferredoxin was excised and the radioactively labeled could not be detected on a dried gel. The stromal fraction from labeled chloroplasts was incubated with [35S]cysteine, [35S]methionine, or [35S]methionine, respectively. They were analyzed by gel electrophoresis under non-denaturing conditions followed by autoradiography. Lanes 3 and 4, the stromal proteins labeled with [35S]cysteine or with [35S]methionine, respectively. They were analyzed by gel electrophoresis under non-denaturing conditions followed by autoradiography. Lanes 5 and 6, the same samples as in lanes 3 and 4, respectively, but the electrophoresis was performed in the NaDodSO₄ gel followed by fluorography. Apo and Hol-Fd refer to the positions on the gel of apoferrredoxin and holoferrredoxin under denaturing (NaDodSO₄ treatment) and non-denaturing conditions, respectively. LS marks the large subunit of rubisco-1,5-bisphosphate carboxylase.

**RESULTS**

To distinguish native holoferrredoxin from apoferrredoxin, an iron- and sulfur-free form, we used polyacrylamide gel electrophoresis under non-denaturing conditions. Many polypeptide bands were found with apoferrredoxin migrating more slowly in the gel than the single band of holoferrredoxin (Fig. 1, lanes 1 and 2), presumably because of the random formation of disulfide bonds in the apoferrredoxin in an intramolecular manner; apoferrredoxin migrated as a single band after carboxymethylation of cysteine residues (data not shown).

When freshly isolated spinach chloroplasts were incubated with [35S]cysteine under light illumination, the soluble fraction of chloroplasts gave many radioactively labeled proteins that could be visualized on an autoradiogram (Fig. 1, lane 3) after gel electrophoresis under non-denaturing conditions. Among them, there was a radioactive protein with an electrophoretic mobility corresponding to holoferrredoxin. However, when the chloroplasts were incubated with [35S]methionine, no such band could be observed (lane 4). The synthesis of proteins by chloroplasts was also analyzed by NaDodSO₄ gel electrophoresis and fluorography, and the results are shown in lanes 5 and 6. Both [35S]cysteine and [35S]methionine were incorporated into the chloroplasts and utilized in the synthesis of several polypeptides, including the large subunit of ribulose-1,5-bisphosphate carboxylase. The ferredoxin radioactively labeled could not be detected on the NaDodSO₄ gel even when the chloroplasts were incubated with [35S]cysteine, in agreement with previous reports indicating the polypeptide moiety of ferredoxin to be synthesized in the cytoplasm but not in the chloroplasts (3, 4, 6). These results suggest that the protein with the same electrophoretic mobility as holoferrredoxin was radioactively labeled by [35S]cysteine without synthesis of the polypeptide chain of ferredoxin.

To confirm the labeled protein as holoferrredoxin, its ability to bind ferredoxin-NADP⁺ reductase was examined. After incubation with [35S]cysteine, the soluble fraction of the chloroplasts was incubated with Sepharose 4B coupled with ferredoxin-NADP⁺ reductase. As shown in Fig. 2, only the radioactive protein that comigrated with holoferrredoxin was adsorbed onto the Sepharose, indicating that this radioactive species had the same conformation as the native ferredoxin, which interacts with the ferredoxin-linked enzyme, ferredoxin-NADP⁺ reductase (23).

The radioactively labeled holoferrredoxin (Fig. 1, lane 3) was eluted from the gel and denatured by adding trichloroacetic acid to a Thunberg tube. The label sulfide of the iron–sulfur cluster in the ferredoxin was expected to be released from the polypeptide in the form of H₂S gas and trapped in the 0.1 M NaOH solution in the side arm. Table 1 shows that 46% of the radioactivity was recovered in the alkaline solution, 42% remained in the trichloroacetic acid solution, and 12% was lost in the atmosphere during the operation. The trichloroacetic acid precipitate containing apoferrredoxin was electrophoresed again, but there was no detectable radioactive band on the autoradiogram (data not shown). Lane 5 in Fig. 1 also shows that the [35S]methionine was released from ferredoxin under denaturing conditions with NaDodSO₄. These observations indicate that label sulfide in the iron–sulfur cluster of ferredoxin was introduced from [35S]cysteine or its metabolic product through formation of an iron–sulfur cluster.

No radioactive sulfide was incorporated into ferredoxin when the reaction with intact chloroplasts proceeded in the dark, as evident from Fig. 3A, but such an incorporation did occur, even in the dark, when ATP was added to the reaction system (Fig. 4, lane 3). Fig. 3B shows that [35S]cysteine was incorporated into chloroplasts under both light and dark conditions, and thus light stimulation of the cluster formation is independent of the transport process of cysteine across the envelope of chloroplasts. Several specific inhibitors were used to determine the process responsible for light stimulation of this sulfide incorporation into ferredoxin. Dichloro-phenyldimethylurea inhibits the electron flow between the two photosystems. Atebrin and gramicidin are uncouplers.
that prevent generation of electrochemical gradients across the thylakoid membranes. As evident in Fig. 4, these inhibitors suppress the light-stimulated formation of the iron-sulfur cluster. When ATP was present in the incubation mixture at a concentration of 10 mM, the inhibitory effects of dichlorophenyldimethylurea, atebrin, and gramicidin on cluster formation were reversed, and, in some cases, their effects were essentially the same as under light. Chloramphenicol, an inhibitor of chloroplast protein synthesis, did not alter light stimulation of the cluster formation (lanes 11 and 12).

**DISCUSSION**

We developed an in vitro system to examine the formation of the iron-sulfur cluster of ferredoxin in spinach chloroplasts. We used conventional polyacrylamide gel electrophoresis at a concentration of 10 mM, the inhibitory effects of dichlorophenyldimethylurea, atebrin, and gramicidin on cluster formation were reversed, and, in some cases, their effects were essentially the same as under light. Chloramphenicol, an inhibitor of chloroplast protein synthesis, did not alter light stimulation of the cluster formation (lanes 11 and 12).

**Table 1.** Distribution of the $^{35}$S moiety of labeled ferredoxin treated with 10% trichloroacetic acid

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<th>cpm</th>
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<tr>
<td>Before treatment</td>
<td>5200</td>
<td>100</td>
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<tr>
<td>After treatment</td>
<td></td>
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<tr>
<td>In 0.1 M NaOH</td>
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<td>46</td>
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<tr>
<td>In 10% trichloroacetic</td>
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<td>11</td>
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<tr>
<td>acid supernatant</td>
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<tr>
<td>In 10% trichloroacetic</td>
<td>1600</td>
<td>31</td>
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<td>acid precipitate</td>
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$^{35}$S-labeled ferredoxin was incubated with 10% trichloroacetic acid in a Thunberg tube. The radioactivities recovered in trichloroacetic acid soluble and insoluble fractions and trapped with 0.1 M NaOH solution in the side arm were measured as described.

**Fig. 2.** Affinity binding of the labeled ferredoxin to ferredoxin--NADP$^+$ reductase Sepharose 4B. The intact chloroplasts were incubated with $[^{35}$S]cysteine and the stromal fraction of chloroplasts, prepared as described in Fig. 1, was incubated with ferredoxin--NADP$^+$ reductase Sepharose 4B. Ferredoxin adsorbed onto the resin was eluted and analyzed by gel electrophoresis under nondenaturing conditions. Lane 1, labeled stromal proteins before treatment with ferredoxin--NADP$^+$ reductase Sepharose; lane 2, the eluate from the ferredoxin--NADP$^+$ reductase Sepharose. Fd refers to the position of the holoferredoxin.

**Fig. 3.** Time course of the formation of the iron-sulfur cluster of ferredoxin in isolated chloroplasts. (A) Intact chloroplasts were incubated with $[^{35}$S]cysteine under light (lanes 1–3) or in the dark (lanes 4–6). At the times indicated above the lanes, the samples were removed and the labeled stromal proteins were analyzed by gel electrophoresis under nondenaturing conditions followed by autoradiography. Fd indicates the authentic holoferredoxin. (B) Time course of the transport of $[^{35}$S]cysteine across the membrane of chloroplasts. Intact chloroplasts were incubated in the light (c) or dark (o) as described above. The chloroplasts were reisolated at an appropriate time, washed with sorbitol-Tricine (pH 8.4) containing 1 mM of nonlabeled cysteine, and lysed. The radioactivity in the soluble fraction of chloroplasts was counted with a liquid scintillation counter. The labeled bands of ferredoxin shown in A were quantified by densitometry of the exposed x-ray film and the data plotted. $\Delta$ and $\triangle$, labeled ferredoxin in the light and dark, respectively.

The present experiments clearly indicate that soluble sulfide in the iron-sulfur cluster of spinach ferredoxin is radioactively labeled when isolated spinach chloroplasts are incubated with $[^{35}$S]cysteine on the basis of the following: (i) under nondenaturing conditions, a radioactively labeled protein showed the same electrophoretic mobility as holoferrredoxin; (ii) this protein showed ability to bind with ferredoxin--NADP$^+$ reductase; (iii) under denaturing conditions such as trichloroacetic acid treatment and NaDodSO$_4$ gel electrophoresis, the $^{35}$S moiety was released from the polypeptide and ferredoxin was converted to apoferredoxin having no radioactivity; and (iv) by trichloroacetic acid treat-
containing O-acetylserylserine (thiol)-lyase [O-acetyl-L-serine acetyl-lyase (adding hydrogen sulfide)]. EC 4.2.99.8], which synthesizes cysteine from bound or free sulfide (24). Such cysteine is utilized in extremely complex metabolic processes (25). That the donor of sulfur to ferredoxin is cysteine or its metabolite in chloroplasts thus seems reasonable to conclude. In the present experiments, endogenous iron in chloroplasts was assumed to be incorporated into ferredoxin. The iron is probably supplied directly or indirectly by phytoferrin, an iron storage protein in chloroplasts (6).

The enzymic reconstitution of holoferedoxin in vitro has been reported by using rhodanese from beef kidney and liver (11, 12). However, the role of this enzyme in chloroplasts still remains uncertain. We are now purifying an enzyme having activity for cluster formation from the soluble fraction of chloroplasts, using ammonium sulfate fractionation, DEAE-cellulose chromatography, and gel filtration. We have obtained evidence that a partially purified enzyme has activity for forming the iron–sulfur cluster in the apoferredoxin without detectable rhodanese activity. The results of these experiments will be presented in detail elsewhere.

The authors express their sincere appreciation to Dr. K. Wada for his interest and advice during the course of the present research. This work was supported in part by Grants-in-Aid for Encouragement of Young Scientists (Grant 6074072) and a Grant-in-Aid for Scientific Research (Grant 60470156) from the Ministry of Education, Science and Culture of Japan.