Roles of ATP and NADPH in Formation of the Fe-S Cluster of Spinach Ferredoxin

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ABSTRACT

Ferredoxin (Fd) in higher plants is encoded by a nuclear gene, synthesized in the cytoplasm as a larger precursor, and imported into the chloroplast, where it is proteolytically processed, and assembled with the [2Fe-2S] cluster. The final step in the biosynthetic pathway of Fd can be analyzed by a reconstitution system composed of isolated chloroplasts and [35S]cysteine, in which [35S]sulfide and iron are incorporated into Fd to build up the 35S-labeled Fe-S cluster. Although a lysed chloroplast system shows obligate requirements for ATP and NADPH, in vitro chemical reconstitution of the Fe-S cluster is generally thought to be energy-independent. The present study investigated whether ATP and NADPH in the chloroplast system of spinach (Spinacia oleracea) are involved in the supply of [35S]sulfide or iron, or in Fe-S cluster formation itself. [35S]Sulfide was liberated from [35S]cysteine in an NADPH-dependent manner, whereas ATP was not necessary for this process. This desulfhydration of [35S]cysteine occurred before the formation of the 35S-labeled Fe-S cluster, and the amount of radioactivity in [35S]sulfide was greater than that in 35S-labeled holo-Fd by a factor of more than 20. Addition of nonradioactive sulfide (Na2S) inhibited competitively formation of the 35S-labeled Fe-S cluster along with the addition of nonradioactive cysteine, indicating that some of the inorganic sulfide released from cysteine is incorporated into the Fe-S cluster of Fd. ATP hydrolysis was not involved in the production of inorganic sulfide or in the supply of iron for assembly into the Fe-S cluster. However, ATP-dependent Fe-S cluster formation was observed even in the presence of sufficient amounts of [35S]sulfide and iron. These results suggest a novel type of ATP-dependent in vivo Fe-S cluster formation that is distinct from in vitro chemical reconstitution. The implications of these results for the possible mechanisms of ATP-dependent Fe-S cluster formation are discussed.

Fd is one of the major Fe-S proteins involved in the electron transport system in chloroplasts of higher plants. It consists of a 10-kD polypeptide carrying a [2Fe-2S] cluster chelated by four cysteine residues (25). Although a considerable amount of information has been accumulated about the physical and chemical properties of Fd and other Fe-S proteins, the mechanism by which an Fe-S cluster is formed in vivo is poorly understood.

Recent progress in the field of molecular biology has made it possible to clone the genes for Fe-S proteins from a wide variety of materials and express them in foreign hosts such as Escherichia coli. The Bacillus subtilis gene encoding glutamine phosphoribosylpyrophosphate amidotransferase has been cloned and expressed in E. coli (13). The Bacillus enzyme was functionally expressed in E. coli after correct processing in two steps; the removal of 11 amino acid residues from the amino-terminus, and assembly of the [4Fe-4S] cluster into its polypeptide. Genes encoding [2Fe-2S]-proteins, human adrenodoxin, and cyanobacterial Fds have also been expressed in E. coli, and the [2Fe-2S] clusters were assembled and incorporated into Fds in these heterogeneous systems (2, 4). The correct assembly of Fe-S clusters into foreign proteins suggests several possibilities for in vivo cluster formation. The Fe-S cluster may be assembled spontaneously from apoprotein, sulfide, and iron by a mechanism similar to that reported in earlier in vitro chemical reconstitutions (14, 16). Alternatively, unknown enzymes may be involved in the assembly process. If the assembly is enzymatic, the enzymes responsible must have broad specificity for protein acceptors, or are involved in preassembly of the Fe-S cluster moiety followed by its spontaneous incorporation into apoprotein. It should also be noted that the expression of active nitrogenase iron protein (nifH-encoded) in E. coli requires expression of an additional gene (nifM) (10), suggesting that the nifH product alone is insufficient for assembly of the [4Fe-4S] cluster in a foreign host.

We have addressed this problem and reported physiological formation of the Fe-S cluster in isolated intact and hypotonically lysed spinach chloroplasts (21, 22). When chloroplasts are incubated with [35S]cysteine, the sulfur atom derived from cysteine is incorporated into the Fe-S cluster of holo-Fd. This process is driven by NADPH and ATP generated by photosynthetic electron transport and phosphorylation. We have also provided evidence that ATP must be hydrolyzed and that NADPH cannot be substituted by NADP or NADH (22). Despite these studies, we still know very little about the putative ATP- and NADPH-dependent enzymes and their role in cluster formation. For example, ATP and NADPH may be involved in the supply of sulfide or iron, or in Fe-S cluster formation itself. Thus, it becomes important to separate the process into various stages and study each step separately. The overall process of formation of the 35S-labeled Fe-S cluster from [35S]cysteine might be composed of the following reactions: (a) liberation of [35S]sulfide from [35S]cysteine; (b) utilization of iron from the endogenous iron pool; (c) assembly and/or insertion of the Fe-S cluster into

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apo-Fd; and (d) conformational change of apo-Fd to holo-Fd. The system utilizing hypotonically lysed chloroplasts described here provides a possible way of separating and analyzing several steps. Examination of the effect of ATP and NADPH at each step might clarify the individual reaction involved.

MATERIALS AND METHODS
Formation of 35S-Labeled Fe-S Cluster of Fd in Lysed Chloroplasts

Radioactive l-[35S]cysteine (>600 Ci/mmol) was purchased from Amersham International. Fresh leaves of spinach (Spinacia oleracea) were obtained from local markets. Intact chloroplasts were isolated from the spinach leaves (21) and then lysed hypotonically by suspension in 5 mM Tricine-KOH, pH 8.0. For formation of the 35S-labeled Fe-S cluster, the lysed chloroplasts were incubated with [35S]cysteine under conditions essentially as described previously (22); minor modifications are detailed in the legends of the relevant figures. In some cases, stromal and thylakoid fractions were prepared from spinach lyzed chloroplasts and tested for their abilities to synthesize the Fe-S cluster of Fd. The hypotonically lysed chloroplasts were centrifuged at 40,000g for 30 min and the thylakoid pellet was washed twice with 5 mM Tricine-KOH, pH 8.0. Aliquots of the resulting stromal and thylakoid fractions (equivalent to 0.1 mg Chl of the original chloroplasts) were incubated with [35S]cysteine under conditions similar to those described previously (22). 35S-labeled holo-Fd was analyzed by non-denaturing PAGE and autoradiography (22). For determination of the radioactivity, the bands corresponding to holo-Fd were cut out of the dried gel, and 35S radioactivity in each was determined as described previously (22).

Measurement of H2S Production

The total amount of [35S]sulfide released from [35S]cysteine was determined essentially by the method described for the radiochemical assay of cysteine desulphydrase (27). The reaction mixture described for Fe-S cluster formation was placed in the main compartment of a Thunberg tube and 0.5 mL of 0.1 N NaOH solution to trap the H2S produced was placed in the side arm. Following the incubation, TCA was added to the main compartment to a concentration of 10%, and at least 1 h was allowed for the H2S to be trapped in the NaOH solution. Acid-volatile 35S-labeled sulfide trapped in the NaOH solution was determined using a scintillation counter with Aquasol 2.

Test for Phosphorylation or Adenylylation of Apo-Fd

Osmotically lysed chloroplasts were prepared as described above. Phosphorylation and adenylylation experiments were carried out in the presence of [γ-32P]ATP and [α-32P]ATP (Amersham), respectively, under the conditions described for 35S-labeled Fe-S cluster formation except that the reaction mixtures received 0.2 mM instead of 5 mM ATP. The final radioactivity concentrations of [γ-32P]ATP and [α-32P]ATP were 20 μCi/100 μL. After incubation at 25°C for 30 min, the samples were centrifuged to remove membranes and then desalted by passing through small columns of Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.0, containing 10 mM 2-mercaptoethanol. 32P-labeled stromal proteins were subjected to PAGE under nondenaturing conditions and the gels were stained with Coomassie blue R. 32P-labeled apo-Fd was analyzed on an autoradiogram of the dried gel. Characterization of phosphoamino acid in 32P-labeled apo-Fd was carried out by acid hydrolysis and subsequent paper electrophoresis by the methods of Lin et al. (11).

Test for Complex Formation between Fd and Chaperonin

Hypotonically lysed chloroplasts (equivalent to 0.1 mg Chl) were incubated in a volume of 0.1 mL with 10 μg of apo-Fd or 10 μg of holo-Fd, or without added Fds in a buffer containing 50 mM Tricine-KOH, pH 8.0, and 0.33 M sorbitol. After incubation at 25°C for 60 min, these samples were divided into aliquots and subjected to four different treatments. The first received ATP to a final concentration of 5 mM. The second received 5 mM ATP and an ATP-regeneration system composed of 20 μg/mL rabbit phosphocreatine kinase (Sigma), 10 mM phosphocreatine, and 2 mM MgCl2. The third received an ATP-consuming system containing 5 units/mL potato apyrase (Sigma), and the fourth was a control with no addition. Following further incubation at 25°C for 30 min, the samples were centrifuged to remove membranes, and the aliquots of the stromal fractions were subjected to nondenaturing PAGE through 5 to 20% polyacrylamide gels using a Tris-glycine buffer system (7). For identification of the Fd-chaperonin complex, Western blot analysis was carried out (9) using a rabbit antibody raised against spinach Fd (20).

RESULTS AND DISCUSSION

Formation of Fe-S Cluster of Fd and Desulfhydration of Cysteine

Previous studies have shown that [35S]sulfide is integrated into the Fe-S cluster of Fd when isolated intact or hypotonically lysed chloroplasts are incubated with [35S]cysteine (21, 22). To understand the metabolic reactions involved in the transfer of cysteine sulfide into Fd, it is necessary to study the degradation of cysteine to release inorganic sulfide. To accomplish this, the total amount of [35S]sulfide liberated from [35S]cysteine was determined as acid-volatile radioactivity released from acidified solution and trapped in NaOH solution. Figure 1 shows the time course of [35S]sulfide emission from [35S]cysteine during the experimental period of 35S-labeled Fe-S cluster formation. The rate of sulfide emission was constant up to about 40 min and then became saturated, whereas the Fe-S cluster formation showed a short lag period and then increased up to 90 min. The release of [35S]sulfide was observed before Fe-S cluster formation, and the total amount of radioactivity in [35S]sulfide was higher than that in 35S-labeled holo-Fd by a factor of over 20. To examine the effect of ATP and NADPH on sulfide emission and Fe-S cluster formation, the total radioactivity in inorganic sulfide was compared with that in holo-Fd labeled under various conditions. Figure 2 shows that [35S]sulfide emission from [35S]cysteine was stimulated by light or by externally added NADPH in the dark, either in the presence or absence of ATP. By contrast, Fe-S
Figure 1. Formation of [35S]-labeled Fe-S cluster of Fd and desulfhydration of [35S]cysteine in lysed spinach chloroplasts; time dependence. Intact chloroplasts were prepared from spinach leaves and lysed by suspending them in a hypotonic solution. The resulting broken chloroplasts (equivalent to 0.1 mg of Chl) were incubated with 5 μCi of [35S]cysteine, 10 μg of apo-Fd, 10 μg of holo-Fd, 5 mM ATP, 50 mM Tricine-KOH (pH 8.0), and 0.33 mM sorbitol in a final volume of 0.1 mL. After incubation at 25°C in the light, the radioactively labeled soluble proteins were analyzed by PAGE under nondenaturing conditions and autoradiography. The radioactivity of [35S]-labeled holo-Fd in the gel slice was determined as described previously (22). For determination of the [35S]sulfide released from [35S]cysteine, the reaction mixture was placed in the main compartment of a Thunberg tube and 0.5 mL of 0.1 N NaOH solution was placed in the side arm. Following the incubation, TCA was added to a concentration of 10% and acid-volatile sulfide trapped in the NaOH solution was determined by scintillation counting as described in “Materials and Methods.”

Figure 2. [35S]-labeled Fe-S cluster formation and [35S]cysteine desulfhydration; ATP- and/or NADPH-dependence. The reaction was carried out for 90 min in the light (L) or dark (D) and in the presence (+) or absence (−) of ATP (5 mM) under the conditions described in Figure 1. Where indicated, 5 mM NADPH, 10 μM DCMU, or 10 mM NH₄Cl was supplied to the reaction mixture.

cluster formation required both ATP and light (NADPH), as characterized previously (22). Addition of DCMU suppressed not only the Fe-S cluster formation but also sulfide emission, whereas NH₄⁺, an uncoupler, stimulated both reactions. These results confirmed that sulfide emission from lysed chloroplasts requires reducing compounds such as NADPH, whereas formation of the Fe-S cluster needs ATP in addition to NADPH. It should be noted again that the total amount of [35S]sulfide was always higher than that in [35S]-labeled holo-Fd under any of the conditions tested. These results suggest that cysteine is degraded, releasing inorganic sulfide into the medium in an NADPH-dependent manner, followed by incorporation of only part of the liberated inorganic sulfide into Fd during the course of the ATP-dependent reaction. Direct transfer of cysteine sulfide into the Fe-S cluster of Fd seems unlikely.

To confirm these hypotheses, we examined the effect of nonradioactive sulfide on formation of the [35S]-labeled Fe-S cluster from [35S]cysteine. As shown in Figure 3, low concentrations (0.1–1 mM) of the nonradioactive cysteine or N₅H₅S completely inhibited the formation of the [35S]-labeled Fe-S cluster from [35S]cysteine. The extent of inhibition by these two substances occurred almost in parallel with increasing concentration. These results indicate that some of the [35S]sulfide released from [35S]cysteine into the medium is incorporated into the Fe-S cluster of Fd in competition with the nonradioactive sulfide. On the other hand, thiosulfate, a substrate for rhodanese, was less inhibitory than the two mentioned above, and addition of rhodanese had no effect, either in the presence or absence of thiosulfate. Rhodanese, one proposed candidate responsible for formation of the Fe-S cluster of Fd (3), seems not to be involved in either the production of sulfide or its transfer to Fd. For further characterization of the light (NADPH)-stimulated sulfide emission

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whether ATP or NADPH might be involved in the supply of a suitable form of iron to the Fe-S cluster of Fd.

Figure 4 shows the effect of externally added ferric chloride on Fe-S cluster formation. When the reactions were carried out in the presence of ATP in the light, externally added iron showed no stimulatory effect relative to the minus iron sample. A higher concentration (≥0.1 mM) of iron was rather inhibitory, probably due to the formation of harmful hydroxyl radicals from hydrogen peroxide through metal-catalyzed reactions (1). When the reactions were carried out in the absence of ATP or in the dark, the Fe-S cluster formation could not be restored by addition of iron. Similar results were also obtained when the reactions were carried out with ferrous sulfate (not shown), and therefore externally added ferric or ferrous salt was unable to substitute for the role of ATP or NADPH in Fe-S cluster formation. Since both forms of iron are effective donors used in the chemical reconstitution of Fd (14, 16), the amount and chemical nature of the endogenous iron contained in chloroplast preparations should be sufficient for Fe-S cluster formation. From these results, ATP and NADPH do not appear to play a role in the supply of iron.

Possible ATP Requirement

In view of the ATP requirement for Fe-S cluster formation, the present studies indicate that the role of ATP is not associated with the supply of inorganic sulfide or iron. [35S]Cysteine was desulphydrated to produce [35S]sulfide in an NADPH-dependent but ATP-independent manner, and ATP could not be substituted by externally added iron. The requirement for ATP was observed even in the presence of sufficient amounts of [35S]sulfide and iron. These results indicate that the overall reaction of 35S-labeled Fe-S cluster

from cysteine, sulfite and sulfate were also examined as to whether they were involved as intermediary metabolites in the light-dependent sulfate assimilation pathway. When nonradioactive Na2SO3 or Na2SO4 was externally added to the reaction mixture, 35S-labeled Fe-S cluster formation was inhibited slightly but not completely even at high concentrations; the residual activities of cluster formation were 28% and 61% in the presence of 10 mM Na2SO3 and Na2SO4, respectively. These results indicate that the [35S]sulfide is derived mainly from the desulphydration of [35S]cysteine, and that the sulfur cycle including the oxidation and reduction process may not operate during the experimental period. NADPH may thus stimulate the activity of the cysteine desulphydrating enzyme, and then some of the liberated [35S]sulfide is incorporated into the Fe-S cluster of Fd.

Utilizable Iron Pool in Chloroplasts

The iron-storage protein ferritin is widely distributed in nature, and an analogous protein named phytoferritin is located in chloroplasts (23). Ferritin plays a key role in the iron metabolism of mammalian cells, so that the iron it stores in ferritin is reductively mobilized as needed for the synthesis of iron-containing proteins (6). NAD(P)H can serve as a source of reducing power for the enzyme-mediated reduction and mobilization of ferritin iron (24). In addition, a transit iron pool composed of iron chelated by ATP has also been found in reticulocytes (26). In plant cells, most of the iron found in leaves is located in the thylakoid and stromal fractions of chloroplasts, although its nature is poorly understood (23). On the basis of these considerations, we examined

Figure 3. Competitive inhibition of 35S-labeled Fe-S cluster formation with nonradioactive sulfur-containing substrates. Fe-S cluster formation assay was done under conditions similar to those described in Figure 1 except that the reactions were carried out with various concentrations of nonradioactive cysteine (O), sodium sulfide (△), or sodium thiosulfate (▽). In some cases, bovine rhodanese (1 unit/mL) was supplied to the reactions with thiosulfate (●). The amount of 35S-labeled holo-Fd was measured by densitometry of the exposed film.

Figure 4. Effect of iron on formation of the 35S-labeled Fe-S cluster of Fd. Lysed spinach chloroplasts were incubated under conditions similar to those described in Figure 1 except for the addition of various concentrations of ferric chloride. Reactions were carried out in either the presence (○) or absence (△) of added ATP (5 mM) in the light, or in the presence of ATP in the dark (●). The radioactivity of the 35S-labeled holo-Fd was determined as described in the legend to Figure 1.
formation from \(^{35}\text{S}\)cysteine can be divided into two sequential reactions. First, \(^{35}\text{S}\)cysteine is desulfhydrated to produce \(^{35}\text{S}\)sulfide by the action of the cysteine desulfhydrating enzyme, which is stimulated by NADPH. Second, the liberated \(^{35}\text{S}\)sulfide is incorporated into the Fe-S cluster of Fd possibly through an ATP-dependent reaction. Little is known about the nature of this ATP-dependent reaction other than the fact that it is a requirement for ATP hydrolysis. However, no such ATP requirement has been found for the in vitro chemical reconstitution of the Fe-S cluster of Fd; apo-Fd can be reconstituted to holo-Fd by chemical reactions involving ferric or ferrous salt, inorganic sulfide, and thiols (2-mercaptoethanol or DTT) (14, 16). Therefore, the ATP-dependent Fe-S cluster formation observed in chloroplasts strongly suggests a novel in vivo mechanism that cannot be explained by the simple analogy of chemical reconstitution. One might speculate that ATP hydrolysis is catalyzed by a protein acting as a synthetase of the Fe-S cluster by coupling the energy released upon ATP hydrolysis. Alternatively, ATP might be required for the phosphorylation of a certain protein that is required for the reaction. To address the question of how ATP is used to drive Fe-S cluster formation in chloroplasts, the following possible functions should be investigated.

**Phosphorylation of Apo-Fd**

A number of chloroplast polypeptides are phosphorylated when chloroplasts are incubated in the light in the presence of \(^{32}\text{P}\) or when stroma or thylakoid preparations are incubated with \(^{32}\text{P}\)ATP. To examine whether the stimulation of Fe-S cluster formation by ATP is associated with phosphorylation or adenylylation of apo-Fd, lysed chloroplasts were incubated with \(^{32}\text{P}\)ATP or \(^{32}\text{P}\)ATP and soluble proteins were analyzed by non-denaturing PAGE and autoradiography. As shown in Figure 5, externally added apo-Fd was phosphorylated by \(^{32}\text{P}\)ATP in the light but not in the dark, whereas phosphorylation of holo-Fd was not observed. The phosphorylation of apo-Fd was confirmed by crossed immuno-electrophoresis using a specific antibody against spinach Fd 20, where the immunoprecipitins lines associated with apo-Fd were radioactively labeled (data not shown). An identical gel was run with samples incubated with \(^{32}\text{P}\)ATP. A prominent labeled protein was observed to migrate on the gel slightly more slowly than apo-Fd, although apo-Fd was revealed not to be adenylylated (not shown).

The phosphorylation of apo-Fd offers some possibilities with regard to ATP-dependent Fe-S cluster formation. A phosphate group linked to cysteinyi thiol (15) might be an intermediate in binding the Fe-S cluster moiety, or phosphorylation might be a signal that stimulates Fe-S cluster formation. However, the first possibility was argued by the following two results: carboxymethylation of cysteine residues in apo-Fd did not prevent the polypeptide from undergoing phosphorylation (Fig. 5, lane 5), and the phosphoamino acid found in apo-Fd was identified to be phosphoserine. As to the second possibility, a pulse-chase experiment was carried out, and this revealed that the \(^{32}\text{P}\)-labeled apo-Fd was not dephosphorylated during the chase period in the presence of excess nonradioactive ATP, cysteine, and iron (data not shown).

These results suggest that the phosphorylation/dephosphorylation cycle of apo-Fd is not operative in chloroplasts. On the basis of these observations, it would be premature to draw a firm conclusion about the role of phosphorylated apo-Fd in formation of the Fe-S cluster. It might be due to nonspecific phosphorylation, since the serine protein kinase activity present in spinach stroma has been reported to phosphorylate exogenous substrates like histones II and III, phosvitin, and casein in addition to the endogenous stromal proteins (5).

**Molecular Chaperones**

Recently, the term “molecular chaperone” has been proposed for a family of proteins whose role is to ensure the folding of certain polypeptides and to aid in their correct assembly into oligomeric structures (8). In chloroplasts, chaperonin (rubisco\(^3\) subunit binding protein) plays an essential role in the assembly of rubisco. Rubisco large subunits, once associated with chaperonin oligomer, can be dissociated and released from it in the presence of ATP, and then assembled into an oligomeric structure with small subunits of rubisco (18). In addition, several chloroplast proteins such as the \(\beta\)-subunit of ATP synthase and glutamine synthetase, have been

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\(^{3}\)Abbreviation: rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase.
evidence for interaction between apo-Fd and ATP-dependent refoldase.

**Intra-Plastid Location of Fe-S Cluster Formation Activity**

An important approach for characterizing the nature of the putative enzyme responsible for Fe-S cluster formation is to determine its location in the plastid compartments. To address this, the thylakoids were separated from the stroma by differential centrifugation and aliquots of these fractions were analyzed for their abilities to synthesize the Fe-S cluster of Fd. When the incubations were carried out in the presence of ATP in the light, no activity was detected in the stroma or thylakoids, and occurred only upon combination of the two fractions. By contrast, when the reactions were supplemented with ATP and NADPH in the dark, the stromal fraction showed considerable Fe-S cluster formation, as did the stroma plus thylakoid fraction (Fig. 6). When the reactions were carried out in the absence of externally added ATP, less than 10% of the activity was found when compared with that in the presence of ATP (not shown). These results confirm the earlier conclusion that both ATP and NADPH are required for the reaction (22). In chloroplasts, the thylakoids are employed only to provide ATP and NADPH via the photosynthetic electron transport and phosphorylation systems. These findings also indicate that the soluble enzyme is responsible for Fe-S cluster formation. A cysteine desulphhydrating enzyme might also exist in the soluble fraction of chloroplasts. Based on these findings, purification and characterization of these enzymes should provide valuable insight into the mechanism involved.

**CONCLUSION**

Formation of the Fe-S cluster of spinach Fd was shown to require both ATP and NADPH. Our previous studies have demonstrated that ATP must be hydrolyzed and NADPH cannot be substituted by NADH or NADP (22). In the present studies, distinct steps in the Fe-S cluster formation pathway demonstrated to form a high-molecular-weight complex with chaperonin (12). If chloroplast chaperonin is involved in the refolding or assembly of a wide range of chloroplast proteins, a wrongly folded structure of apo-Fd might be refolded by the action of chaperonin and ATP hydrolysis. To determine whether chaperonin might be involved in the interaction with Fd, we tried to detect chaperonin-Fd complex using non-denaturing gradient PAGE (5–20%) and immunoblotting. However, no such complex could be found in spinach chloroplast extract. Most of the apo- and holo-Fd were observed to exist in their free forms. Although a small amount of Fd (<5%) was found to be associated with unknown proteins, these Fd complexes migrated on the gel much faster than the chaperonin oligomer, and could not be dissociated by ATP (data not shown). We and others (12) have so far found no
were investigated for their requirement for ATP and NADPH and the results are summarized in Figure 7. The overall reaction responsible for Fe-S cluster formation from cysteine is proposed to be divided into two sequential steps. (a) Cysteine is desulphydrated to produce inorganic sulfide by the action of a cysteine desulphydrating enzyme that is stimulated by NADPH. (b) Some of the liberated sulfide and iron is incorporated into Fd to build up the Fe-S cluster. The second step requires ATP hydrolysis even in the presence of a sufficient amount of sulfide and iron, and therefore a putative ATP-dependent enzyme may facilitate the assembly and/or insertion of the Fe-S cluster into Fd. Enzymes involved in these reactions are located in the stroma fraction of chloroplasts, and NADPH and ATP are supplied via photosynthetic electron transport and phosphorylation. Formation of the Fe-S cluster of chloroplast-type Fd is not the only example in which ATP is needed. A requirement for ATP has been reported for the formation of iron-molybdenum cofactor in nitrogenase molybdenum-iron protein (19). The data presented here are consistent with the proposed role of iron-sulfur iron protein in the ATP-dependent assembly and insertion of iron-molybdenum cofactor into molybdenum-iron protein (17). Additional studies on physiological Fe-S cluster formation for a wide range of Fe-S proteins will have to focus on the role of ATP.

LITERATURE CITED