Iron-Sulfur Cluster Biogenesis in Chloroplasts. Involvement of the Scaffold Protein CpIscA

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The chloroplast contains many iron (Fe)-sulfur (S) proteins for the processes of photosynthesis and nitrogen and S assimilation. Although isolated chloroplasts are known to be able to synthesize their own Fe-S clusters, the machinery involved is largely unknown. Recently, a cysteine desulfurase was reported in Arabidopsis (Arabidopsis thaliana; AtCpNiF) that likely provides the S for Fe-S clusters. Here, we describe an additional putative component of the plastid Fe-S cluster assembly machinery in Arabidopsis: CpIscA, which has homology to bacterial IscA and SufA proteins that have a scaffold function during Fe-S cluster formation. CpIscA mRNA was shown to be expressed in all tissues tested, with higher expression level in green, photosynthetic tissues. The plastid localization of CpIscA was confirmed by green fluorescent protein fusions, in vitro import, and immunoblotting experiments. CpIscA was cloned and purified after expression in Escherichia coli. Addition of CpIscA significantly enhanced CpNiF-mediated in vitro reconstitution of the 2Fe-2S cluster in apo-ferredoxin. During incubation with CpNiF in a reconstitution mix, CpIscA was shown to acquire a transient Fe-S cluster. The Fe-S cluster could subsequently be transferred by CpIscA to apo-ferredoxin. We propose that the CpIscA protein serves as a scaffold in chloroplast Fe-S cluster assembly.

Iron (Fe)-sulfur (S) clusters are cofactors of proteins that perform a number of biological roles, including electron transfer; redox and nonredox catalysis; regulation of gene expression; and as sensors for Fe and oxygen within all living organisms, prokaryotes, and eukaryotes (Beinert, 2000). Although Fe-S clusters can be assembled in proteins in vitro with ferrous Fe and sulfide, it is now clear that the process is not spontaneous in vivo, and proteins have been shown to be required for the biological formation of these clusters (for review, see Lill and Kispal, 2000; Frazzon et al., 2002). Genetic and biochemical studies in microorganisms initially led to identification of two types of Fe-S cluster machinery, termed NIF (nitrogen fixation) and ISC (iron-sulfur cluster; Zheng et al., 1993, 1998). A third machinery (SUF) with homologs in a wide range of organisms was characterized more recently (Takahashi and Tokumoto, 2002).

The proposed mechanism of cluster formation is as follows: (1) S is mobilized from Cys by the action of a Cys desulfurase enzyme (Zheng et al., 1993, 1998); (2) Fe atoms are transferred to a scaffold protein (Urbina et al., 2001). Fe atoms are supplied to the scaffold protein, and the transient Fe-S cluster is assembled; and (3) the Fe-S cluster is inserted into various apo-proteins to form the Fe-S proteins (Yuvaniyama et al., 2000; Agar et al., 2000a; Krebs et al., 2001; Wu et al., 2002; Tong et al., 2003). In addition to Cys desulfurase and scaffold proteins, other factors such as Hsp70- and Hsp40-type chaperones as well as ferredoxin (Fd)/Fd reductase systems may be involved (Lill and Kispal, 2000; Frazzon et al., 2002). There are indications that molecular chaperone proteins interact with the scaffold protein and keep the scaffold protein in a conformation that facilitates the Fe-S cluster assembly and the transfer to an apo-Fe-S protein (Hoff et al., 2000; Silberg et al., 2001; Muhlenhoff et al., 2003).

In the nitrogen-fixing bacterium Azotobacter vinelandii, NifU was shown to provide a scaffold for Fe-S cluster formation (Zheng et al., 1993, 1994; Agar et al., 2000a; Yuvaniyama et al., 2000). In non-nitrogen-fixing bacteria and eukaryotic mitochondria, an IscU scaffold protein is essential and the main protein for Fe-S cluster biosynthesis; this protein shows a high sequence identity with the N-terminal domain of A. vinelandii NifU (Agar et al., 2000a, 2000b). IscU proteins accept S from a NifS-like Cys desulfurase and are also the binding site for Fe to build an Fe-S cluster (Agar et al., 2000b). By contrast, plastids and most non-nitrogen-fixing cyanobacteria whose genome sequence is known do not possess any homolog of IscU. Instead, proteins, termed NFUs, with sequence similarity to the C-terminal domain of A. vinelandii NifU were found to be scaffolds for Fe-S cluster assembly (Leon et al., 2003; Yabe et al., 2004). SyNiF, the NiF of cyanobacterium Synechocystis, serves as a scaffold for Fe-S cluster assembly and delivery (Nishio and Nakai, 2000).

IscA is another protein that may function as a scaffold protein for Fe-S cluster synthesis in Escherichia coli.

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(Takahashi and Nakamura, 1999; Tokumoto and Takahashi, 2001), yeast (Saccharomyces cerevisiae; Jensen and Culotta, 2000; Kaut et al., 2000), and cyanobacteria (Morimoto et al., 2002; Wollenberg et al., 2003). It is proposed that the IscA family of proteins provides alternative scaffolds to the NifU and IscU proteins for mediating Nif-specific and general Fe-S cluster assembly (Krebs et al., 2001). In addition to a scaffold function in cluster formation in E. coli, it was proposed that IscA can provide Fe for the assembly of the transient Fe-S cluster in IscU in the presence of IscS and Cys in vitro (Ding and Clark, 2004). In Synechocystis PCC 6803, IscA1, the product of SLR1417, predominantly binds the Fe ion alone, whereas IscA2, the product of SLR1565, binds a 2Fe-2S cluster (Morimoto et al., 2002). IscA2 forms a complex with a HEAT-repeat-containing protein, IaiH, which stabilizes the Fe-S cluster in IscA2 (Morimoto et al., 2002).

In addition, the SUF gene clusters of E. coli and Erwinia chrysanthemi have genes that encode components similar to IscS and IscA, named SufS and SufA, respectively (Takahashi and Tokumoto, 2002). These proteins are thought to be involved in Fe-S cluster formation under Fe limitation and oxidative stress conditions (Ollagnier-de-Choudens et al., 2001; Outten et al., 2003, 2004).

In plant cells, mitochondria and chloroplasts are believed to originate from endosymbiotic bacteria and therefore are predicted to have their own Fe-S cluster biosynthesis machinery. The structural and physiological differences between mitochondria and chloroplasts suggest the existence of two distinct Fe-S assembly machineries. Mitochondria are a site of oxygen consumption, whereas chloroplasts produce oxygen through the photosynthetic process. Based on sequence homology of putative components, the mitochondrial Fe-S machinery may be most similar to the bacterial ISC machinery, where IscU is regarded as the major and essential scaffold protein for the cluster assembly. In chloroplasts, the components of the Fe-S machinery are starting to be identified. A NifS-like protein (AtCpNifS) from Arabidopsis was shown to have Cys desulfurase activity and to be located in the chloroplast (Leon et al., 2002; Pilon-Smits et al., 2002). To identify another Arabidopsis protein(s) that might be involved in Fe-S cluster formation in plastids, a database search was performed to identify homologs of the cyanobacterial Synechocystis PCC 6803 IscA proteins. BLAST searches in the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/thal/db/) and The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) databases revealed the presence of one IscA-like protein (At1g10500). TargetP predictions suggest that this protein is a chloroplast protein with a 55-amino-acid-long chloroplast transit sequence. The corresponding gene was named AtCpIscA, and its cDNA was cloned using reverse transcription-PCR.

Cloning of CpIscA and Sequence Analysis

In earlier studies, the NifS-like protein AtCpNifS from Arabidopsis was shown to have Cys desulfurase activity and to be located in the chloroplast (Leon et al., 2002; Pilon-Smits et al., 2002). To identify another Arabidopsis protein(s) that might be involved in Fe-S cluster formation in plastids, a database search was performed to identify homologs of the cyanobacterial Synechocystis PCC 6803 IscA proteins. BLAST searches in the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/thal/db/) and The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) databases revealed the presence of one IscA-like protein (At1g10500). TargetP predictions suggest that this protein is a chloroplast protein with a 55-amino-acid-long chloroplast transit sequence. The corresponding gene was named AtCpIscA, and its cDNA was cloned using reverse transcription-PCR. Alignment of the AtCpIscA primary sequence with IscA- and SufA-like proteins from cyanobacteria and E. coli reveals the presence of the three conserved Cys residues (residues 104, 170, and 172), including the characteristic motif CXC and a conserved Asp (residue 149) in the C-terminal region (Fig. 1A). These residues are conserved among all IscA-type proteins from prokaryotic and eukaryotic organisms. In addition to the three Cys conserved in all IscA-type proteins, AtCpIscA contains two other Cys, at residues 94 and 135, which are also present in cyanobacterial IscA1 from Synechocystis PCC 6803. AtCpIscA exhibits higher sequence similarity to IscA1 (Slr1417) from Synechocystis PCC 6803 (58% identity) and IscA from E. coli (47% identity) compared to the mitochondrial IscA-like protein from Arabidopsis (At2g16710; 24%) and SufA from E. coli (25%). Consequently, in a phylogenetic analysis, AtCpIscA was grouped with cyanobacterial IscA1 and E. coli IscA, separate from E. coli SufA and the Arabidopsis mitochondrial NifA-like protein (Fig. 1B).
Ten micrograms of total RNA extracted from roots, stems, leaves, and flowers was electrophoresed, and ethidium bromide staining was used to verify equal loading. Northern-blot analysis reveals that AtCpIscA is expressed in all tested tissues, with higher expression level in green photosynthetic tissues (leaves and stems) than non-green tissues (roots and flowers; Fig. 2).

Intracellular Localization of CpIscA

The TargetP program (Emanuelsson et al., 2000) predicted a chloroplast localization of AtCpIscA and a cleavable transit sequence of 55 amino acids. To examine the subcellular localization, we constructed fusions with the green fluorescent protein (GFP). GFP was fused to the coding region for the predicted chloroplast transit sequence of CpIscA (TP-IscA-GFP) and to the full-length IscA including its transit sequence (Full length-IscA-GFP). GFP alone expressed from the same constitutive promoter was used as a control. The localization in cells was analyzed using confocal laser microscopy. Fluorescence corresponding to GFP expressed without a transit sequence was excluded from the chloroplasts as expected (Fig. 3, top). By contrast, green fluorescence from TP-IscA-GFP was localized to the chloroplast stroma, as indicated by the overlay of green fluorescence and red autofluorescence (Fig. 3, middle). Because chloroplast transit sequences effectively mediate translocation of a passenger protein across the envelope (Keegstra and Froehlich, 1999), this is the expected location for a transit sequence fusion. Interestingly, green fluorescence from the full-length CpIscA coupled to GFP (Full length-IscA-GFP) was localized to discrete locations in the chloroplast stroma, which may be indicative of the inclusion in discretely localized complexes (Fig. 3, bottom).

To investigate the chloroplast localization of CpIscA by an alternative method and to determine the size of the mature CpIscA protein in plastids, we performed...
an in vitro chloroplast uptake experiment (Fig. 4A). Radiolabeled precursor protein was produced by in vitro transcription of the cloned cDNA and subsequent translation of the synthetic mRNA in the presence of $^{35}$S-Met. The translation reaction (Fig. 4A, right, PR) resulted in a radiolabeled protein band with a size expected for the precursor. In addition to the precursor, two translation products with a size smaller than the expected mature protein were present that may result from initiation at either of the two downstream AUG codons corresponding to residues 65 and 85. The translation mixture including the precursor was incubated with purified intact chloroplasts in the light and in the presence of ATP. After import and treatment with protease, the chloroplasts were recovered and proteins were analyzed by SDS-PAGE (Fig. 4A). The E. coli-expressed recombinant mature CplscA was electrophoresed in the same gel to allow a direct comparison of the molecular mass with the imported protein. Chloroplasts incubated with the precursor encoded by CplscA and treated with protease accumulated a protein of about 14 kD in size that has the same electrophoretic mobility as the purified recombinant mature protein. Quantitation of the precursor and the mature bands indicated that 32% of the added CplscA precursor was imported in this assay, which was comparable to the plastocyanin control (data not shown). We conclude that the CplscA precursor contains plastid-targeting information and that the protein is active in the chloroplast. The recombinant protein purified from E. coli corresponds in size to the imported mature CplscA.

To analyze localization and expression levels in Arabidopsis plants, we raised an antibody (see “Materials and Methods”) that was used in immunoblots to analyze the presence of CplscA in total leaf homogenate and chloroplast stroma fractions. The antibody recognized a band corresponding to the size of mature CplscA in total homogenate and isolated stroma, confirming the stromal localization of AtCplscA (Fig. 4B). The mobility of the detected CplscA protein band in
plant fractions is the same as that of purified recombinant CpIscA. Control experiments showed that these bands were not detected with preimmune serum (data not shown). Based on comparisons of the CpIscA staining intensities in stromal fractions and purified protein, we estimate the abundance of CpIscA in stroma to be approximately 0.01% to 0.02% of protein, which is slightly lower than the measured abundance of CpNifS (Ye et al., 2005). To investigate the oligomeric state of CpIscA in the stroma, a gel filtration experiment was performed using a high-resolution column and detection of AtCpIscA using immunoblotting (Fig. 4C). Approximately 90% of the stromal IscA was eluted in a single peak at high molecular mass (approximately 600 kD, fractions 17–20). In addition, a smaller amount of IscA was eluted as a dimer. This result indicates that CpIscA present in stroma interacts with other proteins in vivo and may form a transient complex with them. Interestingly, a fraction of CpNifS was also detected in a complex of approximately 600 kD (Ye et al., 2005), and coelution of CpIscA and CpNifS was confirmed by gel filtration (data not shown).

Expression and Purification of AtCpIscA

The coding sequence for mature CpIscA (without transit sequence) was cloned into the expression vector pET11d, and the mature-sized protein was expressed in BL21 codon+ E. coli cells. Three hours after induction with isopropyl-β-D-thiogalactopyranoside (0.4 mM), a protein with a molecular mass of about 14 kD accumulated to about 5% to 10% of the soluble protein, as shown by Coomassie Brilliant Blue staining (Fig. 5, lane 1). This molecular mass is in agreement with the molecular mass calculated from the DNA sequence (13,971 D). Soluble IscA was purified using cation exchange chromatography and a gel filtration step (Fig. 5, lanes 2 and 3). The protein was eluted from the calibrated S200 gel filtration column with an apparent molecular mass of 54 kD, suggesting that the protein is purified as a tetramer (data not shown). N-terminal sequencing (Macromolecular Resources, Colorado State University) by Edmann degradation yielded the sequence RNRLSV, corresponding to the predicted N-terminal sequence of mature CpIscA. The pure CpIscA protein was colorless, indicating it did not contain an Fe-S cluster while in E. coli.

CpIscA with CpNifS Stimulates Fe-S Cluster Formation in Fd

To study the role of CpIscA in Fe-S cluster formation, an in vitro reconstitution assay was developed. In this assay, apo-Fd was reconstituted to the holo-form by acquiring an Fe-S cluster, which was synthesized in vitro from Cys S and a ferrous Fe salt. Holo-Fd was

Figure 4. Chloroplast targeting of AtCpIscA and gel filtration analysis. A, In vitro import of radiolabeled CpIscA precursor. 35S-Met-labeled Precursor CpIscA protein (PR) was obtained by in vitro translation and incubated with isolated pea chloroplasts. Chloroplasts were reisolated, and proteins in the intact chloroplasts were analyzed either directly (CI) or after treatment of intact chloroplast with protease (CP). Proteins were separated by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (left), and the radiolabeled protein bands were visualized using a PhosphorImager (right). B, Immunoblot analysis of 20 μg of total leaf homogenate protein (TH) and 20 μg of chloroplast stroma (S). Purified CpIscA (5 ng and 20 ng) was applied for comparison in the two right lanes of Figure 4B. AtCpNifS antibody (bottom) was used as a control for stromal protein. C, Gel filtration analysis of AtCpIscA in the stroma. One milligram of stromal protein was separated on a Superdex-S200 gel filtration column. Elution was monitored by the A280 (data not shown) and by immunoblotting of collected fractions with CpIscA-specific antibodies. The void volume of this column corresponds to fraction 11.
separated from apo-Fd and other proteins and quantified by HPLC using an ion-exchange column. The activity of CpNifS proved to be sufficient and required for the Fe-S cluster formation in Fd (Ye et al., 2005). However, other proteins may promote the efficiency of CpNifS-mediated holo-Fd formation in a plant cell. In the presence of all necessary substrates for Fe-S cluster formation, except CpNifS, CpIscA alone did not show any reconstitution activity. However, preincubation of CpIscA with CpNifS significantly increased subsequent Fd reconstitution activity compared to that shown by CpNifS alone \( (P < 0.05; \text{Fig. 6}) \). The observed modest increase in reconstitution activity was a first indication of a possible role of CpIscA in Fe-S cluster formation.

**Incorporation of an Fe-S Cluster into CpIscA**

The stimulation of CpNifS-dependent Fd reconstitution activity by CpIscA suggested possible interactions between CpIscA and CpNifS in the Fe-S cluster formation where CpIscA may be a scaffold for cluster assembly and an intermediate in Fe-S insertion. To investigate this possibility in more detail, CpIscA (300 \( \mu \)g) was incubated with CpNifS (75 \( \mu \)g) in reconstitution buffer (5 mm dithiothreitol [DTT], 1 mm L-Cys, 1 mm ferrous ammonium sulfate, 20 \( \mu \)M pyridoxal 5’-phosphate [PLP]) for 30 min and then applied to a gel filtration column (Fig. 7A, line 3). As controls, apo-CpIscA (300 \( \mu \)g) incubated in gel filtration buffer or in reconstitution buffer, as well as CpNifS alone (75 \( \mu \)g), were incubated and run through the same column (Fig. 7A, lines 1, 2, and 4, respectively). Proteins were detected by \( A_{280} \) (Fig. 7) and 420 nm (data not shown). Purified untreated apo-CpIscA eluted from the column in a peak with a retention time expected for the tetramer but a shoulder was present, suggesting the existence of lower-molecular mass IscA species (Fig. 7A, line 1). Incubation of ApoIscA with DTT-containing buffer resulted in mainly the dimeric form of the protein (line 2). No absorbance was detected at 420 nm, indicating the absence of Fe-S clusters in the apo-IscA fractions (data not shown). As shown in Figure 7A (line 3), incubation of CpIscA with CpNifS resulted in two major peaks corresponding to CpNifS and a CpIscA dimer, according to its calculated molecular mass. SDS-PAGE (Fig. 7B) indicated that the peak of CpNifS eluted at 17.5 min (fraction 28) and the dimeric CpIscA at 20.5 min (fraction 34). Interestingly, the peak corresponding to reconstituted dimeric CpIscA could also be detected by \( A_{420} \) (data not shown), suggesting the presence of an Fe-S cluster. Apo-CpIscA (line 2) and reconstituted dimeric CpIscA (line 3) were collected from gel filtration runs, and their absorption spectra were measured (Fig. 7C). Unreconstituted CpIscA showed only one peak at 280 nm, characteristic for aromatic residues in proteins. Reconstituted CpIscA (fraction 34), however, showed extra maxima at 330 nm, 420 nm (with a shoulder at 470), and 580 nm, indicative of a 2Fe-2S cluster (Fig. 7C). In a typical 2Fe-2S cluster, absorption from 420 nm to 460 nm is attributed to the vibration between Fe and bridging inorganic S (Morimoto et al., 2002).

The presence of Fe in CpIscA was determined chemically (see "Materials and Methods") using spinach \((Spinacia oleracea)\) holo-Fd as control, which contains one 2Fe-2S cluster per protein. We measured 2.0 \( \pm \) 0.2 Fe/protein for spinach holo-Fd. Reconstituted CpIscA contained 1.4 \( \pm \) 0.2 Fe/dimer, while no Fe was detected in the apo-form. Because the reconstituted CpIscA enhanced the Fd reconstitution activity of CpNifS. Purified CpIscA (30 \( \mu \)g) or a combination of both CpNifS (7.5 \( \mu \)g) and CpIscA (30 \( \mu \)g) were incubated with 50 mm Tricine-NaOH, pH 7.5, 5 mm DTT, 1 mm L-Cys, 1 mm ferrous ammonium sulfate, 20 \( \mu \)M PLP for 30 min, followed by addition of 30 \( \mu \)g of apo-Fd. After 20 min, the reconstituted holo-Fd was assayed by HPLC. Data represent the average and \( \pm \) of three replicates.

![Figure 5. SDS-PAGE of IscA at different stages of its purification. Lane 1, Molecular mass marker; lane 2, total lysate from E. coli BL21 (DE3)/codon+ expressing IscA; lane 3, IscA after cation exchange column; lane 4, IscA after HPLC gel filtration column.](image-url)
tuted fraction obtained after incubation with CpNifS (line 3) still may contain some inactive apo-CpIscA, a number slightly lower than two is expected for the number of Fe atoms/dimer. These data indicate that the isolated and reduced CpIscA is a clusterless apo-protein, while the reconstituted dimer may contain a 2Fe-2S cluster. The Fe-S cluster formed in IscA is stable since treatment with Fe chelator (1 mM EDTA) or overnight incubation at 4°C did not induce destruction of the Fe-S cluster, as indicated by the reconstitution efficiency (data not shown).

Fe-S Cluster Transfer from CpIscA to Apo-Fd

As CpIscA appeared to have an Fe-S cluster, the potential transfer of the Fe-S cluster to apo-Fd was investigated. Both apo- and holo-CpIscA (150 μg) were collected from the gel filtration column and incubated with 30 μg of apo-Fd in the presence of 5 mM DTT at 37°C for 30 min. As controls, holo-Fd (30 μg) alone and holo-IscA (150 μg) alone were incubated. Incubation mixtures were used for holo-Fd analysis by HPLC. As shown in Figure 8A, the apo-CpIscA fraction did not lead to any formation of holo-Fd, while 50% (15 μg) of apo-Fd was reconstituted to holo-Fd by acquiring an Fe-S cluster from the dimeric holo-CpIscA. To test if the amount of holo-Fd formation depends on the amount of holo-IscA, various amounts of holo-IscA were incubated with apo-Fd (30 μg) for 30 min and the amount of holo-Fd was assayed (Fig. 8B). The amount of holo-Fd formed increased with increasing the amount of holo-IscA. Importantly, in the same condition when holo-IscA (60 μg) was incubated with apo-Fd (30 μg) in the presence of 1 mM EDTA, reconstitution was still observed with 70% activity of the reaction without EDTA indicating direct transfer from IscA to Apo-Fd (data not shown). Thus, it is proposed that holo-CpIscA can be formed from apo-CpIscA by acquisition of a transient Fe-S cluster, which it can subsequently deliver to apo-Fd. This suggests a role for CpIscA as a scaffold during Fe-S cluster formation in plastids and in transfer of the formed cluster to apo-Fe-S proteins.

DISCUSSION

Recently, it was demonstrated that AtCpNifS, a chloroplastic NifS-like Cys desulfurase of Arabidopsis, is responsible for the release of S from Cys for the biogenesis of Fe-S clusters in vitro (Ye et al., 2005). In this study, we report that Arabidopsis chloroplasts contain an IscA-like protein, CpIscA, which enhances the CpNifS-dependent Fe-S cluster formation in vitro. In addition, we provide evidence that recombinant apo-CpIscA, upon incubation with AtCpNifS and appropriate substrates, can acquire an Fe-S cluster, which in turn can be donated to apo-Fd in vitro. From these observations, we hypothesize that CpIscA functions as a scaffold protein for the assembly of transient Fe-S clusters from Fe and elemental S, which can be donated to Fe-S apo-proteins in the plastids. These results are of significance since Fe-S cluster proteins play crucial roles in essential plant processes, such as photosynthesis, which determine plant productivity.
and nutritional value. Although plastids are known to be able to synthesize their own Fe-S clusters (Takahashi et al., 1986), the machinery involved in Fe-S cluster formation in plastids remains largely to be elucidated. The results presented here contribute to the understanding of this important process.

CpIscA has three conserved Cys residues and a conserved Asp residue (Fig. 1) with the sequence arrangement C-X$_{42-44}$-D-X$_{20}$-C-G-C. These residues are conserved among all IscA-type proteins from prokaryotic and eukaryotic organisms, including nif-specific IscA proteins (Krebs et al., 2001). This sequence motif of IscA scaffold proteins is different from the primary sequence arrangement of Cys in NifU/IscU scaffold proteins (C-G-D-X$_{22-24}$-C-X$_{43}$-C; Krebs et al., 2001). Individual amino acid substitutions for each of the three conserved Cys residues of the yeast Isa1p and Isa2p proteins (IscA homologs) yield the same phenotype as the gene knockout (Jensen and Culotta, 2000). When any one of three conserved Cys residues in *Synechocystis* IscA2 was replaced with Ser, the amount of assembled 2Fe-2S was significantly reduced (Morimoto et al., 2003). It has been suggested that two of the three conserved Cys residues are involved in cluster binding, whereas the third one provides an electron during cluster assembly for the reduction of the Cys persulfide at NifS/IscS (Krebs et al., 2001). Further studies are required to investigate the role of these Cys residues in CpIscA.

Two scaffold proteins for Fe-S cluster formation in nitrogenase, called NifU and NifIscA, were discovered in the Nif operon of *A. vinelandii*. NifU consists of three domains: an N-terminal region that can accept a transient cluster, a central region with stable cluster, and a C-terminal thioredoxin-like domain known as the Nfu region (Agar et al., 2000a; Yuvaniyama et al., 2000). Homologs of the N-terminal domain of NifU as well as of NifA seem to function in the general Fe-S

Figure 8. Fe-S cluster transfer from CpIscA to Fd apo-protein. A, Fe-S cluster transfer from holo-IscA to apo-Fd. Apo-Fd (30 μg) was incubated with 150 μg of holo-CpIscA (collected from gel filtration column, reaction 3, Fig. 7) or 150 μg of apo-CpIscA purified by gel filtration column. After 30 min, the Fd was assayed by HPLC and quantified. Holo-Fd (30 μg) and holo-IscA (150 μg) were used as controls. B, The amount of holo-Fd formed depends on the amount of holo-IscA. Thirty micrograms of apo-Fd was incubated with various amounts of holo-IscA for 30 min, and the amounts of holo-IscA were assayed by HPLC and quantified as before.

A

![Figure 8A](image)

B

![Figure 8B](image)
cluster machinery found in mitochondria (Lill and Kispal, 2000) and are also encoded by the Isc-gene cluster of bacteria such as E. coli (Frazzon et al., 2002). This Isc-type machinery was reported to be sensitive to oxygen in E. coli, A. vinelandii, and Schizosaccharomyces pombe (Krebts et al., 2001; Ollagnier-de-Choudens et al., 2001; Wu et al., 2002). In E. coli, a second Fe-S assembly machinery is encoded by the Suf operon and the gene cluster includes SufA, a homolog of IscA/NifA. The Suf operon may function in Fe-S assembly under conditions of oxidative stress and Fe limitation (Outten et al., 2004).

The CpsIscA-mediated formation of the [2Fe-2S] cluster in apo-Fd proceeds under normal atmospheric (oxygenic) conditions in the presence of a thiol reductant (DTT) in the assembly cocktail. By comparison, the reconstituted Fe-S cluster in either E. coli or A. vinelandii IscA proteins was fairly labile (Ollagnier-de-Choudens et al., 2001; Krebs et al., 2001), and the cluster formed in CpsIscA was stable and insensitive to oxygen, as indicated by the persistence over several days at 4°C. Since photosynthetic activity produces oxygen, this reaction condition may be appropriate for a chloroplast enzyme. By contrast, the mitochondrial Isc-type Fe-S protein maturation machinery requires the exclusion of oxygen in vitro (Muhlenhoff et al., 2002). Chloroplasts are proposed to be derived from an ancestral cyanobacterium-like endosymbiont (Martin et al., 1998). No IscU-type scaffold proteins have been identified in the nine completely sequenced genomes in the Arabidopsis genome (TAIR; www.arabidopsis.org). This suggests that the Arabidopsis chloroplast, like cyanobacteria, does not express an IscU scaffold homolog. Therefore, the CpsIscA protein may provide an alternative scaffold to the IscU proteins for Fe-S cluster formation and delivery in chloroplasts.

In addition to CpsIscA, three chloroplast-localized Cpn56 proteins were identified that may function as molecular scaffolds for Fe-S cluster biosynthesis (Leon et al., 2003; Touraine et al., 2004; Yabe et al., 2004). An interesting question that remains to be answered is whether the CpsIscA and Cpn56 scaffold proteins function complementary, overlapping, or parallel to each other in chloroplasts. Some plausible reasons for the need of alternative scaffolds are that each scaffold protein is optimized for assembly of either [2Fe-2S] or [4Fe-4S] clusters in vivo, that they preferentially transfer clusters to different acceptor proteins, or that each functions optimally under different physiological conditions, e.g. conditions that are more reducing or oxidizing. Complementary or partially overlapping roles in the delivery of Fe-S clusters to various sub-strate apo-proteins in different plastids and/or under different growth conditions were postulated for AtCpn56 (Touraine et al., 2004; Yabe et al., 2004). A scaffold role for CpsIscA in plastid Fe-S cluster formation is similar to the finding that IscA from E. coli (Ollagnier-de-Choudens et al., 2001) and cyanobacterium Synechocystis PCC 6803 (Wollenberg et al., 2003) can serve as a scaffold for formation of a [2Fe-2S] cluster, which can be donated to the Fe-S apo-protein Fd. The homolog from A. vinelandii NiIscA can also serve as a scaffold for 4Fe-4S clusters (Krebts et al., 2001). Once a suitable in vitro plant system for reconstitution of a 4Fe-4S apo-protein has been established, it will be interesting to investigate whether CpsIscA serves as a scaffold for both types of Fe-S cluster.

MATERIALS AND METHODS

Cloning and Plasmid Construction

The Arabidopsis (Arabidopsis thaliana) CpsIscA coding sequence was amplified by PCR using cDNA as a template. cDNA was prepared from DNase-treated total RNA prepared from 2-week-old seedlings as described (Pilon-Smiths et al., 2002). Primers used for IscA amplification were 5′-GCCT-CTAGACGAAAGATAGCCTGCGC-3′ (forward primer) and 5′-GCCGCGTGATGAAACCTCGGTACATC-3′ (reverse primer). Underlined bases indicate XhoI and Smal sites, respectively. The PCR product was digested with XhoI and Smal and then ligated into a PBS (K57) vector (Stratagene, La Jolla, CA), digested with the same restriction enzymes to produce plasmid pPrIscA. To subclone the mature sequence of IscA in pET11d for expression, PCR was performed with another set of nested primers, 5′-CATGCCATGGCTTTCTGCTGACGGCCTGTC-3′ (forward primer) and 5′-CCGGATCTCCTACATCTCAGGACGAAAAG-3′ (reverse primer). Underlined bases indicate Ncol and BamHI sites, respectively. The PCR product was digested with Ncol and BamHI and subcloned in pET11d to produce plasmid pMPrIscA.

Construction of the plasmid for expression of the transit peptide (TP; amino acids 1–55) of IscA fused to GFP was performed as follows. The sequence encoding the predicted TP of IscA was amplified by PCR using flanking primers SalI-N (5′-GAATGTGGCACATGGGCTTTCGCTACTGGA-3′) and Ncol-C-TP (5′-CATGGCATGGACATCTCGGAGCAAAAGACTCTCC-3′). To make a fusion of GFP with the full-length protein, the entire coding sequence of IscA was amplified by PCR using flanking primers SalI-N and Ncol-C-FI (5′-CATGCCATGGCTGTCTCTACTCTTTACTCAGATGGTCA-3′). pPrIscA plasmid was used as a template for PCR. PCR products were digested and inserted into the SalI/Ncol-digested GFP reporter plasmid, 35S-I-SGFP(565T), to create the TP/IscA-GFP and Full length-IscA-GFP, respectively. The inserts in all plasmids were sequenced by the dyeoxy dye terminator method to ensure that no error was introduced during PCR reactions. The plasmids used for protoplast transformation were prepared using the Plasmid Midi kit (QIAGEN, Valencia, CA).

Sequence Analysis and Alignments

Sequence analysis was performed using the Mac Vector sequence analysis software (International Biotechnologies, New Haven, CT). Searches for sequence similarity were performed using the BLAST network service provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using ClustalW at European Bioinformatics Institute, ExPASY Proteomics tools (http://www.ebi.ac.uk/clustalw). Phylogenetic analysis was performed using the PAUP (Sinauer Associates, Sunderland, MA; version 4.0b10) heuristic search method with tree bisection-reconnection branch swapping (Swofford, 1993). Bootstrap analysis with 1,000 random replicates was performed using the heuristic method.

In Vitro Chloroplast Import Assay

The pPrIscA plasmid was linearized with KpnI and transcribed in vitro using T7 polymerase (Epicerent Technologies, Madison, WI) according to the manufacturer’s instructions. Radiolabeled precursors were synthesized in
a wheat germ lysate system in the presence of 35S-Met (25 μCi/50 μL reaction; Amersham/Piscataway, NJ) according to suggested protocols (Promega, Madison, WI). Chloroplasts for import experiments were isolated from 10-d-old pea (Pisum sativum) seedlings (cv Little Marvel) and incubated with radiolabeled precursor as described (Pilon et al., 1992). The postimport thermolysin treatment and reisolation of intact chloroplasts were performed as described (Smeekens et al., 1986). Proteins from import experiments equivalent to 10 μg of chlorophyll were separated by 15% SDS-PAGE, stained with Coomassie Brilliant Blue, fixed in 7% (v/v) acetic acid, 25% (v/v) methanol, dried, and the radiolabeled proteins visualized and quantified using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting, Gel Filtration, and Antibody Production

Total homogentate and intact chloroplasts were isolated from rosette leaves of Arabidopsis plants as described by Rensink et al. (1989). A stromal protein fraction was obtained from chloroplasts as described by Smeekens et al. (1986). Gel filtration analysis was performed as described (Ye et al., 2005). Protein samples were separated by SDS-PAGE, transferred to cellulosic membrane, and probed with CpslscA-specific antibody as described by Pilon-Smiths et al. (2002). Polyclonal antibodies against bacterially expressed CpslscA were raised in rabbits at a commercial facility (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Two rabbits were immunized after collection of immunum. Serum aliquots were stored at −80°C. The optimal antiserum dilutions in phosphate-buffered saline were found to be 1:2,000 for immunoblots. The antibody for CpiNis has been described (Pilon-Smiths et al., 2002).

Subcellular Localization of GFP-Fusion Proteins

GFP fusions were expressed in Arabidopsis protoplast-derived cells from the 35S promoter of cauliflower mosaic virus in the GFP reporter plasmid 35SlSGFP(S65T). For protoplast preparation, Arabidopsis plants (ecotype Columbia) were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) for 3 weeks. Two grams fresh weight of leaf tissue was placed in 30 mL of a buffer containing 1% (w/v) cellulase Onozuka R-10, 0.25% Macerozyme R-10 (Karlan Research Products, Santa Rosa, CA), 8 mM CaCl2, 0.5M mannitol, 170 Plant Physiol. Vol. 138, 2005 of total RNA was electrophoresed on a 1% (w/v) agarose gel containing 4% formaldehyde, transferred to a nylon membrane, and probed with a 32P-labeled AtCpslscA cDNA synthesized with a random primer labeling kit (Amersham Bioscience, Piscataway, NJ) according to suggested protocols (Smeekens et al., 1986). After incubation, the membrane was washed with three volumes of the same buffer and eluted with a 500-mL linear gradient of 0 to 0.5 M KCl in the same buffer, and 6-mL fractions were collected. Peak fractions were concentrated by adding ammonium sulfate to 70%, and the precipitated protein was collected by centrifugation for 20 min at 12,500 rpm and dialyzed overnight in 3 L of the same buffer. The dialyzed protein was filtered through a 0.2-μm filter and applied to a Sephacryl S200 HiPrep 16/60 column (Amersham Bioscience, Piscataway, NJ) equilibrated in 25 mM potassium phosphate, pH 7.5, 100 mM KCl, 1 mM EDTA at room temperature and connected to a Summit HPLC system (Dionex, Sunnyvale, CA). The column was eluted, and fractions of 1 mL were collected. Elution was monitored by detection of the OD at 280 nm. The purified protein was concentrated as before and dissolved in the same buffer, without EDTA, dialyzed, aliquoted, and stored at −80°C with 15% (w/v) glycerol. Typical yields were 5 to 10 mg/L of culture. The N-terminal sequence was determined by Edmann degradation at the Colorado State University Macromolecular Resources facility.

Enhancement of Fe-S Cluster Assembly in Fd by IscA

The role of IscA in Fe-S cluster assembly was assayed by incubating IscA (30 μg) with NiisF (7.5 μg) for 30 min in reconstitution buffer containing 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT (Roche Diagnostics, Palo Alto, CA), 1 mM l-Cys (Sigma, St. Louis), 1 mM ferrous ammonium sulfate, and 20 μM PLP. Apo-Fd (30 μg) was added and incubated for 20 min at 37°C. After incubation, the reaction mixture was centrifuged at 14,000 g for 1 min and directly applied to a 1-mL RESOURCE Q anion-exchange column (Amersham) connected to a summit HPLC system with a UVD170 detector and controlled by Chromeleon software (Dionex). The sample loop size was 100 μL. The column had been equilibrated with 25 mM Tris-HCl, pH 7.5. The following KCl gradient was applied in this buffer at a flow rate of 1.5 mL min−1: 0 to 4.5 min, 0.5 mM KCl; 4.5 to 5 min, 0 to 0.25 mM KCl; 5 to 14 min, 0.25 to 0.55 mM KCl; ramp up to 1 M KCl; 14.1 to 15.6 min, 1 M KCl; ramp down to 0 M KCl; and hold for 2 min to reequilibrate. Holo-Fd eluted at 10.8 min. Elution was monitored by absorbance at both 280 and 420 nm. Holo-Fd was quantified by signal integration at 420 nm, a characteristic absorption maximum for Fd. Spinach holo-Fd (30 μg in 150 μL) was used as a standard. The activity of the holo-Fd recovered from CpiNis/CpslscA reconstitution was assayed by examining NADP reduction (Smillie and Entsch, 1971). The ΔA340 was 0.27 mg−1 Fd min−1, and this activity was light dependent. The absorption spectrum change was the same for both reconstituted Fd and spinach holo-Fd.

Incorporation of a Transient Fe-S Cluster into CpslscA

Incorporation of the Fe-S cluster into CpslscA was achieved by incubating IscA (300 μg) with 75 μg of NiisF in reconstitution buffer (reaction 3; Fig. 7).
Separate incubations of CpsIscA (300 µg) in gel filtration buffer (25 mM Tricine/KOH, pH 7.9, 50 mM KCl, reaction 1; Fig. 7), IscA (300 µg) in reconstitution buffer (reaction 2), and CpsNi5S (75 µg) in reconstitution buffer (reaction 4; Fig. 7) were used as controls. After incubation at 37°C for 30 min, the mixtures were centrifuged at 14,000 g for 1 min and directly applied to a Superdex-200 gel filtration column (1 × 30 cm; Pharmacia) connected to a summit HPLC system with a UV/Vis detector and controlled by Chromeleon software (Dionex). The column was equilibrated in 25 mM Tricine/KOH, pH 7.9, 50 mM KCl. The flow rate was 0.75 mL min⁻¹; and fractions were collected every 0.5 min. A loop size of 500 µL was used. Elution was monitored by A280 and 420 nm. The void volume was determined with blue dextran. Standards used for calibration were IgY, bovine serum albumin, ovalbumin, chymotrypsinogen, and RNase.

Apo-Fd (30 µg) was incubated with hol-IscA, which was collected from a gel filtration column (reaction 3), for 30 min at 37°C in gel filtration buffer with 5 mM DTT. The resulting holoz-Fd was assayed by HPLC as described (Ye et al., 2005). Gel filtration analysis of CpsIscA in stromal fractions utilized the same column, buffer, and elution conditions as described above.

UV-Visible Spectroscopy

UV-visible absorption spectra (250–750 nm) were recorded with a Beckman DU 530 spectrophotometer (Beckman Instruments, Fullerton, CA) in a 100-µL quartz cuvette containing 20 to 30 µg of IscA protein in assay buffer (25 mM Tricine/KOH, pH 7.9, 50 mM KCl) in 1.0-nm scan steps.

General Methods

Protein was assayed according to Bradford using bovine serum albumin as a standard (Bradford, 1976). For CpsIscA, the protein assay by Bradford was calibrated by measuring the protein absorption at 280 nm in 6 M guanidine HCl and calculation of absolute amounts using the molar extinction coefficient predicted by the primary sequence (Gill and von Hippel, 1989). Fe content of proteins was determined according to Fish (1988). Spinach holoz-Fd was used as a control. Statistical analysis was performed with the JMP- IN software (SAS Institute, Cary, NC).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY971959.

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LITERATURE CITED


