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; AICpNifS) 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 CpNifS-mediated in vitro reconstitution of the 2Fe-2S cluster in apo-ferredoxin. During incubation with CpNifS 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 plastid 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 Tokamoto, 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) the S 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). SyNifU, the NifU 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.
(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 provide 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) with Cys desulfurase activity was localized in the chloroplast (Leon et al., 2002; Pilon-Smits et al., 2002). This protein is similar in sequence to a cyanobacterial l-Cys/l-cystine C-S lyase (C-DES; Lang and Kessler, 1999; Kessler, 2004). Other putative components include three NFU proteins (NFU1–3), similar to cyanobacterial NFU-like scaffold proteins and to the C terminus of A. vinelandii NifU (Leon et al., 2003; Yabe et al., 2004). Nfu2 purified from E. coli contained a transient Fe-S cluster (Leon et al., 2003) that could be passed on to apo-Fd in vitro (Yabe et al., 2004). Nfu2 insertion mutants have a dwarf phenotype and are deficient in some but not all plastid Fe-S proteins (Touraine et al., 2004; Yabe et al., 2004). Other potential Fe-S candidate proteins in plastids are the Arabidopsis homologs of SufBCD and E and the high chlorophyll fluorescence 101 protein (HCF101). The putative SufC homolog AtNAP7 is confirmed to be in the chloroplast, and mutants have phenotypes that indicate a role in plastidic Fe-S cluster maintenance and repair during Arabidopsis embryogenesis (Xu and Moller, 2004). HCF101 encodes a NifH-related P-loop ATPase that seems required for 4Fe-4S but not 2Fe-2S clusters in chloroplasts (Lezhneva et al., 2004).

So far, a link of either NFUs or CpSufBCDE with CpNifS has not been made. Here, we report the cloning, purification, and characterization of a chloroplast-localized IscA-like protein (AtCpIscA) from Arabidopsis. CpIscA is shown to act as a molecular scaffold in Fe-S cluster biosynthesis in vitro and to be able to transfer the cluster to apo-Fd producing active holo-Fd.

RESULTS

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 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).

Expression Analysis of CpIscA

The AtCpIscA gene expression pattern in different tissues was analyzed using RNA-blot analysis (Fig. 2).
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 targeting 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 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 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 CpisC 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 CpisC 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 CpisC precursor was imported in this assay, which was comparable to the plastocyanin control (data not shown). We conclude that the CpisC 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 CpisC.

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 CpisC in total leaf homogenate and chloroplast stroma fractions. The antibody recognized a band corresponding to the size of mature CpisC in total homogenate and isolated stroma, confirming the stromal localization of AtCpisC (Fig. 4B). The mobility of the detected CpisC 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 CplscA precursor. 35S-Met-labeled Precursor CplscA 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 radioabeled 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 CplscA (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 CplscA-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; 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 μg) was incubated with CpNifS (75 μg) in reconstitution buffer (5 mM dithiothreitol [DTT], 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM pyridoxal 5’ phosphate [PLP]) for 30 min and then applied to a gel filtration column (Fig. 7A, line 3). As controls, apo-CpIscA (300 μg) incubated in gel filtration buffer or in reconstitution buffer, as well as CpNifS alone (75 μg), were incubated and run through the same column (Fig. 7A, lines 1, 2, and 4, respectively). Proteins were detected by A280 (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 ApoCpIscA 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 A420 (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 ± 0.2 Fe/protein for spinach holo-Fd. Reconstituted CpIscA contained 1.4 ± 0.2 Fe/dimer, while no Fe was detected in the apo-form. Because the reconsti-

![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.](http://www.plantphysiol.org/)

![Figure 6. Purified CpIscA enhances the Fd reconstitution activity of CpNifS. Purified CpNifS (7.5 μg), CpIscA (30 μg), or a combination of both CpNifS (7.5 μg) and CpIscA (30 μg) were incubated with 50 mM Tricine-NaOH, pH 7.5, 5 mM DTT, 1 mM L-Cys, 1 mM ferrous ammonium sulfate, 20 μM PLP for 30 min, followed by addition of 30 μg of apo-Fd. After 20 min, the reconstituted holo-Fd was assayed by HPLC. Data represent the average and SE of three replicates.](http://www.plantphysiol.org/)
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
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 Schizosaccharomycetes pombe (Krebs 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 CplscA-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 CplscA 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 of cyanobacteria (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). However, IscA scaffold proteins were identified in Synechocystis PCC 6803 (Wollenberg et al., 2003). Genes encoding IscU homologs with putative chloroplast-targeting sequences were not found 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 CplscA protein may provide an alternative scaffold to the IscU proteins for Fe-S cluster formation and delivery in chloroplasts.

In addition to CplscA, three chloroplast-localized CplNfu 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 CplscA and CplNfu 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 function 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 AtCpNfus (Touraine et al., 2004; Yabe et al., 2004).

A scaffold role for CplscA 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 NifIscA can also serve as a scaffold for 4Fe-4S clusters (Krebs et al., 2001). Once a suitable in vitro plant system for reconstruction of a 4Fe-4S apo-protein has been established, it will be interesting to investigate whether CplscA serves as a scaffold for both types of Fe-S cluster.

MATERIALS AND METHODS

Cloning and Plasmid Construction

The Arabidopsis (Arabidopsis thaliana) CplscA 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-Smits et al., 2002). Primers used for IscA amplification were 5'-GGCTCTAGACGACAGAGATGGCTTTCGCTACTGGAGA-3' (forward primer) and 5'-TCCCCCGGGGTGTGAACCTGGTTCACATC-3' (reverse primer). Underlined bases indicate Xhol and Smal sites, respectively. The PCR product was digested with Xhol and Smal and then ligated into a PBS (K50 vector) (Stratagene, La Jolla, CA), digested with the same restriction enzymes to produce plasmid pPrIscA. To subclone the mature sequence of IscA in pET1Id for expression, PCR was performed with another set of nested primers, 5'-CATGCCAGGCGCTGTTCGACTCCGGCTCTGCTTCTC-3' (forward primer) and 5'-CGGGATCTCATCCATCTCCGGGACAAAAAAGCTCC-3'. The entire coding sequence of IscA was amplified by PCR using flanking primers Sall-N and Ncol-CF1 (5'-CATGCCAGGCGCTGTTCGACTCCGGGACAAAAAAGCTCC-3').

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 Sall-N (5'-GAATGTTGTCATGGGTCTCGAGA-3') and Ncol-C (5'-CAGGATCTACATCCGGGACAAAAAAGCTCC-3'). The PCR product was digested with SalI and Ncol and subcloned in pET11d to produce plasmid pEGFP.

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.expasy.org/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 (Epicenter 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/PharMacia, 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-Smiths et al., 1992). The postimport thermolysis 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 homogenate and intact chloroplasts were isolated from rosette leaves of Arabidopsis plants as described by Rensing 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). Ten micrograms 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 AtCplSca cDNA synthesized with a random primer labeling kit from Amersham. Prehybridization, hybridization, washing, and detection were done as described previously (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 35S-SCFP(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 (Karakal Research Products, Santa Rosa, CA), 8 mM CaCl2, 0.5 mM mannitol, 5 mM MES, pH 5.5, vacuum infiltrated for 1 min, and incubated for 3 h at room temperature with gentle shaking. The clear digest was filtered through a 37- to 70-μm nylon mesh (Cellular Biological Supply, Burlington, NC), and the protoplasts were harvested by centrifugation for 5 min at 1,000 rpm and washed twice in 10 mL of cold W5 wash solution (154 mM NaCl, 125 mM argon ion laser system and a 1.4 numerical aperture 60× objective lens (Olympus America, Melville, NY). The fluorescence signals were detected at 530 nm for GFP and 660 nm for chlorophyll. Sections of 1-μm thickness were scanned, and images were captured by the Fluoview software as Tiff files.

Preparation of Proteins

Fd was extracted from fresh spinach (Spinacia oleracea) that was obtained at a local health-food store, essentially as described (Yocum, 1982), and further purified as described (Pilon-Smiths et al., 1992). The apo-Fd for reconstitution assays was prepared from holo-Fd by removal of the Fe-S cluster as described (Kato et al., 2000). Apo-Fd (at 0.5 mg/mL) was stored in 100 mM Tris-HCl, pH 8.0, under nitrogen in small aliquots at −80°C. CpNiS was purified and expressed as described (Pilon-Smiths et al., 2002). For overexpression of AtCplSca, Escherichia coli BL21 (DE3) codon (Stratagene) was transformed with pMHsca vector, and 2 L of LB medium containing 50 μg/mL ampicillin was inoculated with 1/100 volume of overnight culture. Cells were grown at 37°C to an OD600 of 0.5, and expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The bacterial pellet was washed with 150 mM NaCl and resuspended in buffer A (25 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA) and passed twice through a French press (8,000 psi) at 4°C to disrupt the cells. All further manipulations were at 4°C unless indicated otherwise. The lysate was centrifuged for 20 min at 12,000g, and the cleared supernatant was loaded onto a cation-exchange SP Sepharose 1.6×20-cm column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 25 mM potassium phosphate, pH 7.5, 1 mM EDTA at a flow rate of 5 mL·min⁻¹. The column 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 12,000g, dissolved in 5 mM buffer A, 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 Sepabead/S200 (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⁻¹ 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 NiSs (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 t-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 14,000g for 1 min and directly applied to a 1-mL RESOURCE Q anion-exchange column (Amersham) connected to a summit HPLC system with a UV/Vis detector and controlled by Chromelon software (Dionex). The sample loop size was 100 μL. The column was 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 KCl, 4.5 to 5 min, 0 to 0.25 KCl, 5 to 14 min, 0.25 to 0.5 KCl, 14.5 to 15 min, 0.5 to 1 M KCl, 15 to 16.5 min, 1 M KCl. The column was reequilibrated for 2 min at 0 KCl, and held for 2 min at reequilibration. 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 CpNiS/CpIscA reconstitution was assayed by examining NADP reduction (Smithile and Ensch, 1971). The ΔA260 was 0.27 μg⁻¹·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 CplSca, Gel Filtration Analysis, and Cluster Transfer

Incorporation of the Fe-S cluster into CpIscA was achieved by incubating IscA (300 μg) with 75 μg of NiSs 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 CpsNiS5 (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 14,000g for 1 min and directly applied to a Superdex-200 gel filtration column (1 x 30 cm; Pharmacia) connected to a summit HPLC system with a UVD170 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 holo-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 holo-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-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 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.

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

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