Identification and Characterization of the Terminal Enzyme of Siroheme Biosynthesis from Arabidopsis thaliana

A PLASTID-LOCATED SIROHYDROCHLORIN FERROCHELATASE CONTAINING A 2FE-2S CENTER

Received for publication, October 5, 2004, and in revised form, November 15, 2004
Published, JBC Papers in Press, November 15, 2004 DOI 10.1074/jbc.M411360200

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Higher plant sulfite and nitrite reductases contain siroheme as a prosthetic group. Siroheme is synthesized from the tetrapyrrrole primogenitor uroporphyrinogen III in three steps involving methylation, oxidation, and ferrochelation reactions. In this paper we report on the Arabidopsis thaliana sirohydrochlorin ferrochelatase At-SirB. The complete precursor protein of 225 amino acids and shorter constructs in which the first 46 or 79 residues had been removed were shown to complement a defined Escherichia coli sirohydrochlorin ferrochelatase mutant. The complete precursor of the protein appeared to consist of only 150 amino acids, making it much smaller than previously characterized ferrochelatases. Green fluorescent protein tagging revealed that it is located in the plastoplast. The enzyme was easily produced in E. coli as a recombinant protein, and the isolated enzyme was found to have a specific activity of 48.5 nmol/min/mg. Significantly, the protein purified as a brown-colored solution with a UV-visible spectrum containing maxima at 415 and 455 nm, suggestive of an Fe-S center. EPR analysis of the recombinant protein produced a rhombic spectrum with G-values of 2.04, 1.94, and 1.90 and with temperature dependence consistent with a 2Fe-2S center. Redox titration demonstrated that the Fe-S center is highly unstable, with an apparent midpoint reduction potential of about −370 mV. This is the first Fe-S center to be reported in a higher plant ferrochelatase. The implications of the Fe-S center in an enzyme that is so closely associated with the metabolism of sulfur and iron are discussed.

In plants siroheme is found as a prosthetic group in just two enzymes, sulfite and nitrite reductases (1). These enzymes catalyze the six electron reduction of sulfite and nitrite, respectively, and since the majority of cellular sulfur and nitrogen is processed via these enzymes, they are essential for sulfur and nitrogen metabolism in all living organisms. Central to the catalytic activity of these enzymes is the role played by siroheme (1), which is an iron-containing isobacteriochlorin (2, 3). This metallo-prosthetic group is a modified tetrapyrrole similar in structure to both heme and chlorophyll (4). The structural commonality among modified tetrapyrroles reflects their synthesis along a branched biosynthetic pathway (Fig. 1).

In higher plants tetrapyrrole synthesis occurs largely in the plastid (5), where it is initiated by the reduction of the glutamyl moiety of glutamyl-tRNA to glutamate-1-semialdehyde (6, 7). Intermolecular transamination of glutamate semialdehyde generates 5-aminolevulinic acid, which is then transformed into the first macrocyclic intermediate of the pathway, uroporphyrinogen III (uro’gen III),1 by the action of the enzymes 5-aminolevulinic acid dehydratase, porphobilinogen deaminase, and uro’gen III synthase (4). Uro’gen III represents the first branch point in the pathway (Fig. 1), as methylation of this intermediate directs it toward siroheme synthesis, whereas decarboxylation steers it toward heme and chlorophyll synthesis (8). For siroheme synthesis, methylation occurs at positions 2 and 7, forming dihydrosirohydrochlorin (9). The enzyme that catalyzes this reaction, uro’gen III methyltransferase (UPM1), requires S-adenosyl-L-methionine as a methyl donor and has been characterized from a wide variety of sources including eubacteria (10, 11), Archaea (12), and higher plants (13, 14). In Arabidopsis thaliana UPM1 has been localized to the chloroplast (14). To complete the synthesis of siroheme, dihydrosirohydrochlorin has to be oxidized to sirohydrochlorin and then chelated with ferrous iron. In yeast, these two steps are catalyzed by a single bifunctional enzyme called Met8p (15), which houses both dehydrogenase and chelatase functionalities within the same active site (16). However, no orthologues of Met8p are found in higher plants. In some bacteria, the transformation of dihydrosirohydrochlorin into siroheme is catalyzed by two separate enzymes called SirC (dihydrosirohydrochlorin dehydrogenase) and SirB (sirohydrochlorin ferrochelatase) (17, 18). The latter inserts ferrous iron into sirohydrochlorin to give siroheme, and the enzyme displays a high degree of similarity to the cobaltochelatase (ChiX) found in E. coli as a recombinant protein, and the iso-

1 The abbreviations used are: Uro’gen III, uroporphyrinogen III; SirA, uroporphyrinogen III methyltransferase; SirB, dihydrosirohydrochlorin ferrochelatase; SirC, dihydrosirohydrochlorin dehydrogenase; At-SirB, A. thaliana dihydrosirohydrochlorin ferrochelatase; CbiK, dihydrosirohydrochlorin chelatase; CbiX, dihydrosirohydrochlorin chelatase; EPR, electron paramagnetic resonance; HemH, protoporphyrin IX ferrochelatase; Met8p, bifunctional dihydrosirohydrochlorin dehydrogenase and sirohydrochlorin ferrochelatase; UPM1, uroporphyrinogen III methyltransferase; GFP, green fluorescent protein.
Arabidopsis Sirohydrochlorin Ferrochelatase

in the anaerobic pathway of cobalamin (vitamin B12) synthesis, which inserts cobalt into sirohydrochlorin (19).

Sirohydrochlorin ferrochelatase, SirB of siroheme biosynthesis (17), is a class II tetrapyrrrole biosynthetic chelatase, defined as a chelatase that exists as either a monomer or homodimer and which does not require ATP for activity (20). Within this class are included the protoporphyrin ferrochelatases (HemH) of heme synthesis (21, 22), the sirohydrochlorin cobaltoclelata- 

ses associated with cobalamin biosynthesis, CbiK (23) and CbiX (19), as well as SirB. The structures of HemH and CbiK have been determined by x-ray crystallography, and these have revealed a high level of structural similarity indicating that the proteins have probably arisen by divergent evolution from a common ancestor (22, 23). This structural similarity is only reflected in an approximate 10% sequence identity. Although SirB and CbiX share about 40% sequence identity, they also display a lower sequence identity (~15%) with CbiK, suggesting that they, too, will be structurally similar.

The structures of HemH and CbiK revealed that the enzymes are composed of two main α/β domains that are related to one another by a pseudo-2-fold symmetry (22, 23). This suggested that the protein structure had arisen from a gene duplication and fusion event. In support of this theory, cobaltoclecellatas (CbiX) have recently been identified in Archaea that are only about half the size of those in eubacteria and which can be aligned to either one of the two structural domains (20). These shorter versions of CbiX have been termed CbiXα to differentiate them from the longer versions (CbiXβ).

In this paper we describe the identification of a plant sirB orthologue in the genome of Arabidopsis by similarity search- ing. We have cloned a cDNA for this gene and have demon- 

strated its function both in vitro and in vivo. Sequence analysis of the mature form of the enzyme reveals that it is only about half the size of the bacterial SirB proteins. We have investi- 
gated the subcellular location of the enzyme within the plant cell and investigated the properties of the recombinant enzyme.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Porphobilinogen and most other chemicals were purchased from Sigma. Other materials were provided by the following suppliers. Restriction enzymes and modification enzymes were from Promega, Chilworth, Southampton, UK; pKK223.3, chelat- ing Sepharose fast flow resin, and gel filtration columns were from Amersham Biosciences; pET14b and pET3a were from Novagen, Madison, WI; pGEX1 and pGEX2 were purchased from Sigma. Other materials were provided by the
glove box.

ELEXSYS E500 spectrometer equipped with an Oxford Instruments ESR900 liquid helium cryostat.

Redox Potentiometry—Redox titrations were performed in a Belle Technology glove box under a nitrogen atmosphere essentially as de- scribed previously (29). All solutions were degassed under vacuum with nitrogen and sealed to prevent glass to glass contamina- tion.

For recombinant protein production, three variants of the full-length cDNA were constructed to generate three different start sites. Thus, constructs starting with amino acid Met1, V46M, and G79M were made (Table I), representing full-length precursor protein and two potential mature protein variants.

In Vivo Complementation of an E. coli cysG Mutant Strain—The E. coli cysG mutant strain 3023a (25) was transformed with pA- 

Cyc184, which harbors the lacP gene as well as the Pseudomonas denitrificans coaA gene cloned under the control of a tac promoter (26).

This resultant strain was then transformed with the different pETac constructs (Table I) and plated onto LB plates containing ampicillin and chloramphenicol. The recombinant strains were finally restreaked on M9 medium or M9 medium supplemented with either cysteine (50 mg/liter), cobalt chloride (5 μM), nickel sulfate (5 μM), or copper sulfate (5 μM) followed by incubation for 24 h at 37 °C (26). The empty pETac vector and the construct containing the Saccharomyces cerevisiae MET8 gene were used as controls (16).

Expression of At-SirB-GFP Fusion in Vivo—The At-SirB-GFP fusion protein construct in pUC118-smRS-GFP vector (27) was used for transient expression in tobacco (Nicotiana tabacum) leaves from sterile-grown 4-week-old plants were used. At-Sir-B-GFP was introduced by biolistic transformation, and the location of GFP fluorescence was de- 

termined by confocal microscopy as described (28).

Purification of At-SirB—E. coli BL21star(DE3)pLysS was transformed with the pET14b constructs harboring the cDNA with start codons at Met1, V46M, and G79M, named pET14b-AtSirB1, pET14b-AtSirB4, and pET14b-AtSirB79, respectively. They were sub-
sequently grown in LB supplemented with ampicillin and chloramphenicol. Isopropyl-β-D-thiogalactopyranoside (0.4 mM) was added when the cells reached an absorbance of 0.6 at 600 nm and were left overnight at 16 °C. The cells were harvested and resuspended in 20 mM Tris-HCl buffer, pH 8.5, containing 0.5 mM NaCl and 5 mM imidazole (buffer A). The cells were lysed by sonication and spun at 15000 × g for 20 min to pellet the cell debris and membranous fraction. The supernatant containing the soluble cell extract was applied to a column (4 ml) of chelating Sepharose resin (Amersham Biosciences) that had previously been charged with 50 mM NiSO4 and equilibrated with buffer A. The column was washed with 10 column volumes of buffer A and a further 10 column volumes of buffer A containing 80 mM imidazole. Finally, the His-tagged protein was eluted from the column in 20 mM Tris-HCl buffer, pH 8.5, containing 0.5 mM NaCl and 1 mM imidazole.

In Vivo Analysis of At-SirB Kinetics—Chelatase assays were performed as described previously (16) by monitoring the conversion of sirohydrochlorin into either cobalt-sirohydrochlorin or siroheme. Sirohydrochlorin was generated in situ under an atmosphere of nitrogen in a glove box with less than 2 ppm oxygen. This was accomplished by charging the column with 5 mg of porphobilinogen in 4 ml of buffer containing 5 mg of purified porphobilinogen deaminase, 1 mg of purified urogen III synthase, 5 mg of purified urogen III methyltransferase, 15 mg of S-adenosyl-l-methionine, 5 mg of a dihydrosirohydrochlorin de-
hydrogenase (SirC), and 10 mg of NADP*. The reaction was omitted overnight at room temperature to allow it to reach completion, and the mixture was filtered before use. The rate of the chelatase reaction was measured by measuring the increase in absorbance at 376 nm using the extinction coefficient of 2.4 × 104 M cm⁻¹. The chelatase activity was measured with sirohydrochlorin (2.5 μM), Co²⁺ (20 μM), or Fe²⁺ (20 μM) and varying amounts of At-SirB in a 1-ml reaction volume in 0.05 M Tris-HCl buffer, pH 8.0. For all reactions initial rates were recorded on a Hewlett Packard 452A photodiode and an Emisspectrophotometer, and assays were performed in triplicate in the glove box.

Preparation of Samples for EPR and Their Analysis—EPR samples were prepared with protein isolated from the strain containing the pET14b-ATSirB79 construct, which was grown in 4 liters of super LB (32 g/liter peptone, 20 g/liter yeast extract, and 5 g/liter sodium chlo-
ride). The purification was carried out as described above except that it was performed under anaerobic conditions in a glove box (Belle tech-
nology) in an atmosphere of less than 2 ppm oxygen. After the metal chelate column, the brown-colored fraction was collected and applied to a gel filtration column equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 100 mM NaCl. EPR samples were prepared in the glove box, loaded into EPR tubes, sealed with a cap, and then immediately frozen in liquid nitrogen. Samples of the protein were prepared plus or minus 500 μM dithionite. EPR spectra were obtained at X-band using a Bruker ELEXSYS E500 spectrometer equipped with an Oxford Instruments ESR900 liquid helium cryostat.
addition of reductant or oxidant to allow stabilization of the electrode. Spectra (300–800 nm) were recorded using a Cary UV-50 Bio UV-visible scanning spectrophotometer via a fiber optic absorption probe (Varian) immersed in the enzyme solution and connected to the external spectrophotometer. The electrochemical potential of the solution was measured using a Hanna pH 211 meter coupled to a Pt/calomel electrode (ThermoRussell Ltd.) at 25 °C. The electrode was calibrated using the Fe$^{3+}$/Fe$^{2+}$/EDTA couple as a standard (108 mV). A factor of 244 mV was used to correct relative to the standard hydrogen electrode. Absorption versus potential data at appropriate wavelengths (reflecting maximal changes in spectral properties between oxidized and reduced enzyme) were fitted to the Nernst equation to derive the apparent midpoint reduction potential ($E_0$) for the one-electron reduction of the bound iron-sulfur cluster.

RESULTS

Identification of a Putative A. thaliana sirB Gene—The transformation of uro'gen III into siroheme requires the action of up to three enzymes, a uro'gen III methyltransferase, a dehydrogenase, and a ferrochelatase (Fig. 1). In E. coli, a single enzyme encoded by cysG catalyzes all three reactions (32). In yeast this transformation requires two enzymes called Met1p (a uro'gen III methyltransferase) and Met8p (a bifunctional dehydrogenase and ferrochelatase) (16). However, in other bacteria such as Bacillus megaterium, these activities are housed within three separate enzymes called SirA (uro'gen III methyltransferase), SirC (dihydrosirohydrochlorin dehydrogenase), and SirB (sirohydrochlorin ferrochelatase) (17). We had already shown that Arabidopsis contains a functional SirA orthologue UPM1 (14). Using the BLAST algorithm we were unable to identify orthologues in the Arabidopsis genome or in any plant EST data base of SirC.

However, a hypothetical protein encoded by At1g50170 (Arabidopsis Genome Initiative, 2000) displayed similarity to SirB (e value, 1e$^{-68}$). The gene is predicted to encode a protein of 225 amino acids, with 28% identity to B. megaterium SirB (Fig. 2a). Primers were designed to the predicted 5′ and 3′ ends of the gene and used to amplify the sequence by PCR from a cDNA library (24). The amplified product was cloned into pETac (Table I), and the resulting plasmid (pETac-AtsirB1) was introduced into E. coli strain 302a::pACYC184-lacIq-PdcobA, which is a sirohydrochlorin ferrochelatase mutant and therefore, unable to grow in the absence of exogenous cysteine (see “Experimental Procedures”). The At1g50170 cDNA was able to complement the mutant phenotype (Table II). Previous work has shown that some cobaltochelases can also complement sirohydrochlorin ferrochelatase deficiency (26). However, cobaltochelases can be differentiated from ferrochelatases on the basis that complementation of ferrochelatase activity by
cobaltchelatases is inhibited in the presence of exogenous cobalt in the medium, presumably due to competitive inhibition of the metal binding site within the chelatase (15). At1g50170 was still able to complement the *E. coli* sirohydrochlorin ferrochelatase-deficient strain in the presence of exogenous cobalt (Table II), indicating that it is specific as a ferrochelatase and can, thus, be assigned as the *Arabidopsis* SirB (At-SirB). Subsequently, further database searching has revealed the presence of conserved histidine residues in the chelation process either in metal binding or as general bases in the removal of two protons from sirohydrochlorin. The cysteines marked with asterisks found in the C-terminal region of the plant SirB sequences are thought to be involved in housing the Fe-S center. Conserved residues are boxed in black, and similar residues are boxed in gray. Os, *Oryza sativa* (rice); At, *Arabidopsis thaliana*; Bm, *Bacillus megaterium*; Meth, *Methanosarcina barkeri*. 

**FIG. 2.** SirB protein alignments. a, alignment between the sirohydrochlorin ferrochelatases (SirB sequences) from *A. thaliana*, *Oryza sativa* (rice), and *B. megaterium* (N terminus) and the ChiXs from *B. megaterium* (N terminus of ChiX2) and *Methanosarcina barkeri* (ChiX3). It is likely that the conserved histidine residues are involved in the chelation process either in metal binding or as general bases in the removal of two protons from sirohydrochlorin. The cysteines marked with asterisks found in the C-terminal region of the plant SirB sequences are thought to be involved in housing the Fe-S center. Conserved residues are boxed in black, and similar residues are boxed in gray. Os, *Oryza sativa* (rice); At, *Arabidopsis thaliana*; Bm, *Bacillus megaterium*; Meth, *Methanosarcina barkeri*. b, protein alignment of the *A. thaliana* SirBS (amino acids 80–225) against the N- and C-terminal regions of the SirBL from *B. megaterium*. The plant SirBS aligns against both the N and C termini of the longer SirBL Eubacterial sequence.
ence of orthologues of SirB in rice (Fig. 2a) and several other plants (www.plantgdb.org).

Subcellular Location of At-SirB—Sequence alignment of the At-SirB sequence against a range of different bacterial SirB proteins and the highly related cobaltchelatase CbiX revealed that significant similarity between the proteins started only at amino acid 79 (Fig. 2a). Furthermore, the N terminus of the Arabidopsis SirB protein was predicted to be a chloroplast transit peptide. By At1g50170 is targeted to plastids, and the N terminus is a functional chloroplast transit peptide.

However, from the SirB sequence alignments it is difficult to judge the exact length of the transit peptide, so two putative mature protein constructs were generated. At-SirB46 encodes a protein that starts from amino acid 46, the cleavage site predicted by ChloroP, whereas At-SirB79 corresponds to the position that aligns with the start of similar bacterial proteins (Fig. 2a).

Burkholderia pseudomallei has been enhanced (Fig. 3, panels E–F) so chlorophyll fluorescence that appears to be inside the perimeter of the cell is probably from underlying mesophyll cells in the same plane. From these data it can be concluded that the protein encoded by At1g50170 is targeted to plastids, and the N terminus is a functional chloroplast transit peptide.

Both of these constructs, cloned into pETac vector, were tested for their ability to complement the E. coli sirohydrochlorin ferrochelatase mutant. Both were able to do so and again were not affected by the presence of cobalt in the medium (Table II). The mature form of At-SirB is predicted, therefore, to contain between 146 and 179 amino acids. All the plant homologues identified in the databases are of similar length to the Arabidopsis SirB. This protein is only about half the size of the bacterial SirB orthologues (Fig. 2a) and closer in size to the recently identified short CbiX enzymes (termed CbiXs) (20). As with the CbiXs sequences, the plant SirB aligns against both the N- and C-terminal halves of bacterial SirB sequences (Fig. 2b), highlighting the fact that these bacterial proteins had evolved as a result of a gene duplication and fusion event (20).

In this respect, the plant SirB can be considered a short version of SirB and should be referred to as SirBs to differentiate it from the longer versions of SirB2.
Production of At-SirB as Recombinant Protein in E. coli—

Recombinant forms of At-SirB containing 225, 179, and 146 amino acids were produced in E. coli after transforming competent cells with the plasmids pET14b-AtSirB1, pET14b-AtSirB46, and pET14b-AtSirB79 (Table I). The recombinant protein forms were produced with N-terminal histidine tags to allow the proteins to be purified by metal chelate chromatography. Analysis of protein isolated from strains harboring these constructs revealed that the full-length protein (encoded by At-SirB) is highly unstable and is rapidly broken down into a number of smaller peptides (Fig. 4a). The middle-sized construct is much more stable and is only partially degraded, whereas the smallest construct produces the most stable protein of all, with a molecular mass of 15 kDa (Fig. 4a).

UV-Visible Spectra and EPR—The production of His-tagged recombinant At-SirB in E. coli allowed the protein to be rapidly purified by metal chelate chromatography. The recombinant At-SirB purified as a brown-colored solution, suggesting that it might contain some bound prosthetic group or redox center. Indeed, toward the C terminus of the protein there is a short sequence containing four cysteine residues between amino acids 198 and 225 (H_{C}LSHVEGDADE_{C}LV_{C}AGT-NK_{C}KLYNSS) (Fig. 2a), which would have the potential of binding such an Fe-S center. A UV-visible spectrum of the protein revealed that it has absorption peaks at 410 and 450 nm (Fig. 4b), a spectrum consistent with the presence of a 2Fe-2S center (34, 35).

To verify that the chromophore was due to the presence of an Fe-S center, the shortest version of the protein was further characterized by EPR. However, the addition of excess sodium dithionite (typically 11.5 mM final concentration) as a reductant yielded a weak spectrum that suggested that excess dithionite was destroying the iron-sulfur center and leading to
the disruption of the binding of the Fe-S center to the protein. The protein was, therefore, reduced with stoichiometric amounts of sodium dithionite, and the EPR spectrum shown in Fig. 5 was obtained. This is a rhombic spectrum with G-values of 2.04, 1.94, and 1.90. This spectrum was observable at 70 K that, together with the g anisotropy, provides further support for the presence of a 2Fe-2S center. The form of the EPR spectrum is, however, not exactly comparable with those previously reported for 2Fe-2S centers (35), which may be indicative of reductant-induced damage or of a new cluster structure that is susceptible to such damage.

Redox Potentiometry—To determine the midpoint reduction potential of the At-SirB<sup>+</sup> iron-sulfur cluster, a spectroelectrochemical potentiometric titration was performed, as described previously (29, 36). Anaerobic reductive titration of At-SirB<sup>+</sup> with sodium dithionite resulted in significant spectral changes, observed as bleaching of the absorption features by a factor of ~60% as the enzyme is converted between the [2Fe-2S]<sup>2+</sup> and [2Fe-2S]<sup>1+</sup> forms (Fig. 6). Although the protein remained stable during the reductive titration and showed no significant degree of aggregation during the ~5 h required to reduce the iron-sulfur cluster fully, subsequent attempts to restore the starting spectrum by oxidation with ferricyanide were unsuccessful. The fully reduced spectrum was not significantly affected by additions of the powerful oxidant. This again suggested that the redox center was unstable during the reduction process. A further reductive titration was undertaken, this time terminating the reduction at the stage at which ~50% of the absorption of the At-SirB<sup>+</sup> iron-sulfur cluster was bleached. Thereafter ferricyanide was added to assess whether the partially reduced cluster could be reoxidized. However, the At-SirB<sup>+</sup> spectrum was again almost completely unaffected by the addition of the oxidant, indicating that the 2Fe-2S cluster was unstable to reduction by dithionite.

Notwithstanding the irreversible nature of the reductive titration, plots of absorption change at various wavelengths across the visible range versus applied potential were sigmoidal and gave reasonable fits to the Nernst equation, producing consistent apparent midpoint potentials (36). Fig. 6 shows the spectra collected during the anaerobic reductive titration of At-SirB<sup>+</sup> from oxidized (uppermost spectrum) to reduced (lowest spectrum), illustrating the absorption decrease across the entire visible range. The inset shows a fit of absorption data at 450 nm to a single electron Nernst function. The data fit produces an apparent midpoint reduction potential (E<sub>v</sub> versus the standard hydrogen electrode (SHE)) of ~372 ± 4 mV. Although this may be a reasonable estimate of the potential of the 2Fe-2S cluster in At-SirB<sup>+</sup>, the fact that the titration is not reversible suggests that the true potential may actually be rather more negative than this value.

In Vitro Analysis of At-SirB<sup>+</sup> Chelatase Activity—The activity of the At-SirB<sup>+</sup> was investigated by assaying the activity of the purified At-SirB<sup>+</sup> in the presence of sirohydrochlorin and

FIG. 5. EPR spectrum of At-SirB<sup>+</sup>. X-band EPR spectrum of dithionate reduced AT-SirB recorded at 15 K using 1 milliwatt microwave power, 100 KHz modulation frequency and 5 G modulation amplitude. G-values are as marked on the figure.

FIG. 6. Potentiometric-reductive titration of At-SirB<sup>+</sup>. Main figure, UV-visible spectral changes associated with the reductive titration of At-SirB<sup>+</sup> with sodium dithionite. The arrow indicates the direction of absorption change occurring as the 2Fe-2S cluster is reduced. The uppermost spectrum is that for the fully oxidized enzyme (200 μM). The lowest spectrum is for the fully reduced enzyme. The inset shows a plot of absorption at 450 nm (reflecting the maximal overall absorption change observed in the visible absorption range) versus the applied potential (standard hydrogen electrode (SHE)). The data are fitted to the Nernst equation to produce an apparent midpoint reduction potential (E<sub>v</sub>) = ~372 ± 4 mV.
either cobalt or ferrous iron. The smallest form of the enzyme was found to have a specific activity of 48.5 nmol/min/mg with cobalt as substrate and 5.4 nmol/min/mg with ferrous iron as substrate. Thus, the enzyme has a higher rate of activity with cobalt rather than iron. It was not possible to measure an accurate $K_m$ value for the metals as at low concentrations of metal the assay was not sensitive enough, whereas at higher metal ion concentrations the enzyme was inactivated.

**DISCUSSION**

In this paper we describe for the first time the identification, cloning, production, and characterization of a plant sirohydrochlorin ferrochelatase. We have shown that this protein has sirohydrochlorin ferrochelatase activity both in *vivo* and *in vitro*, so there can be no doubt that it represents the terminal enzyme of siroheme synthesis. As expected, the protein is translocated to the plastid, since this is where the urogen III methylase (UPM1) is found (13, 14) and is the location of sulfitase and nitrite reductase (1), the enzymes for which siroheme is a prosthetic group. With the description of the UPM1 (13, 14) and At-SirB, there now just remains the sirohydrochlorin dehydrogenase to be found to complete our molecular understanding of siroheme synthesis in higher plants. Being located in the chloroplast does, however, raise some interesting questions, since the protoporphyrin IX ferrochelatase (HemH), the terminal enzyme for heme synthesis, is also found in this organelle. For instance, what is the source of the ferrous ion, and how do these two enzymes compete for the bioavailability of this metal? Despite being one of the most abundant elements on earth, iron is frequently one of the most limiting factors for plant growth.

The mature form of At-SirB contains around 150 amino acids, making it half the size of bacterial orthologues that are about 300 amino acids long. Sequence analysis reveals that the At-SirB aligns with both the N and C termini of the longer bacterial SirB proteins, indicating that the longer version is likely to have evolved as a result of a gene duplication and fusion of a smaller enzyme, which nonetheless persisted in the plant lineage. To discriminate between the shorter plant SirB sequences and the longer bacterial versions, we suggest that they should be referred to as SirB and SirB'. The presence of short and long versions of SirB in plants and eubacteria, respectively, is analogous to the presence of short and long versions of the cobaltocobalamin (ChbiX) in Archaea and eubacteria (20).

Interestingly, the At-SirB' has an integral Fe-S center at the C terminus, which is not found in the longer eubacterial SirB's. Iron-sulfur centers have been reported with animal (21) and some bacterial (37) protoporphyrin ferrochelatases in the past but not on plant protoporphyrin ferrochelatases (21), and the presence of such a center on a sirohydrochlorin-ferrochelatase suggests that it may have a highly significant physiological role. The center in the At-SirB' is very unstable to reduction and rapidly breaks down, indicating that its physiological role may not involve oxidation and reduction during catalysis. Thus, the center could be involved in redox-sensing, signaling, or in mediating protein-protein interactions. For example, might this be involved in the competition for ferrous iron between the sirohydrochlorin and protoporphyrin ferrochelatases within the plastid?

There are some similarities between the protoporphyrin ferrochelatase and the At-SirB' that are worth noting. In the human protoporphyrin ferrochelatase the 2Fe-2S center is very unstable and rapidly degrades in an aerobic environment (38). Second, the protoporphyrin ferrochelatase Fe-S center is housed in a C-terminal region of the protein containing four cysteine residues (38, 39) which have a similar spacing to that observed in the C-terminal region of At-SirB'. Finally, only three of the four cysteine residues in the C-terminal region of the protoporphyrin ferrochelatases contribute to holding the Fe-S center (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40). The fourth ligand is a terminal methyl group (40).

From a wider perspective, the presence of an Fe-S center on plant SirB' is even more intriguing, since it further integrates cofactor synthesis into iron and sulfur metabolism (Fig. 1). The role of siroheme in sulfate and nitrite reductases means that it is responsible for the assimilation of all inorganic sulfur and the majority of nitrogen in the biosphere (1). Thus, without siroheme, there would be no reduced sulfur in the cell required for incorporation into the amino acids cysteine and methionine and for sulfur in Fe-S centers. In this context it is interesting to note that both sulfite and nitrite reductases also have an Fe-S cofactor (1). This intimate relationship between iron and sulfur is likely to be reflected in the signaling processes that respond to environmental factors, including nutrient availability. Much further work is required to understand the basis of this potential signaling system.

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J. Biol. Chem. 2005, 280:4713-4721.
doi: 10.1074/jbc.M411360200 originally published online November 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411360200

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