Role of a NifS-like Protein from the Cyanobacterium *Synechocystis* PCC 6803 in the Maturation of FeS Proteins†

Karin Jaschkowitz and Andreas Seidler*

Biochemie der Pflanzen, Fakultät für Biologie, Ruhr-Universität Bochum, 44780 Bochum, Germany

Received September 15, 1999; Revised Manuscript Received December 13, 1999

ABSTRACT: In *Azotobacter vinelandii* and *Escherichia coli* NifS or NifS-like proteins are involved in FeS protein assembly by mobilizing sulfur from free cysteine. This sulfur together with Fe^{2+} is then incorporated into apo-FeS proteins to form an FeS center. A different activity termed C-DES [for cyst(e)ine desulfurylase] was recently isolated from the cyanobacterium *Synechocystis* PCC 6714 which also mobilized sulfur and which was able to incorporate the FeS center into apoferredoxin. In the genome of the cyanobacterium *Synechocystis* PCC 6803, there are three open reading frames (orfs) that are similar to NifS and one that is similar to C-DES, indicating that this bacterium might contain both activities, NifS and C-DES. One orf from *Synechocystis* PCC 6803 encoding a NifS-like protein, slr0387, was overexpressed in *E. coli* and purified. The molecular mass of the recombinant protein was determined to be about 82 kDa, indicating that it is a homodimer. The absorption spectrum was typical for PLP-containing proteins with an absorption maximum at 390 nm at pH 9.0 and at 425 nm at pH 6.5. The pH dependence of the absorption spectrum correlated with enzyme activity. Maximal activity measured as sulfide production was observed between pH 8.5 and 10. The activity decreased at lower pH values and was undetectable at pH 5.5. pH-dependent changes in the absorption spectrum and activity were attributed to protonation of the Schiff base formed by a lysine side chain and the PLP cofactor. Studies on substrate specificity demonstrated that cysteine derivatives other than cysteine methyl ester and cysteine-sulfinic acid could not serve as substrates for this enzyme. In particular, cystine was not a substrate for the *Synechocystis* NifS-like protein, whereas it is the best substrate for C-DES. In the presence of Fe^{2+}, cysteine, and a reductant, the NifS-like protein was able to produce holoferredoxin from apoferredoxin. The implications of two different activities for FeS center biosynthesis in *Synechocystis* are discussed.

FeS proteins are known to be involved in various cellular processes such as electron transfer and regulation. They are ubiquitous proteins present in all organisms investigated so far. Their structure, function, and spectroscopical properties have been studied in great detail. However, little is known about their biogenesis. The best studied organism with regard to the FeS center assembly is *Azotobacter vinelandii*. The inactivation of the *nifS* gene resulted in a phenotype characterized by a very low nitrogenase activity (*1*). The major breakthrough in the understanding of NifS function was the finding that the protein had a cysteine desulfurase activity (*2*). Cysteine was converted into alanine and an enzyme-bound sulfur. In the absence of a reductant, this sulfur is released as S°, whereas in the presence of DTT, sulfide is formed. Incubation of the apo-Fe protein of nitrogenase in the presence of NifS, cysteine, DTT, Fe^{2+}, and Mg-ATP resulted in an enzymatically active Fe protein (*3*). The *nifS*-deficient mutant was still able to synthesize FeS proteins, but formation of active nitrogenase was strongly inhibited. Further biochemical studies led to the isolation of an activity very similar to NifS. This protein, termed IscS, was very similar in sequence and enzyme characteristics to NifS (*4*). Since *iscS* could not be inactivated by interposon mutagenesis, it was concluded that its gene product is involved in the synthesis of FeS proteins other than nitrogenase which are essential for growth of *A. vinelandii*.

Genes that were similar to *nifS* were found not only in nitrogen-fixing organisms but also in almost all microorganisms of which the genome has been sequenced so far. In addition, a NifS-like protein was isolated from *Escherichia coli* that has FeS cluster assembly activity (*5*). Similarly, NifS-like proteins may be involved in FeS protein biosynthesis in many organisms.

The deletion of another gene, *nifU*, located upstream of *nifS* in the *nif* gene cluster from *A. vinelandii* has consequences similar to those of the *nifS* deletion, i.e., almost no FeS center incorporation into nitrogenase (*1*). The purified protein contained a 2Fe2S cluster and an Fe binding site (*6*). The role of NifU in FeS center assembly has not yet been clarified. It might supply Fe ions for the FeS cluster or be a transmitter of sulfur from NifS to the apoprotein of nitrogenase. In *A. vinelandii*, a second gene upstream of *nifS* is *iscU*, which encodes a protein which is similar to the N-terminal part of NifU. This much smaller protein does not contain a region for binding of a 2Fe2S center (*4*, *6*).

† This work was supported by the Ministry of Sciences and Research of the German federal state of Nordrhein-Westfalen (Bennigsen-Foerder Program to A.S.).

* To whom correspondence should be addressed. Telephone: 49-(0)234-322 45 49. Fax: 49-(0)234-321 43 22. E-mail: Andreas.Seidler@ruhr-uni-bochum.de.

10.1021/bi992147c CCC: $19.00
Published on Web 03/02/2000

From the cyanobacterium *Synechocystis* PCC 6714, another enzyme (C-DES)[1] was isolated and shown to be involved in the assembly of the FeS cluster of ferredoxin (*7*). It resembles NifS in that it has a PLP cofactor and mobilizes sulfur from cysteine. In contrast to NifS, C-DES produces pyruvate and ammonium instead of alanine. Another striking difference between C-DES and NifS is the fate of the sulfur. When cystine was supplied as the substrate for C-DES, cysteine persulfide, pyruvate, and ammonium were produced. The C-DES activity was not sensitive to alkylating agents such as iodine acetamide, indicating that no cysteine side chain of the enzyme was involved in the enzymatic reaction (*7*). Consistent with this finding was the fact that in the sequence of C-DES no conserved cysteine residue was found, but it is slightly but significantly similar to NifS-like proteins (*8*).

In the genome sequence of the cyanobacterium *Synechocystis* PCC 6803, there are three open reading frames (slr0387, sll0704, and slr0077) that are similar to genes encoding NifS and NifS-like proteins and one (slr2143) of which the product is similar to C-DES. However, no orf that is similar to *iscU* was found. Instead, there is an orf (ssl2667) encoding a protein that is similar to the C-terminal part of NifU from *A. vinelandii*. The function of this part of NifU is unknown. This raises the question of whether in *Synechocystis* there is a different mechanism of FeS center assembly involving C-DES, or whether *Synechocystis* (and perhaps also other cyanobacteria) has two different pathways for the synthesis of FeS centers, one involving C-DES and another involving IscS. The latter pathway would then be different from the Isc/Nif pathway in *A. vinelandii* since it would involve a different type of NifU/IscU protein. It should be noted that not all nitrogen-fixing organisms have a NifU protein similar to the one from *A. vinelandii*. For example, NifU from *Rhodobacter capsulatus* is similar in size to the protein predicted to be encoded by ssl2667 and is similar to the C-terminal part of NifU from *A. vinelandii* (*9*).

To investigate whether the three orfs (slr0387, sll0704, and slr0077) encode NifS-like proteins, we overexpressed them in *E. coli*. Here we present the overexpression of slr0387 in *E. coli* and the characterization of the purified protein. The features of this protein are indeed NifS-like. We therefore termed this protein IscS, according to the nomenclature introduced by Zheng et al. (*4*).

EXPERIMENTAL PROCEDURES

Materials. The restriction enzyme *Nde*I was purchased from New England Biolabs, and all other DNA-modifying enzymes were from Lifetechnologies. L-Alanine amino transferase and L-lactate dehydrogenase were obtained from Sigma. IPTG and ampicillin were purchased from Applichem, and all other chemicals were from Sigma at the highest purity available.

Methods. The open reading frame slr0387 was amplified from chromosomal DNA of *Synechocystis* PCC 6803 by PCR using the primers PRiscS11 (5′-GGGAATTCCATATGG-AACGGCCTCTTTACTTCG) and PRiscS12 (5′-AAGATCT-GCAGTTAACTCTGGTTAGAAGCATCG). The PCR product was cleaved with the restriction enzymes *Nde*I and *Pst*I and cloned into the *Nde*I−*Pst*I sites of the expression plasmid pRSET6a (*10*), a kind gift of R. Schöpfer, yielding the expression plasmid pISCS1. In this plasmid, transcription is controlled by the T7 promoter.

Site-directed mutagenesis was carried out by PCR. The codon for cysteine 326 was replaced with an alanine codon by amplifying a 1085 bp fragment using the primers MOiscS12 (5′-ATGGGAAGCTTCAGTGCGGTAGGAGC-TCGCGGCGGAACCGGAAGATAATGC) and PRiscS12. The PCR fragment was cleaved with *Aat*II and *Hin*dIII, and the resulting 433 bp fragment was cloned into pISCS1 which was cleaved with the same enzymes. The presence of the mutations in the expression plasmid pISCS1-C326A was verified by cleavage with *Sac*I. The recognition site for this enzyme was introduced together with the codon change. The entire *isc*S gene of one mutant clone was sequenced.

Expression of IscS and the mutant C326A was carried out in *E. coli* strain BL21(DE3) also containing the plasmid pLysS. Cells were grown at 25 °C in tryptone−phosphate medium [1.5% yeast extract, 2% tryptone, 0.8% NaCl, 0.2% Na_2HPO_4, and 0.1% KH_2PO_4 (*11*)] containing 10 μM PLP, 100 mg/L ampicillin, and 50 mg/L chloramphenicol for 18−22 h without addition of IPTG. Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C and resuspended in a buffer containing 50 mM HEPES/NaOH (pH 7.5), 10 mM EDTA, and 1 mM PMSF in a final volume of 20 mL/L of cell culture. Cells were broken by three cycles of freezing and thawing, and then 25 mM $MgCl_2$ and 0.5 mg/mL DNase were added. The cell debris was sedimented by centrifugation (20000*g* for 20 min at 4 °C). To maximize recovery of recombinant IscS, the cell debris was resuspended in 50 mM HEPES/NaOH (pH 7.5) and 10 mM EDTA in a final volume of 10 mL/L of cell culture and sedimented as described above. Small particles still present in the solution were sedimented by centrifugation at 100000*g* for 1 h at 4 °C. Removal of *E. coli* DNA was carried out by adding streptomycin sulfate to the combined supernatants (crude extract) to a final concentration of 10 mM. After incubation on ice for 1 h, the solution was cleared by centrifugation (20000*g* for 20 min at 4 °C).

For purification of the recombinant proteins, solid ammonium sulfate was added to the protein solution at 0 °C until 25% saturation (1 M) was reached. After incubation for 1 h at 0 °C and subsequent centrifugation at 20000*g* for 20 min at 4 °C, ammonium sulfate was added to the supernatant to a final concentration of 1.65 M (40% saturation). The solution was stirred for about 15 h at 0 °C and then centrifuged as described above. The yellow pellet containing IscS was dissolved in a minimal volume of 20 mM TAPS/NaOH (pH 9.0), dialyzed against the same buffer, frozen in liquid nitrogen, and stored at −70 °C until further use. Further purification was carried out by hydrophobic interaction chromatography. A protein solution containing 80 mg of protein and 250 mM ammonium sulfate was loaded onto a Butyl Sepharose column (2.6 cm × 15 cm, Pharmacia) connected to a BioCad 700E instrument (Perseptive Biosys-

[1] Abbreviations: CSD, cysteine sulfinate desulfinase; C-DES, cyst(e)ine desulfurylase; DEAE, diethylaminoethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; orf, open reading frame; PLP, pyridoxal phosphate; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; Tris, *N*-tris(hydroxymethyl)aminomethane.

tems). The column was washed with 300 mL of 20 mM TAPS/NaOH (pH 9.0) and 250 mM ammonium sulfate at a flow rate of 5 mL/min. IscS protein was eluted with 200 mL of the same buffer but lacking the ammonium sulfate. The elution was monitored at 280 and 390 nm (absorption maxima of IscS).

Reconstitution of purified IscS with PLP was carried out by mixing IscS at a protein concentration of 5 mg/mL with 0.1 mM PLP and subsequent dialysis for 5 h against 100 volumes of 20 mM TAPS/NaOH (pH 9.0) with two buffer exchanges.

The molecular mass of recombinant IscS was determined in two ways. First, for size exclusion chromatography, 0.5 mg of purified protein was loaded onto a Superose 12 column connected to a Waters HPLC system consisting of a model 515 pump and a model 996 photodiode array detector. The elution buffer was either 20 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl, 20 mM TAPS/NaOH (pH 9.0) and 100 mM NaCl, or 20 mM TAPS/NaOH (pH 9.0) and 100 mM $MgSO_4$ with or without 0.03% β-D-dodecyl maltoside. The standard proteins used for calibration were chromatographed with the same buffers. The second method that was used was nondenaturing gel electrophoresis, according to the method of Clark and Critchley (*12*). IscS (8 μg) was loaded onto a 3 to 27% nondenaturing polyacrylamide gel (18 cm $\times$ 18 cm $\times$ 0.1 cm). Electrophoresis was carried out at 20 mA for at least 16 h at 8 °C. In such a gel, a protein migrates until it is caught in the increasingly concentrated polyacrylamide gel. Therefore, the molecular mass of a protein can be estimated when marker proteins with known molecular masses were loaded onto the same gel.

Ferredoxin was isolated from *Synechocystis* PCC 6803 using a method similar to that of Bottin and Lagoutte (*13*). Solid ammonium sulfate was added to soluble extracts from *Synechocystis* to a final concentration of 2 M. After the solution was stirred at 0 °C for 1 h, the precipitated proteins were sedimented by centrifugation at 20000*g* for 20 min at 4 °C. More solid ammonium sulfate was added to a final concentration of 2.8 M. The solution was stirred for 1 h at 0 °C and centrifuged as described above. The supernatant was directly loaded onto a DEAE-Sephacel column (2.6 cm $\times$ 15 cm, Pharmacia) preequilibrated with 20 mM HEPES/NaOH (pH 7.5) containing 2.8 M ammonium sulfate. The column was washed with 160 mL of equilibration buffer and then with 160 mL of 20 mM HEPES/NaOH (pH 7.5). Ferredoxin was eluted with 30 mL of 20 mM HEPES/NaOH (pH 7.5) containing 1 M NaCl. DNase and RNase were added to the eluted ferredoxin to final concentrations of 0.1 and 0.01 mg/mL, respectively. The solution was dialyzed for 15−20 h at 8 °C against 10 mM HEPES/NaOH (pH 7.5) and 5 mM $MgCl_2$, concentrated to about 10 mg/mL, and loaded onto a size exclusion column (Sephadex G50, 3 cm $\times$ 90 cm) preequilibrated with 150 mM Tris-HCl (pH 7.6) and 200 mM NaCl. The column was connected to a Gradifrac 100 system (Pharmacia), and ferredoxin was eluted with 1 L of the same buffer at a flow rate of 0.5 mL/min. The ferredoxin that was obtained was virtually free of nucleic acid, and the ratio of the absorption maxima at 423 and 278 nm of the main fractions was $\geq$0.52.

The enzymatic activity of IscS was measured as sulfide production using cysteine as the substrate according to refs *2* and *14*. In a standard assay, 15 μg of IscS was incubated in 1 mL of 20 mM HEPES/NaOH (pH 8.0) containing 0.5 mM cysteine, 1 mM DTT, and 10 μM PLP. In some experiments, different pH values were applied using the following buffers: 20 mM MES/NaOH for pH 5.5, 6.0, or 6.5, HEPES/NaOH for pH 7.0, 7.5, or 8.0, TAPS/NaOH for pH 8.5 or 9.0, and CAPS, for pH 9.7, 10.0, 10.5, or 11.0. DTT was sometimes exchanged with other reductants such as 2-mercaptoethanol or glutathione. After a 10 min incubation at room temperature, the reaction was stopped by adding 0.1 mL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 0.1 mL of 30 mM $FeCl_3$ in 1.2 N HCl. After further incubation in the dark for 30 min, the absorption of methylene blue was measured at 650 nm.

Pyruvate was assayed photometrically by its reaction with pyruvate dehydrogenase. Alanine was quantified after deamination to pyruvate. Both procedures were carried out essentially as described in ref *2*.

N-Acetylcysteine, D-cysteine, D,L-homocysteine, and L-cysteine methyl ester were tested as substrates for IscS by incubation at a concentration of 0.5 mM with 15 μg of IscS in a buffer containing 20 mM HEPES (pH 8.0) and 1 mM DTT. After a 10 min incubation at 25 °C, the sulfide content was determined as described above. L-Cystine, L-cysteine sulfinic acid, and L-cysteic acid were tested as substrates in a different way. IscS (100 μg) was incubated in 20 mM HEPES (pH 8.0) in the presence of the L-cysteine derivative (0.1 mM), 1 mM DTT, and 10 μM PLP. After incubation for 3 h, the alanine and pyruvate content was measured as described above.

Incorporation of the 2Fe2S center into apoferredoxin was carried out using apoferredoxin from *Synechocystis* as the substrate. Apoferredoxin was obtained from holoferredoxin according to the method described in ref *15*. For the reassembly of the FeS cluster, apoferredoxin was incubated for various times at 30 °C in 0.5 mL of 50 mM Tris-HCl (pH 7.5) in the presence of 0.2 mM cysteine, 2.5 mM DTT, 2 mM $Fe(NH_4)_2(SO_4)_2$, 6 μg of IscS, and 20 nmol of apoferredoxin. In some experiments, DTT was replaced with 2-mercaptoethanol or glutathione. The FeS cluster incorporation was analyzed by nondenaturing gel electrophoresis, absorption, and EPR spectroscopy.

Absorption spectra were recorded with a Beckman DU7400 diode array spectrophotometer. EPR spectra were recorded at the Séction de Bioénergétique, CEA, Saclay, France, on a Bruker EPR200 spectrometer equipped with a helium cryostat from Oxford Instruments.

SDS gel electrophoresis was carried out as described in ref *16* and nondenaturing gel electrophoresis for analysis of FeS incorporation as described in ref *7*.

RESULTS

The open reading frame slr0387 in the genome of *Synechocystis* PCC 6803 was amplified by PCR and cloned into the expression plasmid pRSET6a. One of the clones that was obtained was sequenced, and the sequence was found to be identical with the one deposited in the cyanobase. From this plasmid, IscS was expressed in *E. coli* grown in a very rich, phosphate-buffered medium containing 10 μM PLP without addition of IPTG. Under these conditions, a protein with an apparent molecular mass of 42 kDa accumulated to about 35% of the total soluble protein. This molecular mass

Table 1: Purification of IscS Overexpressed in *E. coli*

purification step	total amount of protein (mg)	total activity[a] (nmol of H_2S min^{-1})	specific activity[a] (nmol of H_2S mg^{-1} min^{-1})	yield (%)
soluble extract[b]	562	41 026	73	100
after spectinomycin sulfate precipitation	547	32 820	60	80
supernatant after ammonium sulfate precipitation at 25% saturation	497	29 820	60	73
pellet of ammonium sulfate precipitation at 40% saturation	294	29 400	100	71
after treatment on a Butyl Sepharose column	101	23 533	233	57

[a] Activity was measured with 15 μg of protein in the presence of 0.5 mM cysteine, 1 mM DTT, and 10 μmol of PLP in 1 mL of 20 mM HEPES/NaOH (pH 8.0). [b] Soluble extract from 1 L of *E. coli* culture.

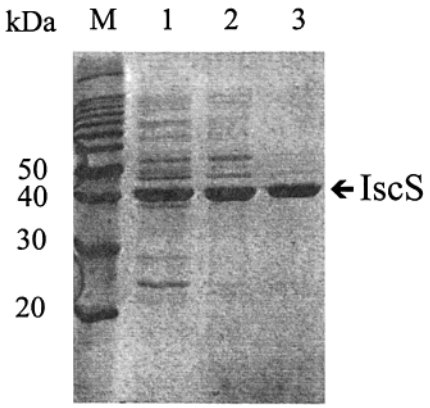

FIGURE 1: SDS gel electrophoresis of IscS at various stages of its purification: lane M, molecular mass standard; lane 1, crude extract from *E. coli* BL21(DE3) in pLysS and pISCS1; lane 2, IscS after ammonium sulfate precipitation; and lane 3, IscS after hydrophobic interaction chromatography.

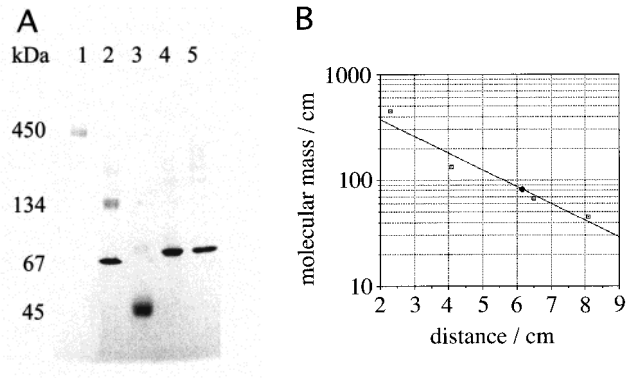

FIGURE 2: Nondenaturing gel electrophoresis on a 3 to 27% polyacrylamide gel of *Synechocystis* IscS and marker proteins (A) and a logarithmic plot of the migration distances vs molecular masses of proteins used for calibration (B): lane 1, ferritin (450 kDa); lane 2, BSA (67 kDa monomer and 134 kDa dimer); lane 3, ovalbumin (45 kDa); lane 4, IscS; and lane 5, IscS variant C236A. The arrow indicates the position of IscS.

is very similar to those of NifS and NifS-like proteins from *A. vinelandii* and *E. coli* and in good agreement with the molecular mass of 41 682 Da deduced from the DNA sequence of orf slr0387. Recombinant IscS was purified by a simple two-step procedure consisting of ammonium sulfate precipitation and hydrophobic interaction chromatography, yielding an essentially pure protein with a yield of more than 100 mg/L of *E. coli* culture. This high yield was mainly due to the high expression level and the ease of purification but also due to the unusually high cell density of the expression culture (OD_{600} = 10−13). The success of the purification was followed by measuring the increase of the cysteine desulfurase activity (Table 1) and by SDS gel electrophoresis (Figure 1).

Estimation of the molecular mass of IscS by the means of gel filtration revealed masses between 57 and 66 kDa, depending on the buffer that was used for chromatography. This is 1.4−1.6 times greater than the mass of the IscS protein derived from the gene sequence (41.7 kDa). If IscS were a monomer, the value of greater than 1.0 might be due to the fact that the protein has an unusual shape which lets it migrate faster through the column than one would expect on the basis of its molecular mass. However, if IscS is a dimer, the slower elution might be due to its hydrophobic interactions with the column material which might not be suppressed by the 0.03% β-D-dodecyl maltoside present in the elution buffer. Therefore, another method, nondenaturing polyacrylamide gel electrophoresis, was used for determination of the molecular mass of the protein. On a nondenaturing 3 to 27% polyacrylamide gel, IscS and the marker proteins migrated until they were trapped by the increasing density of the polyacrylamide network. Assuming that the proteins have approximately the same shape, the molecular mass of IscS can be estimated. With this method, a molecular mass of 82 kDa was obtained (Figure 2A,B). This mass is consistent with IscS being a dimer.

Cells, cell extracts, and the purified IscS were yellow. The absorption spectrum of the purified IscS protein exhibited maxima typical for PLP-containing proteins and very similar to those of NifS and NifS-like proteins from *A. vinelandii* and *E. coli* (Figure 3, spectrum at pH 7.5). In crude extracts, the cysteine desulfurase activity of IscS was found to be almost independent of exogenous PLP. However, after ammonium sulfate precipitation, the activity was stimulated by about 30% when 10 μM PLP was added to the assay buffer. This indicated the loss of the PLP cofactor in a fraction of the enzyme preparation, most likely due to the high ammonium concentration.

The pH optimum was between pH 8.5 and 10.0 (Figure 4). When the pH was lowered, the half-maximum activity was reached at about pH 7.2. At pH 5.5, the protein was virtually inactive. The decrease in activity at pH values lower than 8.5 was accompanied by a change in the absorption spectrum (Figure 3). At pH 9.0, the PLP cofactor gave rise to a relatively sharp absorption maximum at 380 nm. When the pH was lowered, the absorption at this wavelength decreased and a second, broader absorption maximum appeared at about 425 nm. This shift in the absorption maximum was fully reversible down to a pH value of 6.5. When the pH was increased to 9.0, activity as well as the absorption properties were fully restored. However, at pH

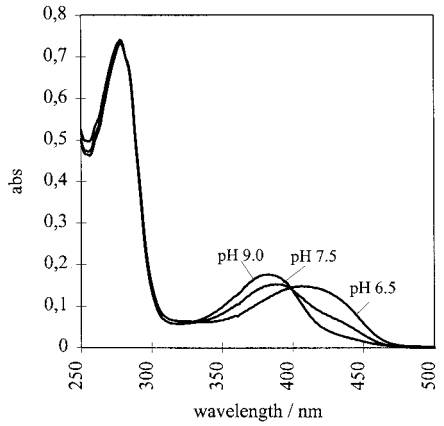

FIGURE 3: Absorption spectra of IscS at different pH values. Purified IscS (5 mg/mL) reconstituted with PLP in 10 mM TAPS/NaOH (pH 9.0) was diluted 5-fold into 20 mM MES/NaOH (pH 6.5), 20 mM HEPES/NaOH (pH 7.5), or 20 mM TAPS/NaOH (pH 9.0).

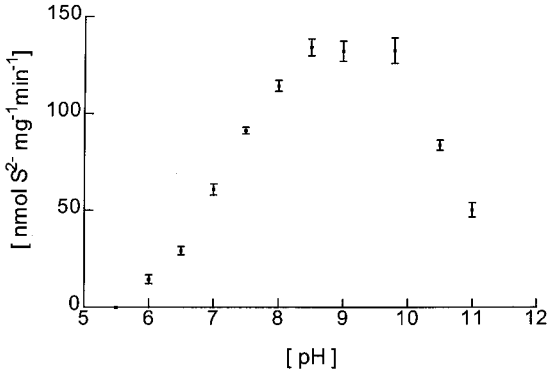

FIGURE 4: pH dependence of the *Synechocystis* IscS cysteine desulfurase activity. Cysteine desulfuration was carried out in 20 mM buffer as described in Experimental Procedures containing 0.5 mM cysteine, 1 mM DTT, and 6 μg of IscS. Each experiment was carried out three times in duplicate.

Table 2: Quantitation of IscS Reaction Products[a]

addition	nmol of sulfide/mL	nmol of alanine/mL	nmol of pyruvate/mL
1 mM DTT	97.0	90.3	0.0
none	0.0	68.0	0.0

[a] The reaction was carried out in 2 mL of 20 mM HEPES/NaOH (pH 8.0) containing 10 μmol of PLP, 0.1 mM cysteine, and 100 μg of *Synechocystis* IscS; DTT was added when indicated, and incubation was carried out for 2.5 h at 30 °C.

$\leq$6.0, the protein started to precipitate irreversibly with a concomitant loss of activity. The decrease in activity with the lowering of the pH is proportional to the decrease in absorption at 380 nm. We therefore concluded that the reason for the lower activity at pH values below 8.5 was due to the inactivation of a fraction of the enzyme rather than due to a slowing of the enzyme turnover.

During purification, the activity of IscS was measured as sulfide production. NifS from *A. vinelandii* produced alanine (*17*), but C-DES from *Synechocystis* PCC 6714 produced pyruvate and ammonium (*7*). However, as shown in Table 2, IscS produced in the presence of DTT almost 1 mol of alanine per mole of sulfide, but no pyruvate was detected. In the absence of DTT, S° is produced instead of sulfide as can be seen by the increasing turbidity of the sample. However, also under these conditions, alanine is produced.

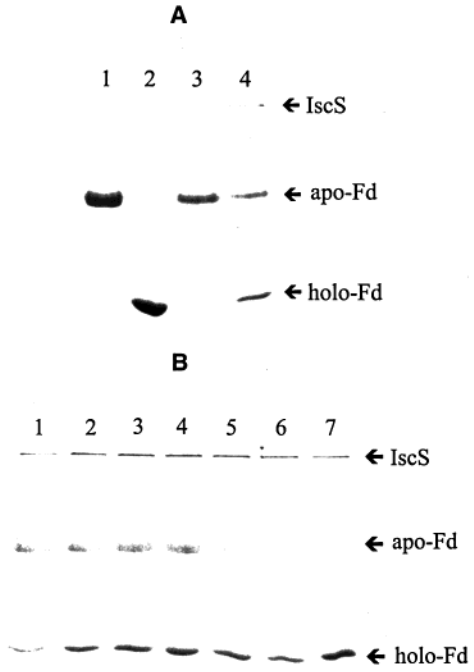

FIGURE 5: Nondenaturing gel electrophoresis of ferredoxin. (A) Lane 1, apoferredoxin; lane 2, holoferredoxin; lane 3, apoferredoxin incubated for 30 min at 30 °C in the reconstitution mixture [0.2 mM cysteine, 2.5 mM DTT, 2 mM $Fe(NH_4)_2(SO_4)_2$, and 10 μM PLP in 20 mM HEPES/NaOH (pH 7.5)] without IscS; and lane 4, same as lane 3 but in the presence of IscS. (B) Lanes 1−7, apoferredoxin incubated for 5, 10, 20, 30, 60, 120, and 180 min, respectively, at 30 °C in the presence of 0.2 mM cysteine, 2.5 mM DTT, 2 mM $Fe(NH_4)_2(SO_4)_2$, 10 μM PLP, and 6 μg of IscS in 20 mM HEPES/NaOH (pH 7.5).

The reason for the production of only 68% alanine might be due to an incomplete reaction of cysteine or due to the occurrence of some side reactions.

To study the substrate specificity of IscS, a variety of cysteine derivatives were incubated with the enzyme and the sulfide production was determined. It turned out that the enzyme is highly specific for cysteine. No reaction was observed with *N*-acetylcysteine, D-cysteine, D,L-homocysteine, or L-cysteic acid. Some residual activity was observed with L-cysteine methyl ester or L-cysteinesulfinic acid.

The ability of IscS to incorporate FeS centers into apo-FeS proteins was investigated by using ferredoxin as a model protein. Ferredoxin was isolated from *Synechocystis* PCC 6803, and the FeS center was quantitatively removed as described in Experimental Procedures. Apoferredoxin was then incubated with cysteine, Fe^{2+}, DTT, PLP, and IscS at pH 7.5 under anaerobic conditions. Different times after the addition of IscS samples were taken and analyzed on nondenaturing polyacrylamide gels. On such gels, holoferredoxin shows a mobility remarkably different from that of apoferredoxin. This therefore provides a very sensitive method for analyzing the degree of FeS center assembly. As shown in Figure 5B, with increasing times of incubation the amount of holoferredoxin increased. After about 3 h, the transformation of apoferredoxin into holoferredoxin was almost complete. However, when no IscS was added, there was still some holoferredoxin formation observed (Figure 5A, lane 3), although the extent was much lower than in the presence of IscS. A similar phenomenon was already observed by Flint (*5*) in the FeS center incorporation into dihydroxy acid dehydratase by an *E. coli* NifS-like protein.

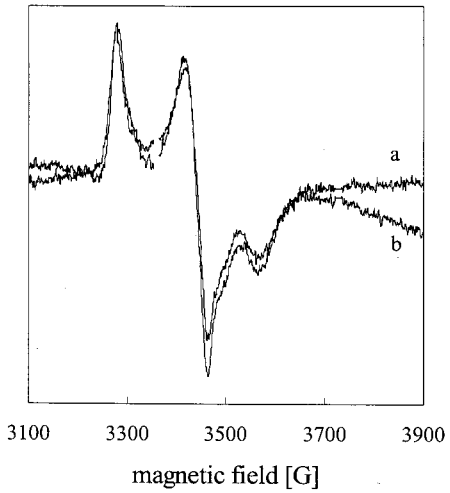

FIGURE 6: Difference EPR spectra of ferredoxin. The spectra were recorded with ferredoxin in its oxidized form, and subsequently, 10 mM sodium dithionite was added to reduce the iron−sulfur cluster. The recorded spectrum of the reduced sample was subtracted from the spectrum of the oxidized ferredoxin (a) as isolated from *Synechocystis* and (b) after acid denaturation and reincorporation of the FeS cluster in the presence of IscS. Instrument settings for both spectra were as follows: temperature, 15 K; microwave frequency, 9.44 GHz; microwave power, 6.3 mW; and modulation amplitude, 10 G.

This nonenzymatic FeS cluster assembly depends on the presence of a reductant but was not specific for DTT (data not shown).

To verify the correct insertion of the FeS center into apoferredoxin, EPR spectroscopy was used. As shown in Figure 6, the ferredoxin isolated from *Synechocystis* PCC 6803 and the in vitro assembled ferredoxin exhibited essentially identical spectra. This again demonstrated the successful FeS center synthesis by IscS.

The mechanism of the cysteine desulfurase activity of IscS was further studied by site-directed mutagenesis. Cysteine 326 is conserved in many proteins that are similar to NifS and was shown to be crucial for the activity of NifS from *A. vinelandii*. In NifS, this cysteine side chain is thought to bind the sulfur mobilized from free cysteine. In IscS from *Synechocystis*, we replaced this residue with alanine. The variant was overexpressed and purified as the wild-type protein. Consistent with the finding for *A. vinelandii*, NifS no activity was observed.

Flint (*5*) has shown that the reaction of NifS-like proteins with cysteine can be followed by absorption spectroscopy. Incubation of *Synechocystis* IscS with cysteine led to similar absorption changes, as observed for the *E. coli* enzyme (Figure 7A). After incubation for 1 min, the absorption at 380 nm had decreased and a new peak at 360 nm with a shoulder at 400 nm appeared. The spectrum remained similar for about 10 min. Then the absorbance increased drastically. About 1 h after addition of cysteine, no further changes were observed. The increased absorbance is in fact due to the increased turbidity of the sample originating from the elementary sulfur produced by disproportionation of IscS persulfide. When the sample was centrifuged 2 h after addition of cysteine, the absorption was essentially the same as after incubation for 10 min (data not shown). Incubation of the variant C326A with cysteine led to spectral changes in the first 10 min of incubation that were identical to those

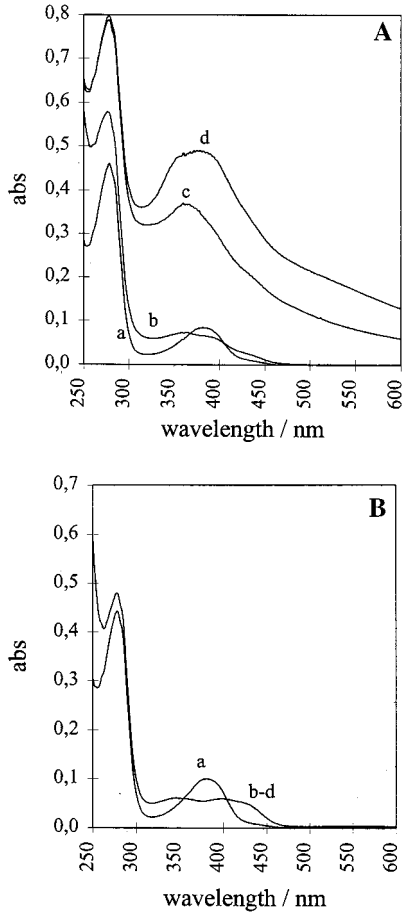

FIGURE 7: Absorption spectra of IscS (A) and IscS C326A (B) at different times after addition of cysteine. Cysteine (0.5 mM) was added to 1 mL of a solution of *Synechocystis* IscS (1 mg/mL) in 20 mM TAPS/NaOH (pH 9.0). Spectra a were recorded before and spectra b−d 10, 30, and 60 min after addition of cysteine.

of the wild-type protein (Figure 7B). However, no later spectral changes occurred. Apparently, the variant C326A can still bind cysteine via the PLP cofactor, but the desulfurase reaction is blocked due to the mutation as no S° is formed.

DISCUSSION

There are numerous sequences in the GenBank database that are similar to NifS. Almost all of the genomes of microorganisms that have been sequenced completely contain several such open reading frames, indicating that the NifS pathway of FeS center incorporation might be used by many organisms. However, in the case of NifS-like proteins, sequence alignments might be misleading because there are proteins that are similar to NifS but which are unlikely to be involved in FeS center synthesis. An example of such a protein is CSD (cysteine sulfinate desulfinase) from *E. coli* (*18*) (see below).

The NifS-like protein from *Synechocystis* PCC 6803 which was studied here was shown to be almost twice as active in the desulfurization of cysteine as NifS from *A. vinelandii* (*2*) and the NifS-like protein isolated by Flint (*5*) when comparable assay conditions (pH 8.0, 0.5 mM cysteine, and 1 mM DTT) were used. In addition, we found that the activity was strongly dependent on the pH. The decrease in activity at lower pH values was accompanied by a change in the

absorption spectrum of the enzyme. It seems that lowering the pH resulted in protonation of a group with a p*K* value of about 7.2 at or in proximity to the PLP cofactor which led to a conformational change and inactivation of the enzyme. The conformation of the protonated enzyme seemed to be rather unstable. When the majority of the protein became protonated, it precipitated irreversibly with a concomitant loss of activity. The nature of the protonated group is likely to be the Schiff base formed by a lysine side chain and the PLP cofactor. Similar spectral changes due to the pH have been observed in other PLP-dependent enzymes such as aspartate amino transferase (*19*) and in model compounds (*20*). For the model compounds and therefore also for other PLP-dependent proteins such as aspartate amino transferase, this phenomenon was attributed to the protonation of the Schiff base itself (*20*). Aspartate amino transferase differs from IscS in that the p*K* of the Schiff base is about 1 unit lower. It seems that for both enzymes the Schiff base must not be protonated to bind their substrate. Indeed, in the case of IscS, the absorption at 425 nm did not decrease upon addition of cysteine at pH 6.5 (data not shown) but the absorption at 390 nm decreased as was observed when IscS was incubated with cysteine at pH 9.0 (Figure 7A).

In contrast to those of IscS and NifS, the absorption maximum of the PLP cofactor in *E. coli* CSD at pH 7.4, where the enzyme is quite active, is about 420 nm, whereas the absorption maximum of active IscS (or NifS) is around 390 nm. If this absorption maximum is indicating a protonated Schiff base, then it is possible that CSD is active even when the Schiff base is protonated.

A puzzling observation was that *E. coli* CSD contains the conserved cysteine residue which in NifS binds the sulfur from free cysteine. However, site-directed mutagenesis showed that this residue is not involved in the cysteine desulfurase reaction and neither is any other cysteine residue (*18*). To investigate the role of cysteine 326 of *Synechocystis* IscS, which corresponds to cysteine 325 in NifS from *A. vinelandii*, in the cysteine desulfurase activity we exchanged this residue for alanine. The protein variant turned out to be completely inactive, although it is still able to bind cysteine as indicated by the change in the absorption spectrum (Figure 7B). We therefore concluded that this cysteine side chain is involved in the reaction mechanism by binding the sulfur atom from free cysteine as is the case for NifS.

The sulfur mobilized from free cysteine was transferred in the presence of Fe^{2+} into apoferredoxin. This reaction was strongly dependent on the presence of a reductant such as DTT. The function of the reductant in our in vitro experiments might be the transfer of sulfur from the IscS persulfide to apoferredoxin. DTT can be replaced by the more physiological reductant glutathione. The physiological concentration of reduced glutathione in *Synechocystis* PCC 6714 was shown to be 9 mM (*7*). However, in vitro at least 20 mM glutathione was required for maximal holoferredoxin formation by IscS, whereas at the physiological concentration, only a few FeS clusters are formed (data not shown). This means either that glutathione is not the natural sulfur transmitter from IscS to the apo-FeS protein or, if glutathione were to be the natural sulfur transmitter, that there is sufficient IscS present in the cell for FeS center assembly which would compensate for the lower activity. Possible proteins for sulfur transfer are NifU-like proteines and thioredoxins. The involvement of such proteins will be investigated in the future.

Another role of the reductant could be the release of sulfur from IscS persulfide as H_2S. However, this is unlikely in vivo since sulfide is quite toxic for the cell and therefore the intracellular sulfide concentration is quite low. In vitro, the reaction of IscS persulfide with a reductant might form sulfide in relatively small quantities, but it is less likely that this sulfide is incorporated into FeS centers. It was shown for at least three proteins, SoxR (*21*), FNR (*22*), and the Rieske FeS protein (D. Schneider, B. Gubernator, K. Jaschkowitz, A. Szcepaniak, and A. Seidler, unpublished results), that for in vitro reconstitution of FeS clusters the cysteine/NifS or IscS system is more efficient than the addition of sulfide in millimolar concentrations.

Our data presented here show clearly that *Synechocystis* PCC 6803 contains at least one NifS-like protein which we termed IscS. Another NifS-like protein encoded by orf sll0704, which also exhibits cysteine desulfurase activity (K. Jaschkowitz and A. Seidler, unpublished results), is able to incorporate the two 4Fe4S centers into photosystem I subunit PsaC (M. L. Antonkine and J. H. Golbeck, personal communication). Therefore, *Synechocystis* might contain three (or more) FeS center assembly pathways, two involving NifS-like proteins and one involving C-DES. Further studies are required to determine whether each of these enzymes is specifically involved in the assembly of one or more FeS proteins or whether all of them are involved in the maturation of the same FeS proteins. Conceivably, the three pathways may also function under different *Synechocystis* growth conditions. The preference of C-DES for cystine over cysteine as a substrate might indicate its function in a more oxidized environment, i.e., in the dark when no photosynthetic electron transport takes place.

ACKNOWLEDGMENT

We thank U. Kokelj for excellent technical assistance and C. König for the soluble extracts of *Synechocystis* PCC 6803. Special thanks to A. W. Rutherford and A. Boussac for the giving A.S. the opportunity to use the EPR spectrometer at CEA, Saclay, France. D. Kessler is gratefully acknowledged for helpful discussions and W. Vermaas for critical reading of the manuscript. We are also indepted to M. L. Antonkine and J. H. Golbeck for sharing data prior to publication.

REFERENCES

1. Jacobson, M. R., Cash, V. L., Weiss, M. C., Laird, N. F., Newton, W. E., and Dean, D. R. (1989) *Mol. Gen. Genet. 219*, 49−57.
2. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2754−2758.
3. Zheng, L., and Dean, D. R. (1994) *J. Biol. Chem. 269*, 18723−18726.
4. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) *J. Biol. Chem. 273*, 13264−13272.
5. Flint, D. H. (1996) *J. Biol. Chem. 271*, 1608−1674.
6. Fu, W., Jack, R. F., Morgan, T. V., Dean, D. R., and Johnson, M. K. (1994) *Biochemistry 33*, 13455−13463.
7. Leibrecht, I., and Kessler, D. (1997) *J. Biol. Chem. 272*, 10442−10447.
8. Lang, T., and Kessler, D. (1999) *J. Biol. Chem. 274*, 189−195.

9. Masepohl, B., Angermüller, S., Hennecke, S., Hübner, P., Moreno-Vivian, C., and Klipp, W. (1993) *Mol. Gen. Gentet. 238*, 369−382.
10. Schöpfer, R. (1993) *Gene 124*, 83−85.
11. Moore, J. T., Uppal, A., Maley, F., and Maley, G. F. (1993) *Protein Expression Purif. 4*, 160−163.
12. Clarke, A. K., and Critchley, C. (1992) *Plant Physiol. 100*, 2081−2089.
13. Bottin, H., and Lagoutte, B. (1992) *Biochim. Biophys. Acta 1101*, 48−56.
14. Siegel, L. M. (1965) *Anal. Biochem. 11*, 126−132.
15. Meyer, J., Moulis, J., and Lutz, M. (1986) *Biochim. Biophys. Acta 871*, 243−249.
16. Seidler, A. (1994) *Biochim. Biophys. Acta 1187*, 73−79.
17. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) *Biochemistry 33*, 4714−4720.
18. Mihara, H., Kurihara, T., Yoshimura, T., Soda, K., and Esaki, N. (1997) *J. Biol. Chem. 272*, 22417−22424.
19. Jenkins, W. T., and Sizer, I. W. (1960) *J. Biol. Chem. 235*, 620−624.
20. Johnson, R. J., and Metzler, D. E. (1970) in *Methods in Enzymology*, pp 433−471, Academic, San Diego.
21. Hidalgo, E., and Demple, B. (1996) *J. Biol. Chem. 271*, 7269−7272.
22. Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J., and Guest, J. R. (1996) *Biochem. J. 316*, 887−892.

BI992147C