Gene Cloning, Purification, and Characterization of Two Cyanobacterial NifS Homologs Driving Iron-Sulfur Cluster Formation

Shin-ichiro Katô,§ Hisaaki Mihara,§ Tatsuo Kurihara, Tohru Yoshimura, and Nobuyoshi Esaki†

Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611-0011, Japan

Received May 24, 2000; Accepted July 10, 2000

Iron-sulfur proteins are essential in the photosynthetic system and many other biological processes. We have isolated and characterized enzymes driving the formation of iron-sulfur clusters from Synechocystis sp. PCC6803. Two genes (sir0387 and sir0704), showing similarity to nifS of Azotobacter vinelandii, were cloned, and their gene products (SsCsd1 and SsCsd2) were purified. They catalyzed the desulfuration of L-cysteine. Reconstitution of a [2Fe-2S] cluster of cyanobacterial ferredoxin proceeded much faster in the presence of L-cysteine and either of these enzymes than when using sodium sulfide. These results suggest that SsCsd1 and SsCsd2 facilitate the iron-sulfur cluster assembly by producing inorganic sulfide from L-cysteine. Synechocystis sp. PCC6803 has no gene coding for a protein with similarity to the N-terminal domain of NifU of A. vinelandii, which is believed to cooperate with NifS to assemble iron-sulfur clusters. Thus, the cluster formation in the cyanobacterium probably proceeds through a mechanism that is different from that in A. vinelandii.

Key words: iron-sulfur cluster; cysteine desulfurase; NifS; cyanobacterium

Cyanobacteria are Gram-negative bacteria with the ability to perform oxygenic photosynthesis. They have much simpler cellular and genomic structures than plants, and have been regarded as a good model for the study of a photosynthetic system. The system comprises photosystems I and II, and the former contains three [4Fe-4S] clusters named FX, FA, and FB. FB provides soluble [2Fe-2S] ferredoxin with electrons, and electrons are further transferred to NADP⁺ to produce NADPH. Iron-sulfur proteins are thus essential in the photosynthetic system. However, it remains largely unknown how iron-sulfur clusters are synthesized in photosynthetic organisms.

Cysteine desulfurase, originally identified in a nitrogen-fixing bacterium, Azotobacter vinelandii, and named NifS, is essential for the production of a holo-form of nitrogenase in vivo. The gene coding for NifS (nifS) is in the nif gene cluster, which contains genes encoding nitrogenase and other proteins involved in nitrogen fixation. NifS was shown to facilitate in vitro reconstitution of iron-sulfur clusters of nitrogenase from A. vinelandii as well as dihydroxy-acid dehydratase, SoxR, and FNR from Escherichia coli. The enzyme catalyzes the degradation of L-cysteine to produce l-alanine and elemental sulfur, which is used for the iron-sulfur cluster assembly. IscS, another enzyme from A. vinelandii, has a sequence similar to NifS, catalyzes the same reaction as NifS, and is involved in the iron-sulfur cluster formation. It is believed that, in vivo, IscS plays a general role in iron-sulfur cluster assembly, whereas NifS is specialized in the maturation of nitrogenase. Genes with sequence similarity to nifS and iscS have been found in a variety of organisms, suggesting that NifS homologs play a role in the iron-sulfur cluster formation in a wide range of organisms.

nifU in the nif gene cluster is another essential gene for the full activation of nitrogenase. It was proposed recently that NifU from A. vinelandii cooperates with NifS in the maturation of iron-sulfur proteins by providing an intermediate iron-sulfur assembly site: NifU and NifS interact with each other, and NifS catalyzes the formation of a transient iron-sulfur cluster on the N-terminal domain of NifU, from which iron and/or sulfur may be transferred to the apo-form of iron-sulfur proteins for their maturation. Proteins with sequence similarity to the N-terminal domain of NifU have been found in many organisms including E. coli, Haemophilus influenzae, and Saccharomyces cerevisiae. Thus these NifU homologs are believed to be crucial for the iron-sul-

† To whom correspondence should be addressed. Phone: +81-774-38-3240; Fax: +81-774-38-3248; E-mail: esaki@scl.kyoto-u.ac.jp
§ These authors contributed equally to the results of this work.

Abbreviations: DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; PLP, pyridoxal 5'-phosphate; C-DES, L-cysteine/C-S-lyase, NifS; cysteine desulfurase
fur cluster formation in general. A. vinelandii has a paralogous gene of nifU, named iscU, which is in a gene cluster containing iscS, suggesting that IscS and IscU are cooperating with each other.

Sequence analysis of the entire genome of a cyanobacterium, Synechocystis sp. PCC6803, has been completed, and revealed the presence of three nifS homologs (srl0387, sl0704, and slr0077), the first two of which show a particularly high similarity to nifS and iscS from A. vinelandii. Surprisingly, however, Synechocystis sp. PCC6803 has no gene coding for a protein with similarity to the N-terminal domain of NifU. In the this study, we cloned two nifS homologs, srl0387 and sl0704, and purified and characterized their gene products, named Sscsd1 and Sscsd2, respectively. We found that these proteins effectively reconstitute a [2Fe-2S] cluster of cyanobacterial ferredoxin. Recently, Jaschkwitz and Seidler also characterized Sscsd1, which they termed IscS, and obtained results comparable with ours. Our findings, together with theirs, suggest that the NifS system is operating in iron-sulfur cluster assembly in Synechocystis sp., and that the cluster formation in this cyanobacterium proceeds through a mechanism different from that in other organisms involving a NifU homolog.

Materials and Methods

Materials. Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA); oligonucleotides from Biologica (Nagoya, Japan); Spirulina sp. ferredoxin and ferredoxin-NADP reductase from Sigma (St. Louis, MO); Superoxide 12 (1 × 30 cm) and molecular weight markers for SDS-PAGE from Amersham Pharmacia Biotech (Uppsala, Sweden); Shodex QA-825 from Showa Denko (Tokyo, Japan); DEAE-Toyopearl, Phenyl-Toyopearl and Butyl-Toyopearl from Tosoh (Tokyo, Japan). L-Selenocysteine was synthesized as described previously. L-Selenocysteine was prepared from L-selenocysteine according to the previous method. Molecular weight markers for gel filtration were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of analytical grade from Nacalai Tesque (Kyoto, Japan).

Construction of expression plasmids. DNA fragments containing srl0387 and sl0704 encoding Sscsd1 and Sscsd2, respectively, were cloned from the chromosomal DNA of Synechocystis sp. PCC6803 by PCR as follows. Each reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.4 mM each dNTP, 0.2 μM each primer (5'-GAATTCAGGAAACGCATATGGAACGGGCACATGGAACTTACGGG-3' and 5'-CGGGATCCGACATGGAAAGGAAACCTTACGGG-3') for srl0387 or 5'-GGAAATTCCAGGAAACGCATATGGAACGGGCACATGGAACTTACGGG-3' and 5'-CGGGATCCGACATGGAAAGGAAACCTTACGGG-3' for sl0704; italic letters indicate EcoRI and BamHI sites, and boldface letters indicate a putative ribosome binding sequence). 2.5 units of LA Taq DNA polymerase (Takara Shuzo), and 100 ng of the chromosomal DNA from Synechocystis sp. PCC6803 as a template. The amplified DNA fragments were digested with EcoRI and BamHI, and then they were ligated into pUC118 digested with the same enzymes to produce plasmids pUSSP1 and pUSSP2 for expression of srl0387 and sl0704, respectively. Plasmids pSSP1 and pSSP2 were constructed by subcloning the cloned DNA fragments of pUSSP1 and pUSSP2, respectively, into pKK223-3 at the EcoRI and PstI sites. The expression of the genes was confirmed by measuring the activity of their products using L-selenocysteine as a substrate as described below and by SDS-PAGE.

Purification of Sscsd1. All operations were performed at 4°C unless otherwise mentioned, and 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA was used as a standard buffer throughout the purification. E. coli JM109 cells harboring pSSP1 were grown in 4.5 liters of 2 × YT medium [1.6% polypeptone, 1% yeast extract, and 0.5% NaCl (pH 7.0)] containing 200 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 12 hours. The cells were harvested by centrifugation, suspended in the standard buffer, and disrupted by sonication. The cell debris was removed by centrifugation, and the supernatant was fractionated on ice by ammonium sulfate precipitation. Sscsd1 was precipitated between 20% and 50% ammonium sulfate saturation. The precipitate was dissolved in the standard buffer and dialyzed against the standard buffer containing 0.65 mM ammonium sulfate. The enzyme was applied to a Phenyl-Toyopearl column (3.0 × 15 cm) equilibrated with the standard buffer containing 0.65 mM ammonium sulfate. The elution was performed with a 1-liter linear gradient of ammonium sulfate (0.65-0 M) in the standard buffer. The active fractions were collected, concentrated by ultrafiltration, and dialyzed against the standard buffer. The enzyme solution was applied to a DEAE-Toyopearl column (3.0 × 12 cm) equilibrated with the standard buffer. After the column was washed with the same buffer (400 ml), the enzyme was eluted with a 1-liter linear gradient of NaCl (0-0.3 M) in the same buffer. The active fractions were concentrated by ultrafiltration and dialyzed against the standard buffer containing 0.65 mM ammonium sulfate. Then the enzyme was applied to a Butyl-Toyopearl column (3.0 × 10 cm) equilibrated with the standard buffer containing 0.65 mM ammonium sulfate. The enzyme was eluted with a 1-liter linear gradient of ammoni-
um sulfate concentration (0.65–0 m) in the standard buffer. The active fractions were collected, concentrated by ultrafiltration, and dialyzed against the standard buffer.

**Purification of SsCsdl.** *E. coli* JM101 cells harboring pKSSP2 were grown in 4.5 liters of 2 X YT medium containing 200 μg/ml ampicillin and 1 mM IPTG at 37°C for 12 hours. The crude extract was prepared as described for the purification of SsCsdl1. The enzyme was fractionated on ice by ammonium sulfate precipitation. SsCsdl2 was precipitated between 15% and 40% ammonium sulfate saturation, and the precipitate was collected by centrifugation. The precipitate was resuspended in the standard buffer and dialyzed against the same buffer. The enzyme solution was applied to a DEAE-Toyopearl column (3.0 x 15 cm) equilibrated with the standard buffer. The enzyme was eluted with the same buffer (400 ml), and the active fractions were concentrated by ultrafiltration and dialyzed against the standard buffer containing 0.65 mM ammonium sulfate. The enzyme solution was applied to a Phenyl-Toyopearl column (3.0 x 12 cm) equilibrated with the standard buffer containing 0.65 mM ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate (0.65–0 m) in the standard buffer. The active fractions were collected, concentrated by ultrafiltration, and dialyzed against the standard buffer. The enzyme was applied to a Superose 12 column (1.0 x 30 cm) with an FPLC system equilibrated with the standard buffer containing 0.15 M NaCl. The enzyme was eluted with the same buffer, and the active fractions were concentrated and dialyzed against the standard buffer.

**Assays.** The standard reaction mixture contained 50 mM dithiothreitol (DTT), 20 μM pyridoxal 5'-phosphate (PLP), 0.12 mM Tricine-NaOH buffer (pH 7.5), enzyme, and a substrate amino acid (10 mM) and was incubated at 37°C. The activity of SsCsdl1 and SsCsdl2 toward various amino acids was examined by measuring the production of alanine with a Beckman amino acid analyzer 7300 (Beckman Coulter, Fullerton, CA). The enzymatic activity toward L-selenocysteine was measured by the production of selenium with lead acetate as described previously.13 DTT was omitted in the assay of desulfination of L-cysteine sulfenic acid, and the sulfite produced was determined with fuchsin.14 Specific activity was expressed as units/mg of protein, with one unit of enzyme defined as the amount that catalyzed the formation of 1 μmol of the product in one minute.

**Other analytical methods.** Protein was measured by the Bradford method15 using Protein Assay CBB Solution (Nacalai Tesque) with bovine serum albumin as a standard. Spectrophotometric measurements were performed with a Shimadzu UV260 (Shimidzu, Kyoto, Japan) spectrophotometer. Terminal amino acid sequences were determined using a Shimadzu PPSQ-10 protein sequencer. The nucleotide sequences were identified with Applied Biosystems 370A DNA sequencer.

**Preparation of apoferredoxin and reconstitution of iron-sulfur cluster.** Apoferredoxin was prepared as described by Meyer et al.10 Spirulina sp. ferredoxin (0.2 mg in 5 ml of 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl) was kept in a nitrogen-flushed stoppered flask on ice. HCl was added to a final concentration of 0.5 M, and the reaction was allowed to proceed with continuous stirring under a nitrogen flow for 60 min. The slurry was centrifuged, and the white pellet was quickly rinsed with deionized water and dissolved in 200 μl of 50 mM Tris-HCl (pH 8.0). This manipulation was repeated once, and ferredoxin was shown to be in an apo-form by the absence of absorption at 332, 337, 415, and 459 nm.

The iron-sulfur cluster synthesis experiment was performed in a reaction mixture containing 20 μg apoferredoxin, 50 mM Tricine-NaOH (pH 7.5), 5 mM DTT, 1 mM L-cysteine, 1 mM ammonium iron (II) sulfate, 20 μM PLP, and 1 μg of SsCsdl1 or SsCsdl2 in 100 μl. Non-enzymatic synthesis of iron-sulfur cluster of ferredoxin was done in a reaction mixture in which 1 mM Na2S was substituted for 1 mM L-cysteine and the enzymes. The reaction mixture was incubated at 37°C, 90 μl of the mixture was applied to an anion-exchange column (Shodex IEC QA-825) equilibrated with 50 mM Tris-HCl (pH 7.5), and the column was washed with the same buffer for 10 min. The protein was eluted with a 20-min linear gradient from 0 M to 1 M NaCl at a flow rate of 1 ml/min using an HPLC system. The elution of holoferredoxin was monitored by measuring the absorbance at 410 nm, and then the total amount was measured. The holoferredoxin form was eluted at around 30 min.

**Results and Discussion**

**Expression of slr0387 and slr0704**

Three genes (slr0387, slr0704, and slr0077) are registered as *nifS* homologs in "Cyanobase", the genome database for *Synechocystis* sp. strain PCC6803 (http://www.kazusa.or.jp/cyano/). However, the protein encoded by slr0077 (SsCsdl3) is more distantly related to NifS and IscS from *A. vinelandii* than the proteins encoded by slr0387 and slr0704 (SsCsdl1 and SsCsdl2, respectively): Sequence similarities of SsCsdl1, SsCsdl2, and SsCsdl3 with *NifS* are 37.2%, 34.8%, and 20.1%, respectively, and those with *IscS* are 43.7%, 32.7%, and 19.8%, respectively. According to the classification by Mihara et al.,12 SsCsdl1 and SsCsdl2 belong to Group I of *NifS* family pro-
teins, which also includes NifS and IscS from *A. vinelandii*, and Sscd3 belongs to Group II of NifS family proteins. Thus we decided to characterize Sscd1 and Sscd2 in this study.

The nucleotide sequences of slr0387 and slr0704 cloned into the expression vectors were identical with those registered in GenBank (accession numbers: D63999 and D90899, respectively). Since bacterial NifS homologs examined so far decompose L-selenocysteine as a substrate to yield L-alanine and selenide derived from elemental selenium in the presence of DTT, we examined the expression of the gene by measuring the selenocysteine lyase activities. The crude extracts of *E. coli* clones, JM109/ pUSSP1 and JM109/pUSSP2, had selenocysteine lyase activity (0.25 units/mg and 0.05 units/mg, respectively). To improve expression levels, pKSSP1 and pKSSP2 were constructed by subcloning the EcoRI-PstI fragments from pUSSP1 and pUSSP2, respectively, into pKK223-3. Selenocysteine lyase activities of JM109/pKSSP1 and JM109/pKSSP2 were 0.6 units/mg and 0.23 units/mg, respectively, indicating that the expression levels in these clones increased to 2.4 times and 4.6 times those in JM109/pUSSP1 and JM109/pUSSP2. SDS-PAGE analysis showed accumulation of a soluble protein with a molecular weight of about 43,000 in the cells carrying pKSSP1 or pKSSP2, whereas the proteins were not produced in the cells containing pKK223-3. The amounts of Sscd1 and Sscd2 corresponded to about 3–4% of the total soluble proteins in the cell extract as judged from the specific activity in the crude extract.

**Physical characterization of Sscd1 and Sscd2**

Purifications of Sscd1 and Sscd2 are summarized in Table 1 and Table 2, respectively. Sscd1 and Sscd2 were purified to homogeneity as judged by SDS-PAGE. The N-terminal sequences of the purified Sscd1 and Sscd2, MERPLYFDNH and MKIYLDYSAT, respectively, agreed with those deduced from the nucleotide sequences of slr0387 and slr0704. The *M*<sub>c</sub> of Sscd1 and Sscd2 were about 42,000 by SDS-PAGE, which agreed with that calculated from the deduced amino acid sequences (41,682 for Sscd1 and 42,485 for Sscd2). The *M*<sub>c</sub> of native Sscd1 and Sscd2 were about 87,000 and 88,000, respectively, by gel filtration using an FPLC system with a Superose 12 column. These results indicate that both are composed of two identical subunits. Three NifS homologs of *E. coli* also consist of two identical subunits. This is one of the common properties among the NifS homologs. The absorption spectrum of Sscd1 and Sscd2 showed a peak at 420 nm in 10 mM KPB (pH 7.4). When the enzymes were incubated with 0.1 mM sodium borohydride, the absorption peak at 420 nm disappeared with a concomitant increase in the absorption peak at 380 nm. These results are consistent with both enzymes containing PLP as a cofactor.

**Catalytic Properties**

As described above, Sscd1 and Sscd2 had the selenocysteine lyase activity. In addition, Sscd1 and Sscd2 acted on L-cysteine sulfinic acid, L-cysteine, L-selenocysteine, and L-cysteine to form L-alanine. Both enzymes decomposed one mol of L-cysteine sulfinic acid to yield one mol of sulfite and one mol of alanine (data not shown). The substrate specificities of Sscd1 and Sscd2 are summarized in Table 3. Sscd1 showed the maximum activity around at pH 7.0 with L-selenocysteine and at pH 7.7 with L-cysteine sulfinic acid. Sscd2 showed maximum activity at around pH 7.0 with both L-selenocysteine and L-cysteine sulfinic acid.

### Table 1. Purification of Sscd1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1800</td>
<td>1100</td>
<td>0.61</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>800</td>
<td>890</td>
<td>1.1</td>
<td>1.8</td>
<td>81</td>
</tr>
<tr>
<td>Phenyl-Toyopearl</td>
<td>250</td>
<td>760</td>
<td>3.0</td>
<td>4.8</td>
<td>69</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>46</td>
<td>640</td>
<td>14</td>
<td>18</td>
<td>58</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>24</td>
<td>500</td>
<td>21</td>
<td>34</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity was measured with L-selenocysteine as a substrate.

### Table 2. Purification of Sscd2

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1500</td>
<td>340</td>
<td>0.23</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>800</td>
<td>280</td>
<td>0.35</td>
<td>1.5</td>
<td>82</td>
</tr>
<tr>
<td>Phenyl-Toyopearl</td>
<td>53</td>
<td>170</td>
<td>3.2</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>Superoxide 12</td>
<td>20</td>
<td>92</td>
<td>4.6</td>
<td>20</td>
<td>38</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity was measured with L-selenocysteine as a substrate.

### Table 3. Substrate Specificity of Sscd1 and Sscd2

<table>
<thead>
<tr>
<th>Substrate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sscd1 Specific&lt;sup&gt;c&lt;/sup&gt; activity</th>
<th>Sscd2 Specific&lt;sup&gt;c&lt;/sup&gt; activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-selenocysteine</td>
<td>units/mg</td>
<td>units/mg</td>
</tr>
<tr>
<td>L-cysteine sulfinic acid</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>4.5</td>
<td>21</td>
</tr>
<tr>
<td>S-selenocysteine</td>
<td>0.92</td>
<td>4.4</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0.0039</td>
<td>0.019</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.0035</td>
<td>0.017</td>
</tr>
</tbody>
</table>

<sup>b</sup> Specific activity was measured by the production of alanine in the presence of 10 mM each substrate in 0.12 M Tricine-NaOH buffer (pH 7.5).

<sup>c</sup> The degradation of the following compounds were not detectable under these conditions: D-cysteine, D-cystine, D,L-methionine, D,L-homocysteine, D,L-homocystine, D,L-cysteine acid, D,L-djenkolic acid, D,L-thialanine, S-methyl-L-cysteine, S-benzyl-L-cysteine, D,L-serine, D,L-aspartic acid, D,L-glutamic acid, D,L-asparagine, D,L-gluatamine, and β-cyano-D,L-alanine.
and L-cysteine sulfinic acid. Jaschkowitz and Seidler reported that maximum activity of SsCsd1 toward L-cysteine was obtained at around pH 9.0. The higher optimum pH of SsCsd1 toward L-cysteine than toward L-selenocysteine and L-cysteine sulfinic acid is probably due to a mechanistic difference between the L-cysteine desulfurase reaction and the decomposition of two other substrates, as described for the reaction mechanism of E. coli NifS homologs. Desulfuration of L-cysteine requires a cysteine residue at the active site of the enzyme, but decomposition of L-selenocysteine and L-cysteine sulfinic acid do not.

Kinetic parameters of the enzymes are shown in Table 4. The parameters for L-cysteine were not measured because both enzymes were inhibited by high concentrations of L-cysteine (Fig. 1). This inhibition was not observed when L-selenocysteine and L-cysteine sulfinic acid were used as substrates. The \( k_{cat}/K_m \) for L-selenocysteine and L-cysteine sulfinic acid indicate that SsCsd1 and SsCsd2 prefer L-selenocysteine and L-cysteine sulfinic acid, respectively, as a substrate. Jaschkowitz and Seidler reported that SsCsd1 is highly specific for L-cysteine and shows only residual activity with L-cysteine sulfinic acid. The observation is apparently different from ours (Table 3 and 4). This inconsistency is likely to arise from a difference in the substrate concentration in the assay mixtures used: Our assay mixture contained 10 mM cysteine sulfinic acid, but Jaschkowitz and Seidler's mixture contained only 0.1 mM substrate, which is far below the \( K_m \) (17 mM).

The specific activity of SsCsd1 for L-selenocysteine (21 units/mg) is much higher than that of NifS homologs from E. coli (CSD, 6.2 units/mg; CsdB, 5.5 units/mg; IscS, 3.1 units/mg) and comparable to that of mouse selenocysteine lyase (29 units/mg), which specifically catalyzes the decomposition of L-selenocysteine to produce elemental selenium and L-alanine and is proposed to participate in the selenoprotein synthesis. However, as far as we know, no selenoprotein containing selenocysteine residues has been reported for Synechocystis sp. PCC6803. By homology-search analysis, we found no gene similar to E. coli genes involved in selenoprotein biosynthesis, i.e. selA, selB, selC, and selD. Accordingly, it is unlikely that the selenocysteine lyase activity of SsCsd1 and SsCsd2 has physiological relevance to selenoprotein synthesis in Synechocystis sp. PCC6803.

Since E. coli NifS homologs are activated by pyruvate, the effects of pyruvate on SsCsd1 and SsCsd2 were examined. The specific activities of SsCsd1 and SsCsd2 with L-selenocysteine (5 mM) were increased 2.0-fold and 6.1-fold, respectively, in the presence of 5 mM pyruvate. The activation was also observed toward L-cysteine sulfinic acid as a substrate: the specific activities of SsCsd1 and SsCsd2 were increased 1.8-fold and 5.7-fold, respectively. The kinetic parameters measured in the presence of pyruvate (1 mM) in the reaction mixture show that both of the \( k_{cat} \) and \( K_m \) values were increased by the addition of pyruvate (Table 4). The activation by pyruvate was not observed when L-cysteine was used as a substrate. Similar observations were reported for E. coli NifS homologs. Pyruvate increased the enzyme activity toward L-selenocysteine and L-cysteine sulfinic acid, but not toward L-cysteine. These results suggest that the enzymatic degradation of L-selenocysteine and L-cysteine sulfinic acid and that of L-cysteine are mechanistically different from each other.

We found that DTT significantly increased the activities of SsCsd1 and SsCsd2 toward L-cysteine: the activity was about 13 times higher in the presence of 50 mM DTT than in the absence of DTT. DTT is probably used for the turnover of the enzyme: An active-site cysteine residue of the enzyme probably at-

**Table 4. Kinetic Parameters of SsCsd1 and SsCsd2 for L-Selenocysteine and L-Cysteine Sulfinic Acid**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SsCsd1</th>
<th>SsCsd2</th>
<th>( k_{cat}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{cat} )</td>
<td>( K_m )</td>
<td>( k_{cat}/K_m )</td>
</tr>
<tr>
<td>L-selenocysteine</td>
<td>15</td>
<td>0.43</td>
<td>35 ( \cdot 10^{-3} )</td>
</tr>
<tr>
<td>L-cysteine sulfinic</td>
<td>3.2</td>
<td>17</td>
<td>0.19 ( \cdot 10^{-2} )</td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td></td>
<td>3.4 ( \cdot 10^{-2} )</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>28</td>
<td>0.70</td>
<td>10 ( \cdot 10^{-1} )</td>
</tr>
<tr>
<td>lyase</td>
<td>25</td>
<td>0.67</td>
<td>10 ( \cdot 10^{-1} )</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of L-Cysteine Concentration on the Rate of Desulfuration of L-Cysteine Catalyzed by SsCsd1 and SsCsd2. Assays were performed at 37°C in 50 mM Tricine-NaOH (pH 7.5), 10 mM DTT, 20 μM PLP, and SsCsd1 (●) or SsCsd2 (○). Activity was measured by the production of alamine with a Beckman amino acid analyzer 7300.
tacks the sulfur atom of the substrate cysteine resulting in the formation of a cysteine persulfide residue, and the sulfane sulfur on this residue is supposed to be removed by nucleophilic displacement by DTT for the next round of the catalytic reaction. Jaschkowitz and Seidler also observed that DTT increases the activity of SsCsd1.9) However, the effect of DTT they observed was much less significant than that observed in our experiment: The enzyme activity in the absence of DTT was 68% of that in the presence of DTT.9) This apparent inconsistency is most likely due to the difference between our assay system and their assay system.9) Two of the major differences are as follows: 1) We measured the initial velocity of the enzyme reaction, but they measured the amount of the product accumulated after a 2.5-h reaction. 2) We used 10 mM cysteine as a substrate, but they used 0.1 mM cysteine. In their assay system, the enzyme reaction in the presence of DTT might have reached a plateau, making the difference between the apparent activities in the presence and absence of DTT smaller.

Iron-sulfur cluster assembly

We examined whether SsCsd1 and SsCsd2 function as iron-sulfur cluster-forming proteins. An HPLC assay was used to measure the amount of [2Fe-2S]ferredoxin according to the method of Nakamura et al.21) The measurement of ferredoxin was possible in the range of 0.5−100 μg. Figure 2 shows the course of the enzymatic formation of [2Fe-2S]ferredoxin by SsCsd1 and SsCsd2. Replacement of sulfide (as Na2S) with L-cysteine and either of the enzymes in the in vitro reconstitution system increased the rate of [2Fe-2S]ferredoxin formation. SsCsd1 showed a higher rate of [2Fe-2S]ferredoxin formation than SsCsd2 when the same amount of SsCsd1 and SsCsd2 were used. The higher rate of the cluster formation with SsCsd1 is correlated to the higher specific activity of SsCsd1 for the cysteine desulfuration: The activity of SsCsd1 toward L-cysteine is 2.8−times higher than that of SsCsd2.

Recently, L-cysteine/cystine C-S-lyase (C-DES) from a cyanobacterium, Synecochystis sp. PCC6714, was shown to direct the [2Fe-2S] cluster formation of ferredoxin in vitro.22,23) It catalyzes β-elimination of L-cysteine to produce pyruvate, ammonia, and sulfide, which is incorporated into ferredoxin to form the [2Fe-2S] cluster. The enzyme acts on L-cysteine as an alternative and better substrate to degrade it to pyruvate, ammonia, and L-cysteine persulfide, which is also believed to be a supplier of inorganic sulfur for iron-sulfur clusters. Thus cyanobacteria have two different systems for iron-sulfur cluster assembly: The cysteine desulfurase system and the C-DES system. This view is supported by the recent observations by Jaschkowitz and Seidler.24)

Fig. 2. Reconstitution of Apoferrodoxin with SsCsd1, SsCsd2, and Na2S.

The reaction mixture contained 10 μg apoferrodoxin, 50 mM Tricine-NaOH (pH 7.5), 5 mM DTT, 1 mM L-cysteine, 1 mM ammonium iron (II) sulfate, 20 μM PLP, and 1 μg of SsCsd1 (●) or SsCsd2 (▲) in 100 μl. For reconstitution with Na2S (●, the enzyme and L-cysteine in the reaction mixture were replaced by 1 mM Na2S. The reconstitution mixture not containing the enzyme, L-cysteine, and Na2S was used for a negative control experiment (×).

Characteristics of the Synechocystis genome relevant to the iron-sulfur cluster assembly

NifU of A. vinelandii has an N-terminal region in which a labile iron-sulfur cluster is assembled transiently and a C-terminal region that binds an iron-sulfur cluster tightly.26) NifS interacts with NifU and directs the formation of a transient [2Fe-2S] cluster within the N-terminal region of NifU. The transient [2Fe-2S] cluster is thought to be delivered to the target apo-form of iron-sulfur protein for its maturation.27) IsCU of A. vinelandii has a sequence similarity with NifU, and has been proposed to cooperate with IscS in the iron-sulfur cluster assembly.28) When compared with NifU, IscU is considerably truncated, bearing sequence similarity only to the N-terminal third of NifU, which corresponds to the region containing the transient iron-sulfur cluster site. Genes coding for IscU homologs have been identified in various organisms including both eukaryotes (S. cerevisiae, Caenorhabditis elegans, etc.) and prokaryotes (E. coli, Bacillus subtilis, etc.), suggesting that IscU homologs play a role in the iron-sulfur cluster assembly in these organisms.

Synechocystis sp. PCC6803 has a gene encoding a NifU homolog (ssl2667). However, the NifU homolog of Synechocystis sp. PCC6803 does not have sequence similarity with IscU. The cyanobacterial gene product is much shorter than NifU, corresponding only to the C-terminal quarter of NifU, which lacks the region for the transient iron-sulfur cluster assem-
bly. Thus, the NiFS-driven iron-sulfur cluster assembly in the cyanobacterium probably proceeds through a mechanism different from that in *A. vinelandii* and other organisms involving NiFUSiscU homologs. Among all the 28 organisms whose entire genome sequence has been identified (http://pedant.mips.biochem.mpg.de/), we found that one bacterium (*Borrelia burgdorferi*), one protozoan (*Plasmodium falciparum*), and four archaea (*Methanococcus thermoautotrophicum*, *Pyrococcus horikoshii* OT3, *Aeropyrum pernix* K1, and *Pyrococcus abyssi*) have a nifS/iscc homolog and yet lack an iscU homolog.

*iscS* of *A. vinelandii* is in the *iscSUAsiscBA-fdx* gene cluster, which encodes IscS and IscU as well as other proteins with sequence similarity to DnaJ, Dnak, and ferredoxin. These proteins are proposed to cooperate with each other for the maturation of iron-sulfur proteins. The gene organization is conserved in *A. vinelandii*, *E. coli*, and *H. influenzae*. Recently, Nakamura *et al.* demonstrated that the co-expression of the gene cluster facilitates the assembly of iron-sulfur clusters of several kinds of ferredoxins overproduced in *E. coli*. In contrast to these organisms, no such gene organization is seen around slr0387 and sll0704 in the genome of *Synechocystis* sp. PCC6803. This also suggests that the mechanism of the iron-sulfur cluster assembly in the *Synechocystis* is different from that in *A. vinelandii* and other organisms such as *E. coli* and *H. influenzae*. Although we could not find any gene around slr0387 or sll0704 that implies the physiological role of SsCsd1 and SsCsd2, these enzymes are supposed to be responsible for maturation of different subsets of iron-sulfur proteins as proposed for IscS and NiFS of *A. vinelandii*.

In conclusion, we have revealed the enzymological properties of two NiFS homologs from *Synechocystis* sp. PCC6803 and demonstrated that both enzymes promote reconstitution of the [2Fe-2S] cluster in the ferredoxin. Our results suggest that SsCsd1 and SsCsd2, in addition to C-DES, are involved in the iron-sulfur cluster assembly in the cyanobacterium. Further investigations are required to find whether and how different kinds of iron-sulfur proteins are allotted among these enzymes.

**Acknowledgments**

We are grateful to Prof. Y. Sawa and Dr. H. Ashida of Shimane University for providing the genomic DNA from *Synechocystis* sp. PCC6803 and Dr. Y. Takahashi of Osaka University for helpful advice on measurement of holoferrredoxin by HPLC.

**References**


13) Milhara, H., Kurihara, T., Yoshimura, T., Soda, K., and Esaki, N., Cysteine sulfinate desulfinase, a NiFS-like protein of *Escherichia coli* with selenocysteine lyase and cysteine desulfurase activities: Gene cloning, purification, and characterization of a novel
Properties of Two NifS Homologs from Synechocystis


