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Characterization of Slr0077 of *Synechocystis* sp. PCC6803, a homolog of chloroplastic cysteine desulfurase of higher plants

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Summary

Plant cells have two different mechanisms for the assembly of iron-sulfur clusters: the mitochondrial mechanism and the chloroplastic mechanism, which is less well characterized. Cysteine desulfurase catalyzes the desulfurization of L-cysteine and is supposed to deliver the sulfur atom for the synthesis of iron-sulfur clusters in both organelles. However, it remains unclear what proteins cooperate with cysteine desulfurase for cluster assembly in chloroplasts. The protein encoded by *slr0077* of *Synechocystis* sp. PCC6803, named SsCsd3, shows a high sequence similarity to chloroplastic cysteine desulfurase from *Arabidopsis thaliana* (AtCpNifS) (60% identity). Thus, the mechanism for cluster assembly involving SsCsd3 can be regarded as a model of the mechanism operating in chloroplasts. In this study, SsCsd3 was overproduced, purified, and characterized. SsCsd3 acted not only on L-cysteine but also on L-selenocysteine, although the physiological significance of its activity toward L-selenocysteine is unknown. The specific activity of purified SsCsd3 toward 10 mM L-selenocysteine (5.4 units/mg) was comparable to that of AtCpNifS (3.7 units/mg), and the activities of these enzymes toward L-selenocysteine were much higher (over 100 times) than those toward L-cysteine. Thus, SsCsd3 is similar to AtCpNifS not only in its primary structure but also in its catalytic properties. The iron-sulfur cluster of ferredoxin was reconstituted *in vitro* by using SsCsd3 as the sulfur delivery protein.

Introduction

Iron-sulfur clusters play essential physiological roles not only in electron transfer, such as that in the photosynthetic pathway, but also in metabolic reactions and gene regulation[1, 2]. Inorganic sulfur atoms of iron-sulfur clusters derive from L-cysteine. Cysteine desulfurase, a pyridoxal-5'-phosphate (PLP)-dependent enzyme, catalyzes the desulfurization of L-cysteine to produce L-alanine, and the sulfur atom released from L-cysteine is incorporated into iron-sulfur clusters with the aid of other proteins that mediate sulfur transfer[3]. Cysteine desulfurase has a catalytic cysteine residue that attacks the sulfur atom of L-cysteine, and the sulfur atom of L-cysteine is transferred onto the Sγ-atom of this residue[3]. The sulfur atom bound to cysteine desulfurase is then transferred to another protein before it is incorporated into iron-sulfur clusters[3].

In eukaryotes, cysteine desulfurase occurs in mitochondria, which are regarded as the major sites for iron-sulfur cluster assembly[4]. Precursors of iron-sulfur clusters of cytosolic iron-sulfur proteins are supposed to be produced in mitochondria and transported to cytosol[4]. However, in plant cells, iron-sulfur clusters are produced not only in mitochondria but also in chloroplasts. Assembly of the iron-sulfur cluster of ferredoxin was shown to occur in isolated chloroplasts[5], indicating that the cluster assembly of this protein does not require mitochondrial cysteine desul-

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furase. Recently, chloroplastic cysteine desulfurase (AtCpNifS) was identified in *Arabidopsis thaliana*[6, 7]. Cysteine desulfurases can be classified into two groups, I and II, according to their sequence similarities[8]. Mitochondrial cysteine desulfurase belongs to Group I, whereas chloroplastic cysteine desulfurase belongs to Group II.

As the chloroplast ancestor is believed to be phylogenetically related to cyanobacteria, it can be postulated that a conserved mechanism of iron-sulfur cluster synthesis operates in cyanobacteria and chloroplasts. Sequence analysis of the entire genome of a cyanobacterium, *Synechocystis* sp. PCC6803, has revealed the presence of three genes coding for cysteine desulfurases: *slr0387*, *sll0704*, and *slr0077* (CyanoBase [http://www.kazusa.or.jp/cyano/]). The *slr0387* and *sll0704* gene products, named SsCsd1 and SsCsd2, respectively, are Group I cysteine desulfurases[9], whereas the *slr0077* gene product, named SsCsd3, belongs to Group II, showing a high sequence similarity to AtCpNifS (60% of the amino acid residues were identical). Studies of SsCsd3 would help to clarify the mechanism of iron-sulfur cluster assembly in chloroplasts, which is distinct from that in mitochondria. In this study, SsCsd3 was overproduced, purified, and characterized.

Experimental procedures

Materials

Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan); KOD plus DNA polymerase from Toyobo (Tokyo, Japan); oligonucleotides from Genset Oligos (Kyoto, Japan); molecular weight markers for gel filtration from Oriental Yeast (Tokyo, Japan); molecular weight markers for SDS-PAGE (LMW calibration kit) and Superose 12 (1 × 30 cm) from Amersham Pharmacia Biotech (Uppsala, Sweden); and Butyl-Toyopearl 650M and DEAE-Toyopearl 650M from Tosoh (Tokyo, Japan). L-Selenocystine was synthesized as described previously[10]. L-Selenocysteine was prepared from L-selenocystine according to the previously described method[10]. The genomic DNA from *Synechocystis* sp. PCC6803 was a kind gift from Dr. H. Ashida and Prof. Y. Sawa (Department of Applied Biochemistry, Faculty of Agriculture, Shimane University, Japan). All other chemicals, of analytical grade, were from Nacalai Tesque (Kyoto, Japan). *Escherichia coli* JM109 was used as a host strain for plasmid construction. Plasmid pHCE19T(I) was obtained from the BioLeaders Corporation (Daejon, Korea).

Cloning and overexpression of the slr0077 gene

The DNA fragment containing *slr0077* was cloned from the chromosomal DNA of *Synechocystis* sp. PCC6803 by PCR. The reaction mixture contained 1 × KOD plus buffer, 1 mM $MgSO_4$, 0.2 mM of each dNTP, 0.3 μM of each primer (5'-cgcggatcc*gccatgg*ttgctctccaaattcc-3' and 5'-aaaa*ctgcag*ttattaaacggtaaagtcgtcatca-3': italic letters indicate *Nco*I and *Pst*I sites), 1.0 unit KOD plus DNA polymerase, and 3.2 μg of the chromosomal DNA from *Synechocystis* sp. PCC6803 as a template. The program used for the PCR was as follows: denaturation at 94℃ for 1 min; 30 cycles consisting of denaturation at 94℃ for 20 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 1.5 min. The amplified DNA fragment was digested with *Nco*I and *Pst*I and then ligated into pHCE19T(I) digested with the same enzymes to produce pHCSD3. Constitutive expression of the cloned gene in *E. coli* is achieved by the promoter included in the vector. Overexpression of the gene was verified by SDS-PAGE and by determining the activity of the crude extract of the recombinant *E. coli* cells using L-selenocysteine as a substrate.

Enzyme assays

The activity of SsCsd3 toward L-selenocysteine was determined as follows. The reaction mixture contained 0.12 M Tricine-NaOH buffer (pH 7.5), various concentrations of L-selenocysteine, 50 mM DTT, 20 μM PLP, and SsCsd3 in 150 μl. The reaction was carried out at 37℃. The selenide produced was quantified with lead acetate as described previously[10]. The molecular turbidity coefficient of PbSe at 400 nm is $1.18 \times 10^4\ M^{-1} \cdot cm^{-1}$.

The activity of SsCsd3 toward L-cysteine was determined by measuring sulfide released from L-cysteine by the method of Siegel[11] as follows. Reactions were carried out at 37℃ in a solution (200 μl) containing 0.5 μM SsCsd3, various concentrations of L-cysteine, 20 μM PLP, and 50 mM Tris-HCl, pH 7.5. Reactions were initiated by the addition of L-cysteine and were terminated by adding 25 μl of 20 mM *N,N*-dimethyl-*p*-phenylenediamine in 7.2 M HCl and 25 μl of 30 mM ferric chloride in 1.2 M HCl. The amount of methylene blue produced from sulfide and *N,N*-dimethyl-*p*-phenylenediamine was determined by measuring the absorbance at 670 nm.

The enzyme activities of SsCsd1, SsCsd2, and AtCpNifS toward L-selenocysteine and L-cysteine were determined by measuring L-alanine produced with a Beckman high-performance amino acid analyzer 7300 (Beckman Coulter, Fullerton, CA).

Specific activity was expressed as unit/mg of protein, with one unit of the enzyme defined as the amount that catalyzed the formation of 1 μmol of the product in 1 minute.

Purification of the slr0077 gene product (SsCsd3)

E. coli JM109 carrying pHCSD3 was cultured aerobically at 37℃ for 11 h in Luria-Bertani broth (3 liters) supplemented with ampicillin (200 μg/ml). All the following operations were done at 4℃, and 20 mM potassium phosphate buffer (pH 7.4) was used as the standard buffer. The cells were harvested by centrifugation, suspended in 60 ml of the standard buffer, and disrupted by sonication. The cell debris was removed by centrifugation, and the supernatant was used as the crude extract.

Ammonium sulfate was added to the crude extract to a final concentration of 20% saturation. The precipitates were removed by centrifugation, and the supernatant was dialyzed against the standard buffer containing 0.5 M ammonium sulfate.

The enzyme solution was applied to a Butyl-Toyopearl 650M column (3 × 18 cm) equilibrated with the standard buffer containing 0.5 M ammonium sulfate. The elution was performed with a 600 ml linear gradient of ammonium sulfate (0.5 - 0 M) in the standard buffer. The active fractions were collected and dialyzed against the standard buffer.

The enzyme solution was applied to a DEAE-Toyopearl 650M column (3 × 18 cm) equilibrated with the standard buffer. The enzyme was eluted with a 600 ml linear gradient of NaCl (0 - 0.5 M) in the standard buffer. The active fractions were collected, dialyzed, and applied to a DEAE-Toyopearl 650M column (3 × 18 cm) once again. The elution was carried out with a 600 ml linear gradient of NaCl (0 - 0.25 M) in the standard buffer. The active fractions were collected, concentrated by ultrafiltration with YM-10 (Millipore), and then dialyzed against the standard buffer.

Determination of molecular weight

The molecular weight of the subunit of the enzyme was determined by SDS-PAGE. The molecular weight of the native enzyme was determined by gel filtration with a Superose 12 column. The buffer used for chromatography was a 20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Analysis of cofactor

Absorption spectra of the enzyme (0.4 mg/ml) were measured in 20 mM potassium phosphate buffer (pH 7.4) before and after the reduction with 1 mM sodium borohydride.

Cloning of the ssl0020 gene coding for ferredoxin (Fdx) and construction of the expression plasmid

A DNA fragment containing *ssl0020* was cloned from the chromosomal DNA of *Synechocystis* sp. PCC6803 by PCR as follows. The reaction mixture contained 1 × KOD plus buffer, 1 mM $MgSO_4$, 0.2 mM of each dNTP, 0.3 μM of each primer (5'-cat*gccatgg*catcctataccgttaa-3' and 5'-cc*gctcgag*gtagaggtcttcttctttgtg-3': italic letters indicate *Nco*I and *Xho*I sites), 1.0 unit KOD plus DNA polymerase, and 3.2 μg of the chromosomal DNA from *Synechocystis* sp.

PCC6803 as a template. The program used for the PCR was as follows: denaturation at 94℃ for 2 min; 30 cycles consisting of denaturation at 94℃ for 15 sec, annealing at 55℃ for 30 sec, and extension at 68℃ for 30 sec. The amplified DNA fragment was ligated into pET-21d(+), and the plasmid constructed was named pE21-FDX. pE21-FDX codes for Fdx with a His_6-tag at its C-terminus.

Purification of Fdx

E. coli BL21 (DE3) pLysS cells harboring pE21-FDX were grown in LB medium containing 100 μg/ml ampicillin at 23℃. After 11.5 h cultivation, the production of Fdx was induced by the addition of 0.3 mM IPTG. The cells were harvested from an 800 ml culture by centrifugation after 12 h induction. All of the following operations were performed at 4℃. The cells were suspended in 20 ml of the binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and disrupted by sonication. The cell debris was removed by centrifugation, and the supernatant was used as the crude extract.

A His·Bind Resin column (1.5 × 2.0 cm) was charged with 12.5 ml of 50 mM $NiSO_4$ and equilibrated with 7.5 ml of the binding buffer. The crude extract was applied to the column. After the column was washed with 25 ml of the binding buffer, 50 ml of wash buffer-1 (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), and 15 ml of wash buffer-2 (100 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), Fdx was eluted with 15 ml of the elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fractions containing Fdx were collected and concentrated by ultrafiltration, and imidazole and NaCl were removed with Sephadex G-25M.

Reconstitution of the iron-sulfur cluster of Fdx with SsCsd3

The cluster synthesis experiments were performed as follows. The reaction mixture contained 20 μg Fdx, 50 mM Tricine-NaOH (pH 7.5), 5 mM DTT, 1 mM L-cysteine, 2 mM $Fe(NH_4)_2(SO_4)_2$, 20 μM PLP, and 1 μg SsCsd3 in 100 μl. The reaction mixture was incubated at 37℃ for 10 min, and 90 μl of the mixture was applied to an anion-exchange column (Shodex IEC QA-825, Showa Denko, Tokyo, Japan) equilibrated with 50 mM Tris-HCl (pH 7.5). The column was washed with the same buffer for 10 min. The protein was eluted with a 20-min linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 1 ml/min using an HPLC system. The elution of holo-Fdx was monitored by measuring the absorbance at 410 nm, and the amount of holo-Fdx was calculated from the peak area. The elution was also monitored with a Waters 990J photodiode array detector (Millipore, Bedford, MA).

Results and discussion

Cloning and expression of the slr0077 gene

The DNA fragment containing the *slr0077* gene from *Synechocystis* sp. PCC6803 was obtained by PCR, and an expression plasmid was constructed by introducing the DNA fragment into pHCE19T(I) as described in "Experimental procedures". The nucleotide sequence of the *slr0077* gene in the expression vector, named pHCSD3, was shown to be identical to the sequence registered in the database (CyanoBase [http://www.kazusa.or.jp/cyano/]). The specific activity of the extract of the recombinant *E. coli* JM109 cells harboring pHCSD3 was 0.24 units/mg of protein when measured with 2.5 mM L-selenocysteine as the substrate. SDS-PAGE showed the overproduction of a soluble protein with a molecular weight of about 43,000 in the cells carrying pHCSD3, whereas the protein was not produced in the control cells containing pHCE19T(I), indicating the overexpression of the *slr0077* gene (Fig. 1, lane 1). The molecular weight of the overproduced protein estimated by SDS-PAGE was in good agreement with the value calculated from the deduced amino acid sequence of the protein encoded by *slr0077* (46,416).

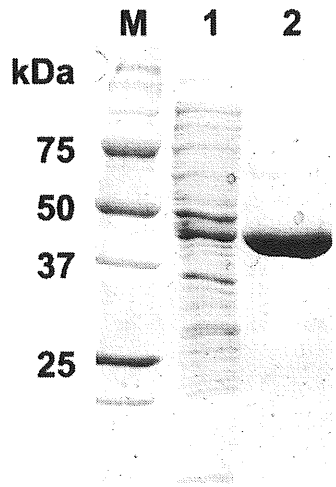

Fig. 1 Purification of SsCsd3. M, molecular weight markers; lane 1, crude extract; lane 2, purified SsCsd3.

Purification of the slr0077 gene product (SsCsd3)

SsCsd3 produced by the recombinant *E. coli* JM109 cells was purified as described in "Experimental procedures." The enzyme was purified about 15-fold with a yield of 40 % (Table 1). The preparation was shown to be homogeneous by SDS-PAGE (Fig. 1, lane 2). The N-terminal sequence of the purified SsCsd3, MVALQIPSLAAT, agrees with that deduced from the nucleotide sequence of *slr0077*. The amount of SsCsd3 produced corresponded to about 6.7% of the total soluble proteins in the cell extract, judging from the specific activity of the crude extract.

Table 1 Purification of SsCsd3

Step	Total Protein	Total Activity[a]	Specific Activity	Purification	Yield
	mg	units	units/mg	-fold	%
Crude extract	490	118	0.24	1	100
Ammonium sulfate	74	104	1.4	5.8	88
Butyl-Toyopearl	60	96	1.6	6.7	81
1st DEAE-Toyopearl	28	73	2.6	11	62
2nd DEAE-Toyopearl	13	47	3.6	15	40

[a] Determined with 2.5 mM L-selenocysteine as a substrate.

Cofactor

At pH 7.4, the enzyme showed an absorption maximum at 425 nm, which is characteristic of bound PLP. Reduction with sodium borohydride resulted in the disappearance of the absorption peak at 425 nm with a concomitant increase in the absorbance at 335 nm. This result shows that the enzyme was purified in the PLP-bound form.

Determination of molecular weight

The molecular weight of the purified SsCsd3 was determined to be about 43,000 by SDS-PAGE, which is in good agreement with that calculated from the deduced amino acid sequence (46,416). The molecular weight of native SsCsd3 was determined to be about 110,000 by gel filtration. The results suggest that SsCsd3 is either a dimer or a trimer. Considering the three-dimensional structures of CsdB[12] and IscS[13] (cysteine desulfurases from *E. coli*) and the fact that the cysteine desulfurases characterized so far are composed of two identical subunits[3], SsCsd3 is likely to be a dimer.

Catalytic properties

The activity of the enzyme toward L-selenocysteine was measured by quantifying the selenide ion produced with lead acetate as described in "Experimental procedures". The specific activity of SsCsd3 toward 10 mM L-selenocysteine was 5.4 units/mg. This value is comparable to that of AtCpNifS (3.7 units/mg)[6]. The specific activities of SsCsd3 and other cysteine desulfurases toward L-selenocysteine and L-cysteine were compared (Table 2). SsCsd3 utilized both substrates. The activity toward L-selenocysteine was much higher than that toward L-cysteine. The discrimination factor was calculated from the specific activity of the enzymes for L-selenocysteine divided by that for L-cysteine. The discrimination factor of SsCsd3 was 491, which is more comparable to that of AtCpNifS (264-fold) (Group II enzyme) than that of other cysteine desulfurases from *Synechocystis* sp. PCC6803, SsCsd1 (23-fold) and SsCsd2 (19-fold) (Group I enzymes). In this respect, SsCsd3 is more closely related to AtCpNifS than to SsCsd1 and SsCsd2. However, SsCsd3 is different from selenocysteine lyases from mammals and *Citrobacter freundii* in its substrate specificity: the latter enzymes are more specific toward L-selenocysteine and showed negligibly small activity toward L-cysteine[10, 14, 15]. Physiological significance of the activity of SsCsd3 and AtCpNifS toward L-selenocysteine is not clear at present, because selenium requirement has not been shown for cyanobacteria and plants so far.

The *K*m and *V*max values of SsCsd3 for L-selenocysteine were 1.7 mM and 6.5 units/mg, respectively. The kinetic parameters for L-cysteine were not determined because of the anomalous kinetic behavior probably because of the substrate inhibition (Fig. 2). If the initial velocities measured at the substrate concentrations of 0.2-5 mM were used, the *K*m value was calculated to be 0.78 mM. Similar anomalous kinetic behavior was observed for other cysteine desulfurases, SsCsd1 and SsCsd2[9].

Table 2 Substrate specificity of cysteine desulfurases

Enzyme	Specific Activity		Discrimination factor
	L-selenocysteine	L-cysteine	
	units/mg	units/mg	Ratio (L-selenocysteine/L-cysteine)
SsCsd3	5.4	0.011	491
SsCsd1	21	0.92	23
SsCsd2	5.8	0.30	19
AtCpNifS	3.7	0.014	264

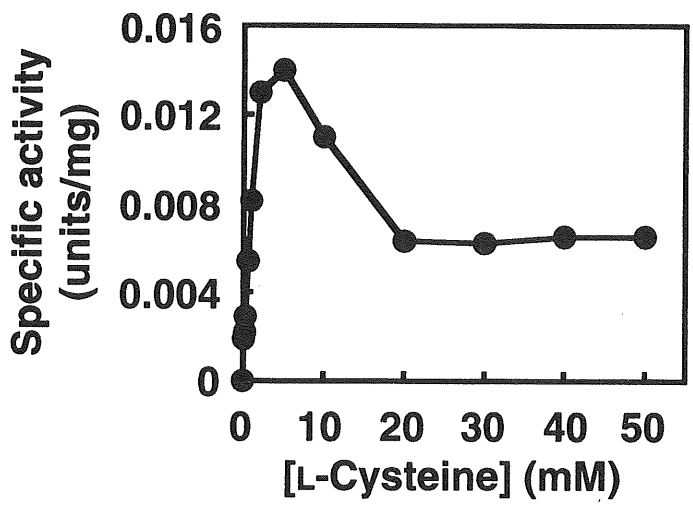

Fig. 2 Dependence of the rates of the desulfurization of L-cysteine catalyzed by SsCsd3 on substrate concentrations.

Iron-sulfur cluster synthesis

The iron-sulfur cluster of ferredoxin (Fdx) was reconstituted *in vitro* as described in "Experimental procedures." The reaction mixture was subjected to HPLC. Holo-Fdx was eluted at 25.2 min. The UV/visible absorption spectrum of the eluate at 25.2 min showed absorption maxima at about 330 and about 410 nm, which are characteristic of holo-Fdx containing a [2Fe-2S] cluster (Fig. 3). The amount of holo-Fdx was estimated by measuring the absorbance of the eluate at 410 nm. The amount of holo-Fdx was increased significantly (17-fold) by the addition of SsCsd3 in the reaction mixture. The result indicates that SsCsd3 facilitates reconstitution of the iron-sulfur cluster of Fdx by mobilizing the sulfur atom of L-cysteine.

The gene coding for SsCsd3 (*slr0077*) constitutes a gene cluster with *slr0074*, *slr0075*, and *slr0076*, which are homologous to *sufB*, *sufC*, and *sufD*, respectively, of the *suf* operon of *E. coli*[16]. Recently, cysteine desulfurase SufS/CsdB encoded by *sufS/csdB* in the *suf* operon of *E. coli* was shown to be activated by the SufE and SufBCD complex encoded by the *suf* operon[17, 18]. Although a gene homologous to *sufE* is missing in the gene cluster containing *slr0077* of *Synechocystis* sp. PCC6803, the gene termed *slr1419*, showing a significant sequence similarity with *sufE*, occurs in a different locus of the chromosome. It would be interesting to see whether the proteins encoded by the *slr0074*, *slr0075*, *slr0076*, and *slr1419* genes cooperate with SsCsd3 to assemble iron-sulfur clusters in *Synechocystis* sp. PCC6803.

In the case of the Suf mechanism, SufA encoded by the *sufA* gene in the *suf* operon functions as a scaffold protein for the iron-sulfur cluster assembly[19]. Although a gene homologous to *sufA* is not found in the vicinity of the *slr0077* gene, both *slr1417* and *slr1565*, which have a significant sequence similarity to *sufA*, are present in the genome of *Synechocystis* sp. PCC6803. It would be interesting to examine whether the sulfur atom released from L-cysteine by the action of SsCsd3 is delivered to the proteins encoded by *slr1417* and *slr1565* for the assembly of a precursor of an iron-sulfur cluster.

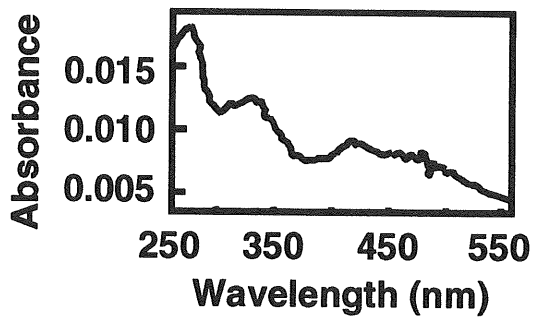

Fig. 3 Absorption spectrum of holo-ferredoxin reconstituted with SsCsd3.

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