A nifS-like Gene, csdB, Encodes an Escherichia coli Counterpart of Mammalian Selenocysteine Lyase

GENE CLONING, PURIFICATION, CHARACTERIZATION AND PRELIMINARY X-RAY CRYSTALLOGRAPHIC STUDIES*

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Selenocysteine lyase is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the exclusive decomposition of L-selenocysteine to L-alanine and elemental selenium. An open reading frame, named csdB, from Escherichia coli encodes a putative protein that is similar to selenocysteine lyase of pig liver and cysteine desulfurase (NifS) of Azotobacter vinelandii. In this study, the csdB gene was cloned and expressed in E. coli cells. The gene product was a homodimer with the subunit Mr of 44,439, contained 1 mol of PLP as a cofactor per mol of subunit, and catalyzed the release of Se, SO₂, and S from L-selenocysteine, L-cysteine sulfinic acid, and L-cysteine, respectively, to yield L-alanine; the reactivity of the substrates decreased in this order. Although the enzyme was not specific for L-selenocysteine, the high specific activity for L-selenocysteine (5.5 units/mg compared with 0.019 units/mg for L-cysteine) supports the view that the enzyme can be regarded as an E. coli counterpart of mammalian selenocysteine lyase. We crystallized CsdB, the csdB gene product, by the hanging drop vapor diffusion method. The crystals were of suitable quality for x-ray crystallography and belonged to the tetragonal space group P₄₂₂ with unit cell dimensions of a = b = 128.1 Å and c = 137.0 Å. Consideration of the Matthews parameter Vₘ (3.19 Å³/Da) accounts for the presence of a single dimer in the crystallographic asymmetric unit. A native diffraction dataset up to 2.8 Å resolution was collected. This is the first crystallographic analysis of a protein of NifS/selenocysteine lyase family.

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The abbreviations used are: SCL, selenocysteine lyase; PLP, pyridoxal 5′-phosphate; KPB, potassium phosphate buffer; PAGE, polyacrylamide gel electrophoresis; Tricine, N-tris(hydroxymethyl)methylglycine; NifS, cysteine desulfurase; CSD, cysteine sulfinate desulfinatease; Mes, 2-(N-morpholino)ethanesulfonic acid.

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can deliver the sulfur from L-cysteine for the in vitro synthesis of the Fe-S cluster of dihydroxyacid dehydratase from E. coli (17). CSD exhibits both selenocysteine lyase and cysteine desulfurase activities in addition to cysteine sulfinate desulfinase activity, and the enzyme is distinct from A. vinelandii NifS in its amino acid sequence, absorption spectrum, and lack of cysteine residues catalytically essential for the decomposition of L-selenocysteine (16). Neither enzyme shows strict specificity for L-selenocysteine, and both act on L-cysteine. Thus, we have explored the possibility that the last nifS homolog (csdB)3 mapped at 37.9 min (23) in the chromosome encodes SCL, which plays a crucial role in selenophosphate synthesis. We have isolated the gene product (CsdB), studied its enzymatic properties, and carried out preliminary x-ray crystallographic studies.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA) and Takara Shuzo (Kyoto, Japan). T4 DNA ligase was from Fermentas (Vilnius, Lithuania). SDS, acrylamide, and gel filtration were from Amershams Pharmacia Biotech (Uppsala, Sweden) and Oriental Yeast (Tokyo, Japan). Oligonucleotides were from Biologica (Nagoya, Japan); Gigapite was from Seikagaku Corporation (Tokyo, Japan); DEAE-Toyopearl, Phenyl-Toyopearl and Butyl-Toyopearl were from Tosoh (Tokyo, Japan). L-Selenocysteine was synthesized as described previously (16). Oligonucleotide primers used were 5'-GGATCCAGAAGGTGCGATATGATTTTTTCCGTCGAC-3' (upstream) and 5'-CCCAAGCTTATTCACAGCAACGGTG-3' (downstream); underlining indicates EcoRI and HindIII sites, respectively, and bold face indicates a putative ribosome binding sequence. The polymerase chain reaction product was ligated into pUC118, and then the resultant expression plasmid, pCSDB, was introduced into E. coli JM109 competent cells.

Enzyme Assays—The enzyme was assayed in 0.12 M Tricine-NaOH buffer at pH 7.5. The enzymatic activities toward L-selenocysteine and L-cysteine were measured with lead acetate as described previously (16). The reported activity value (8) for a molar turbidity coefficient of PbSe at 400 nm was corrected as 1.18 × 104 m2 cm−1 mol−1, which was calculated from the content of tyrosine, tryptophan, and cysteine (26). The subunit and the native M′ of CsdB were determined by SDS-PAGE (29) and gel filtration with Superdex 200 (Amersham Pharmacia Biotech, Uppsala, Sweden), respectively. The PLP content of the enzyme was determined fluorometrically with KCN according to the method of Adams (30).

Crystallography—Crystals of CsdB were grown by the hanging drop vapor diffusion method. Each droplet was prepared by mixing 5 µl of 20 mg/ml enzyme in 10 mM KPB (pH 7.4) with an equal volume of each reservoir solution of the Crystal Screen™ (Hampton Research, CA) initially and of a modified reservoir solution subsequently. The yellow crystals of CsdB were mounted in glass capillaries with the cryostatic-raphic c* axis along the rotation axis of the spindle and subjected to x-ray experiments. Native data for structure determination were collected at 20 °C with a Rigaku R-Axis IIC imaging plate detector using double focusing mirror-monochromated CuKα radiation that was generated with a 0.3-mm focal cup of an x-ray generator RU300 (Rigaku, Tokyo, Japan) operated at 40 kV and 100 mA. The crystal-to-detector distance was set to 130.0 mm. Data reduction was carried out using the R-Axis IIC software package.

RESULTS AND DISCUSSION

Cloning and Expression of the csdB Gene and Purification of the Product—For the production of a large amount of CsdB, expression plasmids were constructed as described under “Experimental Procedures” with chromosomal DNA isolated from E. coli K-12. The nucleotide sequence of csdB in the expression vector (pCSDB) was confirmed to be identical with that registered in GenBank™ accession number D90811 (open reading frame o32017). The clone provided overexpression of the cloned gene: about 10% of the total protein in the extract of E. coli JM109 recombinant cells. In the representative purification (Table I), about 8 mg of homogeneous preparation of CsdB was obtained per liter of culture.

Physical Characterization—CsdB provided a single band corresponding to the M′ of 43,000 on SDS-PAGE (Fig. 1). The N-terminal sequence of the purified enzyme, MIFSVDKVRA,

| Step | Total protein mg | Total activity units | Specific activity units/mg | Purification Yield | Fold |
|------|-----------------|---------------------|---------------------------|--------------------|------|
| Crude extract | 3100 | 1600 | 0.52 | 1 | 100 |
| Ammonium sulfate | 1000 | 920 | 0.92 | 1.8 | 58 |
| DEAE-Toyopearl | 370 | 890 | 2.4 | 4.6 | 56 |
| Phenyl-Toyopearl | 85 | 430 | 5.1 | 9.8 | 27 |
| Gigapite | 70 | 390 | 5.6 | 11 | 24 |

Determined with L-selenocysteine as a substrate.

3 The symbol, csd, was given to designate the postulated cysteine-selenocysteine-decomposition function of a gene product, although the physiological relevance has not been proved.

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agreed with that deduced from the nucleotide sequence of \textit{csdB}. The $M_r$ of the native enzyme was determined to be 88,000 by gel filtration. Consequently, the enzyme is a dimer composed of two identical subunits. The spectrophotometric properties of the enzyme were very similar to those of CSD with an absorption maximum at 420 nm (Fig. 2) at pH 7.4. No significant changes in the absorption spectrum were observed in the range of pH 6–8. This absorption peak is characteristic of PLP enzymes, which contain the cofactor bound to the $\epsilon$-amino group of a lysine residue at the active site. However, CsdB is distinct from either of two \textit{A. vinelandii} proteins, NiS and IscS, and also from IscS of \textit{E. coli}, all of which have an absorption maximum around 390 nm (15, 17, 22). Reduction with sodium borohydride resulted in a decrease in the absorption peak at 420 nm with a concomitant increase in the absorbance at 335 nm (Fig. 2). This result is consistent with that this is a PLP enzyme. The PLP content of CsdB was determined to be 1.0 mol per mol of subunit by the fluorometric method (30).

Catalytic Activity and Substrate Specificity—CsdB catalyzed the removal of a substituent at the $\beta$-carbon of L-selenocysteine, L-cysteine, and L-cysteine sulfinic acid to yield L-alanine. The production of elemental selenium and elemental sulfur from L-selenocysteine and L-cysteine, respectively, in the reaction was confirmed in the same manner as reported previously (31). The optimal pH value for the removal of selenium from L-selenocysteine was between 6.5 and 7.5 in Tricine-NaOH or Mes buffer. The substrate specificity of the enzyme is summarized in Table II; L-selenocysteine was the best substrate followed by L-cysteine sulfinic acid and L-cysteine, in that order. The specific activity of CsdB on L-selenocysteine (5.5 units/mg) was comparable with that of CSD and IscS (Table III) but was about 7 times lower than that of SCL (37 units/mg) (8). The cysteine desulfurase activity of CsdB was about 2 and 5% of that of CSD and IscS, respectively, at a substrate concentration of 12 mM (Table III). The specific activity of CsdB for L-cysteine was about 1/290 of the activity with L-selenocysteine (Table III). This value is much lower than those of CSD and IscS (Table III). In contrast with CsdB, \textit{A. vinelandii} NiS favors L-cysteine as a substrate over its selenium analog (2). CsdB acted on L-cysteine, L-selenocysteine, and L-aspartic acid, although at extremely low rates ($<0.08\%$ of the rate for L-selenocysteine) (Table II).

Crystallization and Preliminary X-ray Characterization—CsdB was crystallized at 25 °C within 2 days by hanging drop vapor diffusion against a 100 mM cacodylate solution (pH 6.8) containing 1.4 M sodium acetate, which corresponds to the solution No.7 in the Crystal Screen™. The crystals were also obtained in 100 mM KPb (pH 6.8) containing 1.4 mM sodium acetate and 10 mM PLP, and these conditions were further used for the crystallization of the enzyme. The yellow crystals (0.5 × 0.5 × 0.4 mm$^3$) had tetragonal-bipyramidal shapes (Fig. 3). They were grown in amorphous debris, which was removed.

### Table II

| Substrate                  | Specific activity$^a$ | Relative activity |
|----------------------------|----------------------|------------------|
| L-Selenocysteine           | 5.5                  | 100              |
| L-Cysteine sulfinic acid   | 0.82                 | 15               |
| L-Cysteine                 | 0.019                | 0.35             |
| L-Selenocysteine           | 0.0044               | 0.080            |
| L-Cysteine                 | 0.0031               | 0.056            |
| L-Aspartic acid            | 0.0044               | 0.080            |

$^a$ Activity was measured in the reaction buffer containing one of the following substrates: L-selenocysteine, 12 mM; L-cysteine sulfinic acid, 12 mM; L-cysteine, 12 mM; L-selenocysteine, 20 mM; L-cysteine, 20 mM; L-aspartic acid, 20 mM.

### Table III

| Enzyme$^b$ | Map position$^c$ | Specific activity | Discrimination factor$^d$ |
|------------|------------------|------------------|--------------------------|
|            |                  | L-Selenocysteine | L-Cysteine               |
|            |                  | min              | units/mg                  |
| CsdB       |                  | 37.9             | 5.5                       |
| CSD        |                  | 63.4             | 6.2                       |
| IscS       |                  | 57.3             | 3.1                       |

$^b$ The amino acid sequence of the proteins can be accessed through NCBI Protein Database under NCBI Accession numbers 1742766 (CsdB), 1789175 (CSD), and 1788879 (IscS).

$^c$ The map positions were from Ref. 23.

$^d$ Discrimination factor was calculated from the specific activity of the enzymes for L-selenocysteine divided by that for L-cysteine. Activity was measured in the reaction mixture containing 120 mM Tricine-NaOH (pH 7.5), 50 mM dithiothreitol, 0.2 mM PLP, and 12 mM substrate.
from the crystals before they were sealed in thin-walled glass capillaries.

The space group of the CsdB was $P4_2_2_2$ with the cell dimensions of $a = b = 128.1$ Å, and $c = 137.0$ Å. The assumption that a single dimer (89 kDa) exists in the asymmetric unit of the crystal gives a $V_n$ value of 3.19 Å$^3$/Da, which is equivalent to a solvent content of 62%. These values lie within the range of values commonly found for proteins (32). A set of native data was collected to 2.8 Å resolution on a Rigaku R-AXIS IIC using 1.5° oscillation over a range of 45° (94.2% complete with 23,770 independent reflections). The $R_{merge}$ value for the intensity data was 7.22%. The data collection statistics obtained for the native CsdB crystals are given in Table IV. The x-ray crystal structure determination of the enzyme is now under way by the multiple isomorphous replacement method.

We also obtained crystals of CSD at 25 °C by hanging drop vapor diffusion against a 100 mM sodium acetate solution (pH 4.6) containing 200 mM ammonium acetate and 30% (w/v) polyethylene glycol 4000. However, these crystals were small and not suitable for x-ray analysis. Further optimization of crystallization conditions by changing pH, polyethylene glycol concentration, and salt has resulted in little improvement.

Comparison with Other PLP-dependent Enzymes—Grishin et al. (33) classified PLP enzymes into seven distinct fold types on the basis of primary structure, secondary structure prediction, and biochemical function. NiF proteins have been classified as "aminotransferases class V" in the fold type I together with serine-pyruvate aminotransferase (EC 2.6.1.51), phosphoserine aminotransferase (EC 2.6.1.52), isopenicillin N epimerase, and the small subunit of the soluble hydrogenase. Recently, three-dimensional structures of phosphoserine aminotransferases from Bacillus circulans sbsp. Alkalophilus$^5$ and E. coli$^6$ were solved and deposited in the Protein Data Bank, Brookhaven National Laboratory, with the codes 1BT4 and 1BJN, respectively. Comparison of the structures of phosphoserine aminotransferases with that of CsdB will contribute to the understanding of how the related proteins confer separate reaction specificities on the same coenzyme.

The reaction of CsdB shares some common features with that of other PLP-dependent enzymes such as aspartate $\beta$-decarboxylase (EC 4.1.1.12) (34), kynureninase (EC 3.7.1.3) (35), and SCL. These enzymes catalyze removal of $\beta$-substituent from the substrate to form alanine. None of their structures have been solved. The solution of the three-dimensional structure of CsdB would contribute to the understanding of the mechanisms of these PLP-dependent enzymes.

TABLE IV

| Data collection statistics |
|-----------------------------|
| Unit cell dimensions (Å)    | $a = b = 128.1; c = 137.0$ |
| Space group                | $P4_2_2_2$ |
| Resolution limit (Å)        | 2.8 |
| Number of reflections       | 73,138 |
| Number of unique reflections| 23,770 |
| Percent completeness (%)    | 94.2 |
| $R_{merge}$ a                | 7.22 |


$^a$ $R_{merge} = \sum I - (I)/\sum I$.

A Possible Role of CsdB in Vivo—Genome sequencing projects have revealed that homologs of A. vinelandii nifS are widespread throughout nature and that some organisms contain more than one copy of a nifS homolog (16, 22). Some of the “NiF-like proteins” characterized so far prefer L-cysteine to L-selenocysteine, and some of them show the opposite preference. Further experiments will need to be done to determine whether putative NiF-like proteins can play a role in Fe-S cluster assembly.

Lacourciere and Stadtman (2) have pointed out that in vivo concentrations of sulfur-containing compounds are on the order of a thousand times greater than those of their selenium analogs (36). Thus, enzymes showing higher activity toward L-cysteine, such as A. vinelandii NiF, will preferentially utilize L-cysteine over L-selenocysteine in vivo (2). Therefore, it may be reasonable to assume that enzymes which are specific toward L-selenocysteine probably function as a physiological selenide delivery system in E. coli. Although CsdB is not strictly specific to selenocysteine, its discrimination factor (290 times over the activity on cysteine) is much higher than those of other NiF homologs of E. coli. Accordingly, the enzyme can be regarded as an E. coli counterpart of mammalian selenocysteine lyase. It would be particularly intriguing to determine whether CsdB is more effective than CSD and IscS as a selenide delivery protein in the formation of selenophosphate catalyzed by E. coli selenophosphate synthetase. The in vivo function of CsdB is now being studied by disrupting its gene.

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FIG. 3. Photograph of crystals of CsdB grown by the hanging drop vapor diffusion method.
Selenocysteine Lyase-like Enzyme of E. coli

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