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Establishment of an *In Vitro* D-Cycloserine-Synthesizing System by Using *O*-Ureido-L-Serine Synthase and D-Cycloserine Synthetase Found in the Biosynthetic Pathway

Narutoshi Uda, Yasuyuki Matoba, Takanori Kumagai, Kosuke Oda, Masafumi Noda, Masanori Sugiyama

Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

We have recently cloned a DNA fragment containing a gene cluster that is responsible for the biosynthesis of an antituberculosis antibiotic, D-cycloserine. The gene cluster is composed of 10 open reading frames, designated *dcsA* to *dcsJ*. Judging from the sequence similarity between each putative gene product and known proteins, DcsC, which displays high homology to diaminopimelate epimerase, may catalyze the racemization of *O*-ureidoserine. DcsD is similar to *O*-acetylserine sulfhydrylase, which generates L-cysteine using *O*-acetyl-L-serine with sulfide, and therefore, DcsD may be a synthase to generate *O*-ureido-L-serine using *O*-acetyl-L-serine and hydroxyurea. DcsG, which exhibits similarity to a family of enzymes with an ATP-grasp fold, may be an ATP-dependent synthetase converting *O*-ureido-D-serine into D-cycloserine. In the present study, to characterize the enzymatic functions of DcsC, DcsD, and DcsG, each protein was overexpressed in *Escherichia coli* and purified to near homogeneity. The biochemical function of each of the reactions catalyzed by these three proteins was verified by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and, in some cases, mass spectrometry. The results from this study demonstrate that by using a mixture of the three purified enzymes and the two commercially available substrates *O*-acetyl-L-serine and hydroxyurea, synthesis of D-cycloserine was successfully attained. These *in vitro* studies yield the conclusion that DcsD and DcsG are necessary for the syntheses of *O*-ureido-L-serine and D-cycloserine, respectively. DcsD was also able to catalyze the synthesis of L-cysteine when sulfide was added instead of hydroxyurea. Furthermore, the present study shows that DcsG can also form other cyclic D-amino acid analogs, such as D-homocysteine thiolactone.

The antituberculosis antibiotic D-cycloserine (D-CS) is produced by several *Streptomyces* species (1). D-CS, which is a cyclic analog of D-alanine, prevents the catalytic activities of both alanine racemase (2, 3) and D-alanyl–D-alanine ligase (4, 5), which are necessary for the biosynthesis of the bacterial cell wall; that is, the antibiotic functions as an inhibitor of bacterial cell wall biosynthesis. D-CS is clinically used as a second line of defense against *Mycobacterium tuberculosis*, especially when the bacterium is resistant to other antituberculosis antibiotics (6, 7). Furthermore, since D-CS was recently shown to be a partial agonist for the *N*-methyl-D-aspartate receptor, the drug may be useful for the treatment of various psychological dysfunctions (8–10).

On the basis of evidence from substrate-feeding experiments conducted by other research groups (11–13), a putative D-CS biosynthetic pathway has been proposed. The first step of D-CS biosynthesis is the transfer of an acetyl residue from acetyl coenzyme A onto the hydroxyl group of L-serine (11, 13). The resulting *O*-acetyl-L-serine (L-OAS) would be converted into *O*-ureido-L-serine (L-OUS) in the presence of hydroxyurea (13). Hydroxyurea was found to be synthesized by the D-CS-producing microorganism (12). L-OUS might then be configurationally changed into D-OUS by a racemase (13). Finally, to generate D-CS, the cyclization of D-OUS and the hydrolysis of the urea moiety would be catalyzed by an ATP- and Mg(II)-dependent enzyme (13).

Recently, our group successfully cloned a D-CS biosynthetic gene cluster from the genomic DNA of the D-CS-producing organism *Streptomyces lavendulae* ATCC 11924 (14). The gene cluster consists of 10 open reading frames, designated *dcsA* to *dcsJ*. Our group has previously found that the *dcsI* and *dcsJ* genes from another D-CS-producing microorganism encode a D-CS-resistant D-alanyl–D-alanine ligase (15) and a putative D-CS efflux pump (16), respectively. These genes are apparently responsible for self-resistance to D-CS. On the basis of a sequence homology search of the open reading frames that were found in the D-CS biosynthetic gene cluster, our group revised the putative D-CS biosynthetic pathway (14), as shown in Fig. 1.

The transfer of the acetyl group onto L-serine may be catalyzed by DcsE, which is a homolog of L-homoserine *O*-acetyltransferase. Hydroxyurea may be generated as the result of the hydrolysis of N^{ω}-hydroxy-L-arginine by DcsB, an arginase homolog. The synthesis of L-OUS from L-OAS and hydroxyurea may be catalyzed by DcsD, since some *O*-acetylserine sulfhydrylases that display a high level of similarity to DcsD can synthesize various derivatives of β-substituted L-alanine from L-OAS and a nucleophilic reagent (17, 18). Based on the sequence similarity between DcsC and known diaminopimelate epimerases, L-OUS might be configurationally changed into D-OUS by DcsC functioning as a putative racemase. Finally, an ATP- and Mg(II)-dependent enzyme may catalyze the cyclization of D-OUS and the hydrolysis of the urea moiety. Three proteins encoded by the D-CS biosynthetic gene cluster (DcsG, DcsH, and DcsI) are suggested to be ATP- and

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Address correspondence to Masanori Sugiyama, sugi@hiroshima-u.ac.jp.
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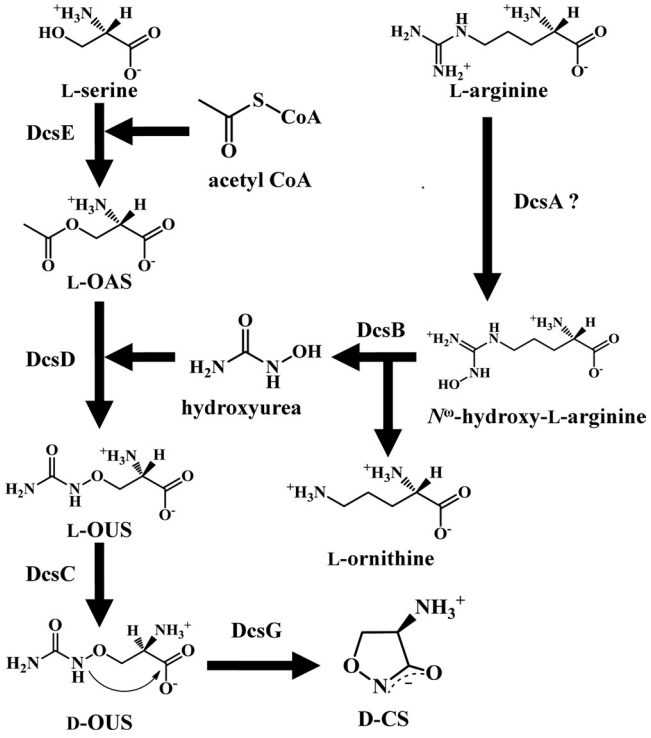

FIG 1 Predicted biosynthetic pathway of D-CS in *S. lavendulae* ATCC 11924. CoA, coenzyme A.

Mg(II)-dependent enzymes with an ATP-grasp fold motif (19, 20). However, DcsI has been demonstrated by our studies to be a D-CS-resistant D-alanyl–D-alanine ligase (15). In addition, our group observed that a *dcsG* disruption mutant from the D-CS-producing organism *S. lavendulae* does not produce D-CS (14). These results indicate that DcsG is the best candidate for the cyclization and hydrolysis of D-OUS.

Our group has already confirmed the catalytic functions of DcsB and DcsE (14). Based on our putative biosynthetic pathway of D-CS (14), another research group recently confirmed that the racemization of OUS is catalyzed by DcsC (21). Furthermore, our recent mutagenesis analysis suggested that DcsA, which exhibits heme-binding-protein-like properties, catalyzes the hydroxylation of L-arginine to yield the substrate for DcsB (22), although the catalytic function of DcsA has not been demonstrated *in vitro*. In the present study, we focus on the catalytic functions of DcsD and DcsG.

DcsD has high sequence similarity with *O*-acetylserine sulfhydrylase and cystathionine β-synthase. *O*-Acetylserine sulfhydrylase is an enzyme that catalyzes L-cysteine synthesis using L-OAS and sulfide (23, 24), which is the last step in L-cysteine biosynthesis, whereas cystathionine β-synthase synthesizes cystathionine using L-serine and L-homocysteine in the transsulfuration pathway from L-methionine to L-cysteine (25, 26). These enzymes commonly require pyridoxal 5′-phosphate (PLP) as a cofactor to express the catalytic activity. PLP is covalently bound to the amino group of the Lys residue at the active center of the enzyme. The absorption spectrum of the PLP-bound enzyme displays a local maximum at about 412 nm, in addition to a local maximum at about 280 nm derived from the Tyr and Trp residues in the enzyme. In the first step of the catalysis of *O*-acetylserine sulfhydrylase and cystathionine β-synthase, the amino group of the first substrate, either L-OAS or L-serine, is covalently attached to PLP instead of to the amino group of the Lys residue at the active site. The Lys residue then acts as a base to catalyze an eliminative double-bond formation, resulting in the generation of an α-aminoacrylate intermediate, which shows an absorption maximum at about 470 nm. The final step is that a nucleophilic reagent (sulfide or L-homocysteine) attacks the β-carbon of the intermediate to release either L-cysteine or cystathionine, respectively.

In contrast, sequence homology searches for DcsG suggested that this protein is a member of the ATP-grasp superfamily (19, 20), which includes various enzymes, such as D-alanyl–D-alanine ligase, glutathione synthetase, biotin carboxylase, and carbamoyl phosphate synthetase. In general, sequence similarities among the ATP-grasp enzymes are low. All of the ATP-grasp enzymes catalyze an ATP-dependent ligation between a carboxyl group carbon of one substrate and an amino or imino group nitrogen or a thiol group sulfur of the second substrate. In the initial step of the reaction mechanism, the acylphosphate intermediates are formed, accompanied by the conversion of ATP to ADP. A covalent bond is then formed, along with the release of phosphate. Although DcsG has been suggested to be an ATP-grasp enzyme, it has been difficult to predict the accurate function of DcsG due to the absence of clear sequence similarity to any functionally defined proteins.

The present study reveals that D-CS is formed when a mixture of purified recombinant DcsC, DcsD, and DcsG is incubated together with L-OAS and hydroxyurea as substrates. Enzymatic kinetic analyses were also performed to characterize DcsD and DcsG.

MATERIALS AND METHODS

Reagents. Most of the reagents used in this study were of analytical grade and purchased from Wako Chemical, Japan. Reagents from other suppliers are indicated in parentheses. L-OUS was chemically synthesized from L-CS (Sigma), and D-OUS and β-aminooxy-D-alanine were synthesized from D-CS, according to methods described previously (27, 28).

DNA manipulations. Genomic DNA of *S. lavendulae* ATCC 11924 was isolated according to a standard protocol (29). Plasmid DNA was isolated from *Escherichia coli* by using the Wizard Plus Minipreps DNA purification system (Promega).

Preparations of DcsC, DcsD, and DcsG. C-terminally His_6-tagged DcsC, DcsD, and DcsG were expressed in *E. coli* and purified to near homogeneity. Recombinant proteins were prepared as described in the supplemental material. Each protein concentration was determined by measuring the absorbance at 280 nm using the molar extinction coefficients 20,700, 26,300, and 35,900 M^{-1} cm^{-1}, respectively.

Analysis of molecular mass. The molecular mass of the purified enzymes was estimated by high-performance liquid chromatography (HPLC), using a Superdex 200 10/300 GL column (GE Healthcare) at 0.75 ml min^{-1} with 100 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl. The protein standards used for the calibration were as follows: ferritin (440 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa). The proteins were detected at 280 nm by using a multiwavelength detector (MD-2010; Jasco). To estimate the molecular mass of the purified proteins, three repetitions of the experiments were done.

Absorption spectroscopy. Prior to spectroscopic analysis, DcsD was dialyzed against 250 mM potassium phosphate buffer (pH 8.0) containing 1 μM PLP. The absorption spectrum of DcsD (15 μM) in the absence or the presence of the given concentrations of L-OAS (Sigma) was recorded at 20°C in a 1-cm-path-length cuvette using a V-550 spectrophotometer (Jasco).

***In vitro* D-CS synthesis.** DcsC, DcsD, and DcsG (0.1 mg ml^{-1} each) were incubated for 2 h at 30°C in 100 mM Tris-HCl buffer (pH 7.8) containing 1 mM L-OAS, 1 mM hydroxyurea, 10 mM KCl, 5 mM $MgCl_2$, and 2 mM ATP. When L- or D-OUS was used as a starting material, the reagent was added at a concentration of 1 mM instead of L-OAS and hydroxyurea. Negative-control experiments without addition of an enzyme, ATP, or Mg(II) were also performed. Enzymatic reactions were stopped by boiling at 100°C for 5 min, and solutions were filtered through a membrane filter (0.2-μm pore size; Advantec). The productivity of D-CS was analyzed by HPLC. Aliquots (10 μl) were injected into a Senshu Pak SCX-1251-N column (250 by 4.6 mm; Senshu Scientific) and eluted with an isocratic mobile phase of 10 mM ammonium acetate buffer (pH 4.6). The flow rate was set to 0.8 ml min^{-1}. D-CS was detected at 210 nm by using a multiwavelength detector (MD-2010; Jasco), and its configuration was conveniently confirmed by using a chiral detector (OR-2090; Jasco). To further confirm the configuration of the product, a 50-μl portion of the reaction mixture was injected into a Senshu Pak SCX-1251-N column and eluted with ammonium acetate buffer (pH 4.6). A peak corresponding to D-CS was collected and then lyophilized. The sample was mixed with 10 μl of a 1 mM L-alanine solution as an internal control and 5 μl of a derivatization solution (30) containing *O*-phthaldialdehyde and *N*-acetyl-L-cysteine. Aliquots were injected into an ODS-A column (100 by 4.6 mm; YMC) equilibrated with 85% solvent A (50 mM sodium acetate at pH 5.2) and 15% solvent B (methanol). The ratio of solvent B was linearly increased from 15 to 66% in 20 min. The flow rate was set to 1.0 ml min^{-1}. A derivative from the product was detected by the absorbance at 340 nm using a multiwavelength detector. Generation of D-CS was further confirmed by a coinjection experiment using authentic D-CS. Coinjection experiments were performed in each of the cases in the present study.

Analysis of the enzyme activity of DcsD. Before the measurement of enzyme activity, stock solutions of L-OAS (HCl form), hydroxyurea, sodium sulfide, sodium thiosulfate, and L-homocysteine (Biosynth) were newly prepared, and the pH of the stock solutions was adjusted to ca. 7 with HCl or NaOH. The reaction mixture, consisting of 50 mM MES (2-morpholinoethanesulfonic acid)-KOH (pH 6.5), 1 mM EDTA, 5 mM L-OAS, 5 mM a second substrate (hydroxyurea, sodium sulfide, sodium thiosulfate, or L-homocysteine), and 20 μg ml^{-1} DcsD, was incubated at 30°C for 2 h. As a control, the same reaction mixture without DcsD was used. The enzymatic reaction was stopped by boiling at 100°C for 5 min, and any precipitate was removed by centrifugation. The supernatant was used for product analysis. The formation of the enzymatic product was conveniently analyzed by thin-layer chromatography (TLC) using silica gel 60 F_{254} (Merck). In the case of detection of L-cysteine, the supernatant was mixed with an equivalent volume of a 5 mM *N*-ethylmaleimide solution and incubated at room temperature for 20 min before TLC analysis. The solvent for the chromatogram was 2-butanone–pyridine–water (18:5:10). Amino acids were visualized by ninhydrin. Furthermore, the enzymatic products were analyzed by electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) using a QSTAR XL spectrometer (Applied Biosystems). It should be noted that although L-OAS in solution was slowly hydrolyzed to L-serine and acetate, the generation of L-serine from L-OAS was increased by the boiling treatment. Therefore, the L-serine that was detected by the TLC and MS analyses appears to have been generated by boiling treatment rather than by any enzyme reaction.

We measured the pH dependence of the L-OUS-synthesizing activity of DcsD. A reaction mixture consisting of 250 mM potassium phosphate (pH 5.0 to 9.0), 100 mM L-OAS, 2 mM hydroxyurea, and 5 μg ml^{-1} DcsD was incubated at 30°C for 10 min. The reaction was started by the addition of hydroxyurea after preincubation for 10 min and stopped by heat treatment at 100°C for 5 min. DcsD enzyme activity was analyzed by HPLC after derivatization with *O*-phthaldialdehyde and *N*-acetyl-L-cysteine. A 10-μl aliquot of the reaction mixture (diluted, if necessary) was mixed with 10 μl of a 1 mM L-threonine solution as an internal control and 5 μl of a derivatization solution (30). Aliquots were injected into an ODS-A column and eluted in a way similar to that described above. In this case, the ratio of methanol was linearly increased from 15 to 49% in 20 min. For the kinetic analysis, peak areas on the HPLC chromatogram were used as indicators.

Next, the kinetic response toward increasing concentrations of L-OAS was measured in the presence of 50 mM hydroxyurea at pH 8.0. A reaction mixture consisting of 250 mM potassium phosphate (pH 8.0), 5 to 300 mM L-OAS, 50 mM hydroxyurea, and 2.0 μg ml^{-1} DcsD was incubated at 30°C for 10 min. To determine the kinetic parameters for hydroxyurea, a reaction mixture that consisted of 250 mM potassium phosphate (pH 8.0), 100 mM L-OAS, 1 to 100 mM hydroxyurea, and 2.0 μg ml^{-1} DcsD was incubated at 30°C for 10 min. The amount of L-OUS formed in the reaction mixture was determined by HPLC analysis.

The enzymatic activity of DcsD to synthesize L-cysteine, *S*-sulfo-L-cysteine, or cystathionine was determined by a colorimetric method established previously (31–33). Experimental procedures are described in detail in the supplemental material.

Analysis of the enzymatic activity of DcsC. A reaction mixture consisting of 50 mM potassium phosphate (pH 8.0), 1 mM L-OUS or D-OUS, and 1.0 μg ml^{-1} DcsC was incubated at 30°C for 20 min. HPLC analysis was carried out after derivatization with *O*-phthaldialdehyde and *N*-acetyl-L-cysteine, as described above.

Analysis of the enzymatic activity of DcsG. Before the measurement of enzyme activity, stock solutions of D-OUS, L-OUS, β-aminooxy-D-alanine, D-2,4-diaminobutyrate (2HCl form; Watanabe Chemical), D-ornithine (HCl form), D-homocysteine (Chemcube), D-homoserine, D-alanine, D-asparagine, and ATP (2Na form) were newly prepared. The pH of the stock solutions was adjusted to approximately 7 with HCl or NaOH. The enzyme product was analyzed by TLC, HPLC, or ESI-TOF MS analysis. The conditions for TLC analysis were the same as those applied for the detection of intact β-substituted L-alanine. The conditions for HPLC analysis to detect products other than D-CS were similar to those applied for the detection of intact D-CS. In this case, however, the elution solution was changed to 100 mM ammonium acetate, and the products were detected by the absorbance at 240 nm.

The pH dependence of DcsG activity to synthesize D-CS from D-OUS was measured. The reaction mixture, which consisted of 250 mM MES-KOH (pH 5.0 to 7.5) or Tris-HCl (pH 7.5 to 10.0), 10 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM D-OUS, and 5 μg ml^{-1} (at pH 7.0 to 9.0) or 50 μg ml^{-1} (at pH 5.0 to 6.5 and 9.5 to 10.0) DcsG, was incubated at 30°C for 10 min. HPLC analysis to detect the amount of D-CS formed was performed as described above. HPLC analysis was also performed to detect the amount of liberated ADP. Aliquots (10 μl) were injected into a Hydrosphere C_{18} column (100 by 4.6 mm; YMC) and eluted with the isocratic mobile phase of 100 mM potassium phosphate buffer (pH 5.5). The flow rate was set to 1.0 ml min^{-1}. ATP and ADP were detected at 260 nm by using a multiwavelength detector.

The kinetic parameters of DcsG for the synthesis of D-CS from D-OUS were determined by an ADP release-coupled assay (34). A reaction mixture consisting of 250 mM Tris-HCl (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 2.5 mM potassium phosphoenolpyruvate, 0.3 mM NADH, 1 mM dithiothreitol, 45 U ml^{-1} pyruvate kinase, 20 U ml^{-1} lactate dehydrogenase (Oriental Yeast), D-OUS and ATP at the given concentrations, and 18 μg ml^{-1} DcsG was incubated at 30°C. The kinetic parameters for D-OUS were determined in the presence of a fixed concentration (5 mM) of ATP and variable concentrations (0.5 to 100 mM) of D-OUS. On the other hand, the kinetic parameters for ATP were determined in the presence of a fixed concentration (50 mM) of D-OUS and variable concentrations (4 to 500 μM) of ATP. The rate of the decrease in absorbance at 340 nm of NADH ($\epsilon = 6{,}220\ M^{-1}\ cm^{-1}$) was monitored by using a V-550 spectrophotometer. The kinetic response of DcsG toward the other amino acids as a substrate was also analyzed by the ADP release-coupled assay.

Enzymatic kinetic study. In general, an appropriate amount of enzyme was added to the reaction mixture. The amount of DcsD and DcsG added in the present study was adjusted so that the amount of the enzymatic products became less than 30% of the maximum amount, which

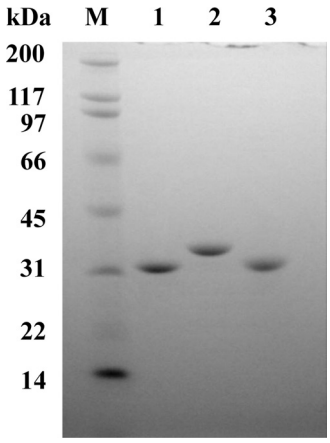

FIG 2 SDS-PAGE analysis. Purities of DcsC (lane 1), DcsD (lane 2), and DcsG (lane 3) were analyzed by SDS-PAGE. Gels were stained by using Coomassie blue.

was estimated from the initial concentrations of two substrates. In the ADP release-coupled assay applied for DcsG, the product concentration could be monitored over time. In this case, the amount of the enzyme was adjusted so that the rate of the decrease in absorbance at 340 nm became less than 1 min^{-1}. To determine the activity at each data point, three sets of experiments were done. The initial reaction rates at different substrate concentrations were analyzed by nonlinear regression fit to the Michaelis-Menten equation. When the K_m value was estimated to be very high, only the k_{cat}/K_m value was calculated from the slope of the linear line in the kinetic graph.

RESULTS

***In vitro* D-CS synthesis.** In the present study, DcsC, DcsD, and DcsG were overexpressed in *E. coli* and purified to near homogeneity (Fig. 2). The molecular masses of DcsC, DcsD, and DcsG monomers are 31, 36, and 34 kDa, respectively, based on the amino acid sequences. However, HPLC analysis using a gel filtration column demonstrated that the molecular masses of DcsC, DcsD, and DcsG are 34 ± 3, 126 ± 2, and 26 ± 4 kDa, respectively, indicating that DcsC and DcsG are monomers, whereas DcsD is a tetramer. Diaminopimelate epimerases and enzymes with an ATP-grasp fold, in general, are known to be monomeric proteins. It should be noted, however, that *O*-acetylserine sulfhydrylases are dimeric or tetrameric. The difference of molecular mass between the observed and predicted values for DcsG was 8 kDa, which accounts for 24% of the predicted molecular mass. Although the reason for the large difference is unclear, DcsG may take the compact structure.

To synthesize D-CS *in vitro*, a mixture composed of the DcsC, DcsD, and DcsG proteins was incubated together with L-OAS and hydroxyurea as substrates. The synthesis of D-CS was detected when Mg(II) ion and ATP were added into a protein mixture supplemented with L-OAS and hydroxyurea as substrates (Fig. 3B). For D-CS production, all three enzymes, Mg(II) ion, and ATP are necessary (Fig. 3C to F). Note that whether D-CS or L-CS was produced from these reactions cannot be distinguished by HPLC analysis using the Senshu Pak SCX-1251-N column, which was used to determine the product being produced, since both compounds are eluted at the same retention time. However, the product appeared to be D-CS, since it showed a positive chirality. The configuration of the product was further confirmed by HPLC analysis after derivatization with *O*-phthaldialdehyde and *N*-acetyl-L-cysteine (Fig. 4).

To verify the enzyme reaction in the D-CS biosynthetic pathway, we prepared L-OUS and D-OUS chemically from L-CS and D-CS, respectively (27, 28). When DcsC and DcsG were incubated with Mg(II), ATP, and either L-OUS or D-OUS as the substrate, D-CS was synthesized (Fig. 5A and B). D-CS was synthesized from D-OUS, even without DcsC (Fig. 5D), but not from L-OUS (Fig. 5C). To synthesize D-CS using D-OUS, Mg(II) and ATP were necessary. These results provide additional evidence in support of the D-CS biosynthetic pathway proposed previously by our group

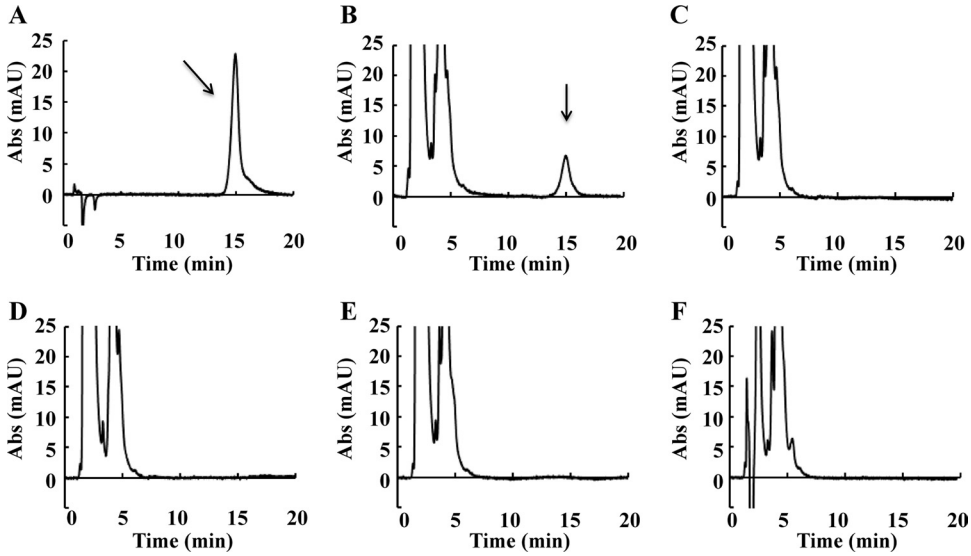

FIG 3 HPLC profile of products formed after incubation of reaction mixtures consisting of L-OAS and hydroxyurea with DcsC, DcsD, and DcsG in the presence of $MgCl_2$ and ATP. (A) HPLC profile of authentic D-CS (1 mM). (B) HPLC profile of the incubation mixture consisting of L-OAS, hydroxyurea, $MgCl_2$, and ATP, which was incubated together with DcsC, DcsD, and DcsG. (C) Incubation without added DcsG. (D) Incubation without DcsD. (E) Incubation without DcsC. (F) Incubation without ATP and $MgCl_2$. Arrows indicate the peak corresponding to D-CS. mAU, milliabsorbance unit.

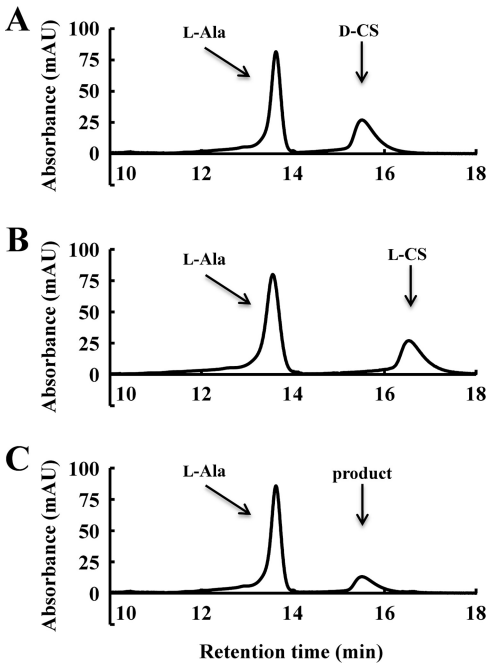

FIG 4 Configuration analysis of the product after derivatization. (A and B) HPLC profile of authentic derivatives from D-CS (A) and L-CS (B) (1 mM each). (C) HPLC profile of the derivative that was formed by incubation with DcsC, DcsD, and DcsG. Derivatives from D- and L-CS have retention times of about 15.5 and 16.5 min, respectively. The peak eluting with a retention time of about 13.5 min corresponds to a derivative from L-alanine used as an internal control.

(Fig. 1); that is, DcsD catalyzes the biosynthesis of L-OUS using L-OAS and hydroxyurea. We also confirmed that DcsC catalyzes the conversion from L-OUS to D-OUS. DcsG was necessary to catalyze the synthesis of D-CS from D-OUS in an Mg(II)- and ATP-dependent manner.

Evaluation of DcsD activity. The ability of DcsD to synthesize L-OUS was conveniently examined by TLC analysis. However, the accurate evaluation of DcsD activity was performed by HPLC analysis after derivatization with *O*-phthaldialdehyde and *N*-acetyl-L-cysteine. The derivatization method (30) employed in this experiment has the advantage of being able to determine the product configuration, whereas determining the configuration by TLC analysis is difficult. In the present study, DcsD was confirmed to catalyze the synthesis of L-OUS, but not D-OUS, by using L-OAS and hydroxyurea as substrates (Fig. 6). Furthermore, when L-serine was used instead of L-OAS, no reaction products were generated.

Like *O*-acetylserine sulfhydrylase and cystathionine β-synthase, purified recombinant DcsD was yellow. When DcsD was preincubated with hydroxylamine, L-OUS-synthesizing activity was completely lost, based on the HPLC analysis. However, the activity of DcsD preincubated with hydroxylamine was restored by the addition of PLP after the dialysis, indicating that DcsD is evidently a PLP-dependent enzyme. The UV-visible (UV-vis) spectrum of DcsD showed an absorption maximum at 412 nm (solid line in Fig. 7A). The addition of sufficient L-OAS (100 mM) quickly caused a change in the spectrum due to the formation of an α-aminoacrylate intermediate (dotted line in Fig. 7A), as found for other *O*-acetylserine sulfhydrylases.

When the concentration of the substrate L-OAS was increased to evaluate the kinetic response of DcsD for L-OUS or L-cysteine synthesis, the Michaelis constant (K_m) for L-OAS was determined to be >200 mM. Since the K_m values of the known *O*-acetylserine sulfhydrylase enzymes for L-OAS are in the low-millimolar range, the K_m value of DcsD is 1 to 2 orders of magnitude higher than that of *O*-acetylserine sulfhydrylases.

The absorption spectra of the DcsD solution (15 μM) were recorded after the addition of various concentrations of L-OAS (Fig. 7B). The rate of the increase in absorbance at 470 nm, which is due to the formation of an α-aminoacrylate intermediate, was

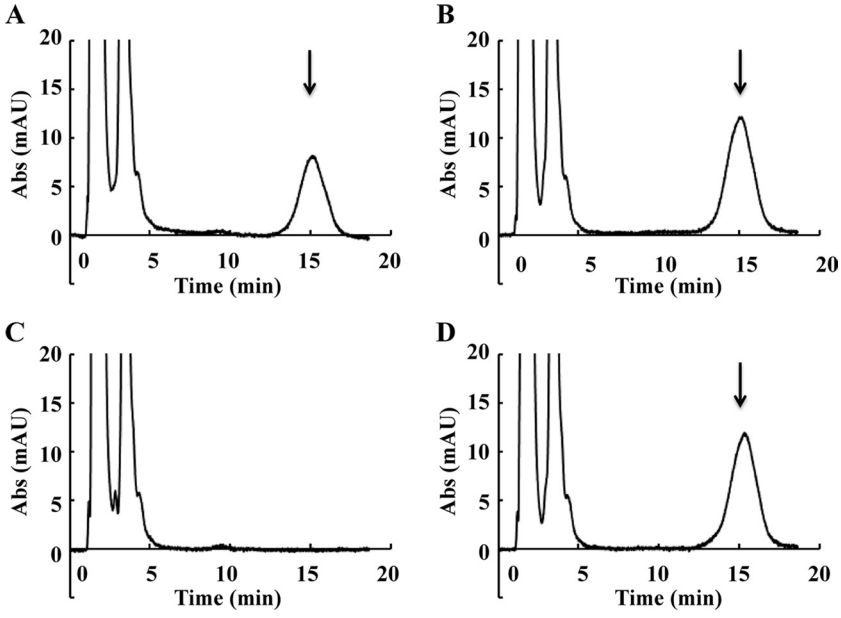

FIG 5 HPLC profile of D-CS formed after incubation of OUS with DcsC and DcsG. (A and C) L-OUS; (B and D) D-OUS. In the case of panels C and D, DcsC was eliminated from the reaction mixture. Arrows indicate the peak corresponding to D-CS.

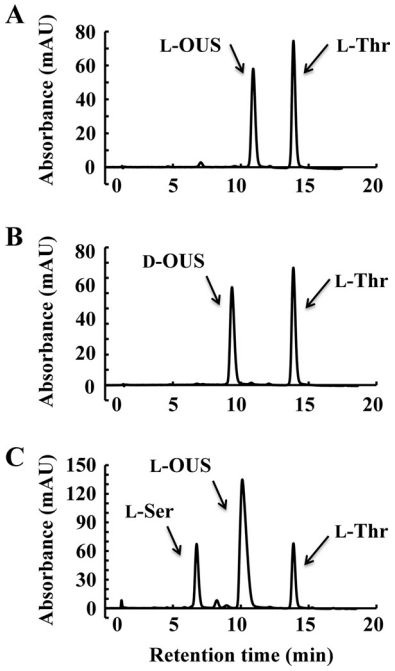

FIG 6 HPLC profile of the reaction mixture of DcsD after derivatization. (A and B) Authentic derivatives from L-OUS (A) and D-OUS (B) (1 mM each). (C) The product by DcsD was analyzed after incubation with 100 mM L-OAS and 20 mM hydroxyurea. Derivatives of D- and L-OUS eluted at about 9.5 and 11 min, respectively. The peak eluting at a retention time of about 14 min corresponds to a derivative of L-threonine used as an internal control. The peak at a retention time of about 7 min corresponds to a derivative of L-serine.

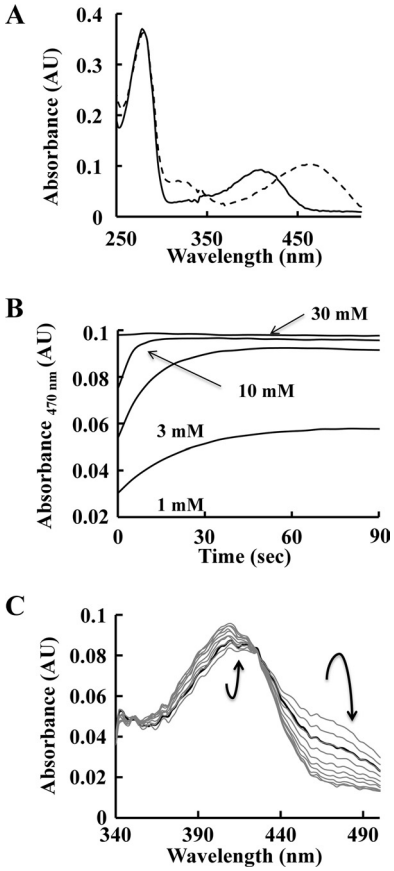

FIG 7 Absorption spectrum of DcsD. (A) Absorption spectrum of solutions containing 15 μM DcsD in the absence (solid line) or the presence (dotted line) of 100 mM L-OAS. (B) Time course of the increase of absorption at 470 nm. The increase of absorption, which is produced by the formation of the α-aminoacrylate intermediate, was monitored after the addition of a given concentration (1, 3, 10, and 30 mM) of L-OAS to a solution containing DcsD. (C) Changes in the spectrum after the addition of 1 mM L-OAS to a solution containing DcsD. The bold line represents the spectrum immediately after the addition of L-OAS. The spectrum was recorded every 3 min. AU, arbitrary units.

found to be dependent on the concentration of added L-OAS. In addition, the absorbance at 470 nm in the plateau phase was also dependent on the concentration of added L-OAS at lower concentrations. The absorbance in the plateau phase was scarcely altered for concentrations of L-OAS over 10 mM, indicating that all of the enzyme molecules were converted into α-aminoacrylate intermediates in this concentration range. However, the rate of increase in absorbance at 470 nm at 30 mM was much higher than that at 10 mM. The increasing rate (k) should be expressed as follows (35):

$$k = \frac{k_{max} \cdot [\text{L-OAS}]}{K + [\text{L-OAS}]}$$

where k_{max} is the value of k at a saturating L-OAS concentration and K is the dissociation constant of the complex between PLP-bound DcsD and L-OAS. Our spectroscopic analysis indicates that K is very high, which may be one of the reasons for the high K_m value of DcsD toward L-OAS.

When L-OAS (1 mM) was added to the DcsD solution, the absorbance at 470 nm initially increased to a maximum within a few minutes, whereas the absorbance at 412 nm decreased to a minimum (Fig. 7C). Following this, the absorbance at 470 nm gradually decreased again as the α-aminoacrylate intermediate degraded, whereas the absorbance at 412 nm gradually increased. This change occurred within 20 min, and the resulting spectrum corresponds with that of the enzyme-bound PLP. Compared with the results from similar experiments using *O*-acetylserine sulfhydrylases (36), the lifetime of the α-aminoacrylate intermediate in DcsD is obviously shorter than that in normal *O*-acetylserine sulfhydrylases. This may be another reason for the high K_m value of DcsD toward L-OAS.

When assayed in 250 mM potassium phosphate buffer (pH 5.0 to 9.0) in the presence of 100 mM L-OAS, L-OUS-synthesizing activity increased with an increase of pH (Fig. 8A). Alkaline pH conditions may be useful to increase the nucleophilicity of hydroxyurea via deprotonation. The kinetic parameters of DcsD for the synthesis of β-substituted L-alanine were determined in 250 mM potassium phosphate buffer at pH 8.0 (Table 1).

Substrate specificity of DcsD. In general, cystathionine β-synthase uses L-homocysteine as a nucleophilic reagent, whereas *O*-acetylserine sulfhydrylase uses sulfide. These enzymes are known to have broad specificity for the second substrate and have been suggested to catalyze the synthesis of various β-substituted L-alanine derivatives (17, 18, 26). *O*-Acetylserine sulfhydrylases are classified into two types, A and B, based on their amino acid sequences. In particular, *O*-acetylserine sulfhydrylase B is known to have high activity for the synthesis of *S*-sulfo-L-cysteine when thiosulfate is used as a nucleophilic reagent.

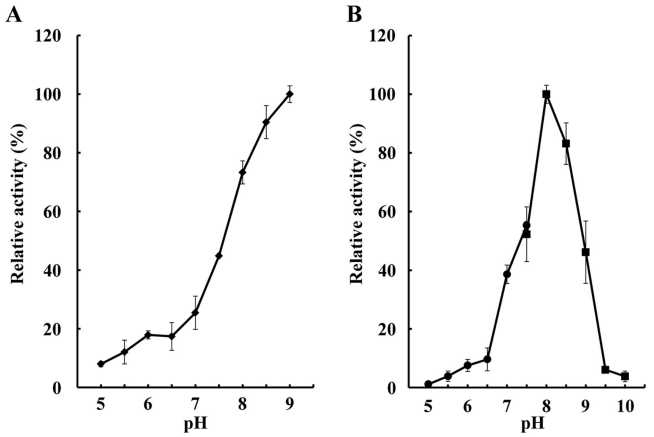

FIG 8 The pH dependencies of DcsD and DcsG. (A) D-OUS-synthesizing activity of DcsD; (B) D-CS-synthesizing activity of DcsG. Potassium phosphate buffer was used for the pH range from 5.0 to 9.0 in panel A, while MES-KOH and Tris-HCl buffers were used for the pH ranges from 5.0 to 7.5 and from 7.5 to 10.0, respectively, in panel B. Bars indicate the standard deviations.

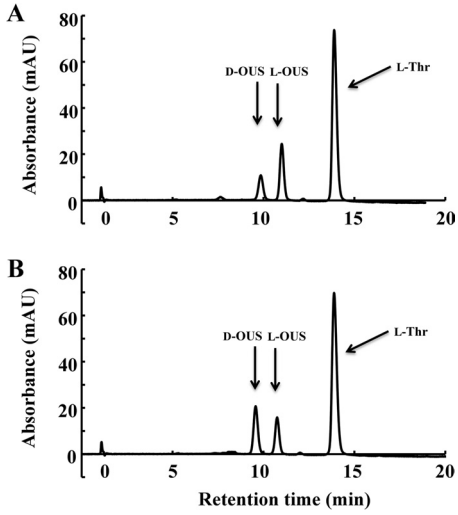

FIG 9 HPLC profile of reaction mixtures of DcsC after derivatization. DcsC was incubated with L-OUS (A) or D-OUS (B).

Using recombinant DcsD, we examined the specificity toward the second substrate. The reagents tested were hydroxyurea, sodium sulfide, sodium thiosulfate, and L-homocysteine, and the expected products were L-OUS, L-cysteine, *S*-sulfo-L-cysteine, and cystathionine, respectively. The TLC and MS analyses demonstrated that the expected products were formed in reaction mixtures consisting of L-OAS, the second substrate, and DcsD. The kinetic parameters for each substrate are summarized in Table 1. Based on the k_{cat}/K_m values (turnover rates), DcsD most prefers sulfide as the second substrate, followed by hydroxyurea, L-homocysteine, and thiosulfate.

Evaluation of DcsC activity. DcsC was recently shown to be a cofactor-independent racemase that strictly reacts with OUS (21). The activity of DcsC was confirmed through the measurement of the exchange of α-hydrogen of the substrate to deuterium or the change of the circular dichroism spectrum of the reaction mixture. In the present study, to evaluate DcsC activity, HPLC analysis was conducted after derivatization with *O*-phthaldialdehyde and *N*-acetyl-L-cysteine, which is the same method used for the evaluation of L-OUS synthesis by DcsD. We confirmed that DcsC can catalyze the configurational conversion of OUS in both the L- to D- and D- to L-directions (Fig. 9).

Evaluation of DcsG activity. As described above, DcsG catalyzes the conversion of D-OUS into D-CS in an Mg(II)- and ATP-dependent manner (Fig. 5D). Since the sequence homology search for DcsG suggested that the protein has an ATP-grasp motif, the reaction is expected to be coupled with the conversion of ATP to ADP. By using HPLC analysis, the stoichiometric ratio of synthesized D-CS to ADP was found to be approximately 1:1. In the first reaction step, DcsG is predicted to catalyze the formation of a D-OUS–phosphate intermediate accompanied by hydrolysis of ATP to ADP. Next, the N-δ atom of the D-OUS–phosphate intermediate would attack its own carbonyl carbon to form a five-membered ring, with the release of phosphate. It is worth noting that in the catalysis step, due to the activity of DcsG, a covalent bond is formed intramolecularly (Fig. 1) but not intermolecularly, as found in normal ATP-grasp enzymes. In addition, to synthesize D-CS, hydrolysis of the urea moiety must occur (Fig. 1).

Substrate specificity of DcsG. When the synthesis of D-CS from D-OUS by DcsG was measured at various pH values, activity was highest at pH 8.0 (Fig. 8B). To examine the substrate specificity of DcsG, an ADP release-coupled assay was performed with various substrates at pH 8.0 and 30°C (Table 2). The k_{cat}/K_m for D-OUS was $2.1 \times 10^5 \pm 0.3 \times 10^5$ M^{-1} s^{-1}. Furthermore, we confirmed that L-OUS was not a substrate for DcsG, suggesting that DcsG reacts exclusively with the D-amino acid.

Since D-alanyl–D-alanine ligase is classified with ATP-grasp enzymes, like DcsG, we investigated whether DcsG reacts with D-alanine, but no activity was detected with this substrate. When DcsG reacted with D-OUS, D-CS was formed, which is a cyclic D-amino acid analog with a five-membered ring and an amide linkage. We tried to clarify whether DcsG catalyzes the synthesis of cyclic D-amino acid analogs from other D-amino acids. β-Aminooxy-D-

TABLE 1 Kinetic parameters of DcsD

2nd substrate	Product	Mean K_m^{app} (mM)[a] ± SD	Mean k_{cat}^{app} (s^{-1})[a] ± SD	Mean k_{cat}/K_m (M^{-1} s^{-1}) ± SD	Mean k_{cat}/K_m for L-OAS (M^{-1} s^{-1})[b] ± SD
Hydroxyurea	L-OUS	9.0 ± 2.1	11 ± 1	1,200 ± 300	150 ± 10
Na_2S	L-Cysteine	0.12 ± 0.02	12 ± 1	100,000 ± 20,000	140 ± 20
$Na_2S_2O_3$	*S*-Sulfo-L-cysteine	22 ± 2	0.72 ± 0.03	33 ± 3	
L-Homocysteine	Cystathionine	ND[c]	ND[c]	190 ± 10	

[a] K_m^{app} and k_{cat}^{app} were determined in the presence of 100 mM L-OAS as the first substrate.
[b] k_{cat}/K_m for L-OAS was determined in the presence of 50 mM hydroxyurea or 1 mM sodium sulfide.
[c] Since K_m^{app} was too high, K_m^{app} and k_{cat}^{app} were not determined (ND).

TABLE 2 Kinetic parameters of DcsG

Substrate	Mean K_m (mM)[a] ± SD	Mean k_{cat} (s^{-1})[a] ± SD	Mean k_{cat}/K_m (M^{-1} s^{-1}) ± SD	Mean K_m^{app} for ATP (μM)[b] ± SD
D-OUS	10 ± 1	2,200 ± 200	210,000 ± 30,000	73 ± 8
β-Aminooxy-D-alanine	ND[c]	ND[c]	14,000 ± 1,000	37 ± 3
D-2,4-Diaminobutyrate	ND[c]	ND[c]	2,100 ± 100	45 ± 7
D-Ornithine	ND[c]	ND[c]	670 ± 40	45 ± 2
D-Homocysteine	19 ± 3	860 ± 60	45,000 ± 9,000	23 ± 6
D-Homoserine	ND[c]	ND[c]	6,100 ± 300	54 ± 10

[a] K_m and k_{cat} were determined in the presence of 5 mM ATP.
[b] K_m^{app} for ATP was determined in the presence of 50 mM D-amino acid.
[c] Since K_m was too high, K_m and k_{cat} were not determined (ND).

alanine, which lacks the carbamoyl group in D-OUS, was synthesized chemically by hydrolyzing D-CS (27). TLC, HPLC, and MS analyses showed that DcsG synthesizes D-CS from β-aminooxy-D-alanine. However, the k_{cat}/K_m value for this compound was shown to be an order of magnitude lower than that for D-OUS (Table 2).

Other reagents tested were D-asparagine, D-2,4-diaminobutyrate, D-ornithine, D-homoserine, and D-homocysteine. In the case of D-asparagine, the formation of D-amino-succinimide, which has a five-membered ring with an amide linkage, like D-CS, is expected. In the case of D-2,4-diaminobutyrate and D-ornithine, five- and six-membered rings with amide linkages would be produced, respectively. However, if DcsG reacts with D-homocysteine and D-homoserine, five-membered rings with ester and thioester linkages will be produced, respectively. On the basis of the ADP release-coupled assay, D-asparagine was not a substrate for DcsG, probably due to the low nucleophilicity of the amide group nitrogen. However, the assay indicated that the other compounds serve as substrates of DcsG. Furthermore, MS analysis demonstrated the generation of the monodehydrate products, probably due to ring formation. Ring formation in products was further confirmed by TLC and HPLC analyses, and the configuration was confirmed by using a chiral detector. The k_{cat}/K_m value for these compounds is lower than that for D-OUS (Table 2). These results indicated that DcsG most prefers D-OUS as a substrate, followed by D-homocysteine, β-aminooxy-D-alanine, D-homoserine, D-2,4-diaminobutyrate, and D-ornithine.

DISCUSSION

DcsD. The DcsD protein has a high level of sequence similarity with *O*-acetylserine sulfhydrylase and cystathionine β-synthase. The first substrate for *O*-acetylserine sulfhydrylase is L-OAS, whereas that for cystathionine β-synthase is L-serine. Both enzymes are known to be able to synthesize various derivatives of β-substituted L-alanine, depending on their specificities toward the second substrate (17, 18, 26). Cystathionine β-synthase, whose normal function is to generate cystathionine using L-homocysteine as the second substrate, is also known to synthesize L-cysteine by using sulfide (26), although the detailed substrate specificity of the enzyme has not been reported. In contrast, *O*-acetylserine sulfhydrylases have been reported to exhibit their highest activities for the synthesis of L-cysteine among the β-substituted L-alanine derivatives (17, 18).

Our present study shows that DcsD uses L-OAS, but not L-serine, as the first substrate. Furthermore, we found that DcsD catalyzes the synthesis of various β-substituted L-alanine derivatives, including L-OUS. The k_{cat}/K_m value of DcsD for L-cysteine synthesis is the highest, 80-fold higher than that for L-OUS synthesis (Table 1). The DcsD protein exhibits lower activities for the synthesis of *S*-sulfo-L-cysteine and cystathionine than for L-OUS synthesis. These results are in agreement with the fact that the amino acid sequence of DcsD is more homologous to that of *O*-acetylserine sulfhydrylase A than to those of *O*-acetylserine sulfhydrylase B and cystathionine β-synthase. In fact, when a sequence homology search for DcsD was done against structurally defined proteins, in order of similarity, *O*-acetylserine sulfhydrylase A, *O*-acetylserine sulfhydrylase B, and cystathionine β-synthase were retrieved. For example, the amino acid sequence of DcsD has 48% identity with that of *Mycobacterium tuberculosis O*-acetylserine sulfhydrylase A, 43% identity with that of *E. coli O*-acetylserine sulfhydrylase B, and 38% identity with that of the catalytic domain of human cystathionine β-synthase.

Based on the kinetic parameters shown in Table 1, DcsD would preferentially synthesize L-cysteine if hydroxyurea and sulfide coexisted at the same low concentrations. Although previous whole-genome sequence analyses suggested that *Streptomyces* species should possess a sulfate-reducing pathway to generate sulfide (37, 38), it has been reported that bacteria from this genus display low activity for the reduction of sulfate to sulfide (39). Therefore, in cells of the D-CS-producing organism *S. lavendulae*, the concentration of sulfide might be kept at a low level, such that DcsD would catalyze the synthesis of L-OUS to produce D-CS.

The *O*-acetylserine sulfhydrylase enzymes from plant sources were reported previously to display L-OUS-synthesizing activity (40–45). However, L-OUS-synthesizing activity is generally low even in the presence of a high concentration of hydroxyurea. Relative velocities of the enzymes from *Allium tuberosum* (45), *Brassica juncea* (42), *Citrullus vulgaris* (42), *Lathyrus latifolius* (44), and *Leucaena leucocephala* (43) for the synthesis of L-OUS are less than 1% of the velocity to synthesize L-cysteine. Exceptionally, although the enzyme from *Spinacia oleracea* (40) has a high rate of synthesis of L-OUS, it is only approximately 10% of its L-cysteine-synthesizing velocity. However, the rate of synthesis of L-OUS by DcsD in the presence of a high concentration of hydroxyurea is comparable to the rate of synthesis of L-cysteine. Therefore, we concluded that DcsD must be a specific enzyme for the synthesis of L-OUS in the D-CS biosynthetic pathway.

The kinetic and spectroscopic analyses of DcsD indicate that the enzyme has a low affinity for L-OAS and that the lifetime of the α-aminoacrylate intermediate formed in DcsD is short. These observations suggest that the active site of DcsD is more accessible to a solvent than that of *O*-acetylserine sulfhydrylases. Therefore,

DcsD may have a binding preference for the bulky compounds as the second substrate. In other words, DcsD may be better able to react with hydroxyurea, which is bulkier than sulfide, due to the greater accessibility of the substrate to the active site.

DcsG. The DcsG protein catalyzes the synthesis of D-CS from D-OUS, and the reaction includes covalent bond formation and hydrolysis of the urea moiety. At present, it is uncertain whether the urea moiety is hydrolyzed before or after the formation of the covalent N-C bond. However, since nucleophilic attack on the carbonyl carbon by the N-δ atom of D-OUS is not favored, the urea moiety of D-OUS may be hydrolyzed before the formation of the covalent N-C bond. Although DcsG also catalyzes the synthesis of D-CS from β-aminooxy-D-alanine, the k_{cat}/K_m value for this compound was 1 order of magnitude lower than that with D-OUS (Table 2). The carbamoyl group attached in D-OUS may be an important factor for DcsG to catalyze the reaction effectively. Specifically, the K_m for β-aminooxy-D-alanine was estimated to be much higher than that for D-OUS, suggesting that the affinity of DcsG toward β-aminooxy-D-alanine is lower than that toward D-OUS. The side chain of β-aminooxy-D-alanine is expected to exhibit a positive charge under physiological pH conditions, whereas the side chain of D-OUS is uncharged. The low K_m for D-OUS indicates that DcsG would preferentially bind to the D-amino acid having a neutral charge at the side chain. In addition, it indicates that the carbamoyl group may form hydrophilic interactions with the atoms in DcsG.

DcsG can catalyze the formation of five- and six-membered rings with amide linkages, from D-2,4-diaminobutyrate and D-ornithine, respectively. The k_{cat}/K_m values for these compounds are 2 to 3 orders of magnitude lower than that for D-OUS. In particular, D-2,4-diaminobutyrate was less reactive than β-aminooxy-D-alanine, indicating that reactivity of the terminal amino groups is dependent on their adjacent atoms (carbon in D-2,4-diaminobutyrate and oxygen in β-aminooxy-D-alanine). Furthermore, since D-ornithine was less reactive than D-2,4-diaminobutyrate, the normal function of DcsG is likely to form a five-membered ring. Similarly, the k_{cat}/K_m values for D-homocysteine and D-homoserine are 1 and 2 orders of magnitude lower than that for D-OUS, respectively, but higher than that for D-2,4-diaminobutyrate. The terminal group of D-homocysteine displays a neutral or negative charge at physiological pH, while that of D-homoserine is neutral. In contrast, the terminal group of D-2,4-diaminobutyrate exhibits a positive charge at physiological pH. Therefore, it was also suggested that DcsG appears to bind preferentially to substrates in which the terminal group does not display a positive charge.

Conclusion. In the present study, we were able to determine the enzymatic functions of the DcsC, DcsD, and DcsG proteins, which are responsible for D-CS biosynthesis. Another research group recently showed that DcsC is a cofactor-independent racemase utilizing OUS as the substrate (21). Based on the experimental evidence presented in this study, we conclude that DscD and DscG are an *O*-ureido-L-serine synthase and a D-cycloserine synthethase, respectively. Since the DcsG protein needs ATP as an energy source, it may be right to call it a synthetase.

Through the present and previous (14) studies, we have identified five enzymes responsible for D-CS biosynthesis, and studies are currently in progress to characterize DcsA, which may catalyze the conversion of L-arginine to N^{ω}-hydroxy-L-arginine (22). The functions of the *dcsF* and *dcsH* genes found in the D-CS biosynthetic operon are unknown at this moment, but no obvious role is apparent for either protein in D-CS biosynthesis. The results of sequence homology searches of DcsF suggest that the protein is a dehydrogenase harboring an NAD-binding motif. In contrast, DcsH seems to have an ATP-grasp fold motif, like DcsG and DcsI, and DcsH displays low sequence similarity with DcsG (16%) and DcsI (15%). To clarify the functions of DcsF and DcsH, the expression of the recombinant proteins in *E. coli* and gene disruption analyses are now under way.

By using the methods described here, we can now synthesize D-CS *in vitro* using commercially available L-OAS and hydroxyurea as substrates (Fig. 3B). Considering that many chemical or pharmaceutical companies have successfully synthesized D-CS in large quantities, our enzymatic D-CS production method may seem insignificant. However, by modifying the enzymes in the D-CS biosynthetic pathway, it may be possible to generate novel antibacterial compounds with the D-CS-like structure. Furthermore, our present study provides a new route for the production of amino acids and their analogs by using DcsC, DcsD, and DcsG.

In recent years, unnatural amino acids have been used frequently as building blocks for the development of new pharmaceutical drugs (18, 46). As shown in this study, although the substrate specificity of DcsC has been reported to be limited to OUS (21), those of DcsD and DcsG are very broad (Tables 1 and 2). DcsD may be useful to produce unnatural L-amino acids, while DcsG may be available as an enzyme to produce cyclic D-amino acid analogs and to separate L-amino acids from racemic mixtures. For example, by using DcsG together with inexpensive racemic homocysteine, it may be possible to produce both L-homocysteine and D-homocysteine thiolactone at a much lower cost than is available by current means.

DcsG is expected to possess a novel structure, since the protein has a low level of sequence similarity to structurally elucidated proteins of related function. The determination of the crystal structure of DcsG is under way. However, we can predict the structures of DcsC and DcsD. Mutation analysis based on the predicted structure could lead to the identification of the amino acid residues that are important for substrate specificity. Furthermore, by introducing site-specific variations based on the structural information, more useful enzymes exhibiting improved substrate specificity characteristics may be created. Mutation analyses based on the predicted structures of DcsC and DcsD are now under way.

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