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Kinetic and Mutational Studies of Three NifS Homologs from *Escherichia coli*: Mechanistic Difference between L-Cysteine Desulfurase and L-Selenocysteine Lyase Reactions[1]

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We have purified three NifS homologs from *Escherichia coli*, CSD, CsdB, and IscS, that appear to be involved in iron-sulfur cluster formation and/or the biosynthesis of selenophosphate. All three homologs catalyze the elimination of Se and S from L-selenocysteine and L-cysteine, respectively, to form L-alanine. These pyridoxal 5′-phosphate enzymes were inactivated by abortive transamination, yielding pyruvate and a pyridoxamine 5′-phosphate form of the enzyme. The enzymes showed non-Michaelis-Menten behavior for L-selenocysteine and L-cysteine. When pyruvate was added, they showed Michaelis-Menten behavior for L-selenocysteine but not for L-cysteine. Pyruvate significantly enhanced the activity of CSD toward L-selenocysteine. Surprisingly, the enzyme activity toward L-cysteine was not increased as much by pyruvate, suggesting the presence of different rate-limiting steps or reaction mechanisms for L-cysteine desulfurization and the degradation of L-selenocysteine. We substituted Ala for each of Cys358 in CSD, Cys364 in CsdB, and Cys328 in IscS, residues that correspond to the catalytically essential Cys325 of *Azotobacter vinelandii* NifS. The enzyme activity toward L-cysteine was almost completely abolished by the mutations, whereas the activity toward L-selenocysteine was much less affected. This indicates that the reaction mechanism of L-cysteine desulfurization is different from that of L-selenocysteine decomposition, and that the conserved cysteine residues play a critical role only in L-cysteine desulfurization.

Key words: cysteine desulfurase, NifS, pyridoxal enzyme, selenocysteine lyase, transamination.

Selenium is an essential trace element in mammals and other organisms. Selenoproteins, such as formate dehydrogenase (*1*) from *Escherichia coli*, contain selenocysteine residues, which play an essential role in catalysis. The chemical properties of selenium and sulfur are similar, and most enzymes cannot distinguish selenium compounds from their sulfur analogs (*2*). Nevertheless, several enzymes, such as selenophosphate synthetase (*3*), strictly distinguish these two elements. The enzymatic discrimination between selenium compounds and the corresponding sulfur compounds is important in order for cells to metabolize selenium compounds without disturbing sulfur metabolism. However, the mechanism for the enzymatic discrimination between these two congeneric elements has not yet been elucidated.

Selenocysteine lyase (SCL) catalyzes the elimination of selenium from L-selenocysteine to yield L-alanine (*4*, *5*). The enzyme has the ability to distinguish selenium and sulfur: it is specific for L-selenocysteine, and its activity toward L-cysteine is negligibly small. Recently, it was proposed that selenium derived from L-selenocysteine by the action of SCL serves as a substrate *in vivo* for selenophosphate synthetase, which catalyzes the production of selenophosphate, an essential precursor molecule in selenoprotein biosynthesis (*6*). We have determined the primary structure of SCL from mouse (*7*), and found that the enzyme shares sequence similarity with NifS, a cysteine desulfurase from *Azotobacter vinelandii*, which indiscriminately acts on L-selenocysteine and L-cysteine to produce selenium and sulfur, respectively, and L-alanine (*6*, *8*).

NifS catalyzes the desulfurization of L-cysteine to supply inorganic sulfur for the formation of iron-sulfur clusters in nitrogenase (*9*). Several proteins with sequence homology to NifS have been purified and characterized. IscSs from *A. vinelandii* (*10*) and *E. coli* (*11*) have cysteine desulfurase activity and are proposed to play a general role in the formation of iron-sulfur clusters in proteins other than nitrogenase. In addition to *iscS*, which encodes IscS, *E. coli* has two more *nifS*-related genes, *csdA* and *csdB* (*12*). The *csdA* gene product, CSD, has been shown to act on L-selenocysteine, L-cysteine, and L-cysteine sulfinate (*12*). The *csdB* gene product, CsdB, shows much higher activity toward L-selenocysteine than L-cysteine (290 times), and is similar to SCL in this respect (*13*). Although the physiological func-

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Abbreviations: KPB, potassium phosphate buffer; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SCL, selenocysteine lyase.

tions of these enzymes remain to be elucidated, it is reasonable to assume that they are involved in iron-sulfur cluster formation or the biosynthesis of selenophosphate.

A reaction mechanism for L-cysteine desulfurization catalyzed by NifS was proposed by Zheng *et al.* (*8*). In the proposed mechanism, the active-site residue, Cys325, first protonates the C4′ atom of the cysteine-pyridoxal quinonoid intermediate, and the thiol group is converted to a thiolate anion. The anion then attacks the sulfur atom of the substrate, L-cysteine, to yield the enzyme-bound persulfide intermediate (Scheme 1). The mutant NifS^{C325A}, in which Cys325 is replaced with Ala, shows no activity toward L-cysteine (*14*). The mutation also abolishes the enzyme activity toward L-selenocysteine. This cysteine residue is conserved among all NifS homologs analyzed so far, suggesting the critical role of this residue. However, in our previous experiment, we obtained an apparently conflicting result: we found that the conserved cysteine residue of CSD is not essential for the activity toward L-selenocysteine (*12*).

In the present study, we analyzed the reaction mechanisms of CSD, CsdB, and IscS from *E. coli* in order to elucidate how the enzyme discriminates between selenium and sulfur. Using pyruvate, which was found to regulate enzyme activity, we revealed that cysteine desulfurization and selenocysteine degradation proceed through different pathways. Moreover, by site-directed mutagenesis, we clarified that the conserved cysteine residues of these NifS homologs are essential only for cysteine desulfurization. This is the first report on the mechanism of enzymatic discrimination between selenium and sulfur.

EXPERIMENTAL PROCEDURES

Construction of an Expression Plasmid for IscS—The construct for the expression of *iscS* was made by PCR with the ordered clone No. 430 by Kohara (*15*). *Nde*I and *Hin*dIII restriction sites were incorporated into the 3′ and 5′ ends of the gene, respectively. The oligonucleotide primers used for the PCR were 5′-GGAATTCGGAGTTTATAGAGCACATATGAAATTACCGATT-3′ (upstream) and 5′-CCCAAGCTTAATGATGAGCCCATTCG-3′ (downstream). The 1.2-kbp product was digested and subcloned into pET21a-(+) to form pEF1. The amount of IscS produced corresponded to about 16% of the protein in the crude extract.

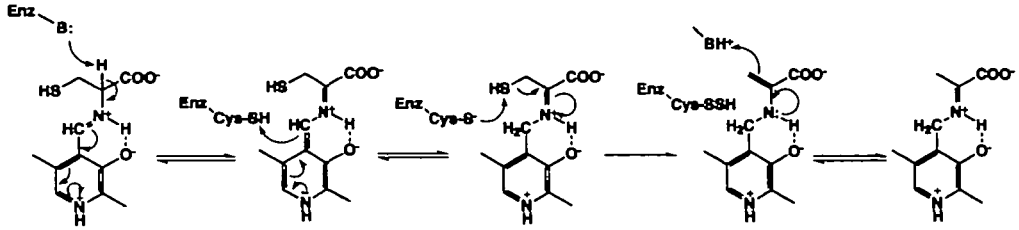
Mutagenesis—Mutations were introduced into pCSDB (*13*) and pEF1 for CsdB^{C364A} and IscS^{C328A}, respectively, by PCR according to the overlap extension method of Ito *et al.* (*16*). The following primers were used: 5′-GCATTGCGGCGTGATGTC-3′ and 5′-CATGATTACGAGTTCAGGAGGTGC-3′ for CsdB^{C364A} and 5′-GCTGACGTAGCCGCGGAACC-3′ and 5′-TAAGAAGGAGATATACGTATGAAATTA-3′ for IscS^{C328A}. DNA sequencing verified that the mutations were introduced only into the desired positions. The plasmid encoding CSD^{C358A} was constructed previously (*12*).

Purification of IscS—Potassium phosphate buffer (KPB) (pH 7.4) was used as the standard buffer throughout the purification. *E. coli* BL21(DE3) pLysS cells harboring pEF1 were grown in 0.8 liter of LB medium containing 100 μg/ml ampicillin at 37°C for 11.5 h. The culture was inoculated into 8 liters of medium containing ampicillin and then permitted to grow for 1 h. At this point, isopropyl-β-thiogalactoside (1 mM) was added. After 3 h the cells were harvested by centrifugation, frozen and thawed in 30 mM KPB (pH 7.4) containing 5 mM DTT, and disrupted by sonication.

The cell debris was removed by centrifugation, and the supernatant was fractionated with ammonium sulfate (25–65% saturation). The enzyme was dissolved in 30 mM KPB and applied to a Phenyl-Toyopearl column (3 × 19 cm). IscS was eluted with a 2.6-liter linear gradient of 0.65–0 M ammonium sulfate in buffer, pooled, and concentrated with ammonium sulfate (70% saturation). The enzyme was dissolved in 10-mM buffer, dialyzed against the same buffer, and applied to a DEAE-Toyopearl column (3 × 23 cm). IscS was eluted with a 1.6-liter linear gradient of 0–0.15 M NaCl in buffer. The active fractions were pooled and concentrated by ultrafiltration. IscS was dialyzed against 10-mM buffer containing 0.9 M ammonium sulfate and applied to a Butyl-Toyopearl column (3 × 17 cm). The enzyme was eluted with a 1.2-liter linear gradient of 0.9–0 M ammonium sulfate in buffer and concentrated by ultrafiltration. The enzyme was dialyzed against 5-mM buffer and applied to a Gigapite column (3 × 10 cm). The enzyme was eluted with a 0.75-liter linear gradient of 5–100 mM KPB and concentrated by ultrafiltration. The enzyme was dialyzed with 10-mM buffer and applied to a Superose 12 FPLC column (1 × 10 cm) (Amersham Pharmacia Biotech, Uppsala, Sweden).

Purified IscS was judged to be homogeneous by SDS-PAGE analysis. IscS did not have an associated protein, although the enzyme isolated from the non-overproducing strain copurified together with the acyl carrier protein bound to IscS *via* a disulfide linkage (*11*). The N-terminal sequence of the purified IscS was MKLPIYLDY, which matches the sequence deduced from the nucleotide sequence deposited in GenBank (accession number D90883). Recombinant IscS exhibited a specific activity of 0.38 unit/mg with 12 mM of L-cysteine. The previously reported value for native IscS was 0.078 unit/mg (*11*). This difference in specific activity is probably due to differences in the assay conditions (buffer, pH, and substrate concentration used).

Purification of Other Proteins—CSD and CsdB were purified as described previously (*12*, *13*). CSD^{C358A} and IscS^{C328A} were purified by ammonium sulfate fractionation



Scheme 1. **Proposed reaction mechanism for the desulfurization of L-cysteine catalyzed by *A. vinelandii* NifS (*8*).**

and chromatographies on DEAE-Toyopearl and Phenyl-Toyopearl columns. CsdB^{C364A} was purified by chromatographies on DEAE-Toyopearl, Phenyl-Toyopearl, and Superose 12 columns. Buffers and elution conditions used for chromatographies were identical to those for the purification of the wild-type enzyme.

Enzyme Assays—All enzymatic activities were assayed in 0.12 M Tricine-NaOH (pH 7.5). The standard reaction mixture contained 50 mM DTT, 0.2 mM PLP, 0.12 M Tricine-NaOH (pH 7.5), the enzyme, and the substrate amino acid. The enzymatic activities toward L-selenocysteine and L-cysteine were measured as described previously (*12*). DTT was omitted from the reaction mixture for the assay of desulfination of L-cysteine sulfinate, and the sulfite produced was determined with fuchsin (*17*). The production of alanine from the substrates was determined with a Beckman high-performance amino-acid analyzer 7300 (Beckman Coulter, Fullerton, CA). Specific activity was expressed as units/mg of protein, with 1 unit of enzyme defined as the amount that catalyzed the formation of 1 μmol of product in 1 min. The concentration of the purified enzymes was determined spectrophotometrically (*18*) using $A^{1\%}_{280}$ = 11.3 (CSD), 11.2 (CsdB), and 9.15 (IscS).

Pyruvate Assays—A reaction mixture consisting of 92 nmol of enzyme, 20 μmol of KPB (pH 7.4), and 8 μmol of L-cysteine sulfinate in a total volume of 0.4 ml was incubated at 37°C for 24 h. The reaction mixture was loaded onto Microcon-10, and the products were separated from the enzyme by ultrafiltration. The sample (50 μl) was added to 25 μl of 0.066% 2,4-dinitrophenylhydrazine in 2 M HCl and incubated for 5 min at ambient temperature. The solution was neutralized by adding 30 μl of 5 M NaOH, and the absorbance at 520 nm was recorded. The pyruvate concentration was calculated based on a standard curve.

Analytical Methods—The formation of PMP in the enzyme reactions was monitored as follows: 10 mM L-cysteine sulfinate was added to the enzymes in 10 mM KPB (pH 7.4), and the increase in the absorbance at 325 nm, showing the presence of PMP, was monitored (*19*). N-terminal amino-acid sequences were determined using a Shimadzu PPSQ-10 protein sequencer (Shimadzu, Kyoto). The nucleotide sequences were confirmed with a Dye Terminator sequencing kit FS (Perkin-Elmer) and an Applied Biosystems 373A DNA sequencer.

RESULTS

CSD, CsdB, and IscS Catalyze Abortive Transamination—Incubation of CSD with L-selenocysteine in the absence of PLP led to time-dependent inactivation as shown in Fig. 1A (●). The presence of PLP prevented inactivation (Fig. 1A, ○). When PLP was added to the inactivated enzyme, the enzyme regained its original catalytic activity (Fig. 1A, △). This result indicates that CSD undergoes reversible inactivation. Similar results were obtained using L-cysteine sulfinate as a substrate (data not shown). CsdB and IscS were also inactivated by incubation with L-selenocysteine in the absence of added PLP (Fig. 1B, ●; 1C, ●). The addition of PLP restored enzyme activity, showing that CsdB and IscS also undergo reversible inactivation.

Several pyridoxal enzymes are known to catalyze abortive transamination as a side reaction, where the substrates (amino acids) are converted into α-keto acids (Scheme 2). This reaction results in the conversion of PLP into PMP and the inactivation of the enzyme. During the 24-h incubation of CSD, CsdB, and IscS in the presence of L-cysteine sulfinate without added PLP, the following amounts of pyruvate were found to be produced: 0.3 mol/mol-CSD, 0.1 mol/mol-CsdB, and 0.4 mol/mol-IscS. The formation of PMP was confirmed spectroscopically as described in "EXPERIMENTAL PROCEDURES" (CSD, Fig. 2; CsdB and IscS, data not shown). The formation of PMP and pyruvate indicates that the enzymes are inactivated by abortive transamination (Scheme 2).

Activation of CSD, CsdB, and IscS by Pyruvate—Abortive transamination may be reversed or prevented by adding α-keto acids due to reverse transamination between the added α-keto acid and the PMP-enzyme to yield the PLP-enzyme. Figure 1A shows that the addition of pyruvate to inactivated CSD leads to more activation than the addition

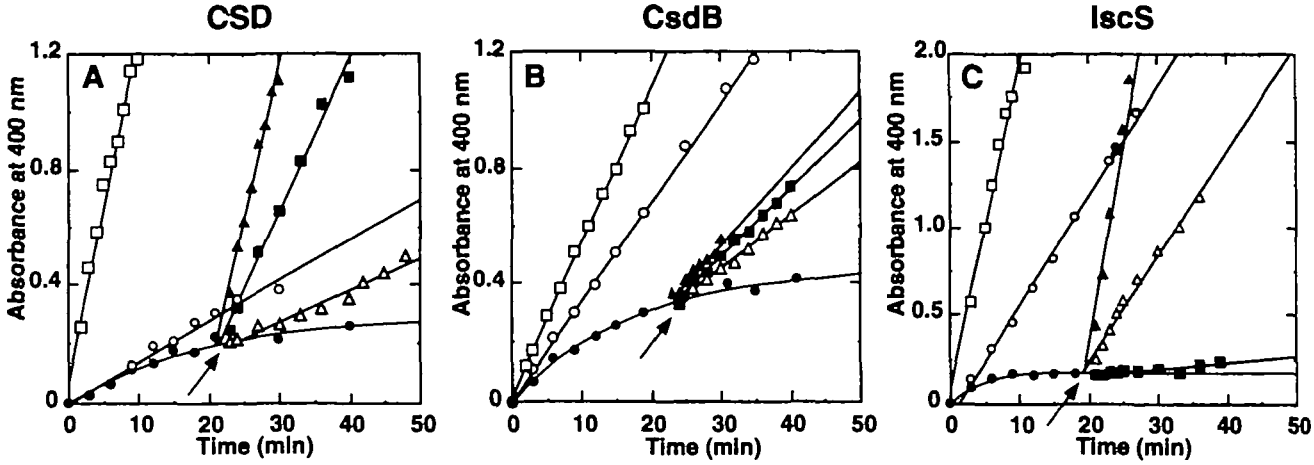


Fig. 1. **Effects of PLP and pyruvate on the activities of CSD, CsdB, and IscS.** Effects of the addition of PLP and pyruvate on the reactions catalyzed by CSD (A), CsdB (B), and IscS (C) are shown. The reaction mixture contained 5 mM L-selenocysteine, 50 mM DTT, and 0.12 M Tricine-NaOH (pH 7.5). The reactions were carried out under the following conditions: no additives (●), in the presence of 0.1 mM PLP (○), and in the presence of 0.1 mM PLP plus 1 mM pyruvate (□). At the point indicated by the arrow, 0.1 mM PLP (△), 1 mM pyruvate (■), or 0.1 mM PLP plus 1 mM pyruvate (▲) was added.

of PLP (Fig. 1A, ■). The reaction rate was found to culminate by the addition of pyruvate plus PLP (Fig. 1A, ▲, □). Pyruvate plus PLP also increased the rate of the reactions catalyzed by CsdB and IscS (Fig. 1B, ▲, □; 1C, ▲, □). The addition of pyruvate to the inactivated CsdB and IscS caused different levels of reversion of activity: the activating effect of pyruvate on CsdB was greater than that of PLP (Fig. 1B, ■, △), whereas PLP was found to lead to greater activation of IscS than pyruvate (Fig. 1C, ■, △).

In order to determine whether pyruvate endogenously formed from L-selenocysteine through abortive transamination has an influence on the rate of the selenocysteine lyase reaction catalyzed by CSD, lactate dehydrogenase and NADH were added to the mixture to deplete endogenous pyruvate (*20*). The depletion markedly decreased the rate of selenocysteine decomposition (data not shown). Therefore, pyruvate formed *via* abortive transamination plays a role in alleviating the inactivation.

Figure 3 shows the effect of added pyruvate on the initial rates of enzymatic selenocysteine decomposition. The results clearly show that the enzyme activity is enhanced by the addition of pyruvate, and the extent of the activation of each of the three enzymes differs significantly: the initial rate of the reaction increased 12-fold (CSD), 1.5-fold (CsdB), and 7-fold (IscS) by the addition of 1 mM pyruvate. The same concentration of pyruvate also accelerated the enzymatic desulfination of L-cysteine sulfinate (initial concentration: 12 mM): ~4-fold (CSD), ~2-fold (CsdB), and ~2-fold (IscS). However, the effect of pyruvate on the desulfurization of L-cysteine (60 mM) catalyzed by CSD, CsdB, and IscS was much less, only ~1.2-fold activation was observed for all three enzymes.

Relationship between Substrate Concentration and Reaction Velocity—Steady-state kinetic analyses of the reactions catalyzed by CSD, CsdB, and IscS were performed with L-selenocysteine, L-cysteine sulfinate, and L-cysteine as substrates in the absence or presence of added pyruvate. All the data obtained are shown in Figs. 4 and 5. CSD shows anomalous behavior with L-selenocysteine in the absence of pyruvate (Fig. 5A, ●). The plot of the initial velocity in the absence of pyruvate *vs.* selenocysteine concentration for CSD is a hyperbolic curve at low substrate concentrations and an upward curve at higher substrate levels (Fig. 4A, inset). No anomalous kinetic characteristics of CSD with L-selenocysteine were observed in our previous study (*12*). This is because the L-selenocysteine concentration varied from 0.2 to 2.5 mM in that study, and the anomalous feature is observed only above 2.5 mM (Fig. 4A). The addition

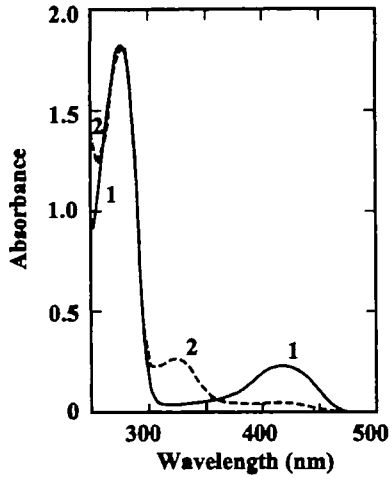
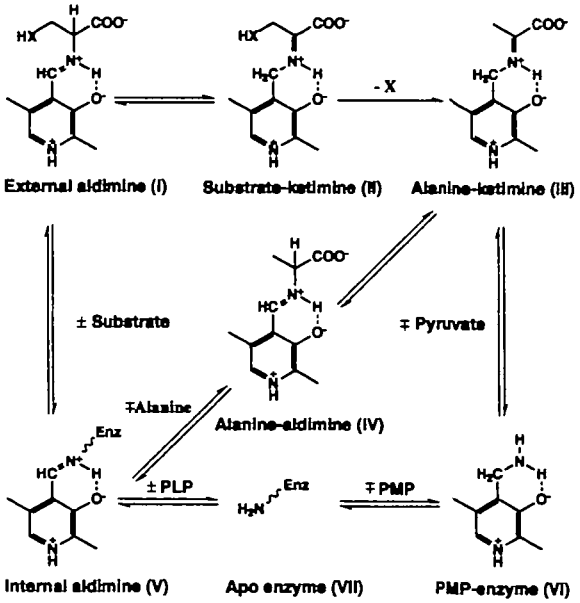


Fig. 2. **Spectral change in CSD during the catalytic reaction.** Curve 1, spectrum of the holoenzyme (1.6 mg/ml in 10 mM KPB, pH 7.4); curve 2, spectrum of the enzyme shown in Curve 1 after 12-h incubation with 10 mM L-cysteine sulfinate. The spectrum of Curve 2 was normalized by adjusting the peak absorbance at 280 nm to that in Curve 1.



Scheme 2. **A possible path for the main reaction and side-transamination catalyzed by *E. coli* NifS homologs (X = Se, S, SO_2), aspartate β-decarboxylase (X = CO_2), and kynureninase (HX = 2-aminobenzoyl).**

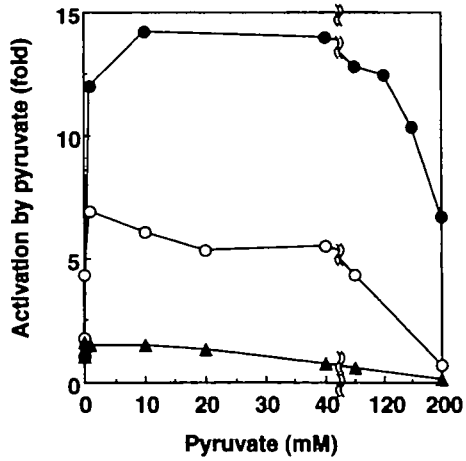


Fig. 3. **Effect of pyruvate concentration on the initial rates of elimination of selenium from L-selenocysteine.** The reaction mixture contained 5 mM L-selenocysteine, 50 mM DTT, 0.2 mM PLP, 0.12 M Tricine-NaOH buffer (pH 7.5), and pyruvate as indicated. Initial rates of the reactions by CSD (●), CsdB (▲), and IscS (○) are shown as relative activity, where the specific activity of each enzyme in the absence of added pyruvate (CSD: 3.4 units/mg, CsdB: 2.6 units/mg, IscS: 1.7 units/mg) is set at 1.

of 1 mM pyruvate made the enzyme show apparent Michaelis-Menten behavior (Figs. 4A, ○; 5A, ○). CsdB and IscS also exhibited non-Michaelis-Menten behavior with L-selenocysteine in the absence of pyruvate and Michaelis-Menten behavior in the presence of pyruvate (Fig. 5, B and C). In short, pyruvate not only increases the activity of CSD, CsdB, and IscS but also changes the kinetic behavior of these enzymes with L-selenocysteine.

Unusual behavior of CSD was also observed using L-cysteine sulfinate as a substrate. A remarkable downward curvature was observed in the double reciprocal plot for CSD with L-cysteine sulfinate in the absence of pyruvate (Fig. 5D), indicating the presence of negative cooperativity. Again, Michaelis-Menten kinetics was observed when pyruvate was added to the reaction mixture (Fig. 5D). In contrast, the double reciprocal plots for CsdB and IscS with L-cysteine sulfinate show only a slight, if any, deviation from Michaelis-Menten kinetics irrespective of the absence or presence of pyruvate (Fig. 5, E and F).

The kinetic behaviors of CSD and CsdB with L-cysteine are similar with: upward curvatures are seen in the double reciprocal plots (Fig. 5, G and H). The plots of the initial velocity *vs.* cysteine concentration of each enzyme (Fig. 4,G and H) are all similar to that for *A. vinelandii* NifS (*6*). Therefore, this type of kinetic behavior with L-cysteine is a common feature among several NifS homologs and is not likely to be due to a technical problem with our assay. These enzymes are probably inhibited by cysteine at high concentrations. In contrast, the double reciprocal plot for IscS with L-cysteine exhibits a downward curvature (Fig. 5I). It is notable that the addition of pyruvate to the cysteine desulfurase reactions caused no significant changes in the kinetic behaviors of any of these enzymes.

Apparent kinetic constants were obtained when the data

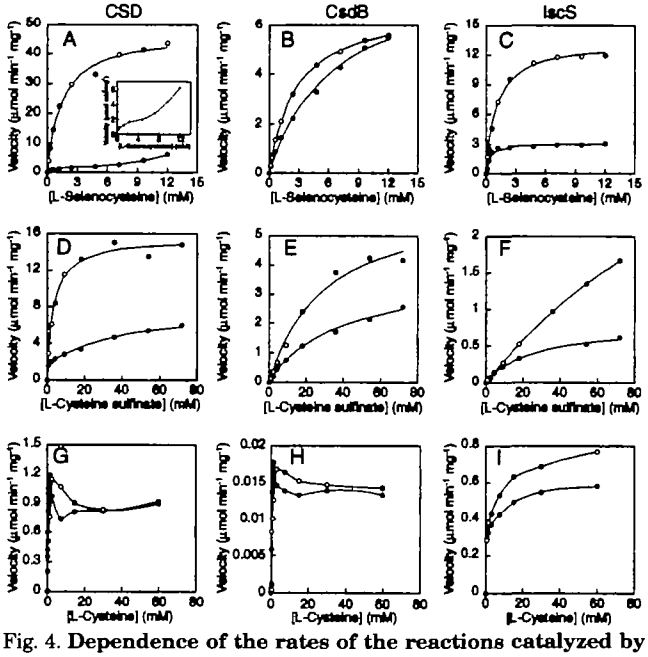


Fig. 4. **Dependence of the rates of the reactions catalyzed by CSD, CsdB, and IscS on substrate concentration.** Assays were performed at 37°C in 0.12 M Tricine-NaOH (pH 7.5), 50 mM DTT, and 0.2 mM PLP in the absence (●) or presence (○) of 1 mM pyruvate. L-Selenocysteine (A, B, C), L-cysteine sulfinate (D, E, F), and L-cysteine (G, H, I) were used as substrates, and the productions of selenide, sulfite, and sulfide were determined, respectively, as described in "EXPERIMENTAL PROCEDURES." The plots for CSD (A, D, G), CsdB (B, E, H), and IscS (C, F, I) are shown. The lines represent the best fits generated with KaleidaGraph (Synergy Software) based on the equation $V = V_{max}^{app}[S]/(K_m^{app} + [S])$, except that the lines in panels G–I are added to guide the eye and have no theoretical significance. Inset: the plot is magnified to show the anomalous kinetic behavior of CSD with L-selenocysteine in the absence of added pyruvate. The lines are added to guide the eye and have no theoretical significance.

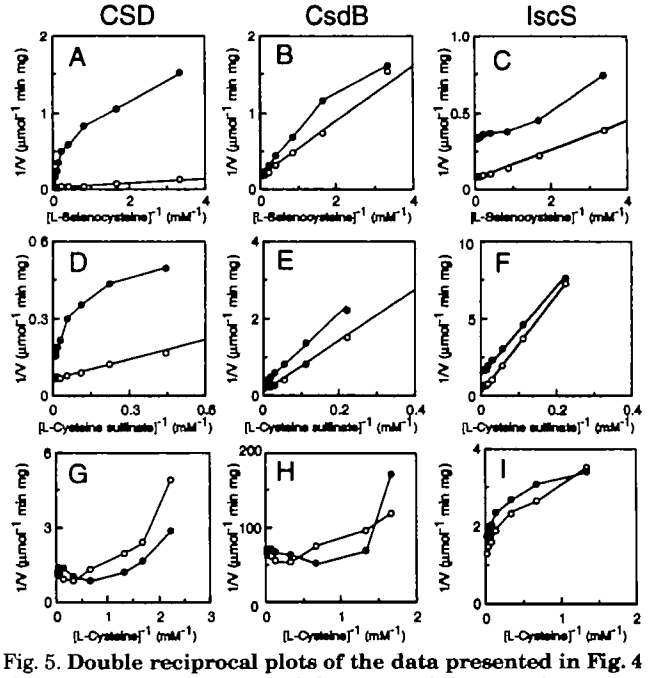


Fig. 5. **Double reciprocal plots of the data presented in Fig. 4 showing the dependence of the rates of the reactions catalyzed by CSD, CsdB, and IscS on substrate concentration.** L-Selenocysteine (A, B, C), L-cysteine sulfinate (D, E, F), and L-cysteine (G, H, I) were used as substrates. The plots for CSD (A, D, G), CsdB (B, E, H), and IscS (C, F, I) are shown. The linear lines are drawn only when there is linearity with a correlation coefficient greater than 0.99. Other lines are added to guide the eye and have no theoretical significance.

TABLE I. **Apparent kinetic constants.**

Substrate	CSD				CsdB				IscS			
	None		+Pyruvate[a]		None		+Pyruvate[a]		None		+Pyruvate[a]	
	V_{max}^{app} (units/mg)	K_m^{app} (mM)	V_{max}^{app} (units/mg)	K_m^{app} (mM)	V_{max}^{app} (units/mg)	K_m^{app} (mM)	V_{max}^{app} (units/mg)	K_m^{app} (mM)	V_{max}^{app} (units/mg)	K_m^{app} (mM)	V_{max}^{app} (units/mg)	K_m^{app} (mM)
L-Selenocysteine	–[b]	–[b]	47	1.4	–[b]	–[b]	6.8	2.6	–[b]	–[b]	13	1.1
L-Cysteine sulfinate	–[b]	–[b]	16	3.5	3.8	39	6.4	30	0.80	26	6.2	190

[a]Assays were performed in the presence of 1 mM pyruvate. [b]Not determined because of the anomalous kinetic behavior.

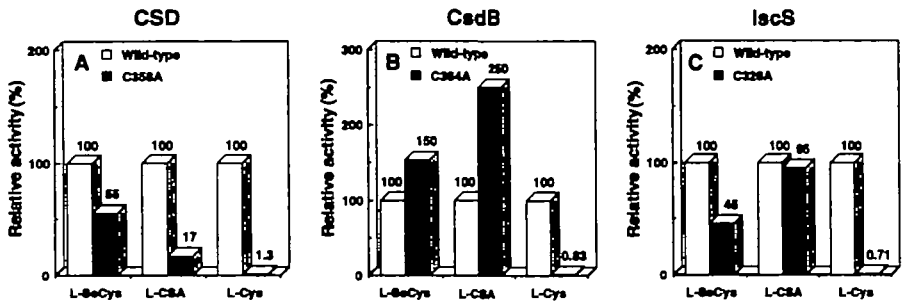
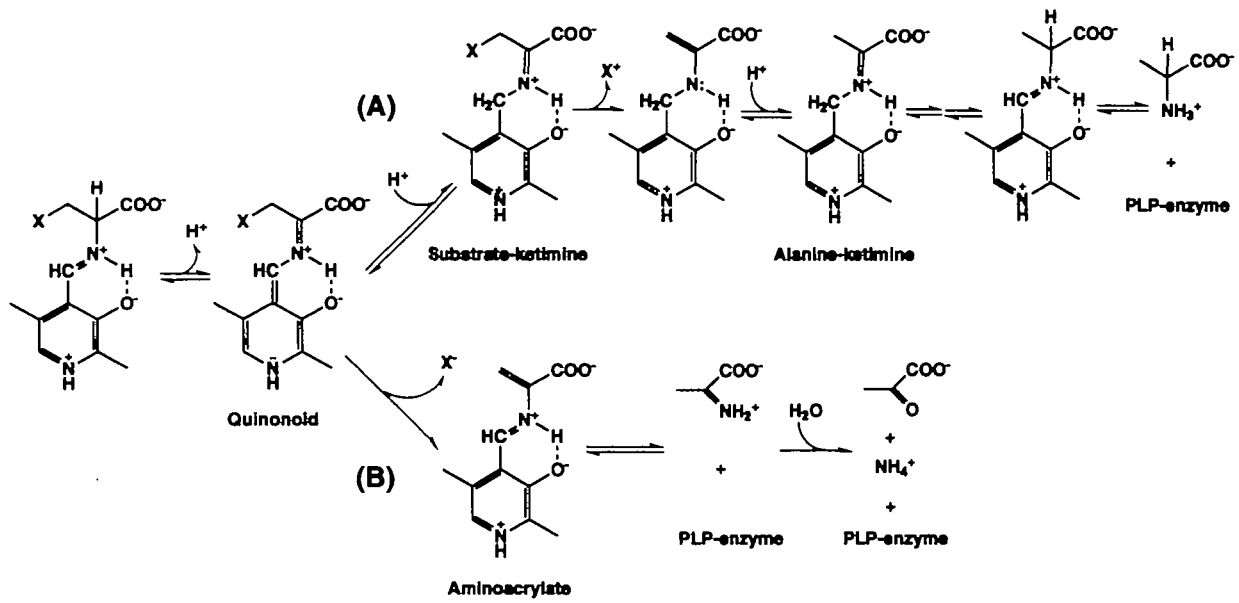


Fig. 6. **Activities of the wild-type and Cys → Ala mutant CSD (A), CsdB (B), and IscS (C).** The specific activities were determined in a reaction mixture containing 12 mM L-selenocysteine (L-SeCys), 90 mM L-cysteine sulfinate (L-CSA), or 60 mM L-cysteine (L-Cys) as a substrate. The specific activity of the wild-type enzyme with each substrate is set at 100%. The number above each column represents the relative activity. Wild-type and mutant enzyme activities are shown in striped and black bars, respectively.



Scheme 3. **Mechanisms for the elimination of β-substituent of α-amino acid catalyzed PLP-dependent enzymes.**

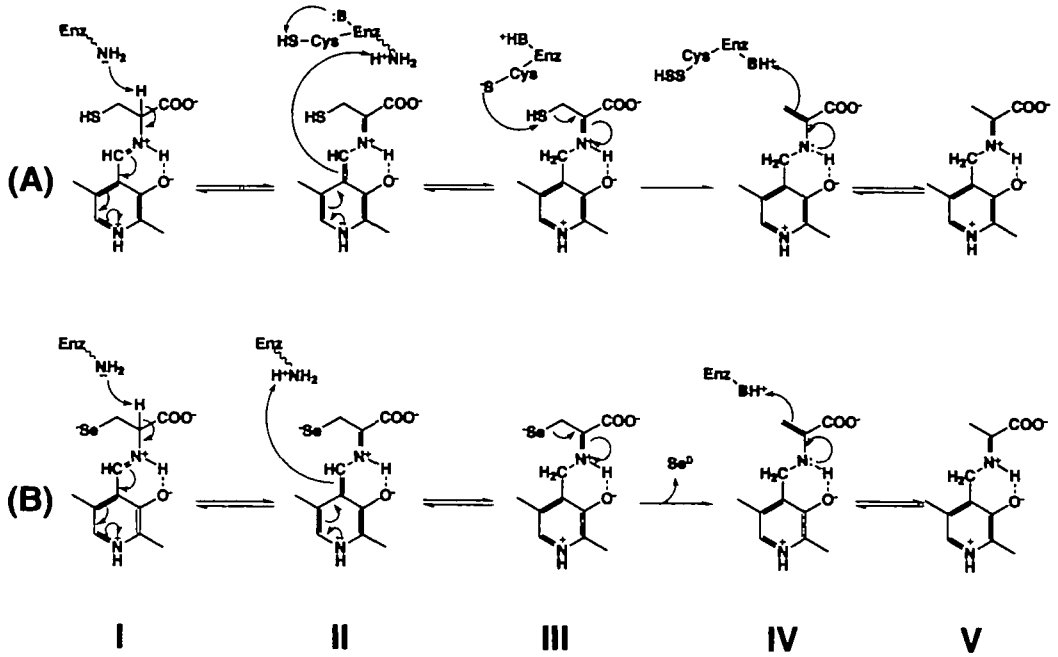
fit the equation $V = V_{max}^{app}$ [S]/(K_m^{app} + [S]). These constants are summarized in Table I. Because of the anomalous relationships between the substrate concentration and velocity of the reaction described above, several values could not be determined. Difficulties in determining the kinetic parameters of NifS and IscS from *A. vinelandii* were also noted by Lacourciere and Stadtman (*6*) and Zheng *et al.* (*8*, *10*).

In our previous study, the kinetic parameters for the desulfination of L-cysteine sulfinate by CSD were determined by the coupled-assay system using alanine dehydrogenase, in which a significant amount of pyruvate is formed from alanine. The activities toward L-selenocysteine and L-cysteine, on the other hand, were assayed by another method that does not produce pyruvate. Since the desulfination catalyzed by CSD is activated by pyruvate (Fig. 4D), the desulfination activity was overestimated in the coupled-assay system. The present study, which did not employ the coupled-assay system for L-cysteine sulfinate, shows that CSD preferentially catalyzes the decomposition of L-selenocysteine as well as the desulfination of L-cysteine sulfinate (Fig. 4, A and D).

Role of the Cysteine Residue—The cysteine residue conserved among NifS homologs (*12*, *14*) has been suggested to play a crucial role in catalysis (Scheme 1) (*8*). Because the conserved Cys325 of *A. vinelandii* NifS has been reported to be essential for activity toward both L-cysteine and L-selenocysteine (*8*), the degradation of these substrates was thought to proceed through the same mechanism (*6*). However, in our previous study, we found that Cys358 in CSD, which corresponds to Cys325 in *A. vinelandii* NifS, is not essential for the decomposition of L-selenocysteine (*12*). In the present work, we substituted Ala for the conserved Cys of CsdB and IscS by site-directed mutagenesis. The mutant enzymes, CSD^{C358A}, $CsdB^{C364A}$, and $IscS^{C328A}$ were purified, and their activities were determined with L-selenocysteine, L-cysteine sulfinate, and L-cysteine. The activities of all enzymes toward L-cysteine were lost almost completely by the mutation (Fig. 6). However, we found that the conserved cysteine residues are not essential for catalytic activity toward L-selenocysteine and L-cysteine sulfinate (Fig. 6). This demonstrates that the conserved cysteine residue plays a crucial role only in the decomposition of L-cysteine. These results suggest that L-cysteine desulfurization and L-selenocysteine/L-cysteine sulfinate decomposition proceed through different mechanisms.

DISCUSSION

Cysteine desulfurase, SCL, kynureninase (*21*), and aspartate β-decarboxylase (*22*) catalyze the electrophilic dis-



Scheme 4. **Proposed reaction mechanisms for the cysteine desulfurase reaction (A) and the selenocysteine lyase reaction (B) catalyzed by CSD, CsdB, and IscS.**

placement of the substituent at C_β of α-amino acids to yield L-alanine (Scheme 3A), whereas other β-lyases and β-synthases catalyze the elimination of a β-substituent to generate α,β-unsaturated aldimine (generally the aminoacrylate-PLP Schiff base) leading to the formation of pyruvate and ammonia (Scheme 3B). Recent studies on the catalytic mechanism of kynureninase from *Pseudomonas fluorescens* (*23*, *24*) have demonstrated the existence of both quinonoid and ketimine intermediates in the reaction. Evidence for the ketimine intermediate has also been presented for the catalytic mechanism of cystathionine γ-synthase (*25*). Thus the reactions catalyzed by NifS homologs and other PLP-dependent enzymes whose reactions involve the formation of β-carbanionic intermediates are thought to proceed *via* a ketimine intermediate (*25*) (Scheme 3A).

In addition to their main reactions, kynureninase and aspartate β-decarboxylase catalyze transamination as a side reaction (*26*, *27*). We have shown in the present study that CSD, CsdB, and IscS also catalyze abortive transamination, where the alanine-ketimine intermediate (Scheme 2, **III**) is hydrolyzed to yield pyruvate and the PMP-enzyme (**VI**). If the affinity between the enzyme and PMP is low, the PMP-enzyme releases PMP to become the apo-enzyme (**VII**). The addition of pyruvate and PLP alleviates the inactivation (Fig. 1), probably by shifting the equilibrium between **III** and **VI** to the formation of **III** and by converting the apo-enzyme to the holo-enzyme (**V**), respectively. The addition of PLP to the inactivated IscS had a much greater activating effect than the addition of pyruvate (Fig. 1C). It is likely that the inactivated IscS easily releases PMP to become an apo-form that is not converted to the active form by the addition of pyruvate. In contrast, added pyruvate had a greater effect on CSD and CsdB than PLP (Fig. 1, A and B). These enzymes probably have much higher affinities for PMP so that the added pyruvate can be utilized to convert the PMP-enzyme (**VI**) to the alanine-ketimine intermediate (**III**). The dissociation constant of the apo-enzyme for PMP and the rate constant of the conversion between **III** and **VI** vary for each enzyme, and thus the effects of pyruvate on CSD, CsdB, and IscS are different.

Kinetic characterization revealed that most of the reactions catalyzed by the NifS homologs in the absence of added pyruvate exhibit non-Michaelis-Menten features (Fig. 5). Non-Michaelis-Menten behavior has also been observed for a variety of enzymes (*28–32*). A line that curves downward near the 1/*V* axis of the double reciprocal plot (Fig. 5) is found in the case of enzyme reactions regulated by negative cooperativity (*28–30*). One model explaining negative cooperativity involves an induced allosteric response, in which high-affinity binding of the first ligand to one subunit of the dimer elicits a conformational transition in the other subunit, thus reducing the affinity for subsequent ligands (*31*). In another model, known as the hysteretic kinetic model (*32*), anomalous behavior is the consequence of slow isomerization between two forms of an enzyme that differ from each other in their catalytic properties. It is difficult, however, to account for the kinetic behavior of the NifS homologs until further data concerning the structural basis for the reactions is obtained. We are now trying to solve the three-dimensional structures of these enzymes (*13*, *33*).

Cys325 in NifS from *A. vinelandii* has been reported by Zheng *et al.* (*8*) to be essential for the activity toward both L-cysteine and L-selenocysteine. They proposed that the cysteinyl thiolate anion derived from Cys325 performs a nucleophilic attack on the sulfur atom of a substrate-PLP adduct, resulting in the formation of a cysteinyl persulfide, as shown in Scheme 1. In this reaction, Cys325 is believed to protonate the C4′-atom of the PLP. The L-selenocysteine lyase reaction was thought to proceed through a similar mechanism (*6*). However, the present study demonstrates that Cys358 in CSD, Cys364 in CsdB, and Cys328 in IscS,

which correspond to Cys325 in *A. vinelandii* NifS, are not required for the activity toward L-selenocysteine and L-cysteine sulfinate in the present study (Fig. 6). The finding that Cys328 in IscS is not essential is rather unexpected because IscS exhibits high-sequence similarity to *A. vinelandii* NifS (44% identity).

One of the important consequences of this observation is that the cysteine residue is not involved in the protonation of the C4′-atom of the quinonoid intermediate, at least in the mutant enzyme with no active-site cysteine residue. This is probably also the case for wild-type CsdB, whose Cys364 has been shown to be located far from the C4′-atom of the PLP bound to the enzyme by X-ray crystallographic analysis (*33*). The protonation is probably carried out by the lysine residue bound to PLP (Scheme 4A) as in the case of aspartate aminotransferase (*34*).

Our results clearly demonstrate that the conserved cysteine residue is essential for cysteine desulfurase activity but not absolutely required for the activities toward L-selenocysteine and L-cysteine sulfinate (Fig. 6). Although it is possible that the decompositions of L-selenocysteine and L-cysteine sulfinate proceed through a mechanism similar to that shown in Scheme 4A in the wild-type enzyme, a catalytic reaction not involving the cysteine residue must take place at least in the mutant enzyme that has no active-site cysteine residue. Scheme 4B illustrates a possible mechanism for the decomposition of L-selenocysteine that does not require a catalytic cysteine residue. The selenohydryl group of L-selenocysteine is probably deprotonated and present in an anionic form (Scheme 4B, **I**) because the pK_a of the selenohydryl group is 5.2, which is much lower than the pH of the reaction mixture (pH 7.4) (*35, 36*). The deprotonation of the selenohydryl group may be facilitated by His55, which has been shown to be located in the active site by X-ray crystallographic analysis (*33*). Subsequently, selenium is released spontaneously from intermediate **III** (Scheme 4B). Desulfination from L-cysteine sulfinate may also proceed through a mechanism similar to that in Scheme 4B without the aid of the active-site cysteine residue, as seen for the mechanism of desulfination of L-cysteine sulfinate catalyzed by aspartate β-decarboxylase (*37*).

One of the intriguing results in this study is that the addition of pyruvate to CSD greatly increases the rate of degradation of L-selenocysteine and L-cysteine sulfinate, but not that of L-cysteine (Fig. 4, A, D, and G). This result is explained by assuming that the mechanism or rate-limiting step for the desulfurization of L-cysteine is different from that for the decomposition of L-selenocysteine and L-cysteine sulfinate. Flint (*11*) suggested that the rate-limiting step in the desulfurization of L-cysteine catalyzed by IscS is the restoration of the cysteine residue from the cysteine persulfide formed in the active sites. If this is the case for CSD and pyruvate does not facilitate the restoration of the cysteine residue, no activating effect of pyruvate on cysteine desulfurization should be observed. This is not the case for the degradations of L-selenocysteine and L-cysteine sulfinate, which do not require the cysteine residue at the active site. Accordingly, pyruvate would have a significant activating effect only on the decomposition of L-selenocysteine and L-cysteine sulfinate.

In conclusion, our present study indicates that the mechanisms for L-cysteine desulfurization and the degradation of L-selenocysteine and L-cysteine sulfinate by NifS homologs are different. The conserved cysteine residue plays an essential role only in L-cysteine desulfurization. This implies that a particular NifS homolog can function as an SCL if the location of the conserved cysteine residue in the active site of the enzyme is not suitable for cysteine desulfurization. The active-site structures of NifS homologs with various substrate specificities are now being analyzed by X-ray crystallography to clarify the relationship between the location of the conserved cysteine residue and the substrate specificity of the enzyme.

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