D-Selenocystine α,β-Lyase of *Clostridium Sticklandii*

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SUMMARY

We have found a novel enzyme that catalyzes α,β-elimination of D-selenocystine to produce pyruvate, ammonia and elemental selenium in *Clostridium sticklandii* cells, and named it D-selenocystine α,β-lyase. The enzyme purified to homogeneity from *C. sticklandii* has a molecular weight of about 74,000, and consists of two subunits identical in molecular weight (35,000). The enzyme requires pyridoxal 5'-phosphate as a coenzyme. In addition to D-selenocystine, D-cystine, D-lanthionine, mesolanthionine, and D-cysteine serve as substrate, but D-selenocysteine is inert. D-Selenocystine α,β-lyase catalyzes also β-replacement reaction between D-selenocystine and various thiols to produce the corresponding *S*-substituted D-cysteines. Selenols also act as substituent donors for D-cystine to produce *Se*-substituted D-selenocysteines.

INTRODUCTION

Various selenium-dependent enzymes contain selenocysteine (2-amino-3-hydroselenopropionic acid) residue in the polypeptide chain. In nature, various other selenium amino acids occur, and most are physiologically active. However, little is known about the metabolism of selenium amino acids. We have studied the selenocysteine synthesis in rat liver[1], and found a novel pyridoxal 5'-phosphate (pyridoxal-P) enzyme that decomposes exclusively L-selenocysteine into L-alanine and elemental selenium to name it L-selenocysteine β-lyase.[2] The enzyme occurs in various mammalian tissues[2] and bacterial cells;[3] the enzyme has been purified to homogeneity from pig liver[2] and *Citrobacter freundii*.[4] During the course of study of L-selenocysteine β-lyase distribution in anaerobic bacteria, we have found that not only L-selenocysteine but also D-selenocystine is completely degraded by the extracts of a few clostridial strains. The decomposition of D-selenocystine has been subsequently found due to the action of a novel pyridoxal-P enzyme, D-selenocystine α,β-lyase. We have chosen *C. sticklandii* ATTC 12662, in which the enzyme occurs most abundantly, for the present study. We have describe the purification of D-selenocysteine α,β-lyase and its enzymological properties.

METHODS

Enzyme purification was carried out as follows. *Step 1 and 2*: The dialyzed extract was applied to a DEAE-Toyopearl 650M (Toyo Soda Manufacturing, Tokyo, Japan) column (8.0 × 60 cm) equilibrated with the buffer. The enzyme was eluted with a linear gradient of KCl (0 to 0.2 M) in the same buffer (2.0 1 each) at a flow rate of 500 ml/h. Active fractions were combined, and protein was precipitated with ammonium sulfate at a final concentration of 80% saturation. The precipitate was dissolved in a small volume of the buffer. *Step 3*: The enzyme solution was applied to a

Butyl-Toyopearl 650M (Toyo Soda Manufacturing, Tokyo, Japan) column (6.0 × 30 cm) equilibrated with the buffer supplemented with 14% ammonium sulfate. The column was washed with 3.0 l of this buffer. The enzyme was eluted with a linear gradient of ammonium sulfate (14–0%) in the buffer (2.0 l each) at a flow rate of 500 ml/min. The active fractions were combined, and concentrated by precipitation with ammonium sulfate at a final concentration of 80% saturation. The precipitate was dissolved in a small volume of the buffer. *Step 4*: The enzyme solution was applied to a Cellulofine GLC 2000 (Chisso Inc., Tokyo, Japan) column (3.0 × 130 cm). The enzyme was chromatographed with 20mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 20 μM pyridoxal-P at a flow rate of 9 ml/h. The active fractions were combined, and concentrated with an Amicon PM10 membrane. *Step 5*: The enzyme solution was applied to a Mono Q HR 10/10 column of the Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The enzyme was eluted with a linear gradient of NaCl (0.3–0.5 M) in 10 mM Bistris propane (pH 8.2) containing 0.01% 2-mercaptoethanol at a flow rate of 1 ml/min. The active fractions were combined, and concentrated with a Centricon 30 (Amicon). *Step 6*: The enzyme solution was applied to a TSK G3000 SW column (7.53 × 600 mm). The enzyme was eluted with 50 mM potassium phosphate buffer (pH 6.8) containing 0.01% 2-mercaptoethanol, 10 μM pyridoxal-P, and 200 mM NaCl at a flow rate of 18 ml/h. The active fractions were combined and concentrated with centricon 30. *Step 7*: The enzyme solution was applied to an Ultron 300X column (4.6 × 150 mm, Shinwa Kako Inc., Kyoto, Japan). The enzyme was eluted with a linear gradient of NaCl (0–0.8 M) in 20 mM potassium phosphate buffer (pH 6.5) containing 0.01% 2-mercaptoethanol at a flow rate of 1.0 ml/min. The active fractions were combined and concentrated with a Centricon 30. *Step 8*: The enzyme was subjected to preparative slab gel electrophoresis (Davis, 1964) to remove a trace amount of impurity protein. The gel was crashed with a Teflon homogenizer, and the enzyme was extracted with 10 mM potassium phosphate buffer (pH 7.2). After centrifugation, the enzyme was concentrated with a Centricon 30, and chromatographed in the same manner as Step 6. The enzymatic α,β-elimination of D-selenocystine was followed by the method of Katsuki *et al.*[5] The standard reaction mixture contained 1.6 μmol of D-selenocystine, 100 μmol of Tricine/NaOH buffer (pH 8.0), 50 nmol of pyridoxal-P, and enzyme in a final volume of 0.5 ml, and was incubated at 37°C for 5 min. For the β-replacement reaction, the reaction system consisted of 0.5 μmol of D-selenocystine, 0.7 mmol of a thiol, 50 nmol of pyridoxal-P, 100 μmol of Tricine/NaOH (pH 8.0) and the enzyme in a final volume of 0.5 ml. After incubation at 37°C for 20 min, *S*-substituted cysteines produced were determined with a Hitachi high performance amino acid analyzer model 835. Protein was determined by the method of Lowry *et al.*[6] with bovine serum albumin as a standard. H_2Se was determined with lead acetate as described previously.[2] Elemental selenium was determined in the same manner after reduction with dithiothreitol to H_2Se. Pyruvate was determined with lactate dehydrogenase or alanine dehydrogenase in the following reaction mixture (1 ml) at 25°C: lactate dehydrogenase (2 units), potassium phosphate buffer (pH 7.2) (100 μmol), and NADH (4 μmol); alanine dehydrogenase (4 units), CHES buffer (pH 10.0) (100 μmol), NH_4Cl (100

μmol), and NADH (4 μmol). Ammonia also was determined spectrophotometrically with glutamate dehydrogenase. The reaction mixture contained 100 μmol of Tricine/NaOH (pH 8.0) 20 μmol of α-ketoglutarate, 4 μmol of NADH, and 2 units of glutamate dehydrogenase. The molar absorption coefficient of NADH at 340 nm,6,220 M^{-1} cm^{-1}, was used for the calculations.

RESULTS

Enzymatic Cleavage of D-*Selenocystine*

When D-selenocystine was incubated with an extract of *C. sticklandii* ATCC12662, pyruvate, ammonia, and red elemental selenium were produced. Balance studies showed that 1.58 μmol of pyruvate, 1.63 μmol of ammonia, and 1.47 μmol of elemental selenium were produced from 0.75 μmol of D-selenocystine. We have termed the enzyme D-selenocystine α,β-lyase.

Physicochemical Properties

The results of enzyme purification are summarized in Table 1. The purified enzyme was found to be homogeneous by sodium lauryl sulfate slab gel electrophoresis. The molecular weight of the enzyme was estimated to be about 74,000 by gel permeation chromatography with a TSK G3000 SW column (7.53 × 600 mm) (Toyo Soda Manufacturing, Tokyo, Japan). Polyacrylamide gel electrophoresis in the presence of 0.1% sodium lauryl sulfate gave a single protein band that had an estimated molecular weight of 35,000. These results suggest that the enzyme is composed of two subunits with identical molecular weight. The isoelectric point of the enzyme was determined to be 4.5 with a FPLC MonoP HR 5/20 column (Pharmacia). The enzyme showed the maximum activity at pH 8.0 for both α,β-elimination and β-replacement (see below) reactions when assayed in Tricine NaOH (pH 7.5–9.0).

Cofactor

We incubated the enzyme with 25 mM hydroxylamine at 25°C for 30 min, and then dialyzed it against 1,000 volumes of the standard buffer (see above) at 4°C for 10 h. The enzyme thus treated had no activity in the absence of added pyridoxal-P. However, activity was restored more than 95% by addition of 20 μM pyridoxal-P. Thus, D-selenocystine α,β-lyase requires pyridoxal-P as a coenzyme, and pyridoxal-P binds to the enzyme through a Schiff base as found for other pyridoxal-P enzymes so far studied.

Substrate Specificity

D-Selenocystine served as the best substrate (relative Vmax, 100%; Km, 1.0 mM). D-cystine (79%, 0.65 mM), D-lanthionine (40%, 0.91 mM), and meso-lanthionine (28%, 2.0 mM) were also effective substrates. Although D-cysteine underwent α,β-elmininationslowly, it showed the highest affinity (Km=0.11 mM) for the enzyme. However, we observed a strong substrate inhibition by D-cysteine at concentrations more than 0.67 mM. D-Selenocysteine was found to be inert when examined at various substrate concentrations (0.1–25 mM). L-Isomers of selenocystine, selenocysteine, cystine, and cysteine were not the substrates.

Table 1. Purification of D-Selenocystine α, β-lyase[a]

Step	Total protein (mg)	Total activity (μmol/min.)	Specific activity (μmol/min./mg)	Yield (%)
1. Extract[b]	32,600	2,490	0.076	100
2. DEAE-Toyopearl	3,850	1,870	0.48	75
3. Butyl-Toyopearl	629	1,580	2.5	63
4. Cellulofine	189	925	4.9	37
5. FPLC MonoQ	29	340	12	14
6. TSK G3000 SW	6	272	45	11
7. Ultron 300X	0.8	181	230	7
8. Gel Electrophoresis	0.28	124	440	5

[a]Bacteria were grown in a medium containing 2% trypton, 1% yeast extract, 0.15% sodium formate, 0.18% KH_2PO_4, and 0.014% sodium thioglycolate in tap water (pH 7.0) at 37°C. Cells were sonicated at 4°C for 5 min. All operations for purification were performed at 0 to 5°C unless otherwise stated. Potassium phosphate buffer (20 mM, pH 7.2) containing 0.01% 2-mercaptoethanol, 20 μM pyridoxal-P, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM *p*-toluenesulfonyl L-phenylalanine chloromethyl ketone, and 1 mM EDTA was used as the standard buffer. [b]*C. sticklandii* cells (wet weight, 700 g) were suspended in 1,500 ml of the buffer, and sonicated.

β-Replacement Reaction

The enzyme catalyzes the β-replacement reaction between D-selenocystine and various thiols to yield the corresponding *S*-substituted D-cysteines. The relative activities of thiols are as follows: ethanethiol, 100%; benzyl thioalcohol, 379; 2-mercaptoethanol, 2,950. The products were quantitatively deaminated with D-amino acid oxidase; they are D-isomers.

DISCUSSION

Various pyridoxal-P enzymes that catalyze elimination and replacement reactions have been purified and characterized.[7] However, most act specifically on the L-isomers of amino acids. Only three pyridoxal-P enzymes acting on D-amino acids have been found: D-serine dehydratase, β-chloro-D-alanine hydrogenchloridelyase, and D-cysteine desulfhydrase.[7,8] D-Selenocystine α, β-lyase is unique in acting on the D-enantiomer of selenium amino acids as the best substrate.

We have shown that D-selenocystine α, β-lyase can decompose D-cysteine also, though slowly. D-cysteine desulfhydrase of *Chlorella fusca*[9] and spinach leaves[10] are dufferebt from D-selenocystine α, β-lyase in the substrate specificity; the plant enzymes do not act on D-cystine. D-Selenocystine α, β-lyase resembles D-cysteine desulfhydrase of *E. coli* in molecular weight, subunit structure, and other physicochemical properties. However, the *E. coli* enzyme is significantly different from D-selenocystine α, β-lyase in the relative activity for D-cystine and D-selenocystine. The reactivity of D-selenocystine corresponds to only 28% of that of D-cystine for the

E. coli enzyme, whereas D-selenocystine is α,β-eliminated 1.3-fold higher than D-cystine by D-selenocystine α,β-lyase. Alkanethiols act as good *S*-substituent donors in β-replacement reaction catalyzed by D-selenocystine α,β-lyase, but are inactive for the *E. coli* enzyme. Thus, D-selenocysine α,β-lyase is a new enzyme.

REFERENCES

1. Esaki, N., Nakamura, T., Tanaka, H., Suzuki, T., Morino, Y. and Soda, K. (1981): Enzymatic synthesis of selenocysteine in rat liver. Biochemistry, 20, 4492–4496.
2. Esaki, N., Nakamura, T., Tanaka, H. and Soda, K. (1982): Selenocysteine lyase, a novel enzyme that specifically acts on selenocysteine. J. Biol. Chem., 257, 4386–4391.
3. Chocat, P., Esaki, N., Nakamura, T., Tanaka, H. and Soda, K. (1983): Microbial distribution of selenocysteine lyase. J. Bacteriol., 156, 455–457.
4. Chocat, P., Esaki, N., Tanizawa, K., Nakamura, K., Tanaka, H. and Soda, K. (1985): Purification and characterization of selenocysteine β-lyase from *Citrobacter freundii*. J. Bacteriol., 163, 669–676.
5. Katsuki, H., Yoshida, T., Tanegashima, C. and Tanaka, S. (1971): Improved direct method for determination of keto acids by 2, 4–dinitrophenylhydrazine. Anal. Biochem., 43, 349–356.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193 265–275.
7. Miles, E. W. (1986): Pyridoxal phosphate enzymes catalyzing β-elimination and β-replacement reactions. in Vitamin B6. Pyridoxal Phosphate. Chemical, Biochemical, and Medical Aspects, Part B., ed. by Dolphin, D., Poulson, R. and Avramovic, O., John Wiley & Sons, New York, pp.253–310.
8. Nagasawa, T., Ishii, T., Kumagai, H. and Yamada, H. (1985): D-Cysteine desulfhydrase of *Escherichia coli*, purification and characterization. Eur. J. Biochem., 153, 541–551.
9. Schmidt, A. and Erdle, I. (1983): A cysteine desulfhydrase specific for D-Cysteine from the green alga *Chlorella fusca*. Z. Naturforsch. Sec. C Biosci., 38, 428–435.
10. Schmidt, A. (1987): D-Cysteine desulfhydrase from spinach. Methods Enzymol., 143, 449–453.