0273–2289/85/1103–0071$02.40

REVIEW

Synthesis of Optically Active Sulfur and Selenium Amino Acids with Microbial Enzymes

HIDEHIKO TANAKA, NOBUYOSHI ESAKI, AND KENJI SODA*

Institute for Chemical Research, Kyoto University, Uji Kyoto-Fu 611, Japan

Index Entries: Synthesis, of S- and Se-amino acids; optical activity, synthesis of S- and Se-amino acids with; sulfur amino acids, synthesis of optically active; selenium amino acids, synthesis of optically active; amino acids, synthesis of optically active S- and Se-; microbial enzymes, in synthesis of optically active amino acids; enzymes, microbial, in synthesis of optically active amino acids.

A variety of sulfur compounds occur in nature, and most are physiologically active. Methionine, cysteine, and some other sulfur amino acids particularly play an important metabolic role. For example, L-methionine is not only an essential amino acid, but also the main methyl donor in various methyltransferase systems through the intermediary *S*-adenosyl-L-methionine. It is also an important precursor of polyamines and ethylene, a plant hormone. A large amount of DL-methionine is widely used as an additive in soybean meal and other sulfur amino acid-deficient feed. Methionine, cysteine, and others are very important also as starting materials for pharmaceuticals, cosmetics, and other chemicals. Various L-amino acids, including glutamate and lysine, are at present

*Author to whom all correspondence and reprint requests should be addressed.

produced with bacteria from simple carbon and nitrogen sources. Sulfur amino acids, however, have been produced microbially with little success (*1*).

Selenium is a metallic, and also nonmetallic, element as a homolog of sulfur and tellurium. It is recognized as an essential micronutrient for mammals, fish, and several bacteria, although toxic in a high concentration. Organic selenium compounds including selenium amino acids in general are chemically and biologically active. Selenocysteine (2-amino-3-hydroselenopropionic acid) and selenomethionine [2-amino-4-(methylseleno)butyric acid] have been demonstrated in polypeptide chains of several mammalian and bacterial enzymes, such as glutathione peroxidase of rat liver, glycine reductase of *Clostridium sticklandii*, and thiolase of *Cl. kluyveri* (*2*). Selenium amino acids have been reported to give protection to animals from radiation injury and carcinogenesis. However, no facile synthetic procedures of optically active selenium amino acids have been available. Recently, the enzymatic methods of producing various amino acids (*e.g.*, L-lysine from DL-α-amino-ε-caprolactam, and L-tryptophan from L-serine and indole) have been extensively studied, and several have been industrialized (*1*). We here review the feasible synthesis of the optically active sulfur and selenium amino acids with microbial enzymes, a hydrolytic one and pyridoxal 5′-phosphate (pyridoxal-P)-containing ones.

PRODUCTION OF OPTICALLY ACTIVE CYSTEINE

2-Amino-Δ^2-thiazoline-4-carboxylate Hydrolase

Sano et al. (*3*) have found that several bacteria are capable of producing L-cysteine from DL-2-amino-Δ^2-thiazoline-4-carboxylate (ATC), an intermediate in the chemical synthesis of DL-cysteine. The bacteria include several strains of *Pseudomonas, Escherichia coli, Bacillus brevis,* and *Micrococcus sodenensis. Pseudomonas thiazolinophilum* isolated from soil is the most active. The L-cysteine-forming activity is enhanced by addition of DL-ATC to the medium. Probably three enzymes, L-ATC hydrolase, *S*-carbamoyl-L-cysteine hydrolase, and ATC racemase, participate in the L-cysteine production (Fig. 1), although their enzymological aspects have not been elucidated. Optimum conditions for the production of L-cysteine have been studied with the reaction system (pH 8.2) containing 1% DL-ATC and intact cells of *Ps. thiazolinophilum* (*4*).

The degradation of L-cysteine by L-cysteine desulfhydrase or other pyridoxal-P enzymes in the cells is successfully inhibited by addition of hydroxylamine or semicarbazide. The addition of detergents such as cetyltrimethyl ammonium chloride and sodium lauryl sulfate, and a native cell lytic factor also increases L-cysteine production. These compounds probably increase permeability of the cell membrane to DL-ATC. Thus, 6.1 mg/mL of L-cysteine is produced from 10 mg/mL of DL-ATC

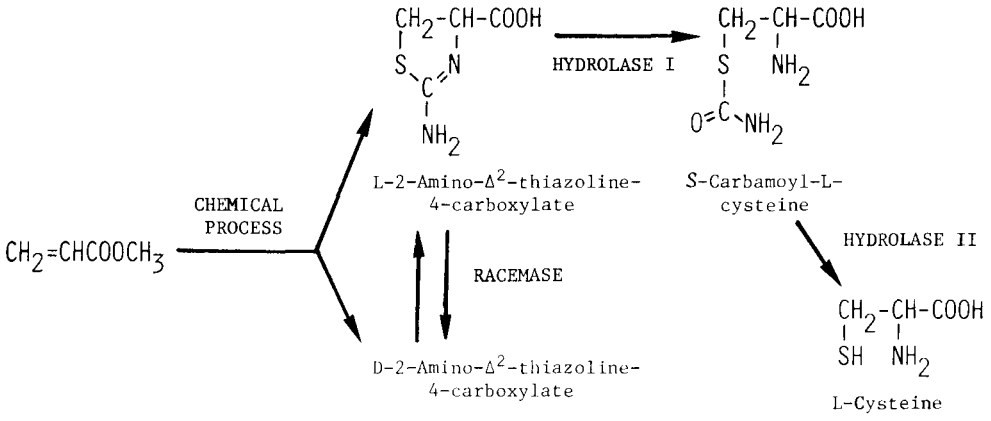


Fig. 1. Enzymatic synthesis of L-cysteine from D-2-amino-Δ^2-thiazoline-4-carboxylate.

with a molar yield of 100% under optimum conditions. When a mutant of *Ps. thiazolinophilum* lacking L-cysteine-decomposing enzymes is used, 31.4 mg/mL of L-cysteine is obtained (*5*).

Cysteine Desulfhydrase

L-Cysteine hydrogensulfide lyase (deaminating) (cysteine desulfhydrase, EC 4.4.1.1), which contains pyridoxal-P as a cofactor, catalyzes the α,β-elimination of L-cysteine as follows.

$$HSCH_2CH(NH_2)COOH + H_2O \rightarrow H_2S + CH_3COCOOH + NH_3$$

The enzyme occurs widely in bacteria (especially *Enterobacteriaceae*), yeasts, and plants, and has been purified to homogeneity from *Salmonella typhimurium* (*6*) and *Aerobacter aerogenes* (*7*). The enzyme is produced inducibly by addition of L-cysteine to the medium, and has molecular weights of approximately 229,000 (*S. typhimurium*) and 252,000 (*A. aerogenes*). It consists of six subunits with identical molecular weights, and contains 6 mol pyridoxal-P/mol enzyme.

In addition to L-cysteine, L-serine, *S*-methyl-L-cysteine, and L-tryptophan serve as substrates in the α,β-elimination. β-Chloro-L-alanine also is α,β-eliminated with 167% of the elimination rate of L-cysteine (*7*).

The enzyme catalyzes also the β-replacement reaction between L-alanine and various thiols to produce the corresponding *S*-substituted L-cysteines (*8*) (Table 1). The reverse reaction of α,β-elimination also is catalyzed; *S*-alkyl-L-cysteines are produced from pyruvate, ammonia, and alkane thiols (*9*). The optimum pH for the reverse reaction is 10.0.

Kredich et al. (*10*) have found that the amount of pyruvate from L-cysteine is about 20% of that of hydrogen sulfide produced. Based on this finding, they have proposed a mechanism for cysteine desulfhydrase

TABLE 1
Relative Velocity of Synthesis of L-Cysteine-Relate d Amino Acids from β-Chloro-L-alanine and Thiol Compounds by Cysteine Desulfhydrase

Thiol compound	Relative rate
Hydrogen sulfide	100
Methanethiol	0.6
Ethanethiol	2.0
2-Propanethiol	0.49
2-Propene-1-thiol	7.2
1-Butanethiol	0.13
2-Methyl-1-propanethiol	0.09
1-Methyl-1-propanethiol	0.05
2-Methyl-2-propanethiol	0.04
Benzenethiol	0.02
Toluenethiol	0.17

reaction in which a pyridoxal-P-α-aminoacrylate Schiff base reacts with L-cysteine to form α-methyl-α,γ-thiazoline dicarboxylic acid (Fig. 2).

Yamada and Kumagai (*11*) have studied the enzymatic synthesis of L-cysteine from β-chloro-L-alanine by replacement reaction with resting

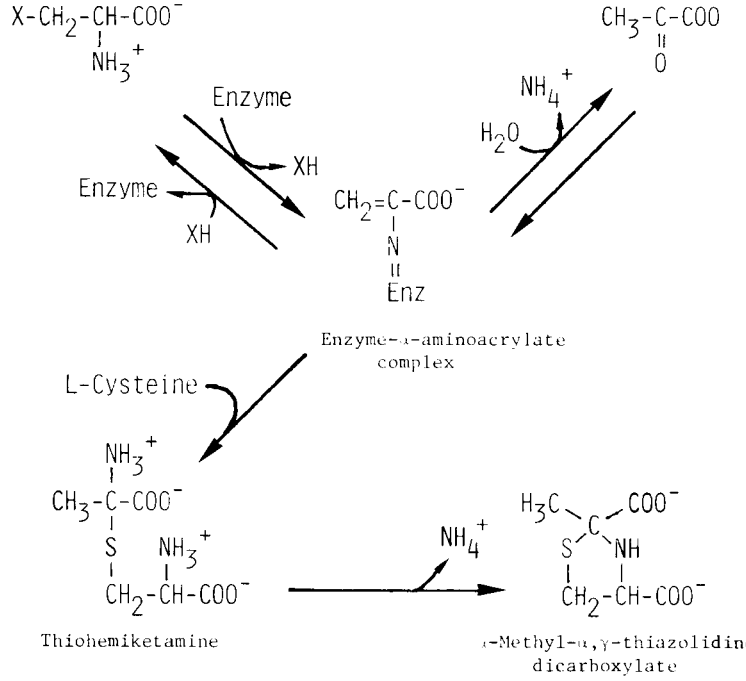


Fig. 2. Schematic representation of the mechanism for the formation of α-methyl-α,γ-thiazolidine dicarboxylate catalyzed by cysteine desulfhydrase.

cells of *Enterobacter cloaceae*, which was selected as the best producer of cysteine desulfhydrase. L-Cysteine is produced effectively by addition of acetone to the reaction system, which prevents L-cysteine from producing an adduct with the α-aminoacrylate intermediate. L-Cysteine is synthesized at a concentration of 5.1 g/dL with a molar yield of more than 80%. L-Cysteine is readily isolated in a form of L-cystine after oxidation with $FeCl_3$.

β-Chloro-D-alanine Chloride Lyase

Growth of various microorganisms is inhibited by β-chloro-DL-alanine. Kaczorowski et al. (*12*) have shown that β-chloro-D-alanine rapidly inactivates an active transport system coupled with a membrane-bound D-alanine dehydrogenase in *E. coli*. Manning et al (*13*) have suggested that the antibacterial action of β-chloro-D-alanine results from the inactivation of D-amino acid transaminase and alanine racemase, which results in the inhibition of biosynthesis of the peptidoglycan layer of cell wall.

Recently, Ohkishi et al. (*14*) isolated bacterial strains resistant to β-chloro-DL-alanine, and found the occurrence of a novel pyridoxal-P enzyme, β-chloro-D-alanine chloride lyase (deaminating), that catalyzes the α,β-elimination of β-chloro-D-alanine.

$$\text{(D)-ClCH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \rightarrow \text{HCl} + \text{CH}_3\text{COCOOH} + \text{NH}_3$$

The enzyme is inducibly formed only by addition of β-chloro-D-alanine to the medium, and has been purified and crystallized from the cell extract of *Pseudomonas putida* CR1-1 (*15*). It has a molecular weight of about 76,000 and consists of two subunits with identical molecular weights (38,000). The absorption spectrum of the enzyme exhibits maxima at 278 and 418 nm and is independent of pH. The enzyme contains 2 mol pyridoxal-P/mol enzyme.

In addition to β-chloro-D-alanine, which is the preferred substrate (V_{max} = 310 μmol/min/mg, K_m = 61 μ*M*), D-cysteine, D-cystine, and D-serine are α,β-eliminated, though slowly. β-Chloro-L-alanine, D- and L-alanine, and other amino acids are inert. The pH optimum for the elimination of β-chloro-D-alanine is 9.0.

The enzyme catalyzes the β-replacement reaction between β-chloro-D-alanine and NaHS to yield D-cysteine with V_{max} of 1620 μmol/min/mg.

$$\text{(D)-ClCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaHS} \rightarrow \text{(D)-HSCH}_2\text{CH(NH}_2\text{)COOH} + \text{NaCl}$$

The K_m value for β-chloro-D-alanine and NaHS are 8.1 and 45 m*M*, respectively.

Nagasawa et al. (*16*) have studied optimization of D-cysteine production with resting cells. The optimum pH and temperature are 7.5–8.0 and about 30°C, respectively. The addition of 2–4% acetone to the reaction

systems is effective to enhance the production as found for L-cysteine production by cysteine desulfhydrase (Fig. 2). The ratio of the α,β-elimination rate to the β-replacement rate depends on the concentration of β-chloro-D-alanine to that of NaHS. The occurrence of a high concentration of NaHS in the reaction mixture is required to convert effectively D-alanine to D-cysteine. When the resting cells are incubated with 200 m*M* D-alanine and 500 m*M* NaHS in potassium phosphate buffer (pH 8.0) containing 2% acetone at 30°C for 2 h, 2.2 g of D-cysteine/DL of the reaction mixture is produced with a molar yield of 100%.

The enzymatic procedure probably facilitates the industrial production of D-cysteine, which is regarded as an important starting material for a semisynthetic cephalosporin, MT-141 (*17*), though β-chloro-D-alanine is now not cheaply available.

PRODUCTION OF *S*-SUBSTITUTED L-HOMOCYSTEINES OR L-CYSTEINES AND SELENIUM AMINO ACIDS

L-Methionine γ-Lyase

L-Methionine γ-lyase (EC 4.4.1.11), a pyridoxal-P enzyme, catalyzes the α,γ-elimination of L-methionine as follows:

$$CH_3SCH_2CH_2CH(NH_2)COOH + H_2O \rightarrow CH_3SH + CH_3CH_2COCOOH + NH_3$$

Screening was carried out to find bacterial strains that would produce a high activity of the enzyme under aerobic and anaerobic conditions. *Pseudomonas putida* (=*Ps. ovalis*) was selected as the best producer (*18,19*). The enzyme is inducibly formed by addition of L-methionine to the medium, and has been purified to homogeneity and crystallized (*20*). It has a molecular weight of about 172,000 and consists of four subunits with an identical molecular weight (43,000) (*19*). It contains 4 mol pyridoxal-P/mol enzyme.

In addition to L-methionine, which is the preferred substrate (V_{max} = 20.4 μmol/min/mg, K_m = 0.99 m*M*), several derivatives of L-methionine and L-cysteine are substrates, whereas D-methionine, D-cysteine, L-cystathionine, and L-norleucine are inert (Table 2). These results show that the enzyme catalyzes both α,γ- and α,β-elimination reactions of L-sulfur-containing monoamino monocarboxylic acids.

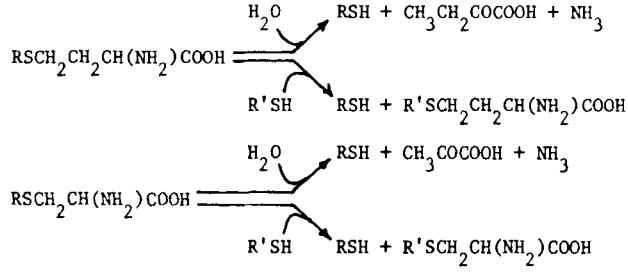
L-Methionine γ-lyase also catalyzes γ-replacement reactions between the sulfur amino acids and thiols (*18*). L-Ethionine is synthesized from L-methionine and ethanethiol. Successive addition of enzyme and ethanethiol is effective to enhance a yield of the product, which attains to more than 95% under the optimum conditions. Ethionine is produced also from the sulfur amino acids that are the substrates for α,γ-elimination (e.g., homocysteine, methionine sulfone, and methionine sulfoxide), and ethanethiol. When various alkanethiols (C_3–C_7) and

TABLE 2
Substrate Specificity of L-Methionine γ-Lyase from *Pseudomonas putida*

Substrate	Relative Activity
L-Methionine	100
D-Methionine	0
L-Ethionine	90
DL-Methionine sulfone	87
DL-Methionine sulfoxide	35
L-Methionine-DL-sulfoximine	27
S-Methyl-L-methionine	8
DL-Homocysteine	71
L-Cystathionine	0
L-Norleucine	0
L-Norvaline	0
DL-α-Aminobutyrate	0
L-Cysteine	11
D-Cysteine	0
L-Cystine	0
S-(β-Aminoethyl)-L-cysteine	14
S-Methyl-L-cysteine	67

arylthioalcohols (e.g., benzenethiol and β-naphthalenethiol) are used, the new sulfur amino acids corresponding to the thiols are synthesized (Table 3). Higher homologs than octanethiol, however, are inert. The derivatives of ethanethiol (e.g., β-mercaptoethanol and cysteamine) also serve as *S*-substituent donors to methionine. *S*-(β-Hydroxyethyl)-L-homocysteine and *S*-(β-aminoethyl)-L-homocysteine are produced from β-mercaptoethanol and cysteamine, respectively. Thiols with a charged group are generally poor *S*-substituent donors.

The enzyme catalyzes the β-replacement reactions of sulfur amino acids as well. For example, *S*-methyl-L-cysteine reacts with ethanethiol to produce *S*-ethyl-L-cysteine. Thus, L-methionine γ-lyase catalyzes the following reactions:

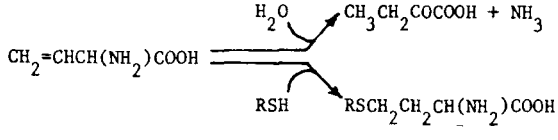


Various sulfur amino acids are synthesized from thiols and methionine, or some other cheaply available sulfur amino acids by enzymatic replacement reactions.

TABLE 3
Relative Velocity of Synthesis of *S*-Substituted Homocysteines from L-Methionine and Thiols by L-Methionine γ-Lyase

Thiol compound	Relative rate
Ethanethiol	100
1-Propanethiol	81
2-Propanethiol	38
1-Butanethiol	43
2-Methyl-1-propanethiol	75
1-Methyl-1-propanethiol	27
2-Methyl-2-propanethiol	26
1-Pentanethiol	43
3-Methyl-1-butanethiol	52
2-Methyl-1-butanethiol	12
2-Methyl-2-butanethiol	9
1-Hexanethiol	17
2-Methyl-2-pentanethiol	5
1-Heptanethiol	4
2-Methyl-2-hexanethiol	0
2-Methyl-2-heptanethiol	0
2-Methyl-2-octanethiol	0
1-Decanethiol	0
Cyclohexanethiol	10
Toluenethiol	71
Benzenethiol	43
β-Naphthalenethiol	6
Thioglycolic acid	11
Thioglycolic acid ethyl ester	111
β-Mercaptoethanol	84
Thiolactic acid	7
Cysteamine	44
N-Acetylcysteamine	267

Vinylglycine is regarded as a common key intermediate in the α,γ-elimination and γ-replacement reactions. The enzyme catalyzes also the deamination of vinylglycine, and γ-addition reaction of various alkanethiols to vinylglycine as follows (*21*):



The relative reactivity of alkanethiols for the γ-addition reaction

The relative reactivity of alkanethiols for the γ-addition reaction of vinylglycine is close to that for their γ-replacement reactions with methionine.

In addition, L-methionine γ-lyase catalyzes the α,γ-elimination of selenomethionine to yield α-ketobutyrate, ammonia, and methaneselenol, and γ-replacement reaction with various thiols to produce *S*-substituted homocysteines (*22*). Selenomethionine is a better substrate than methionine for α,γ-elimination, but is less effective for γ-replacement based on their V_{max} and K_m values. Methionine and its derivatives react with selenols to form the corresponding *Se*-substituted selenohomocysteines, although selenols are less effective donors than thiols. The enzymatic β-replacement reaction also occurs between *S*-substituted cysteines and selenols (*22*). Thus, these enzymatic replacement and γ-addition reactions are applicable to industrial synthesis of various selenium amino acids.

The mechanism of methionine γ-lyase reaction is shown in Fig. 3. The formation of the ketimine quinoid intermediate of *S*-substituted homocysteine (or *Se*-substituted selenohomocysteine) (I) is followed by a loss of a β-hydrogen and a γ-substituent to form a β,γ-unsaturated intermediate (III). This is derived also from a pyridoxal-P aldimine intermediate of vinylglycine (II). Intermediate (III) undergoes γ-addition of thiols (or selenols) to form a product (IV). It alternatively undergoes tautomerization in the absence of thiols (or selenols), followed by hydrolytic deamination to α-ketobutyrate.

L-Methionine γ-lyase shows another function: the exchange of the α and β-hydrogens of L-methionine, *S*-methyl-L-cysteine, and several other substrate amino acids with deuterium or tritium of solvents (*23*). The α-hydrogen exchange occurs about 40 times faster than the elimination reaction. The enzyme catalyzes the α- and β-hydrogen exchange of the following straight-chain amino acids that are not substrate for the elimination: L-alanine, L-α-aminobutyrate, L-norvaline, and L-norleucine. Glycine, phenylalanine and tryptophan, the nonsubstrate amino acids are α-deuterated (or tritiated) enzymatically. The following nonsubstrate amino acids are inert: D-enantiomers, L-valine, L-leucine, L-glutamate, L-asparagine, L-lysine, and L-arginine. This enzymatic hydrogen exchange reaction is useful for the preparation of amino acids labeled with ^2H and ^3H.

Tryptophan Synthase

Tryptophan synthase (EC 4.2.1.20) is a pyridoxal-P enzyme with a multiple catalytic function (Table 4, Reactions 1–6), where Reaction 1, the synthesis of tryptophan from indole glycerol phosphate, is of physiological importance. Reaction 2 and 3 are regarded as partial reactions of Reaction 1 (*24*). Tryptophan synthase is found widely in various bacteria, yeasts, molds, and plants. The enzyme of *E. coli* is composed of two kinds of protein, α (MW, 29,000) and β (MW, 44,200). Pyridoxal-P is bound to the β-subunit. Two α-subunits combine with one β_2-dimer to form an $\alpha_2\beta_2$-complex (MW, 147,000) that catalyzes the physiological reaction. Each of the subunits catalyzes their own specific reactions as well

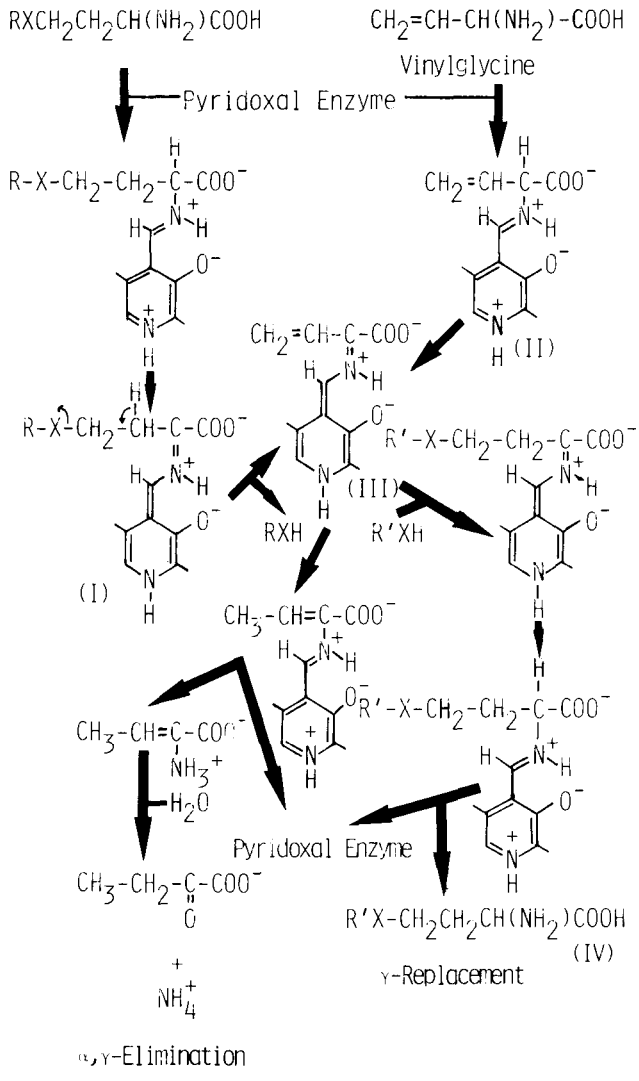


Fig. 3. Proposed mechanism of the α,γ-elimination and the γ-replacement reactions catalyzed by L-methionine γ-lyase. X = S or Se.

(Table 4). The crystalline $\alpha_2\beta_2$-complex was obtained after a sixfold purification from *E. coli* trp R⁻ΔtrpED102/F'ΔtrpED102 induced by Yanofski et al. (*24*). About 16% of the intracellular soluble protein of this mutant is the tryptophan synthase complex. Goldberg and Baldwin (*25*) and Miles and her coworkers (*26*) have shown that methanethiol and β-mercaptoethanol serve as *S*-substituted donors to serine to yield L-cysteine and *S*-(β-hydroxyethyl)-L-cysteine, respectively, by the $\alpha_2\beta_2$- and β_2-complexes (Reaction 5). Esaki et al. (*27*) have studied the enzymatic synthesis of various *S*-substituted L-cysteines from L-serine and its derivatives (e.g., β-chloro-L-alanine and *O*-methyl-L-serine) with the $\alpha_2\beta_2$-complex. Thiols such as toluenethiol, 1-propanethiol, and 1-butanethiol are much more efficient as *S*-substituent donors than the

TABLE 4
Reactions Catalyzed by *Escherichia coli* Tryptophan Synthase and Its Subunits

Reaction	Catalyzed by
1. Indole-3-glycerol phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O	$\alpha_2\beta_2$
2. Indole-3-glycerol phosphate → indole + D-glyceraldehyde 3-phosphate	α; $\alpha_2\beta_2$
3. Indole + L-serine → L-tryptophan + H_2O	β_2; $\alpha_2\beta_2$
4. L-Serine → pyruvate + ammonia	β_2
5. β-Mercaptoethanol + L-serine → *S*-(β-hydroxyethyl)-L-cysteine + H_2O	β_2; $\alpha_2\beta_2$
6. β-Mercaptoethanol + L-serine + pyridoxal-P → *S*-pyruvylmercaptoethanol + pyridoxamine-P + H_2O	β_2

physiological substrate, indole. When L-threonine and L-vinylglycine are used as *S*-substituent acceptors of thiols, the corresponding *S*-substituted β-methyl-L-cysteines are synthesized (*27*).

The enzyme catalyzes also the β-replacement reactions of L-serine with selenols to produce the corresponding *Se*-substituted L-selenocysteines (*28*). Thus, these enzymatic reactions can be used for synthesis of various sulfur and selenium amino acids.

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