Role of Tyrosine 114 of L-Methionine γ-lyase from Pseudomonas putida

Hiroyuki INOUE,¹,† Kenji INAGAKI,¹ Naoki ADACHI,¹ Takashi TAMURA,¹ Nobuyoshi ESAKI,² Kenji SODA,³ and Hidehiko TANAKA¹,*

¹Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama, Okayama 700-8530, Japan
²Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan
³Department of Biotechnology, Faculty of Engineering, Kansai University, Suita, Osaka, 564-8680, Japan

Received April 21, 2000; Accepted June 9, 2000

L-Methionine γ-lyase from Pseudomonas putida has a conserved tyrosine residue (Tyr114) in the active site as in all known sequences of γ-family pyridoxal 5'-phosphate dependent enzymes. A mutant form of L-methionine γ-lyase in which Tyr114 was replaced by phenylalanine (Y114F) resulted in 910-fold decrease in kcat for α,γ-elimination of L-methionine, while the Km remained the same as the wild type enzyme. The Y114F mutant had the reduced kcat by only 28- and 16-fold for substrates with an electron-withdrawing group at the γ-position, namely O-acetyl-L-homoserine and L-methionine sulfone, respectively, and also the similar reduction of kcat for α,β-elimination and deamination substrates. The hydrogen exchange reactions of substrate and the spectral changes of the substrate-enzyme complex catalyzed by the mutant enzyme suggested that γ-elimination process for L-methionine is the rate-limiting determination step in α,γ-elimination overall reaction of the Y114F mutant. These results indicate that Tyr114 of L-methionine γ-lyase is important in γ-elimination of the substrate.

Key words: general acid catalyst; L-methionine γ-lyase; reaction mechanism; site-directed mutagenesis

L-Methionine γ-lyase (EC 4.4.1.11) is a pyridoxal 5'-phosphate (pyridoxal-P) dependent enzyme that catalyzes α,γ-elimination and γ-replacement reactions of L-methionine and its derivatives, and also α,β-elimination and β-replacement reactions of S-substituted L-cysteines.¹,² This enzyme has been found in various bacteria, such as Pseudomonas putida,³,⁴ Aeromonas sp.,⁵ and Clostridium sporogenes.⁶ and in the primitive protozoan Trichomonas vaginalis.⁷ We purified the enzyme to homogeneity from P. putida.³,⁴ and cloned its gene.⁸ The enzyme is composed of 398 amino acid residues, and its amino acid sequence is very similar to γ-family pyridoxal-P enzymes that catalyze α,γ-elimination and γ-replacement reactions,⁹ such as cystathionine γ-lyase,¹⁰¹¹ cystathionine γ-synthase,¹² and O-acetylhomoserine O-acetylserine sulfhydrylase.¹³

The γ-family pyridoxal-P enzyme has multicyclic functions as represented in L-methionine γ-lyase,¹³ suggesting that there are many catalytic residues that is specifically required for each reaction. However, the detailed reaction mechanisms including the role of these residues remain unclear. The general mechanism for α,γ-elimination and γ-replacement reactions by γ-family pyridoxal-P enzyme has been proposed as follows: α- and β-hydrogens of the substrate amino acid are initially removed, and then the γ-substituent is eliminated to yield a vinylglycine-pyridoxal-P intermediate, which is a common key intermediate in α,γ-elimination and γ-replacement reactions.¹⁴¹⁵ Inactivation mechanism studies of L-methionine γ-lyase by suicide substrates, such as L-2-amino-4-chloro-4-pentanooate and 2-amino-4-chloro-5-(p-nitrophenylsulfanyl)pentanooate, have suggested that a cysteine residue is located at the active site in the enzyme, which functions as a nucleophilic residue for an enzymatically activated 3,4-allenic intermediate from these inactivators.¹⁶,¹⁷ The cysteine residue was modified and identified with N-(bromoacetyl)pyridoxamine 5'-phosphate, 2-nitro-5-thiocyanobenzoic acid, and iodoacetate.¹⁸,¹⁹ and was found to correspond to Cys116 from the primary structure of the enzyme.⁵ The equivalent cysteine residue was not conserved in other γ-family enzymes except for trichomonad L-methionine γ-lyase,²⁰ but the tyrosine residue corresponding to Tyr114 was conserved in all known sequences (Fig. 1). It is assumed that Tyr114 and Cys116 are located at the approximate position on a spatial structure in the active site, since a highly conserved Gly115 is positioned be-

¹ Present address: Marine Biological Technology Section, Chugoku National Industrial Research Institute, Kure, Hiroshima 737-0197, Japan
* To whom correspondence should be addressed. Tel: +81-86-251-8298; Fax: +81-86-251-8388; E-mail: htanaka@cc.okayama-u.ac.jp


<table>
<thead>
<tr>
<th>PpMGL</th>
<th>112</th>
<th>TVGCTAPFL</th>
<th>121</th>
</tr>
</thead>
<tbody>
<tr>
<td>TvlMGL1</td>
<td>109</td>
<td>CLYGENTHALF</td>
<td>118</td>
</tr>
<tr>
<td>TvlMGL2</td>
<td>112</td>
<td>CLYGENTHALF</td>
<td>121</td>
</tr>
<tr>
<td>ScAAS</td>
<td>109</td>
<td>TVGCTYNQF</td>
<td>118</td>
</tr>
<tr>
<td>ScCGL</td>
<td>102</td>
<td>DYGONHRF</td>
<td>111</td>
</tr>
<tr>
<td>HsCGL</td>
<td>112</td>
<td>DYGONHRF</td>
<td>121</td>
</tr>
<tr>
<td>EccG</td>
<td>99</td>
<td>DCGGSRFL</td>
<td>108</td>
</tr>
<tr>
<td>AtCLB</td>
<td>179</td>
<td>DYGGSDDRL</td>
<td>188</td>
</tr>
<tr>
<td>BacBL</td>
<td>117</td>
<td>CAYSPVHELH</td>
<td>126</td>
</tr>
<tr>
<td>EccBL</td>
<td>109</td>
<td>TAREPSQDFC</td>
<td>118</td>
</tr>
</tbody>
</table>

Fig. 1. Sequence Comparison of γ-Family Pyridoxal-P Dependent Enzymes.

Each individual sequence is numbered accordingly. Highly conserved residues in all sequences compared with that of \( \text{L-methionine γ-lyase from } \text{P. putida} \) are boxed. Asterisk indicates the residue (Tyr114) that is mutated in this study. PpMGL, \( \text{L-methionine γ-lyase from } \text{P. putida} \); TvlMGL1 and TvlMGL2, two \( \text{L-methionine γ-lyases from } \text{T. vaginalis} \); ScAAS, \( \text{O-acetylhomoserine L-sulphydrylase from \text{Succinocystis cerevisiae}} \); ScCGL and HsCGL, cystathionine γ-lyase from \( \text{S. cerevisiae} \) and human, respectively; EccG, cystathionine γ-synthase from \( \text{E. coli} \); AtCLB, BacBL, and EccBL, cystathionine β-lyase from \( \text{Arabidopsis thaliana} \), \( \text{Bordetella avium} \), and \( \text{E. coli} \), respectively.

Between both residues. Furthermore, the X-ray structure analysis of cystathionine β-synthase and cystathionine β-lyase with molecular modeling of enzyme-substrate complexes has been reported recently that the conserved tyrosine residue is present in the active site and may be important for a reaction mechanism. Hence, we were interested to identify the role of Tyr114 in \( \text{P. putida} \) \( \text{L-methionine γ-lyase} \).

In this study, we have replaced Tyr114 in \( \text{L-methionine γ-lyase with phenylalanine (Y114F)} \) using site-directed mutagenesis, and investigated the catalytic properties of Y114F mutant enzyme based on the individual putative steps of \( \alpha, \gamma \)-elimination and \( \gamma \)-replacement reaction mechanisms. The results indicate that Tyr114 specifically serves to facilitate \( \gamma \)-elimination as one of residues that is required to the multicalytic functions of \( \gamma \)-family pyridoxal-P enzyme.

### Materials and Methods

**Materials.** \( \text{L-Methionine sulfone, L-vinylglycine, S-ethyl-L-cysteine, and S-methylL-cysteine were purchased from Sigma. O-Acetyl-L-homoserine was prepared by the method of Nagai and Flavin.} \)

The other amino acids were purchased from Nacalai Tesque, and D-O (99.8%) was from Merck. A pKK223-3 expression vector was obtained from Amersham Pharmacia Biotech. Synthetic oligonucleotide for site-directed mutagenesis was from Biologica (Nagoya). Restriction enzymes and other DNA modifying enzymes were from Takara Shuzo, and Nippon Gene. The other chemicals were analytical grade reagents.

**Construction of expression plasmid.** The recombinant plasmid pYH103 encoding \( \text{L-methionine γ-lyase gene from } \text{P. putida} \) ICR3460 was constructed as described previously. The 1.35-kb HindIII-BamHI fragment of pYH103 containing the entire coding region was excised from 0.7% agarose gel. The fragment was blunt-ended with Klenow fragment and subcloned into the Smal site of pKK223-3 to yield the expression plasmid pYH301.

**Site-directed mutagenesis.** The 0.6-kb HindIII-SalI fragment of pYH103 corresponding to the 5'-terminal half of the coding sequence was subcloned into pUC119 digested with HindIII and SalI, and then mutagenized by the method of Kunkel using Mutan-K site directed mutagenesis kit (Takara Shuzo). A synthetic mutagenic primer to cause mutation was as follows (the underlined letter indicates the mutagenized nucleotide): 5'-AGGTGCAGCCG-AACAGGGTGTTG-3'. Clones obtained after mutagenesis were screened by sequencing the gene in the mutant region using BacBEST sequencing kit (Takara Shuzo) and M13-specific primer radiolabeled with [\( \gamma \)-\(^{32}\)P]ATP. The clones that were selected for sequencing contained the desired mutation. To construct the entire mutational gene of Y114F mutant of \( \text{L-methionine γ-lyase} \), the resultant plasmid containing the mutational site was digested with HindIII-SalI, and the fragment replaced on same region of pYH103 to give pYH103(Y114F). Finally, HindIII-BamHI fragment of this plasmid was inserted into pKK223-3 as described above, to construct mutated pYH301.

**Expression and purification of the wild type and mutant enzymes.** The wild type and mutant enzymes were produced by *Escherichia coli* JM109. Recombinant *E. coli* cells were cultivated at 37°C for 16 h in Terrific broth. The wild type enzyme was purified using the method of Nakayama et al. The mutant enzyme was purified using the same method with some modifications.

After DEAE-Toyopearl 650M column chromatography, the Y114F mutant enzyme was put on a DEAE-Sephadex A-50 column equilibrated with 0.02 M sodium pyrophosphate buffer (pH 8.3) containing 0.1 M KCl. The enzyme was washed with the same buffer and eluted with the buffer containing 0.2 M KCl. The enzyme solution was then put on a Q-Sepharose FF column equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 0.1 M KCl. Elution of the enzyme was done with a linear gradient of 0.1 M–0.2 M KCl. The buffers used throughout contained 0.02 M pyridoxalphosphate and 0.01% 2-mercaptoethanol. Purity of the enzyme was
Table 1. Steady-state Kinetic Constants for the Wild Type and Y114F Mutant of l-Methionine y-lase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild Type</th>
<th></th>
<th>Y114F</th>
<th></th>
<th>(kcat)Y114F/(kcat)WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mm)</td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(K_m) (mm)</td>
<td>(k_{cat}) (s(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>l-Methionine</td>
<td>0.90</td>
<td>48.6</td>
<td>1.13</td>
<td>0.053</td>
<td>0.0011</td>
</tr>
<tr>
<td>l-Ethionine</td>
<td>0.27</td>
<td>33.4</td>
<td>1.18</td>
<td>0.065</td>
<td>0.0019</td>
</tr>
<tr>
<td>l-Methionine sulfone</td>
<td>8.22</td>
<td>40.4</td>
<td>0.84</td>
<td>2.52</td>
<td>0.062</td>
</tr>
<tr>
<td>O-Acetyl-l-homoserine</td>
<td>2.22</td>
<td>78.0</td>
<td>0.74</td>
<td>2.84</td>
<td>0.036</td>
</tr>
<tr>
<td>S-Methyl-l-cysteine</td>
<td>0.40</td>
<td>5.53</td>
<td>4.18</td>
<td>0.42</td>
<td>0.076</td>
</tr>
<tr>
<td>S-Ethyl-l-cysteine</td>
<td>0.48</td>
<td>5.79</td>
<td>0.72</td>
<td>0.38</td>
<td>0.066</td>
</tr>
<tr>
<td>l-Vinylglycine</td>
<td>7.22</td>
<td>44.4</td>
<td>1.96</td>
<td>2.37</td>
<td>0.053</td>
</tr>
</tbody>
</table>

Enzyme activity was measured by production of \(\alpha\)-ketobutyrate or pyruvate.

measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).\(^{26}\)

Enzyme and protein assays. The enzymatic \(\alpha\), \(\gamma\)- and \(\alpha,\beta\)-elimination reactions were routinely followed by the determination of \(\alpha\)-ketobutyrate and pyruvate, respectively, with 3-methyl-2-benzothiazolone hydrazono hydrochloride as described previously.\(^{25,27}\) For the replacement reaction,\(^{40}\) the reaction system consisted of 100 \(\mu\)mol of potassium phosphate (pH 8.0), 25 \(\mu\)mol of l-methionine sulfone or S-methyl-l-cysteine, 40 \(\mu\)mol of 2-mercaptoethanol, 10 \(\mu\)mol of pyridoxal-P, and enzyme (wild type; 15 \(\mu\)g, Y114F; 65 \(\mu\)g) in a final volume of 0.25 ml. After 30 min of incubation at 37°C, the reaction was stopped by addition of 0.03 ml of 50% trichloroacetic acid. S-(\(\beta\)-Hydroxyethyl)-l-homocysteine or S-(\(\beta\)-hydroxyethyl)-l-cysteine formed was measured with an automated amino acid analyzer JCL-300 (Jeol, Japan). Protein was measured by the method of Lowry et al.\(^{28}\) with bovine serum albumin as a standard.

1\(^H\) NMR analysis. The exchange reaction of the substrate hydrogens with deuterium of solvent D\(_2\)O was followed by 1\(^H\) NMR analysis as described by Esaki et al.\(^{16}\) The reaction mixture contained 1500 \(\mu\)mol of potassium phosphate buffer (pD 8.1), 0.14 \(\mu\)mol pyridoxal-P, 500 \(\mu\)mol of the substrate and enzyme in 7 ml of D\(_2\)O. The reaction was started by addition of 0.5 ml of the enzyme solution (0.1-4.0 mg/ml) and done at 37°C. 1\(^H\) NMR spectra were recorded immediately after addition of 0.1 ml of 2% NaOD to 0.6 ml of the reaction mixture at appropriate intervals. The 1\(^H\) NMR spectra and peak integrals were analyzed with a Varian VXR 200 spectrometer (200 MHz).

Spectroscopic studies. Absorption spectra were obtained by using a Beckman model DU65 spectrophotometer at an enzyme concentration of 1.3 mg/ml in 25 mm potassium phosphate (pH 8.0) containing 25 \(\mu\)M pyridoxal-P. Steady-state spectra of enzyme-substrate complexes were recorded after addition of substrates (final concentration, 25 mm) to the enzyme solution.

Results

Expression, and purification of the wild type and mutant enzymes of l-methionine \(\gamma\)-lyase

The genes encoding the wild type and mutant enzymes were highly expressed under the control of the \(\tau\)ag promoter of pKK223-3. The amounts of the produced enzymes corresponded to about 5% of the soluble proteins. The recombinant wild type enzyme was purified by the same procedure as l-methionine \(\gamma\)-lyase from \(P.\ putida\) ICR3460.\(^4\) As the Y114F mutant enzyme behaved differently from the wild type enzyme during DEAE-Sephadex A-50 column chromatography, they were purified by Q-Sepharose FF column chromatography as the final purification step. The purified enzymes were found to be homogeneous upon SDS-PAGE. The purified mutant enzyme was confirmed to be immunologically indistinguishable from the wild type enzyme by Ouchterlony double diffusion analysis with the antiserum raised against the enzyme from \(P.\ putida\) (data not shown).

Properties of elimination and deamination reactions with the Y114F mutant enzyme

The steady state kinetic parameters of the wild type and mutant enzymes for various substrates are shown in Table 1. The \(k_{cat}\) for l-methionine of Y114F was reduced by 910-fold without changing the \(K_m\) compared with that of the wild type enzyme, suggesting that Tyr114 plays a critical role in the catalytic mechanism. The \(k_{cat}\) for l-ethionine also decreased significantly, while the \(k_{cat}\) for l-methionine sulfone and O-acetyl-l-homoserine, which have good leaving groups at the \(\gamma\)-positions, had only 16- and 28-fold reductions, respectively. Furthermore, Y114F also showed similar reduced \(k_{cat}\) (13 to 19-folds) for l-vinylglycine, which is served as a substrate of deami-
Table 2. Rates of Exchange of α- and β-Hydrogens of l-Amino Acids with the Wild Type and Y114F Mutant Enzymes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial velocity of exchange (μmol/min/mg protein)</th>
<th>Wild Type</th>
<th>Y114F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-H</td>
<td>β-H</td>
<td>α-H</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>841</td>
<td>1080</td>
<td>9.45</td>
</tr>
<tr>
<td>l-Norleucine</td>
<td>33.6</td>
<td>33.6</td>
<td>5.17</td>
</tr>
<tr>
<td>S-Methyl-l-cysteine</td>
<td>76.9</td>
<td>120</td>
<td>3.74</td>
</tr>
</tbody>
</table>

*Initial velocities were calculated from peak integration value of H NMR spectra at various times.*

Hydrogen exchange reaction of substrate in D₂O

To clarify the significant reduction of kcat for l-methionine of the Y114F mutant, we compared the hydrogen exchange rates for l-methionine, S-methyl-l-cysteine and l-norleucine (a non-substrate for elimination reaction) by wild type and mutant enzymes, which influence on transaldimination and α, β-hydrogens removal processes in α, γ-elimination overall reaction. The hydrogens exchange rate was measured by following the disappearance of the ¹H NMR signal corresponding to α- and β-hydrogens of the substrate incubated in D₂O (Table 2). The α-hydrogen exchange rate for l-methionine of Y114F was only 89-fold slower than that for the wild type enzyme, although the α,γ-elimination reactions producing α-ketoacetylurea was 910-fold slower (Table 1). The reductions of α-hydrogen exchange rate were also observed with S-methyl-l-cysteine and l-norleucine. The β-hydrogen exchange rates of all substrates seem to follow their α-hydrogen exchange rates since the ratio of α- to β-hydrogens exchange rate for each substrate were similar in both enzymes. In addition, we found that the hydrogen exchange reactions of l-methionine sulfone and O-acetyl-l-homoserine by both enzymes were not detected under the same conditions.

In the course of study, we also found that the Y114F mutant was stereoselective in the β-hydrogen exchange of l-methionine, although the stereochemistry has not been identified. The ¹H NMR spectrum of β-hydrogen was observed as a triplet in 1.2 ppm after 120 min incubation and the γ-hydrogen signal was transformed into a doublet at 1.85 ppm (Fig. 2). This result was not observed with the wild type enzyme.

Spectral analysis

The Y114F mutant enzyme had a slightly shifted absorption maxima at 424 nm compared to the wild type enzyme (420 nm) in the absence of substrates (Fig. 3). Their absorption spectra were stable to exhaustive dialyses (pH 7.2), suggesting that pyridoxal-P is tightly bound in both enzyme forms. Addition of l-methionine or O-acetyl-l-homoserine to the wild type enzyme resulted immediately in the absorption maximum being shifted to 460 nm and a new absorption shoulder appeared at about 480 nm as described by Esaki et al. (Fig. 3(A)). The absorption shoulder at 480 nm is probably due to a pyridoxalaldimine intermediate of α-aminocrotonate as proposed by Johnston et al. (refer to Fig. 4(VI)), suggesting that the deamination process is a rate-determining step for α, γ-elimination reaction of wild type enzyme. In contrast, addition of l-methionine to Y114F caused no appreciable spectral changes and a little quenching of the 424-nm band (Fig. 3(B)). The result was

Fig. 2. ¹H-NMR Spectra of l-Methionine with the Y114F Mutant Enzyme in D₂O.

The reaction mixture contained 135 μg of enzyme. The ¹H-NMR spectra were recorded immediately after addition of 100 μl of 2% NaOD to the reaction mixture. The deuterium labeled l-methionine, which is expected from the spectrum after incubation (210 min), is shown.
The Y114F mutant enzyme was found to catalyze the \( \gamma \)- (or \( \beta \))-replacement reaction between \( \text{L-methionine sulfone (or \( S \)-methyl-\text{L}-cysteine)} \) and \( \text{2-mercaptoethanol, which is an} \ S \)-substituted donor, to yield \( S \)-\( \beta \)-hydroxyethyl) amino acid derivatives. The relative production rates of these derivatives were decreased by 121-fold (\( \gamma \)-replacement reaction) and 134-fold (\( \beta \)-replacement reaction) compared with that of the wild type enzyme. In addition, the \( K_m \) for \( \text{2-mercaptoethanol, which is} \ S \)-substituent donor, on \( \gamma \)-replacement (16 \( \text{mM} \) and 17 \( \text{mM} \) for wild type and \( \text{Y114F}, \) respectively) and \( \beta \)-replacement reaction (7.1 \( \text{mM} \) and 14.7 \( \text{mM} \), respectively) were little changed.

**Discussion**

Recently, the spatial structures of \( \gamma \)-family pyridoxal-P enzyme, cystathionine \( \beta \)-lyase, and cystathionine \( \gamma \)-synthase have been identified by X-ray crystallography. Clausen et al. have found that a phenolic ring of a highly conserved tyrosine residue, corresponding to Tyr114 of \( \text{L-methionine} \ \gamma \)-lyase, in \( \gamma \)-family pyridoxal-P enzyme is nearly parallel to the plane of the pyridine ring of the pyridoxal-P and leads to stacking interaction with the pyridine ring. Furthermore, molecular-modeling study of substrate-binding cystathionine \( \gamma \)-synthase have suggested that this residue serves to release the \( \gamma \)-substituent of the substrate. In this study, we found that the activity of Tyr114 mutant enzyme of \( \text{L-methionine} \ \gamma \)-lyase was significantly decreased for \( \text{L-methionine}, \) but changed little for \( \alpha \)-\( \beta \)-elimination and deamination substrates (Table 1). Furthermore, the hydrogen exchange reaction study (Table 2) and the spectral analysis (Fig. 3) suggested that the \( \gamma \)-elimination process for \( \text{L-methionine} \ \gamma \)-lyase was the rate-determining step in the \( \text{Y114F} \) mutant. These results are compatible with the above hypothesis that the conserved tyrosine residue plays an important role to facilitate the \( \gamma \)-elimination of substrate. These finding also indicate that the \( \gamma \)- and \( \beta \)-elimination processes in the multicatalytic function of \( \gamma \)-family pyridoxal-P enzyme should be catalyzed by different residues. Therefore, Tyr114 is an essential residue to understand the \( \alpha \),\( \gamma \)-elimination mechanism of \( \text{L-methionine} \ \gamma \)-lyase.

However, it should be noted that the \( \alpha \),\( \gamma \)-elimination reaction of the \( \text{Y114F} \) mutant for \( \text{L-methionine sulfone} \) and \( \text{O-acetyl-L-homoserine, having an} \ \text{electron-withdrawing substituent at the} \ \gamma \)-position, showed reduction of the \( K_m \) values by only 16- and 28-fold compared with that of the wild type enzyme (Table 1). Furthermore, the \( \gamma \)-elimination processes for these substrates did not seem to be the major rate-determining step in the \( \text{Y114F} \) mutant (Fig. 3(B)). These results are in clear contradiction to the role of the tyrosine residue in the \( \alpha \),\( \gamma \)-elimination reaction of \( \text{L-methionine}. \)

**Fig. 3.** Absorption Spectra of Steady-state Reaction Mixtures of the Wild Type and \( \text{Y114F} \) Mutant Enzymes.

\( \text{L-Methionine or O-acetyl-L-homoserine (final concentration,} \) 25 \( \text{mM} \) \) was added into the reaction mixture (pH 8.0) containing 1.3 \( \text{mg/ml} \) enzymes. The wild type enzyme (A) was measured immediately after addition of these substrates. The \( \text{Y114F} \) mutant enzyme (B) was measured after 2-hr \( \text{L-methionine} \) and 30-min \( \text{O-acetyl-L-homoserine} \) incubation at 37\( ^\circ \)C. Curve 1, 1.3 \( \text{mg/ml} \) enzyme without substrate; curve 2, the reaction mixture with \( \text{L-methionine} \); curve 3, the reaction mixture with \( \text{O-acetyl-L-homoserine} \).

Properties of replacement reactions with the \( \text{Y114F} \) mutant enzyme

\( \text{L-Methionine \ \gamma \)-lyase also catalyzes the enzymatic} \ \gamma \)- or \( \beta \)-replacement reactions in the presence of various thiols.\)
homoserine had no apparent hydrogen exchange reaction in both the wild type and Y114F mutant enzymes. As the proposed mechanism for α,β-hydrogen exchange of substrate catalyzed by l-methionine γ-lyase, it has been suggested that the γ-substituent of substrate having the electron-withdrawing substituent is rapidly eliminated. Therefore, it is expected that there are two α,γ-elimination mechanisms: Tyr114-dependent and Tyr114-independent (Fig. 4); (i) Tyr114 is required to destabilize the enamine intermediate IV during a reversible tautomerization between intermediates III and IV; and (ii) the γ-substituent, having an electron-withdrawing group, is chemically eliminated with or without participation of Tyr114 to form directly intermediates V from III by-passing IV. It remains unclear whether Tyr114 of the wild type enzyme is capable of interacting with the electron-withdrawing group. However, the increased $K_a$ of wild type enzyme for l-methionine sulfone and O-acetyl-l-homoserine compared to that of Y114F suggest that O-acetyl or sulfonyl group may rather cause steric volume exclusion with the hydroxyl group of Tyr114 (Table 1), and which is also expected from the short distance between the hydroxyl group of conserved tyrosine residue of cystathionine γ-lyase and the γ-position of substrate. A tyrosine residue, functioning as an essential catalytic residue, has been reported in the mechanisms of tyrosine phenol-lyase, which is a pyridoxal-P enzyme that catalyzes α,β-elimination of l-tyrosine. It has been suggested that the tyrosine residue (Tyr71) in Citrobacter freundii tyrosine phenol-lyase serves as a general acid catalyst in β-elimination process of the substrate, and its hydroxyl group is donated to C-γ of the substrate (l-tyrosine) to form the cyclohexa-dienone tautomer. The mutation of Tyr71 to phenylalanine of tyrosine phenol-lyase resulted in no detectable activity. However, for substrates with electron-withdrawing substituent (S-(o-nitrophenyl)-l-cysteine, β-chloro-l-alanine, and O-benzoyl-l-serine), Y71F mutant of tyrosine phenol-lyase had 1.85–7% of the $k_{cat}$ values of wild-type enzyme. Furthermore, the $k_{cat}$ for S-substituted l-cysteine derivatives of Y71F mutant enzyme also decreased by $10^1$–$10^4$-fold. In this study, we have also found that the $k_{cat}$ of l-methionine γ-lyase Y114F mutant enzyme reduced by approximately $10^1$ fold for l-methionine. The detectable activity of each mutant enzyme for S-substituted substrates may be attributed to the low p$K_a$ values of conjugate acids (for example, a p$K_a$ of 10.5 for ethanethiol) of the leaving groups produced by C-S bond cleavage as compared to C-C bond cleavage of l-tyrosine. Although Tyr114 of l-methionine γ-lyase is essen-
tial for the γ-elimination process, it remains unclear whether this residue serves directly as a general acid catalyst. The slightly red-shifted absorption maxima (424 nm) of Y114F mutant enzyme suggests that a subtle environmental change occurred in the pyridoxal-P binding site. Thus, a changed stacking interaction with the pyridine ring of the pyridoxal-P and benzene ring of the mutated residue may have some effect on the γ-elimination process. Tai et al. have reported that in the β-replacement reaction of Salmonella typhimurium O-acetylserine sulfhydrylase-A, HS−, which undergoes a nucleophilic attack on the α-aminoacylate intermediate, is hydrogen-bonded to the enzyme group that assists in the β-elimination of acetate. 19 However, Tyr114 does not seem to be important in the orientation of an external thiol, since Kα values for substituent of the wild type and Y114F mutant enzymes were little changed. Interestingly, cystathionine β-lyase has the conserved tyrosine residue (Fig. 1), which is located in a similar position with cystathionine γ-synthase based on X-ray structure, 20,21 although this enzyme cannot catalyze an α,γ-elimination reaction. Clausen et al. have suggested that another function of the tyrosine residue is to deprotonate the amino group of the substrate in a transaldimination process, 22,23 but our results from the hydrogen exchange reaction study have suggested that Tyr114 does not play a crucial role in transaldimination and α,β-hydrogens removal processes. Further studies are required to reveal whether the Tyr114 is a general acid catalyst to facilitate γ-elimination process or not. To this end, l-methionine γ-lyase from P. putida has recently been crystallized, and structural studies are under way.

Acknowledgment

We are grateful to the laboratory of SC-NMR for the spectrometric analysis.

References

20) McKie, A. E., Edlind, T., Walker, J., Mottram, J. C., and Coombs, G. H., The primitive protozoan


