^{1}H NMR Studies of Substrate Hydrogen Exchange Reactions Catalyzed by L-Methionine γ-Lyase

Nobuyoshi Esaki,[‡] Toru Nakayama,[‡] Seiji Sawada,[§] Hidehiko Tanaka,[‡] and Kenji Soda*[,‡]

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan, and Department of Science, Kyoto University of Education, Fushimi-Ku, Kyoto-Fu 612, Japan

Received July 11, 1984; Revised Manuscript Received January 4, 1985

ABSTRACT: Hydrogen exchange reactions of various L-amino acids catalyzed by L-methionine γ-lyase (EC 4.4.1.11) have been studied. The enzyme catalyzes the rapid exchange of the α- and β-hydrogens of L-methionine and *S*-methyl-L-cysteine with deuterium from the solvent. The rate of α-hydrogen exchange was about 40 times faster than that of the enzymatic elimination reaction of the sulfur-containing amino acids. The enzyme also catalyzes the exchange reaction of α- and β-hydrogens of the following straight-chain L-amino acids which are not susceptible to elimination: norleucine, norvaline, α-aminobutyrate, and alanine. The exchange rates of the α-hydrogen and the total β-hydrogens of L-alanine and L-α-aminobutyrate with deuterium followed first-order kinetics. For L-norvaline, L-norleucine, *S*-methyl-L-cysteine, and L-methionine, the rate of α-hydrogen exchange followed first-order kinetics, but the rate of total β-hydrogen exchange decreased due to a primary isotope effect at the α-position. One β-hydrogen of *S*-methyl-L-cysteine was exchanged faster than the other, although both the β-hydrogens were exchanged completely with deuterium ultimately. L-Phenylalanine and L-tryptophan slowly underwent α-hydrogen exchange. The *pro-R* hydrogen of glycine was deuterated stereospecifically. None of the following amino acids were susceptible to the enzymatic hydrogen exchange: D isomers of the above amino acids, branched chain L-amino acids, acidic L-amino acids, and basic L-amino acids. L-Methionine derivatives such as methionine sulfoxide having a good leaving group at the γ-carbon, which are substrates in α,γ-elimination, were also not susceptible to the hydrogen exchange. In this case, the β-^{2}H,γ-^{2}H species of α-ketobutyrate was exclusively formed. The enzyme catalyzes deamination of L-vinylglycine in ^2H_2O to give also the same α-ketobutyrate species. These results are consistent with the proposed mechanism that the α,γ-elimination proceeds through a Schiff base of vinylglycine with pyridoxal 5′-phosphate [Davis, L., & Metzler, E. (1972) *Enzymes*, *3rd Ed. 7*, 33].

L-Methionine γ-lyase (EC 4.4.1.11) is a pyridoxal 5′-phosphate (pyridoxal-P)[1] 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 (Tanaka et al., 1977). We have purified the enzyme from *Pseudomonas putida* (=*P. ovalis*) to elucidate its enzymological properties (Tanaka et al., 1976, 1977; Esaki et al., 1977, 1979; Johnston et al., 1979b, 1980). According to the general mechanism for α,γ-elimination and γ-replacement reactions by pyridoxal-P enzymes, 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 quinoid intermediate, which is a common key intermediate in α,γ-elimination and γ-replacement reactions (Davis & Metzler, 1972; Walsh, 1979). During the course of studies on the catalytic mechanism of L-methionine γ-lyase, we have demonstrated that vinylglycine serves as a substrate of the enzyme undergoing deamination and γ-addition reactions (Esaki et al., 1977); this supports the above reported mechanism through vinylglycine.

In this paper, we have applied ^{1}H NMR spectroscopy to a mechanistic study of reactions catalyzed by L-methionine γ-lyase. This technique permits a direct kinetic analysis of the hydrogen-exchange reactions of the substrate and non-substrate amino acids catalyzed by the enzyme and provides detailed information on the enzyme reaction mechanism.

Experimental Procedures

Materials. L-Methionine sulfone (Toeniss & Kolb, 1941) and L-methionine sulfoxide (Lavine, 1947) were prepared from L-methionine according to the methods given in the literature. L-Vinylglycine was a generous gift of Dr. C. Walsh, Massachusetts Institute of Technology, Cambridge, MA. [α-^{2}H]-DL-Methionine was prepared from DL-methionine in ^2H_2O with amino acid racemase (EC 5.1.1.10) which catalyzes the exchange of the α-hydrogen of various amino acids (e.g., methionine, *S*-methylcysteine, lysine, and norvaline) with the deuterium of the solvent (^2H_2O). L-[α-^{2}H]Methionine was prepared by resolution of *N*-acetyl-DL-[α-^{2}H]methionine with acylase I of pig kidney (Sigma). *S*-Methyl-L-[α-^{2}H]cysteine and L-[α-^{2}H]norvaline were obtained in the same manner. Trifluoro-L-methionine was purchased from Vega-Fox, ^2H_2O (99.75%) and acetic acid-d_4 were from Merck, and *S*-methyl-L-cysteine was from Fluka. L-Methionine and other amino acids were products of Ajinomoto Co., Tokyo. The other chemicals were analytical grade reagents.

Enzyme Purification. L-Methionine γ-lyase was purified from a cell-free extract of *P. putida* (IFO 3738) by a modification of the previous method (Tanaka et al., 1976). The enzyme prepared was found to be homogeneous by disc gel electrophoresis, and its specific activity in α,γ-elimination of L-methionine was 4.0 units/mg under the conditions described below. Crystalline amino acid racemase with low substrate

[‡] Institute for Chemical Research.
[§] Department of Science.

[1] Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; NMR, nuclear magnetic resonance.

0006-2960/85/0424-3857$01.50/0

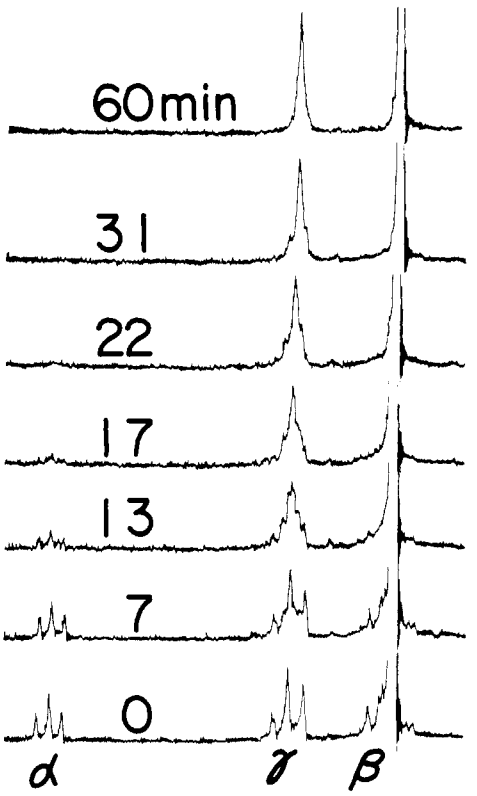

FIGURE 1: ^1H NMR spectra of L-methionine during incubation with L-methionine γ-lyase in ^2H_2O. The reaction mixture contained 56 μg of enzyme.

specificity (EC 5.1.1.10) was obtained from a cell-free extract of *P. putida* (=*P. striata*, IFO 12996) according to the method of Soda & Osumi (1971).

Enzyme Assay. The enzymatic α,γ- and α,β-eliminations were routinely followed by determination of α-ketobutyrate and pyruvate, respectively, with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) (Soda, 1968). The standard reaction system contained 40 μmol of potassium phosphate buffer (pH 8.0), 20 μmol of L-methionine or other amino acids, and 20 nmol of pyridoxal-P and enzyme in a final volume of 0.9 mL. The reaction was carried out at 37 °C for 10 min and terminated by addition of 0.1 mL of 50% trichloroacetic acid. In a blank, the enzyme was added to the reaction mixture after addition of trichloroacetic acid. The standard assay systems and conditions for the β- and γ-replacement reactions were described in a previous paper (Tanaka et al., 1977).

^1H NMR Analysis. The exchange reaction of the substrate hydrogens with deuterium of solvent ^2H_2O was followed by ^1H NMR analysis of the reaction mixture containing 100 μmol of potassium phosphate buffer (p^{2}H 8.6) and 50 μmol of the substrate and enzyme in 0.5 mL of ^2H_2O. The enzyme solution in ^2H_2O was prepared by repeated concentration and dilution with 10 mM potassium phosphate buffer (p^{2}H 7.8) containing 20 μM pyridoxal-P in an Amicon 202 ultrafiltration unit. The reaction was initiated by addition of 0.05 mL of the enzyme solution (0.1–5.0 mg/mL) and performed at 28 °C. At appropriate intervals, the ^1H NMR spectra and peak integrals were taken with a JEOL MH 100 spectrometer (100 MHz) under full-scale irradiation for deuterium decoupling.

RESULTS

Deuterium Incorporation into L-Methionine and S-Methyl-L-cysteine. Figure 1 shows the ^1H NMR spectral change of L-methionine observed during incubation with L-methionine γ-lyase in ^2H_2O. Signals of the α and the β protons disappeared substantially after 31 min, but those of the γ and the *S*-methyl protons were retained under the employed conditions. These spectral changes are not ascribable to the α,γ-elimination of L-methionine, because more than 95% of the initial amount of methionine was determined in the reaction mixture after 60 min by the method of Soda et al. (1961). The loss of α- and β-hydrogens of methionine is attributed to an exchange with deuterium of solvent ^2H_2O. As the signal due to the γ protons was transformed into a singlet after 60 min, both the nonequivalent β-hydrogens were exchanged with deuterium. Thus, L-methionine was converted to the α-^{2}H,-β,β-$^2H_2$ species. In order to compare the exchange rate of each hydrogen, we plotted the logarithm of peak integral vs. time (Figure 2). A linearity was observed for the α-hydrogen exchange until more than 90% of the α-hydrogen was lost, whereas the time course of the β-hydrogen loss deviated from a straight line. The peak integrals of the γ and *S*-methyl protons decreased at the rate of about 2.5% of the α-hydrogen loss. This decrease is ascribed to the α,γ-elimination of methionine, because α-ketobutyrate was formed at the same velocity as the loss of γ-hydrogens and *S*-methyl hydrogens of methionine when α-ketobutyrate was determined with MBTH by the method of Soda (1968).

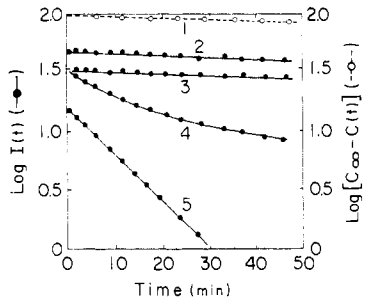

FIGURE 2: Time course of disappearance of α (line 5), β (line 4), γ (line 3), and *S*-methyl (line 2) protons of L-methionine and formation of α-ketobutyrate (line 1). $I(t)$, peak integration value at time t; C_∞, concentration (mM) of α-ketobutyrate that will be produced at infinite time; $C(t)$, concentration (mM) of α-ketobutyrate produced at time t. The multiplet of the β protons, which overlaps with the singlet of the *S*-methyl protons at p^{2}H 8.6, can be separated in the alkaline region. Therefore, ^1H NMR spectra were recorded immediately after addition of 0.05 mL of 2% NaO^{2}H to the reaction mixture (see Experimental Procedures) at appropriate intervals. The reaction mixture contained 34 μg of enzyme.

We observed a similar α- and β-hydrogen exchange for *S*-methyl-L-cysteine, a substrate for α,β-elimination. In semilogarithmic plots of each peak integral vs. time, linear and nonlinear relationships also were obtained for the α and β protons, respectively (see Figure 5). The rate of decrease in the *S*-methyl protons was much slower than those in the α and β protons and was the same as that of the pyruvate formation due to the α,β-elimination. Thus, the exchange rates of α- and β-hydrogens proceed much faster than the net elimination reaction for both L-methionine and *S*-methyl-L-cysteine. We determined initial velocities of elimination reactions of L-methionine and *S*-methyl-L-cysteine in both ^1H_2O and ^2H_2O. A solvent isotope effect $[k(^1H_2O)/k(^2H_2O)]$ of 2.2 was obtained for both substrates.

Deuterium Incorporation into Other Substrates. Hydrogen-exchange reactions of other substrates that undergo enzymatic α,γ-elimination were also examined. The relative V_{max} values for these substrates to that for L-methionine were as follows: L-methionine sulfone, 87%; trifluoro-L-methionine, 180%; L-vinylglycine, 101%. After a 16-h incubation with the enzyme, the integration value of each substrate proton was

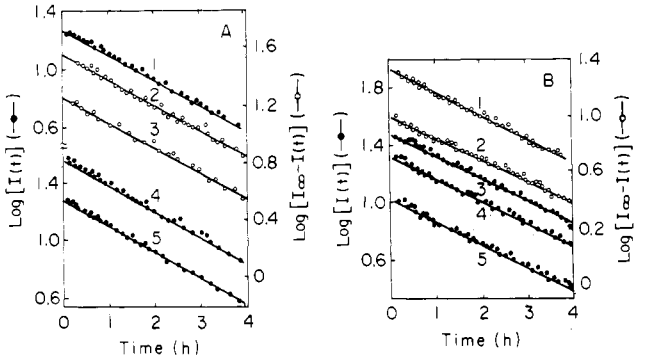

FIGURE 3: (A) Time course of disappearance of α (line 5), β (line 1), and γ (line 4) protons of L-vinylglycine and appearance of β (line 3) and γ (line 2) protons of α-ketobutyrate. (B) Time course of disappearance of α (line 5), γ (line 4), and *S*-methyl (line 3) protons of L-methionine sulfone and appearance of β (line 2) and γ (line 1) protons of α-ketobutyrate. $I(t)$, peak integration value at time t; I_∞, peak integration value at infinite time. Both the reaction mixtures contained 0.24 mg of enzyme.

compared with that of a control in which enzyme was replaced by ^2H_2O. None of the substrates tested underwent α- and β-hydrogen exchange. A decrease in the relative integration value of each proton is ascribed to elimination, because the ratio of relative integration values such as α proton:γ proton, β proton:γ proton, and α proton:*S*-substituent proton did not change apparently after incubation with the enzyme. To confirm this, the rates of disappearance of substrate protons and appearance of α-ketobutyrate protons were determined with L-vinylglycine and L-methionine sulfone (Figure 3). The rate of disappearance of the vinylglycine proton signals coincided with that of appearance of the α-ketobutyrate proton signals. Transformation of the β proton of L-methionine sulfone was obscured by the superposition of the product proton (methanesulfonate), but the rate of disappearance of the α and γ proton signals coincided with that of the appearance of α-ketobutyrate proton signals. These results indicate that the hydrogen exchange of these substrates occurred negligibly slowly.

Deuterium Incorporation into α-Ketobutyrate Formed. We measured deuterium incorporation into the β and γ positions of α-ketobutyrate produced by α,γ-elimination of various amino acids in ^2H_2O. The scale of reaction was 40 times larger (final volume 20 mL in ^2H_2O) than that shown under Experimental Procedures. After incubation at 28 °C for 120 min, α-ketobutyrate was isolated as its 2,4-dinitrophenylhydrazone essentially by the method of Posner & Flavin (1972b). The number of hydrogen atoms at the β and γ position of the α-ketobutyrate was calculated from the relative value of peak integrals against the phenyl proton at C-3. α-Ketobutyrate formed from L-methionine sulfone, trifluoro-L-methionine, and L-vinylglycine, which are not susceptible to α- and β-hydrogen exchange, was assigned as the β-^{2}H,γ-^{2}H species. In contrast, α-ketobutyrate from L-methionine was produced as a mixture of the β-^{2}H,γ-^{2}H and β,β-$^2H_2$,γ-^{2}H species. These results indicate that hydrogen-transfer reactions from α or β positions of the substrate amino acids to the γ position of α-ketobutyrate formed, as reported in the process of α,γ-elimination of *O*-succinyl-L-homoserine by cystathionine γ-synthase (EC 4.2.99.9) (Posner & Flavin, 1972b), were not mediated by L-methionine γ-lyase during α,γ-elimination.

Deuterium Incorporation into Nonsubstrate Straight-Chain L-Amino Acids. A series of straight-chain L-amino acids that are nonsubstrates for elimination and replacement reactions also underwent the α and β hydrogen exchange. Table I summerizes the kinetic parameters of the enzyme-catalyzed

Table I: Rates of Exchange of α- and β-Hydrogens of Straight-Chain L-Amino Acids

substrate	K_i or K_m (mM)	initial velocity of exchange[c] (μmol/min) α-H	β-H	no. of β-H exchanging
L-alanine	5.1[b]	0.20	0.15	3
L-α-aminobutyrate	8.4[b]	2.70	1.98	2
L-norvaline	3.0[b]	14.8	5.20	2
L-norleucine	0.5[b]	18.8	6.00	2
S-methyl-L-cysteine	0.8[a]	28.6	15.0	2
L-methionine	1.3[a]	31.6	15.8	2

[a] K_m values were determined in elimination reactions. [b] K_i values are inhibition constants for the α,γ-elimination reaction of L-methionine. Similar K_i values were obtained for the α,β-elimination reaction of *S*-methyl-L-cysteine. [c] Initial velocities were determined as described under Experimental Procedures; 0.53 mg of the enzyme was added in the reaction mixture.

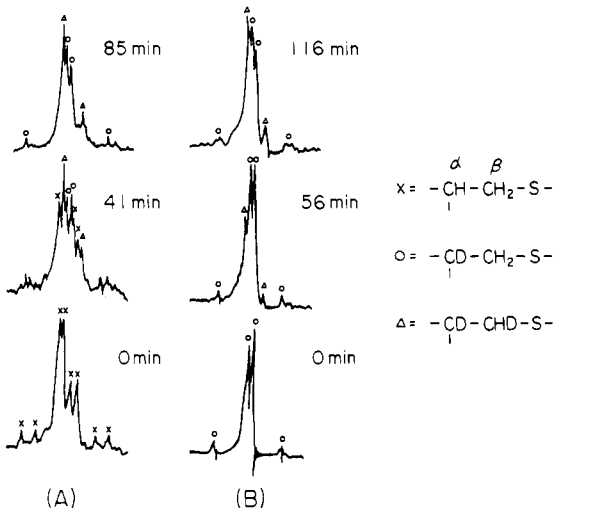

FIGURE 4: ^{1}H NMR spectra by deuterium decoupling of the β protons of *S*-methyl-L-cysteine with L-methionine γ-lyase. (A) *S*-Methyl-L-[α-^{1}H]cysteine was used as a substrate. (B) *S*-Methyl-L-[α-^{2}H]-cysteine was used as a substrate. Both the reaction systems contained 24 μg of enzyme.

exchange of the α- and β-hydrogens of several straight-chain L-amino acids including L-methionine and *S*-methyl-L-cysteine. All the nonsubstrate amino acids listed in the table inhibited both the α,γ-elimination of L-methionine and α,β-elimination of *S*-methyl-L-cysteine competitively with substrates. L-Norleucine showed the highest affinity and was followed by L-norvaline, L-alanine, and L-α-aminobutyrate in this order. The rate of decrease in relative integration values of α proton was exponential vs. time (i.e., first-order kinetics) for all the amino acids. However, kinetics of the β hydrogen loss varied with the kind of amino acids. The β-hydrogen exchange of L-alanine and L-α-aminobutyrate followed first-order kinetics through more than twice the half-life. When L-norleucine and L-norvaline were used, the rate decreased with time, which is similar to the β-hydrogen exchange of L-methionine and *S*-methyl-L-cysteine described above. Therefore, the rates of total β-hydrogen loss of the latter class of amino acids were obtained from a straight line drawn through the initial part of the curve for the β-hydrogen loss plotted semilogarithmically vs. time. For all the amino acids tested, the rate of total β-hydrogen loss did not exceed that of α hydrogen loss, and both rates increased with increase in length of the alkyl side chain of the amino acid.

Kinetics of β-Hydrogen Exchange. The change in the exchange rates of β-hydrogens with time probably occurs due

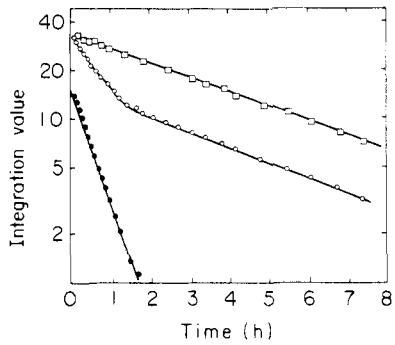

FIGURE 5: Disappearance of α (●) and β (○) protons of *S*-methyl-L-[α-^{1}H]cysteine and β (□) protons of *S*-methyl-L-[α-^{2}H]-cysteine. Conditions were the same as for Figure 4.

to stereospecificity for nonequivalent β-hydrogens by the enzyme or due to a kinetic isotope effect at the α position. Figure 4 shows the ^{1}H NMR spectra of β protons of *S*-methyl-L-[α-^{1}H]- and [α-^{2}H]cysteines taken by deuterium decoupling at intervals. The spectrum of the reaction mixture incubated with *S*-methyl-L-[α-^{1}H]cysteine for 85 min was essentially identical with that of the *S*-methyl-L-[α-^{2}H]cysteine incubated for 116 min. Two peaks that were superimposed on the AB type spectrum of the β-methylene protons appeared with incubation time. They are assigned to the spectra of (3*R*)- and (3*S*)-*S*-methyl-L-[α-^{2}H,β-^{2}H]cysteines, any of which gives rise to a singlet of the β proton. The signal at lower field was stronger than the other one at every stage of incubation, indicating that the β proton whose signal appears upfield is exchanged faster than the other, although we have not yet determined their stereochemistry. Figure 5 shows the time course of the β-hydrogen exchange of *S*-methyl-L-[α-^{1}H]- and [α-^{2}H]cysteines. The exchange rate of total β-hydrogens of *S*-methyl-L-[α-^{1}H]cysteine decreased with time and became constant after 90 min, when virtually no *S*-methyl-L-[α-^{1}H]-cysteine species remained. However, the β-hydrogens of *S*-methyl-L-[α-^{2}H]cysteine were lost at a constant rate throughout the time course, and the velocity coincided with that obtained from the linear part of the curve for the β-proton loss of *S*-methyl-L-[α-^{1}H]cysteine. The same relationship between α-^{1}H and α-^{2}H compounds in the time course of β hydrogen exchange was observed for L-methionine and L-norvaline. The kinetic isotope effect for the removal of β-hydrogens by replacement of α-hydrogen with deuterium were determined as follows: L-methionine, 2.1; *S*-methyl-L-cysteine, 2.6; L-norvaline, 1.9. We can demonstrate the kinetics of β-hydrogen exchange mainly in terms of a primary isotope effect since the enzyme probably has a similar affinity for these *S*-methyl-L-cysteine species in the substrate hydrogen exchange reaction; kinetic parameters for various *S*-methyl-L-cysteine species in α,β-elimination were determined as follows: *S*-methyl-L-cysteine, K_m = 0.77 mM and V_{max} ratio = 1; α-^{2}H species, K_m = 0.88 mM and V_{max} ratio = 0.38; β,β-$^2H_2$ species, K_m = 0.85 mM and V_{max} ratio = 0.91; α-^{2}H,β,β-$^2H_2$ species, K_m = 0.92 mM and V_{max} ratio = 0.33.

Deuterium Incorporation into Other Nonsubstrate Amino Acids. When glycine was incubated with L-methionine γ-lyase in ^2H_2O for 10 h, the peak integral of the proton diminished to half of that of the control in which enzyme was replaced by ^2H_2O. The signals of prochiral α protons were converted to a singlet by deuterium decoupling, indicating that one of the methylene protons was replaced by deuterium. To determine the stereochemistry in this reaction, glycine was separated from a ten times larger scale reaction mixture after 10 h and then converted to phenylalanylglycine according to the method of Kainosho et al. (1975). The ^{1}H NMR spectrum of the dipeptide revealed a singlet due to (*R*)-glycine-d_1 that was assigned on the basis of the reported chemical shift (Kainosho et al., 1975) in addition to a quartet due to nondeuterated glycine, but no signal due to (*S*)-glycine-d_1 was observed. Therefore, L-methionine γ-lyase exchanges the *pro-R* hydrogen of glycine with the deuterium of ^2H_2O.

The hydrogen-exchange reactions of L-tryptophan and L-phenylalanine also were examined with a large amount of the enzyme (1.6 mg/2 mL of the reaction mixture). The spectra of both amino acids showed no signal due to an α proton, but the relative integration values of β protons to the corresponding aromatic side-chain protons remained almost unchanged. Thus, these aromatic amino acids underwent only the α-hydrogen exchange.

We were unable to detect the exchange of α- and β-hydrogens for the following amino acids after a 16 h-incubation with 0.4 mg of L-methionine γ-lyase: D isomers of methionine, *S*-methylcysteine, alanine, α-aminobutyrate, norvaline, and norleucine. The following L-amino acids were also undetected: α-methyl methionine, *N*-acetylmethionine, *N*-methylalanine, serine, threonine, proline, leucine, isoleucine, valine, glutamate, γ-methylglutamate, glutamine, aspartate, asparagine, lysine, ornithine, histidine, and arginine.

Discussion

Figure 6 shows a proposed mechanism for L-methionine γ-lyase catalyzed incorporation of solvent deuterium into the α and β positions of various amino acids. After conventional transaldimination, the α-hydrogen is abstracted as a proton from the resulting amino acid–pyridoxal-P Schiff base (I) by a basic side chain at the active site followed by the formation of intermediate II. If the proton attached to the base is exchanged with a solvent deuteron rapidly, reversal of the process will result in liberation of a species deuterated in the α position. The β-hydrogen exchange probably occurs through a reversible tautomerization between intermediates II and III. Thus, an amino acid species deuterated in both α and β position is produced. Intermediate III can eliminate the γ-substituent X to produce a vinylglycine intermediate (IV), the key intermediate of α,γ-elimination and γ-replacement reactions (Davis & Metzler, 1972; Walsh, 1979). We could not observe the hydrogen exchange at α and β positions for L-methionine sulfone and trifluoro-L-methionine. The γ-substituents of these amino acids are good leaving groups and therefore were eliminated rapidly from intermediate III. The electron-donating nature of the thiomethyl group of methionine may stabilize the enamine intermediate III and facilitate the α-hydrogen exchange. However, the V_{max} value of L-methionine in α,γ-elimination differs only a little from the V_{max} values of the above substrates. Therefore, we can assume that the rate-determining step in α,γ-elimination of these amino acids occurs after the formation of intermediate IV as is suggested by Johnston et al. (1979a). In the α,β-elimination of *S*-methyl-L-cysteine, α-hydrogen abstraction is at least rate limiting since a deuterium isotope effect was observed for α-^{2}H and α-^{2}H,β,β-$^2H_2$ species, but not for β,β-$^2H_2$ species (see Results).

For all amino acids that undergo the hydrogen-exchange reaction, α-hydrogen exchange proceeds linearly with time. For L-methionine, *S*-methyl-L-cysteine, L-norleucine, and L-norvaline, biphasic kinetics was observed for β-hydrogen exchange, which can be explained in terms of a primary kinetic isotope effect. For L-alanine and L-α-aminobutyrate, the rate of total β-hydrogen loss was comparable with that of α-hydrogen loss. For longer straight-chain L-amino acids, the

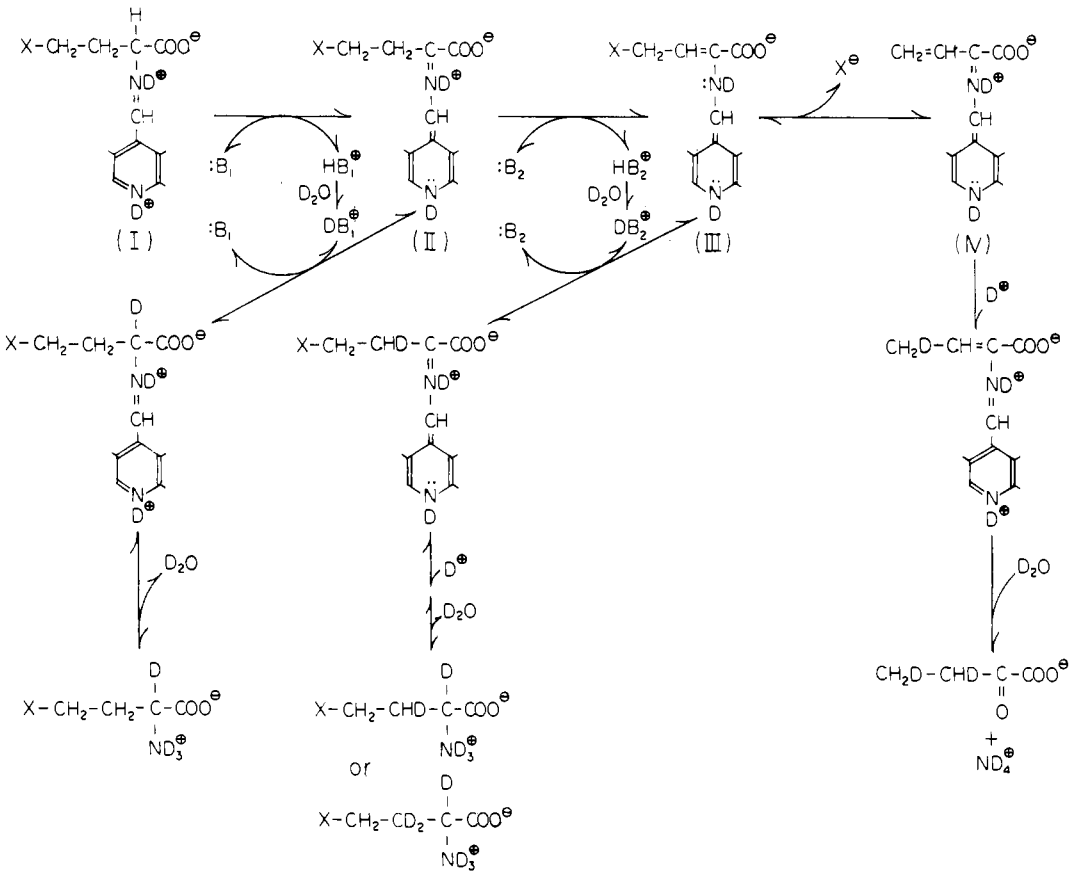

FIGURE 6: Proposed mechanism for L-methionine γ-lyase catalyzed deuterium exchange at the α and β positions of the amino acid.

exchange rate was much higher than that for L-alanine or L-α-aminobutyrate. The alkyl side chain of these amino acids possibly fits the binding site that normally interacts with the thiomethyl moiety of L-methionine. Thus, the action of the base abstracting α- and β-hydrogens will be enhanced.

Alanine aminotransferase (EC 2.6.1.2) catalyzes a rapid exchange of both the α- and β-hydrogens of L-alanine with deuterium in ^2H_2O to yield two types of alanine: [α-^{2}H]- and [β-^{2}H]alanines (Cooper, 1976; Babu & Johnston, 1976; Golichowski et al., 1977). This is attributed to the effective conservation of α-hydrogen once abstracted by the basic group at the active site. Cystathionine γ-synthase also catalyzes α- and β-hydrogen exchange of many kinds of amino acids with deuterium or tritium of solvents (Guggenheim & Flavin, 1969). The rate of tritium incorporation in tritiated water is greater into the β position than into the α position for the L-homocysteinyl moiety of L-cystathionine but vice versa for the L-cysteinyl moiety of L-cystathionine (Posner & Flavin, 1972a). L-Methionine γ-lyase showed no such variation with amino acids. This finding indicates that an α-hydrogen once removed is scarcely conserved at the active site and exchanged instantly with deuterium of solvent. Therefore, the rate of total β-hydrogen loss cannot exceed the rate of α-hydrogen loss.

L-Methionine γ-lyase showed stereoselectivity in the β-hydrogen exchange of *S*-methyl-L-cysteine. However, the following results suggest that the two nonequivalent β hydrogens differ only a little in exchange rate: (a) total β-hydrogens of *S*-methyl-L-[α-^{2}H]cysteine were lost at a constant rate throughout the time course, and (b) the height ratio of the downfield signal to the upfield one due to protons of (3*R*)- or (3*S*)-*S*-methyl[α-^{2}H,β-^{2}H]cysteine did not change apparently at various time intervals. After one hydrogen is exchanged with deuterium by stereoselectivity to form (3*R*) or (3*S*) α-^{2}H,β-^{2}H species, there will be a competition between the hydrogen and the deuterium at the β position of this species for abstraction by the base at the active site. Presumably, the remaining β-hydrogen is abstracted and exchanged with deuterium more rapidly than the β-deuterium, although further investigation is needed. Both cystathionine γ-synthase (Posner & Flavin, 1972a) and cystathionine γ-lyase (Washtien et al., 1977) have been reported to show distinct stereoselectivity in the β-proton exchange of various amino acids. Washtien et al. (1977) observed biphasic kinetics for the β-hydrogen exchange of L-norvaline by cystathionine γ-lyase and showed that one β-hydrogen of L-norvaline is exchanged 24 times faster than the other. Posner & Flavin (1972a) demonstrated that cystathionine γ-synthase can exchange one β-hydrogen of L-homoserine with deuterium more than 100 times faster than the other. They observed that the γ-proton signal of homoserine is completely transformed into a doublet at the early stage of incubation (i.e., formation of α-^{2}H,β-^{2}H species) and then a new singlet appeared between the doublet peaks very slowly (i.e., formation of α-^{2}H,β,β-$^2H_2$ species). We could not observe such a sequential transformation in the β-proton signal of *S*-methyl-L-cysteine (see Figure 4) or in the β-proton signal of L-methionine (see Figure 1) during incubation with L-methionine γ-lyase in ^2H_2O.

Registry No. H_2, 1333-74-0; L-alanine, 56-41-7; L-α-aminobutyrate, 1492-24-6; L-norvaline, 6600-40-4; L-norleucine, 327-57-1; *S*-methyl-L-cysteine, 1187-84-4; L-methionine, 63-68-3; L-vinylglycine, 70982-53-5; L-methionine sulfone, 7314-32-1; L-methionine γ-lyase, 42616-25-1.

REFERENCES

Babu, U. M., & Johnston, R. B. (1976) *Biochemistry 15*, 5671–5678.

Cooper, A. J. L. (1976) *J. Biol. Chem. 251*, 1088–1096.

Davis, L., & Metzler, D. E. (1972) *Enzymes, 3rd Ed. 7*, 33.

Esaki, N., Suzuki, T., Tanaka, H., Soda, K., & Rando, R. R. (1977) *FEBS Lett. 84*, 309.
Esaki, N., Tanaka, H., Uemura, S., Suzuki, T., & Soda, K. (1979) *Biochemistry 18*, 407-410.
Golichowski, A., Harruff, R. C., & Jenkins, W. T. (1977) *Arch. Biochem. Biophys. 178*, 459-467.
Guggenheim, S., & Flavin, M. (1969) *J. Biol. Chem. 244*, 6217—6227.
Johnston, M., Marcotte, P., Donovan, J., & Walsh, C. (1979a) *Biochemistry 18*, 1729-1738.
Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., & Walsh, C. (1979b) *Biochemistry 18*, 4690-4701.
Johnston, M., Raines, R., Walsh, C., & Firestone, R. A. (1980) *J. Am. Chem. Soc. 102*, 4241-4250.
Kainosho, M., Ajisaka, K., Kamisaku, M., & Murai, A. (1975) *Biochem. Biophys. Res. Commun. 64*, 425-432.
Lavine, T. F. (1947) *J. Biol. Chem. 169*, 477.
Posner, B. I., & Flavin, M. (1972a) *J. Biol. Chem. 247*, 6402-6411.
Posner, B. I., & Flavin, M. (1972b) *J. Biol. Chem. 247*, 6412-6419.
Soda, K. (1968) *Anal. Biochem. 25*, 228.
Soda, K., & Osumi, T. (1971) *Methods Enzymol. 17B*, 629-636.
Soda, K., Tochikura, T., & Katagiri, H. (1961) *Agric. Biol. Chem. 25*, 811.
Tanaka, H., Esaki, N., Yamamoto, T., & Soda, K. (1976) *FEBS Lett. 66*, 307.
Tanaka, H., Esaki, N., & Soda, K. (1977) *Biochemistry 16*, 100.
Toennis, G., & Kolb, J. J. (1941) *J. Biol. Chem. 140*, 131.
Walsh, C. (1979) in *Enzymatic Reaction Mechanism*, pp 777-827, W. H. Freeman, San Francisco.
Washtien, W., Cooper, A. J. L., & Abeles, R. H. (1977) *Biochemistry 16*, 460-463.

Light-Scattering Investigation of the Dissociation Behavior of *Lunatia heros* and *Littorina littorea* Hemocyanins†

Theodore T. Herskovits* and L. John Mazzella
Department of Chemistry, Fordham University, Bronx, New York 10458
German B. Villanueva
Department of Biochemistry, New York Medical College, Valhalla, New York 10595
Received November 27, 1984

ABSTRACT: The subunit structure and dissociation of the hemocyanins of two marine snails, *Lunatia heros* and *Littorina littorea*, were investigated by light-scattering molecular weight methods. The hemocyanins of both species of snails are readily dissociated to fragments of one-tenth and one-twentieth of the parent proteins of close to 9×10^6 daltons by either increasing the pH or using dissociating reagents of the hydrophobic urea series or some of the Hofmeister salts. The lower members of the latter group of reagents, NaCl, and to some extent also NaBr were found to have only marginal effects on the observed molecular weight transitions, suggesting that the two hemocyanins investigated possess β-type subunits, which are known to be resistant to NaCl dissociation. The molecular weight profiles obtained with the various dissociating reagents were single inverted sigmoidal-shaped curves for both *Lunatia* and *Littorina* hemocyanins, suggesting overlapping transitions. The ultracentrifugation patterns and the species-distribution plots based on the urea dissociation data of *Littorina* hemocyanin suggest the presence of whole, half, and one-tenth molecular weight species in the dissociation transition region. Fitting of the urea dissociation data of *Littorina* hemocyanin obtained at both pH 5.7 and pH 8.0, assuming a sequential two-step dissociation scheme used in our previous studies [Herskovits, T. T., & Russell, M. W. (1984) *Biochemistry 23*, 2812-2819], was found to be consistent with a model of a few hydrophobic binding sites at the contact areas of the half-molecules and a much larger apparent number of binding sites (N_{app}) at the side to side contacts of the one-tenth molecules. Model calculations also showed that with much larger N_{app} values for the first dissociation step, approaching that of the second step of the dissociation reaction (N_{app} = 50-60), biphasic curves would be produced. This suggests that the stabilizing interactions between the basic decameric units of the marine hemocyanins are largely nonhydrophobic in origin and therefore must be due primarily to polar and ionic interactions. The much larger N_{app} estimates characterizing the second step of the dissociation reaction are comparable to those obtained with the α component of *Helix pomatia* hemocyanin. Hydrophobic stabilization of each half-molecule through side to side contact of the dimeric subunits is suggested by both the latter observations and also our finding that the dissociation of *Littorina*, as well as *Lunatia*, hemocyanin by the urea series follows the expected order of increasing effectiveness with increasing hydrophobicity of the dissociating reagent.

The hemocyanins are copper-containing multisubunit proteins that serve as oxygen carriers in the circulatory systems of many arthropods and molluscs. The hemocyanins of the latter phylum are large cylindrical particles of decameric assemblies consisting usually of one or two such assemblies. The hemocyanins of octopi and squids are the simpler single-assembly particles while the land and marine snail hemocyanins are the

† Supported in part by Grant RR-0715 from the National Institutes of Health, U.S. Public Health Service.

0006-2960/85/0424-3862$01.50/0