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Mechanism of α-Amino-ε-caprolactam Racemase Reaction

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ABSTRACT: α-Amino-ε-caprolactam racemase catalyzes the exchange of the α-hydrogen of the substrate with deuterium during racemization in deuterium oxide. The rate of the hydrogen exchange measured by ^{1}H NMR is lower than that of racemization in deuterium oxide for both the enantiomers. Both the enantiomers of α-amino-ε-caprolactam show an overshoot of the optical rotation during the enzymatic racemization in deuterium oxide (but not in water). This phenomenon may be attributable to a primary deuterium isotope effect at the α-position: α-deuterium isotope effects of 3.6 and 2.0 were observed for the racemization of the D and L enantiomers of α-amino-ε-caprolactam, respectively. Results of tritium-labeling experiments showed that the enzyme catalyzes both retention and inversion of configuration of the substrate with a similar probability in each turnover. Conversion of [α-^{2}H]-D-α-amino-ε-caprolactam in water and unlabeled D-α-amino-ε-caprolactam in deuterium oxide into the L isomer under nearly single turnover conditions with the enzyme showed significant internal return of the α-hydrogen. These results support a single base mechanism for the racemization reaction catalyzed by the enzyme.

Although mechanisms of various pyridoxal-P[1]-dependent enzyme reactions have been studied in some detail (Dunathan, 1971; Dunathan & Voet, 1974; Vederas & Floss, 1980), those of the pyridoxal-P-dependent racemases are not yet well understood. Unlike the other pyridoxal-P enzymes, the racemases have to operate on both sides of a planar substrate–pyridoxal-P complex formed at their active sites: they have to remove the C_α proton on one face and have to add a proton on the opposite face to accomplish the racemization of the substrate. Two mechanisms have been proposed for this unique catalysis by analogy with the proposed mechanisms for non-pyridoxal-P-dependent racemases (Cardinale & Abeles, 1968; Findlay & Adams, 1970; Kenyon & Hegeman, 1979): two acid–base groups may be situated on the opposite sides of the substrate–pyridoxal-P complex at the active site; alternatively, a single base may mediate both the abstraction of the substrate α-hydrogen and protonation of the resonance-stabilized carbanion on the opposite face. Recently, Shen et al. (1983) showed that the racemization of L-alanine catalyzed by pyridoxal-P-dependent amino acid racemase with low substrate specificity of *Pseudomonas putida* proceeds with a substantial internal retention of the α-hydrogen removed from the substrate and returned to the product under nearly single turnover conditions. This was demonstrated by an instant transformation of the product into the *N*-acetyl derivative, a non-substrate, to avoid the next turnover. Thus, they showed a single base mechanism for the enzymatic reaction.

α-Amino-ε-caprolactam racemase is different from other racemases and epimerases and also from other pyridoxal-P-dependent enzymes in acting exclusively on the synthetic intramolecular cyclic amides of lysine (α-amino-ε-caprolactam) and ornithine (α-amino-δ-valerolactam) (Ahmed et al., 1983a; 1983b). We have purified the enzyme to homogeneity from a cell-free extract of *Achromobacter obae* and have characterized it (Ahmed et al., 1982, 1983b). The monomeric enzyme has a molecular weight of 50000 and contains 1 mol of pyridoxal-P. We here present evidence for a single base mechanism for the racemization reaction catalyzed by this unique enzyme. This experiment has been facilitated by the use of a novel enzyme, L-α-amino-ε-caprolactam hydrolase, which catalyzes the stereospecific hydrolysis of L-α-amino-ε-caprolactam to L-lysine (Fukumura et al., 1978), to give a single turnover condition.

Experimental Procedures

Materials. L-α-Amino-ε-caprolactam hydrolase of *Cryptococcus laurentii* (Fukumura et al., 1978) and α-amino-ε-caprolactam racemase of *A. obae* (Ahmed et al., 1982) were purified as described previously. [α-^{2}H]-D- and [α-^{2}H]-L-α-amino-ε-caprolactams were prepared as follows. L-α-Amino-ε-caprolactam hydrochloride (33 g) was dissolved in a minimum volume of ^2H_2O (99.85%) (Merck) and lyophilized. After the process was repeated twice, ^2H_2O was added to give a final volume of 300 mL, and the p^{2}H was adjusted to 8.8 with 0.4 M NaO^2H. α-Amino-ε-caprolactam racemase (sp act. 75 units/mg; total 2500 units) was concentrated by ultrafiltration through an Amicon PM-10 membrane and washed 3 times with ^2H_2O in the same manner. The enzyme (3 mL) was added to the above α-amino-ε-caprolactam solution, and the mixture was incubated at 37 °C with continuous stirring. When the α-proton of α-amino-ε-caprolactam disappeared completely by ^{1}H NMR analysis, the reaction was stopped by addition of 1 N HCl. After neutralization with NaOH, 16.0 g of lyophilized cells of *C. laurentii* was added to the mixture. Then, incubation was continued until all the L-α-amino-ε-caprolactam was hydrolyzed to L-lysine. The reaction mixture was centrifuged, and ethanol (350 mL) was added to the supernatant. The precipitate formed was removed by filtration, and the filtrate was concentrated under reduced pressure. [α-^{2}H]-D-α-Amino-ε-caprolactam in the solution (pH 9.0) was extracted with ethyl acetate and was crystallized as its HCl salt from ethanol. The recrystallized compound (1.3 g) gave a single spot upon silica gel thin-layer chromatography with solvent systems of 1-butanol–acetic acid–water (6:2:2 v/v/v) and acetone–methanol (1:1 v/v). The remaining aqueous solution was brought to pH 1 by addition of HCl and was then treated with activated charcoal. [α-^{2}H]-L-Lysine was crystallized as its HCl salt (6.0 g) and then cyclized to [α-

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

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^2H]-L-α-amino-ϵ-caprolactam essentially by the method of Adamson (1943) followed by crystallization from ethanol as its HCl salt. The recrystallized compound (1.1 g) was free from lysine or other contaminant when analyzed by thin-layer chromatography. Both the enantiomers of [α-^2H]-α-amino-ϵ-caprolactam contained more than 99% deuterium in the α-position when analyzed by ^1H NMR. Their optical purities were measured with a polarimeter in 1 N HCl: D enantiomer, 100%; L enantiomer, 94%. [U-^{14}C]-L-α-Amino-ϵ-caprolactam hydrochloride (35 mCi/mmol) was prepared with [U-^{14}C]-L-lysine (New England Nuclear) in the same manner as described above.

Methods. The enzymatic racemization of α-amino-ϵ-caprolactam was followed by measurement of the rate of decrease in optical rotation at 365 nm with a Perkin-Elmer polarimeter Model 241. The standard assay systems and conditions were described previously (Ahmed et al., 1982).

The enzymatic reaction in ^2H_2O was conducted with a mixture (2.0 mL; p^{2}H 8.8) containing 200 μmol of the substrate, 40 nmol of pyridoxal-P, and enzyme (8.0 units) in 99.85% ^2H_2O. The solvent of the enzyme was exchanged with potassium phosphate (p^{2}H 7.8) in ^2H_2O. The α-proton exchange of the substrate with deuterium was followed by measurement with a JEOL JNM MH 100 NMR spectrometer.

Tritium incorporation into the enzymatic product was followed with a reaction mixture (0.1 mL, pH 8.2) containing 25 μmol of D- and L-α-amino-ϵ-caprolactam, 2 nmol of pyridoxal-P, 0.5 mCi of ^3H_2O (New England Nuclear), and 0.1 unit of α-amino-ϵ-caprolactam racemase. After incubation at 37 °C for 20 min, the reaction was stopped by immersing the tube in a boiling water bath for 10 min, followed by centrifugation to remove the denatured protein. The mixture was then evaporated to dryness and redissolved in 5 mL of water. After repetition of cycles of evaporation to dryness and dissolution in water 5 times, 10 μL of L-α-amino-ϵ-caprolactam hydrolase (3 units) and 0.1 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM $MgCl_2$ were added to the residue. The resulting solution was incubated at 37 °C for 4 h. Another 3 units of the hydrolase was added, and the incubation was continued for another 2 h. L-α-Amino-ϵ-caprolactam present can be converted into L-lysine completely under the conditions. D-α-Amino-ϵ-caprolactam remaining and L-lysine were separated by paper chromatography with a solvent system of 1-butanol–acetic acid–water (6:1:2 v/v/v). The areas corresponding to α-amino-ϵ-caprolactam (R_f 0.48) and L-lysine (R_f 0.17) were cut out to measure the radioactivities.

Results

Comparison of the Kinetic Constants. We have determined the initial velocity for α-amino-ϵ-caprolactam racemase by the polarimetric assay in both the D to L and the L to D directions. The K_m values for D- and L-α-amino-ϵ-caprolactam were 4.9 ± 0.5 and 5.4 ± 0.5 mM, respectively. The V_{max} values for the D to L and L to D directions were 490 ± 25 and 440 ± 20 μmol min^{-1} mg^{-1}, respectively, under identical conditions at pH 8.0. The chemically symmetric reactions should give an equilibrium constant of unity (Briggs & Haldane, 1925) as has been validated with other amino acid racemases (Adams, 1976; Wasserman et al., 1984). The K_{eq} of the α-amino-ϵ-caprolactam racemase was calculated from the derived V/K values and found to be consistent with the theoretical value:

$$K_{eq} = \frac{(V_{max}/K_m)_{D\ isomer}}{(V_{max}/K_m)_{L\ isomer}} = 0.99 \pm 0.1$$

Table I: Rates of Racemization and α-H Exchange of α-Amino-ϵ-caprolactam Catalyzed by α-Amino-ϵ-caprolactam Racemase[a]

substrate	solvent	rate (×10^2 min^{-1}) of α-H exchange	rate (×10^2 min^{-1}) of racemization	V_H/V_D
D-[α-^1H]	^1H_2O		3.0	
D-[α-^2H]	^1H_2O		0.88	3.4
L-[α-^1H]	^1H_2O		2.8	
L-[α-^2H]	^1H_2O		1.1	2.5
D-[α-^1H]	^2H_2O	1.7	2.2	
D-[α-^2H]	^2H_2O		0.60	3.7
L-[α-^1H]	^2H_2O	0.98	1.7	
L-[α-^2H]	^2H_2O		0.87	2.0

[a] The reaction conditions and the methods of determination of α-H exchange and racemization are given in the text.

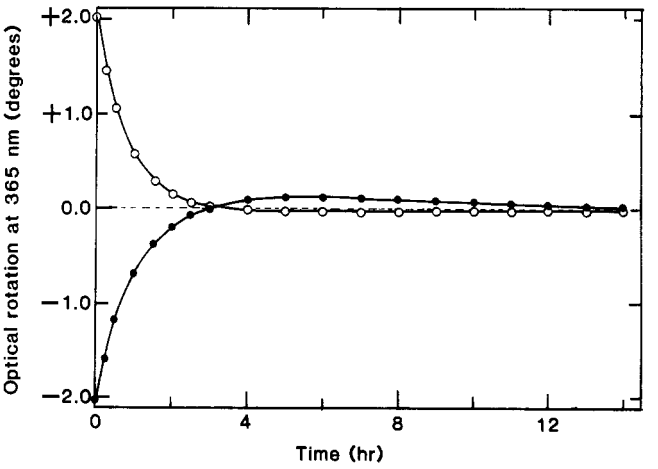

FIGURE 1: Racemization of D- (○) and L-α-amino-ϵ-caprolactam (●) in ^2H_2O. The substrates (200 μmol) were incubated with 40 nmol of pyridoxal-P and α-amino-ϵ-caprolactam racemase (8 units) in 2.0 mL at pH 8.2 and 37 °C.

Deuterium Isotope Effect. As reported previously, α-amino-ϵ-caprolactam racemase catalyzes the exchange of substrate α-hydrogen with deuterium in deuterium oxide during racemization (Ahmed et al., 1983b). Therefore, the exchange rate can be easily determined by ^1H NMR analysis and compared with the rates of racemization for both D and L enantiomers. We have found that the rates of α-hydrogen exchange are distinctly lower than those of racemization for both the substrate enantiomers under the same conditions (Table I). We have determined the rates of racemization of α-amino-ϵ-caprolactam and [α-^2H]-α-amino-ϵ-caprolactam under identical conditions to compare with each other with a substrate concentration of 100 mM, which is about 20 times higher than the K_m value. The observed deuterium isotope effects in both the directions of racemization (i.e., from D to L and from L to D) indicate that α-hydrogen labilization is at least partially rate limiting in the racemization. When the optical rotation change of the L isomer was followed in deuterium oxide, the value, which was initially negative, increased with time to reach to an extremum beyond zero and then approached zero on prolonged incubation (Figure 1). However, such an overshoot of the optical rotation was not observed when the reaction was carried out in water with either of the enantiomers. This is most probably due to deuterium isotope effects at the α-position as was shown by Cardinale & Abeles (1968) and Cleland (1977) for the proline racemase reaction.

Tritium Incorporation into the Product Enantiomers during Racemization in Tritiated Water. There are two possible mechanisms for the enzymatic racemization reactions: the enzyme catalyzes (1) only the inversion of configuration of the substrate (i.e., from the L enantiomer to the D enantiomer

Table II: Tritium Incorporation into Both the Enantiomers of α-Amino-ε-caprolactam during the Initial Period of Incubation with α-Amino-ε-caprolactam Racemase in Tritiated Water

substrate	radioactivity (dpm) of D-α-amino-ε-caprolactam	radioactivity (dpm) of L-lysine[a]
D-α-amino-ε-caprolactam	1900	2100
L-α-amino-ε-caprolactam	1500	1200

[a] L-α-Amino-ε-caprolactam was converted into L-lysine with L-α-amino-ε-caprolactam hydrolase as described in the text.

and vice versa) or (2) both retention and inversion of configuration with a similar or different probability [i.e., from the L enantiomer (or the D enantiomer) to the D and L enantiomers]. In order to clarify the mechanism of racemization, we determined tritium incorporation into the substrate and its enantiomer produced during incubation with the enzyme in tritiated water. The reaction was terminated before less than 10% of the substrate was racemized, and the tritium incorporation was analyzed as described under Experimental Procedures. As shown in Table II, tritium was incorporated into both the enantiomers of α-amino-ε-caprolactam, whichever enantiomer was used as a substrate. This indicates that both the enantiomers are produced with almost an equal probability in each catalytic event. However, tritium was incorporated a little preferentially into the antipode of the substrate used.

Internal Return of α-Hydrogen during Racemization. In order to demonstrate the number of bases at the active site participating in the catalysis of α-amino-ε-caprolactam racemization, we attempted to determine whether the α-hydrogen (or the α-deuterium) of the substrate is retained at the α-position of the product after a single catalytic event. If the α-hydrogen of the substrate is retained at the α-position of the product, a single base probably participates in both removal of the α-hydrogen from the substrate and incorporation of hydrogen atom into the α-position of the product. If different bases participate individually in these two processes, one will not observe the α-hydrogen of substrate to be retained at the α-position of the product. [α-^{2}H]-D-α-Amino-ε-caprolactam in ^1H_2O and [α-^{1}H]-D-α-amino-ε-caprolactam in ^2H_2O were incubated separately with the enzyme, and the L enantiomer formed was immediately converted into L-lysine, a nonsubstrate of the racemase, with L-α-amino-ε-caprolactam hydrolase. After all the L-α-amino-ε-caprolactam formed was converted into L-lysine, protein was removed with an Amicon PM-10 ultrafiltration membrane. The filtrate was evaporated to dryness, and the residue was washed with acetone and deuterium oxide successively. The dried residue was finally redissolved in a small volume of deuterium oxide, and the product was analyzed by ^{1}H NMR. The trapping efficiency was examined by addition of a trace amount of [U-^{14}C]-L-α-amino-ε-caprolactam to the reaction mixture and by measurement of the partition of radioactivity between L-lysine and L-α-amino-ε-caprolactam. An average of more than 94% of trapping efficiency was observed. As shown in Table III, 11–20% of the α-hydrogen (or the α-deuterium) of D-α-amino-ε-caprolactam was retained at the α-position of L-lysine in two separate sets of experiment. The results thus strongly suggest a single base mechanism for the α-amino-ε-caprolactam racemase reaction.

We have shown that the rates of racemization are higher than those of α-hydrogen exchange for both of the substrate enantiomers (Table I). This might be reasonable if the enzyme catalyzes the inversion of configuration much more frequently than the retention in each catalytic event. But this is not the case as validated in Table II. The internal return of the α-hydrogen from the substrate to the product may be responsible at least partially for the observed difference between the rates of racemization and α-hydrogen exchange.

Table III: Internal Retention of α-Hydrogen (or α-Deuterium) during Racemization of α-Amino-ε-caprolactam

expt	α-position of substrate	solvent	racemase (unit): hydrolase (unit)	internal retention (%)
I[a]	^{1}H	^2H_2O	8.0:40	17
	^{2}H	^1H_2O	20:40	11
II[b]	^{1}H	^2H_2O	1.2:6.0	18

[a] The reaction mixtures (2.0 mL, pH 8.2 or p^{2}H 8.8) containing 200 μmol of the substrate, 2 μmol of $MgCl_2$, 5 nmol of pyridoxal-P, and the indicated amounts of the enzymes were incubated at 37 °C with gentle shaking for 5 h. [b] Substrate, 100 μmol; incubated time, 15 h.

Discussion

Racemization is the simplest of the reactions catalyzed by pyridoxal-P enzymes (Dunathan, 1971). However, little attention has been paid to comprehensive understanding of the mechanisms and the active site geometries for pyridoxal-P-dependent racemases. A single base mechanism for alanine racemase of *Bacillus subtilis* named the "swinging door mechanism" has been proposed on the basis of kinetic studies with a partially purified preparation of the enzyme (Henderson & Johnston, 1976). Recently, Shen et al. (1983) showed for the first time that a pyridoxal-P-dependent amino acid racemase with low substrate specificity catalyzes the racemization of alanine through the participation of a single active site base. The results presented here indicate that deprotonation and reprotonation of a pyridoxal-P–substrate complex are accomplished by a single active site base in the same manner as in the amino acid racemase. An internal return of 11–18% of the α-hydrogen (or α-deuterium) suggests the participation of a polyprotic base such as an ε-amino group of a lysine residue, although rapid equilibration of the hydrogen (or deuterium) abstracted by a monoprotic base with the solvent deuterium (or hydrogen) also can explain the phenomena. Considerable difference in the degree of retention of α-^{1}H in a ^2H_2O environment vs. α-^{2}H in a ^1H_2O environment is a feature also observed for the amino acid racemase with low substrate specificity (Shen et al., 1983). There may be discrimination between ^{1}H and ^{2}H due to an isotope effect in the protonation from the active site base to the C_α carbanion of the substrate–pyridoxal-P complex if a polyprotic base participates in the protonation and deprotonation at C_α.

The tritium-labeling experiments indicate that the resonance-stabilized pyridoxal-P carbanion derived from the substrate partitions between both D and L forms of the products. However, the probabilities of the formation of the two enantiomers were not equal, and the enzyme showed a little higher affinity for the D isomer. Such a preference for one enantiomer may reflect the presence of distinct binding sites for the two enantiomers. Alternatively, in the swinging door model one of the two positions for interaction of the bound pyridoxal-P with the substrate (Vederas & Floss, 1980) may be favored by the position of the active site base as has been proposed for alanine racemase (Henderson & Johnston, 1976). The preferential incorporation of tritium into the product enantiomer compared to the substrate enantiomer (Table II) also could be explained by the swinging door model: the acid form of the base, which provides a proton to the α-carbon of the substrate–pyridoxal-P complex, might have access to the tritiated solvent when the swinging door is open. The unusual kinetic behavior of a racemase reaction in deuterium oxide in producing an overshoot of optical rotation was first reported by Cardinale & Abeles (1968) in the racemization of proline by proline racemase. Cleland (1977) showed on the basis of

the results reported by Cardinale and Abeles that the observed minimum value of optical rotation of proline is consistent with the observed deuterium isotope effect at the α-position. The overshoot produced by α-amino-ε-caprolactam racemase is relatively low, but the isotope effects at the α-position are higher than those observed in the proline racemase reaction. This could be related to the fact that both D and L enantiomers are produced from either of the two enantiomers of α-amino-ε-caprolactam and that the proton abstracted from the substrate is mostly exchanged with solvent while the substrate is present on the enzyme.

Registry No. H_2, 1333-74-0; D_2, 7782-39-0; α-amino-ε-caprolactam racemase, 52652-64-9; D-α-amino-ε-caprolactam, 28957-33-7; L-α-amino-ε-caprolactam, 21568-87-6; [α-^{2}H]-D-α-amino-ε-caprolactam hydrochloride, 99560-23-3; [U-^{14}C]-L-α-amino-ε-caprolactam hydrochloride, 99560-25-5; [α-^{2}H]-L-α-amino-ε-caprolactam hydrochloride, 99560-24-4.

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Xylose-Containing Common Structural Unit in N-Linked Oligosaccharides of Laccase from Sycamore Cells†

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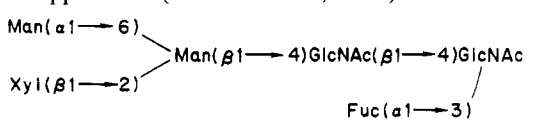
ABSTRACT: The structures of asparagine-linked oligosaccharides of laccase excreted by sycamore (*Acer pseudoplatanus* L.) cells are reported. Peptic glycopeptides obtained from the laccase were treated with *N*-oligosaccharide glycopeptidase (EC 3.5.1.52) to release the oligosaccharide moieties. The oligosaccharides thus obtained were fractionated into six components by gel filtration, thin-layer chromatography, and high-performance liquid chromatography. The structures of the isolated oligosaccharides were determined by sugar analysis, exoglycosidase digestion, and methylation analysis in combination with high-resolution proton nuclear magnetic resonance spectroscopy. It was found that (1) the six oligosaccharides are a series of compounds of xylose-containing biantennary complex types that share as the core a common structural unit, i.e., Xylβ1→2(Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc, and (2) mannose, *N*-acetylglucosamine, galactose, and fucose residues are additionally linked to the core as the outer chain moieties.

We have previously reported the complete structure of the asparagine-linked oligosaccharide of bromelain isolated from the pineapple stem (Ishihara et al., 1979):



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