Properties of Aspartate Racemase, a Pyridoxal 5'-Phosphate-independent Amino Acid Racemase*

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Aspartate racemase from Streptococcus thermophilus contains no pyridoxal 5'-phosphate or other cofactors such as FAD, NAD⁺, and metal ions. It was affected by neither carboxyl reagents such as hydroxylamine nor sodium borohydride but was strongly inhibited by iodoacetamide and other thiol reagents. Aspartate, cysteate, and cysteine sulfinate were the only substrates. The K₅ values for L- and D-aspartate were 35 and 8.7 mM, respectively. The enzyme catalyzed the exchange of α-hydrogen of the substrate with the solvent hydrogen. Racemization of L-aspartate in ²H₂O showed an overshooting in the optical rotation of aspartate before the substrate was fully racemized. This shows that the removal of α-hydrogen of the substrate is at least partially rate-determining. When L- or D-aspartate was incubated with aspartate racemase in tritiated water, tritium was incorporated preferentially into the product enantiomer. The results strongly suggest that aspartate racemase contains two hydrogen acceptors.

Racemization is superficially a simple reaction. For example, it is accomplished by the removal of α-hydrogen bound to a chiral carbon of the substrate and subsequent nonspecific return of a hydrogen to the carbon. However, amino acids are racemized only slowly under ordinary conditions: half-lives of aspartic acid and alanine in the racemization at 25 °C are 3,500 and 12,000 years, respectively (Bada, 1985). This is because of the high dissociation energy of the C=H bond.

Amino acid racemases and epimerases catalyze the racemization and epimerization of amino acids, respectively, and most of them require PLP as a coenzyme. The racemization proceeds through the formation of aldime Schiff bases between the substrate amino acid and PLP (Snell and Di Mari, 1970). However, several other amino acid racemases require no coenzymes. Proline racemase (EC 5.1.1.4) catalyzes the proline racemization effectively without PLP by action of a pair of cysteinyl residues at the active site of the enzyme (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975). Knowles and his co-workers studied the energetics and mechanism of the enzyme reaction (Albery and Knowles, 1986a, 1986b, 1986c; Fisher et al., 1986a, 1986b, 1986c; Belasco et al., 1986a, 1986b, 1986c). Hydroxyproline epimerase (EC 5.1.1.8) is also PLP-independent (Finley and Adams, 1970). It has been demonstrated clearly that proline racemase and hydroxyproline epimerase, which act on α-imino acids, do not require PLP. However, there exist a few PLP-independent racemases and epimerases acting on α-amino acids as well; diaminopimelate epimerase (EC 5.1.1.7) (Wiseman and Nichols, 1984; Higgins et al., 1989) and glutamate racemase (EC 5.1.1.3) (Nakajima et al., 1986, 1988) require neither coenzymes nor metals.

Aspartate racemase was discovered in Streptococcus faecalis and purified partially (Lamont et al., 1972). Recently, Yohda et al. (1991) cloned the enzyme gene from Streptococcus thermophilus, expressed it in Escherichia coli cells, and purified the enzyme to homogeneity from S. faecalis (Okada et al., 1991) and the E. coli clone cells (Yohda et al., 1991). We here show that the enzyme is independent of coenzymes and metals and involves two independent bases participating in the abstraction and return of α-hydrogen of the substrate.

**EXPERIMENTAL PROCEDURES**

**Purification of Aspartate Racemase**—We purified aspartate racemase from E. coli HB101-pAG6-2 (Yohda et al., 1991) to homogeneity by the same method as reported previously (Yohda et al., 1991) except that a step of gel filtration with Cellulose CCL1000m was added after the butyl-Toyopearl hydrophobic chromatography.

**Enzyme and Protein Assay**—Aspartate racemase was assayed at 30 °C with a mixture (0.2 ml) containing 0.1 m potassium phosphate buffer (pH 8.0), 24 mM 4-aminooantipyrine, 24 mM N'-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (from Dojin), 2 units of horseradish peroxidase (from Toyobo), 28 units of L-glutamate oxidase (from Yamasa Shoyu), 0.2 mM D-aspartate, and aspartate racemase in a final volume of 0.2 ml. L-Aspartate formed was determined with L-glutamate oxidase, and the hydrogen peroxide produced reacted with 4-aminooantipyrine, N'-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine, and peroxidase to form a condensation product which absorbs at 555 nm. The production of L-aspartate from the D-enantiomer was followed in the same manner except that L-glutamate oxidase and D-aspartate were replaced by D-amino acid oxidase (2.2 units) and L-aspartate, respectively. Racemization of aspartate, cysteate, cysteine sulfinate, and other amino acids was also followed by measurement of a change in optical rotation at 365 nm with a Perkin-Elmer polarimeter model 241. The reaction mixture containing 0.2 mM substrate, 0.1 M Tris-HCl (pH 8.0), and aspartate racemase was incubated in a polarimeter cell (1 ml) at 30 °C.

Protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard, or from the absorption coefficient of the enzyme [A]_280, which was calculated according to the method of Kurmishtu et al. (1990). One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of L-aspartate from the D-enantiomer/min.

**Metal Analysis**—Metals were determined by the method of standard additions with a Shimadzu AA-670G atomic absorption spectrophotometer equipped with a graphite furnace atomizer. Absorption was monitored at an appropriate wavelength with deuterium arc background correction. All solutions used for metal analysis were

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passed through a Chelex (Bio-Rad) column (1 x 10 cm) to remove free metal ions and were stored in polyethylene bottles.

**Modification of Thiol Group**—The enzyme (0.1 mg, 8 nmol) was incubated with 0.1-2 mM NTCB in a final volume of 0.2 ml at room temperature. The modification was followed by measurement of the absorbance at 412 nm. The amount of TNB formed from NTCB and aspartate racemase was estimated with the absorption coefficient of 13,600 M⁻¹ cm⁻¹. An aliquot of the above reaction mixture was removed at appropriate time intervals and added to the assay mixture to make a final concentration of NTCB lower than 0.2 μM.

**H-H Exchange**—The reaction mixture containing 0.1 μM L-aspartate, 0.1 M Tris-HCl (pH 8.0), and 0.05 mM of aspartate racemase in 4H₂O was incubated at 30 °C. The α-H exchange of the substrate was monitored with a Varian XL-200 NMR spectrometer. Proton incorporation into the α-position of [α-3H]aspartate was examined in a similar manner by incubation in 4H₂O.

**Trinitrobenzenesulfonic Acid Incorporation**—Trinitrobenzenesulfonic acid incorporation into the racemization product was examined in a mixture (1 ml) containing 0.5 mM L- or D-aspartate adjusted at pH 8.0, 0.8 unit of aspartate racemase, and 5 ml of tritiated water. After incubation at 30 °C for 30 or 60 min, the reaction was stopped by boiling for 10 min, followed by centrifugation to remove denatured protein. The supernatant solution was again treated with aspartate aminotransferase as described above to make a final concentration of NTCB lower than 0.2 μM.

**Cofactor and Prosthetic Group Requirement**—Electronic absorption spectra of the enzyme showed no characteristic absorption except for a peak at 277 nm. Addition of PLP, pyridoxal 5'-phosphate, PAP, ATP, NAD⁺, NADP⁺, NADH, and NADPH at a final concentration of 1 mM did not affect the enzyme activity. This indicates that the enzyme requires none of the cofactors. The enzyme retained its original activity after incubation with 1 mM hydroxylamine HCl or sodium borohydride in 0.1 M Tris-HCl (pH 8.0) at 30 °C for 30 min. This excludes the possibility that the enzyme contains a carbonyl group participating in catalysis such as pyruvoyl residue.

**Metal Cation Requirement**—The enzyme was not inactivated by incubation with chelating reagents such as EDTA and a &dipyridyl at a final concentration of 10 mM. Atomic absorption spectra of the enzyme showed the occurrence of about 0.05 g atom of zinc and 0.001-0.005 g atoms of calcium, vanadium, manganese, iron, cobalt, nickel, copper, selenium, and molybdenum per mol of subunit; the enzyme contains no significant amounts of the metals. Moreover, the addition of Ca²⁺, Mg²⁺, Mn²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ at a final concentration of 0.1 mM did not increase the enzyme activity. Thus, the enzyme requires none of these metal ions.

**Substrate Specificity**—L-Cysteine sulinate and L-cysteate were found to be good substrates of the enzyme; they were racemized at a rate of 51 and 88%, respectively, relative to that of L-aspartate. However, the presence of the acidic group at the β-carbon of the substrate is essential; asparagine, cysteine, and alanine were not the substrates. The carbon chain length of the substrate is also important; both isomers of glutamate were inert. L-Proline and L-hydroxyproline were not racemized. The KE values for L- and D-aspartate were 35 and 8.7 mM, respectively. The Ymax values of the racemization of L- and D-aspartate were 8.0 and 1.9 mM/ml/min, respectively. When these values were used, the calculated Kmax for aspartate racemization was 1.05, in good agreement with the theoretical value (1.0) for the chemical symmetric reaction.

Proline racemase is inhibited by proline at high concentrations because of oversaturation (Cardinale and Abeles, 1968). However, aspartate racemase is not influenced by high concentrations of aspartate up to 500 mM.

**Modification of Thiol Group**—The enzyme was inactivated completely by incubation with sulfhydryl reagents such as iodoacetate, iodoacetamide, p-chloromercuribenzoate, N-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoate), and NTCB at a final concentration of 1 mM in 0.1 M Tris-HCl (pH 8.0) at 30 °C for 30 min. The enzyme most probably contains a cysteine residue essential for catalysis.

Thiol groups of proteins are converted quantitatively to S-cyano derivatives specifically with NTCB, and the modification can be easily monitored by determination of TNB. Therefore, NTCB was used to determine the number of reactive cysteine residues of the enzyme and the rate of modification. Aspartate racemase is composed of two identical subunits as described previously (Okada et al., 1991), and six thiol groups were modified per dimer with a 50 x molar excess of NTCB; the value is consistent with the total number of cysteine residues of the enzyme which was deduced from the DNA sequence of the structural gene (Okada et al., 1991). These indicate that the enzyme contains no disulfide bonds. When TNB release was monitored upon incubation of the enzyme (2.4 mM) with 100 mM NTCB at 25 °C and pH 8.0, a fast TNB release of 2.0-2.6 mol/mol of enzyme (t½ < 0.2 min) was followed by a slow one (t½ = 13.8 min) corresponding to 3.4-4.0 mol/mol. When a 5-fold molar excess of NTCB was used for the thiol alkylation, 1 cysteine residue/dimer was modified with a concomitant loss of over 95% activity (Fig. 1). However, upon incubation of the NTCB-inactivated enzyme with an excess amount of thiol (e.g. 10 mM dithiothreitol), the enzyme was reactivated almost fully; the inactivation by NTCB is reversible (Fig. 2). The enzyme was protected from inactivation with NTCB by the addition of 10 mM DL-aspartate (Fig. 2).

**Isotope Incorporation and Time Course of the Reaction in 2H₂O**—We examined by 1H NMR whether deuterium is in-

![Fig. 1. Relationship between residual activity and thiol modification with NTCB. Aspartate racemase (8 nmol) was incubated with 0.2 mM NTCB in 0.2 ml of 50 mM Tris-HCl (pH 7.6) at room temperature (about 20 °C). An aliquot was removed and assayed for aspartate racemase activity (○). The number of cysteine residues modified (©) was estimated by the amount of TNB anion released upon the reaction of NTCB with aspartate racemase.
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**Tritium Incorporation**—We determined the amount of tritium incorporated into the substrate and product enantiomers during incubation with the enzyme in tritiated water. The enzyme acts on the product enantiomer accumulated in the reaction mixture, and the tritium labeling is probably based on the primary, secondary, and their derived reactions. Therefore, the reaction was stopped at its early stage when only a part (from 2.5 to 9.4%) of the substrate enantiomer was converted to the antipode. We analyzed the tritium distribution in the L-isomer with aspartate aminotransferase, which acts specifically on the L-isomer to exchange the α-hydrogen with hydrogen of solvent water. Thus, we determined the amount of tritium incorporated into both isomers by comparison of radioactivity of aspartate before and after the treatment with aspartate aminotransferase. The amount of tritium in L-aspartate was determined specifically by measurement of the radioactivity of water containing tritium liberated from the L-isomer with aspartate aminotransferase. As shown in Table I, tritium was incorporated almost specifically into the product enantiomer regardless of isomerism of the substrate used.

**DISCUSSION**

Two types of mechanisms for amino acid racemization have been proposed: one-base and two-base mechanisms. In the one-base mechanism, an α-hydrogen of substrate amino acid is abstracted by a single acceptor site of a racemase. The intermediate is an α-carbanion derived from the substrate and is kept at the active site until a proton is translocated from one face to the other of the substrate. The one-base mechanism is typical of PLP-dependent amino acid racemases (Snell and Di Mari, 1970). In the two-base mechanism, an α-hydrogen of amino acid is abstracted on one face as a proton on the other face is incorporated concertedly. Proline racemase (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975) and diaminopimelate epimerase (Wiseman and Nichols, 1984) reactions are proposed to proceed by the two-base mechanism.

A mechanism of tritium incorporation into substrate and product enantiomers in tritiated water with a single base is shown in Fig. 4. When L-aspartate was incubated with aspartate racemase, tritium was incorporated almost specifically into the product enantiomer regardless of isomerism of the substrate used.

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**TABLE I**

| Substrate | % Reaction | Total $^3$H incorporated (× 10°) | $^3$H incorporated into L-Aspartate (× 10°) | $^3$H incorporated into D-Aspartate (× 10°) |
|-----------|------------|---------------------------------|---------------------------------------------|---------------------------------------------|
| D-Aspartate | 2.5 | 143 | 130 (91) | 13 (9) |
| D-Aspartate | 5.0 | 268 | 242 (90) | 26 (10) |
| L-Aspartate | 4.6 | 260 | 41 (16) | 219 (84) |
| L-Aspartate | 9.2 | 387 | 42 (11) | 345 (89) |

*The percent reaction was determined by measurement of D- or L-aspartate formed under the standard conditions ("Experimental Procedures").
tate racemase in tritiated water, tritium was incorporated preferentially into \( \text{d-aspartate} \). This indicates that the rate of formation of tritiated \( \text{d-aspartate} \) is much faster than that of the antipode \( \text{d-aspartate} \). \( \text{d-aspartate} \) is racemized also through the common intermediates (i.e., E-BH\(-\)Im and E-BH\(^{3}\)H\(-\)Im). Therefore, when \( \text{d-aspartate} \) is used as a substrate, tritium should be incorporated preferentially into the \( \text{d-aspartate} \) isomer. However, this was not the case. Thus, the one-base mechanism is excluded.

Fig. 5 shows a two-base mechanism for tritium incorporation into substrate and product enantiomers. \( \text{d-[\( \alpha \)-\( ^3 \)H]Aspartate} \) is produced from L-aspartate through the route \( \text{a-b-c or a-b-d-e} \). The removal of \( \text{a}-\text{hydrogen} \) from the substrate (i.e., steps \( \text{b, b', and f} \)) is probably rate-determining as evidenced by the reaction in deutierium oxide. Therefore, the rate of the reaction through \( \text{a-b-c or a-b-d-e} \) should be much faster than that through \( \text{a-b-d-f-e'} \). In the \( \text{D to L} \) direction, the rate of \( \text{a'-b'-c'} \) or \( \text{a'-b'-d'-e'} \) is faster than that of \( \text{a'-b'-d'-f-e'} \) in the same manner. Thus, the scheme is compatible with the result that tritium is incorporated preferentially into the product enantiomers irrespective of the substrate enantiomers used.

In proline racemase (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975) and diaminopimelate epimerase (Wiseman and Nichols, 1984), a thiol group of cysteine residue serves as essential for the enzyme activity as described elsewhere. Cys\(^{151}\) is most probably the residue that is subject to selective modification by NTCB. The alkylation of 1 cysteine residue/dimer with NTCB resulted in a loss of 95% of the activity; the enzyme shows a half-of-the-sites-reactivity as well as proline racemase (Rudnick and Abeles, 1975) and hydroxyproline epimerase (Ramaswamy, 1984), both of which consist of two identical subunits. It is likely that aspartate racemase has a composite active site formed at the interface of two identical subunits in the same manner as proposed for proline racemase (Rudnick and Abeles, 1975).

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