Schachman, H. K. (1957), *Methods Enzymol.* 4, 32.
Schumaker, V. N., and Schachman, H. K. (1957), *Biochim. Biophys. Acta 23*, 628.
Siegel, L. M. and Monty, K. J. (1966), *Biochim. Biophys. Acta 112*, 346.
Smiley, K. L., Berry, A. J., and Suelter, C. H. (1967), *J. Biol. Chem. 242*, 2502.
Svedberg, T. (1940), The Ultracentrifuge, London, Oxford University, p 298.
van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Wolfenden, R. (1966), *J. Am. Chem. Soc. 88*, 3157.
Wolfenden, R., Sharpless, T. K., and Allan, R. (1967), *J. Biol. Chem. 242*, 977.

Purification and Mechanism of Action of Proline Racemase*

G. J. Cardinale and R. H. Abeles

ABSTRACT: Proline racemase was purified to near homogeneity from *Clostridium sticklandii*. The racemization of proline is accompanied by deuterium incorporation from the solvent into the α position of proline. In the initial phase of the reaction, the rate of deuterium incorporation is essentially equal to the rate of product formation, regardless of which isomer is used as substrate. These results indicate the participation of two equivalent hydrogen acceptor sites. No cofactor requirement could be detected.

The structures of inhibitors and the substrate suggest the possible involvement of a metal ion at the active center. The enzyme is a sulfhydryl enzyme and is inactive in the oxidized form. It is rapidly activated by reducing agents such as mercaptans or $NaBH_4$. When the racemization of L-proline is carried out in D_2O, the optical rotation is initially negative. As the reaction proceeds, it becomes positive, and finally zero when equilibrium is reached. This "overshoot" is attributed to a primary isotope effect.

A partially purified preparation of proline racemase was first described by Stadtman and Elliot (1957). The sulfhydryl nature of the enzyme was established and some information concerning cofactor requirements was obtained. Proline racemase appeared to differ from other amino acid racemases in that no requirement for pyridoxal phosphate could be shown. In that respect it resembles hydroxyproline epimerase which has recently been purified (Adams and Norton, 1964). We have now obtained a highly purified preparation of proline racemase and have investigated some aspects of the mechanism of action of this enzyme. Initially we showed that the α-carbon–hydrogen bond is broken in the course of racemization. Subsequent experiments were designed to establish the chemical mechanism by which the α-carbon–hydrogen bond is broken and how the inversion of configuration is brought about. The latter problem is concerned with the mechanism whereby hydrogen is transferred from one side of the α-carbon to the other and can be considered separately from the chemical mechanism by which the bond is dissociated and re-formed. Two mechanisms were considered for the hydrogen-transfer process: a "one-hydrogen-acceptor" and a "two-hydrogen-acceptor" mechanism. In the first mechanism hydrogen is transferred from the substrate to a hydrogen acceptor at the active site with the concomitant formation of an intermediate derived from the substrate. The intermediate could be a carbanion or an oxidized form of the substrate depending upon the chemical mechanism by which the α-carbon–hydrogen bond is broken. In a subsequent step hydrogen is transferred from the acceptor to the intermediate so that either the original substrate isomer or the product isomer is formed. If a single hydrogen-acceptor site participates, it must be flexible enough to interact with the α-hydrogen of both isomers, or the substrate must change its position relative to the hydrogen acceptor during the course of the racemization. The alternative mechanism is the "two-hydrogen-acceptor" mechanism. Here two equivalent sites are involved. The sites are so located that one interacts with the α-hydrogen of one substrate–isomer and the other site with the α-hydrogen of the other isomer. Inversion of configuration is achieved by transferring the α-hydrogen from the substrate to one of the acceptor sites and hydrogen from the other acceptor site to the substrate-derived intermediate. The question concerning the number of hydrogen acceptor sites has not been experimentally investigated for any amino acid racemase. The general problem has been considered by Rose (1966) for enzymes which carry out proton shifts. Evidence in favor of the two-site mechanism is avail-

* Publication No. 598 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received June 17, 1968*. This work was supported by National Institutes of Health Grant 12633. Part of this work was carried out in the Department of Chemistry, The Ohio State University, and was supported by funds from the Research Corporation.

TABLE I: Purification of Proline Racemase.

Fraction	Protein (mg)	Act. (units)	Sp. Act. (units/mg)	Purifcn	% Yield
I. Sonic extract	36,800	77,500	2.1		100
II. Protamine sulfate and $(NH_4)_2SO_4$	1,990	33,600	16.9	8.0	43
III. 70° heating	1,070	33,900	31.8	15.1	44
IV. DEAE column	96.2	26,100	272	130	34
V. $CaPO_4$ column + $(NH_4)_2SO_4$ precipitate	17.7	10,600	600	286	14

able for isopentenyl pyrophosphate isomerases (Agranoff *et al.*, 1960; Shah *et al.*, 1965; Rose, 1966).

Experimental Procedures

Enzyme Purification. Cells from 200 l. of 24-hr cultures of *Clostridium sticklandii* (ATCC 12662) (Wright and Stadtman, 1956) were harvested and washed with a 0.5% NaCl–0.5% KCl solution. Approximately 400 g of wet packed cells was suspended in 800 ml of 0.01 M Tris-HCl buffer (pH 8.7) and sonicated for 10 min in an MSE 500-W sonic oscillator. The cell debris, removed by centrifugation at 25,000*g* for 1 hr, was washed with 0.01 M Tris-HCl (pH 8.7) and the supernatant fluids were combined. In successful preparations, the 280-mμ:260-mμ ratio at this point was always above 0.595.

The protein concentration was adjusted to 20–25 mg/ml by addition of 0.01 M Tris-HCl (pH 8.7). The resultant solution was then treated with a 2% solution of protamine sulfate (about 110 mg of protamine sulfate/g of protein) until the 280-mμ:260-mμ ratio of the supernatant solution was 0.85–0.95.

To each 100 ml of the protamine superatant solution was added 42 g of solid $(NH_4)_2SO_4$. The precipitate was discarded. The addition of a further 21 g of $(NH_4)_2SO_4$/100 ml of solution resulted in a precipitate which was dissolved in a minimal amount of 0.05 M Tris-HCl buffer (pH 8.7). This fraction contained the proline racemase activity.

The ammonium sulfate fraction (usually about 140 ml) was placed in a 500-ml erlenmeyer flask and immersed in a 70° bath for 15 min. After cooling and centrifugation, the supernatant solution was dialyzed for 15 hr against 5 l. of 0.1 M Tris-HCl buffer (pH 7.5).

A DEAE-cellulose column was prepared by suspending 20 g of DEAE-cellulose in 0.1 M Tris-HCl (pH 7.5), readjusting the pH to 7.5 with dilute NaOH, and pouring the slurry into a column with a diameter of 3.8 cm. The DEAE-cellulose was then equilibrated by washing with 4 l. of 0.1 M Tris-HCl (pH 7.5). The dialyzed enzyme was introduced into the column, which was then washed with 0.1 M Tris-HCl (pH 7.5) until all of the protein which was not initially absorbed had been eluted. Proline racemase was eluted with a gradient generated by adding 0.6 M Tris-HCl (pH 7.5) to a constant-volume mixing chamber containing 2 l. of 0.1 M Tris-HCl (pH 7.5). The contents of the mixing chamber were stirred magnetically; 20-ml fractions were collected every 10 min. The enzyme was eluted when the Tris concentration reached about 0.27 M.

The fractions containing the racemase activity were combined and dialyzed successively against two 5-l. quantities of 0.01 M secondary sodium phosphate. This buffer, along with the rest of the buffers used for this column, was prepared in the following way. Sufficient secondary sodium phosphate for 5 l. of a 0.01 M solution was dissolved in 4800 ml. The solution was then brought to pH 6.8 with concentrated phosphoric acid and the volume adjusted to 5 l. A 22 × 185 cm calcium phosphate gel column was prepared using a mixture of $Ca_3(PO_4)_2$ gel and cellulose powder (Swingle and Tiselius, 1951; Massey, 1960). The column was washed overnight with 250 ml of 0.01 M phosphate (pH 6.8) and the dialyzed enzyme was introduced. Washing with 0.01 M phosphate (pH 6.8) was continued until no further protein was eluted at which time 0.03 M phosphate (pH 6.8) was used. As soon as racemase activity was detected, 0.045 M phosphate (pH 6.8) was substituted This buffer was continued until all the proline racemase was eluted. The column flowed at about 10 ml/hr and was completed in about 5 days.

Fractions containing racemase activity were concentrated by imbedding a dialysis bag containing the protein solution in dry silicic acid powder until the protein concentration was about 0.5 mg/ml. Proline racemase was then precipitated by adding 6.3 g of $(NH_4)_2SO_4$ for each 10 ml of solution. A minimal volume of 0.045 M phosphate (pH 6.8) was used to dissolve the precipitate and the specific activity was measured.

All steps in the purification were carried out at 0–5°. With the exception of the dilute effluent from the $Ca_3(PO_4)_2$ column, all fractions could be frozen. The final concentrated enzyme could be stored at 4° for several months without activity loss. Table I summarizes the purification procedure.

Proline Racemase Assay. Racemase assays were carried out in 10-ml volumetric flasks. Enzyme solution (1 ml) was added to a preincubated stock solution (5 ml) which contained 80 μmoles/ml of L-proline, 240 μmoles/ml of Tris-HCl buffer (pH 8.0), 24 μmoles/ml of β-mercaptoethanol, and 10 μmoles/ml of EDTA. The pH of the final solution was always 8.0 ± 0.1. After incubation for 15 min at 37°, the racemization was stopped by the addition of 1 ml of 20% trichloroacetic acid. NaOH

(1 ml of 1 N) was added to readjust the pH close to 8.0 and the flasks were made up to volume with H_2O. The optical rotation at 366 mμ was then measured on a Rudolph photoelectric polarimeter, Model 200. A proline racemase unit is defined as that amount of enzyme required to convert 1 μmole of L-proline into D-proline per min under the above conditions. A change in optical rotation of +0.012° resulted from the conversion of 1 μmole of L-proline into D-proline.

The relationship between enzyme concentration and per cent racemization is linear up to 20% racemization. Therefore, a racemization falling in this range could be converted into enzyme concentration.

The amount of proline before and after racemization was measured. The results showed that racemization was not accompanied by any proline loss.

Analytical Procedures. Proline was measured colorimetrically (Chinard, 1952). D-Proline was determined in a conventional Warburg apparatus using D-amino acid oxidase. Protein concentration was usually determined from the absorbancy ratio, 280 mμ:260 mμ (Warburg and Christian, 1941). For very low protein concentrations the method of Lowry *et al.* (1957) was employed. Deuterium analyses were performed by Mr. Josef Németh of Urbana, Ill.

Preparation of Deuterio-L-proline. Sodium pyrophosphate buffer (25 ml of 0.02 M) (pH 8.2) was lyophilized, dissolved in 5 ml of D_2O, and lyophilized again. L-Proline (5 g), 0.48 M β-mercaptoethanol (5 ml) (in D_2O), D_2O (25 ml), and proline racemase (500 units) (fraction IV), which had been lyophilized and dissolved in 5 ml of D_2O, were added to the pyrophosphate buffer. After incubation at 37° for 24 hr, the solution was taken to dryness and treated with hot methanol. The precipitated protein was removed by filtration and the methanolic solution was concentrated. On the addition of acetone, deuterio-DL-proline precipitated. After recrystallization from absolute methanol–acetone, 4.6 g of product was obtained. The product melted at 199–202°. Nonisotopic DL-proline has a melting point of 203° dec.

Deuterio-DL-proline was resolved by a modification of the method of Stetten and Schoenheimer (1944). Deuterio-DL-proline (1 g) was added to D-amino acid oxidase (300 mg) in 220 ml of sodium pyorphosphate buffer (pH 8.2) and stirred at 37° under a positive oxygen pressure. After 18 hr, the protein was precipitated by the addition of 60 ml of a 20% trichloroacetic acid solution and removed by centrifugation. The solution was heated to 70° on a steam bath and, while still hot, mixed with a solution of 1 g of 2,4-dinitrophenylhydrazine in 20 ml of absolute methanol and 2 ml of concentrated HCl. The phenylhydrazones which precipitated on cooling were removed by filtration and the yellow filtrate was decolorized by charcoal treatment (Darco G-60, Matheson Coleman and Bell). After concentrating to dryness, 25 ml of 0.25 N HCl and 2.5 g of ammonium rhodanilate (Bergmann, 1935) in 34 ml of methanol were added. The red crystals which separated were recrystallized from methanol–0.01 N HCl to give 1.64 g of L-proline rhodanilate: mp 133–134°, lit. (Stetten and Schoenheimer, 1944) mp 134–136°.

The L-proline rhodanilate was mixed thoroughly with 0.6 ml of pyridine and 10 ml of H_2O and, after standing for 1 hr, filtered with suction. A few drops of acetic acid were added to the faintly red filtrate and the small amount of red precipitate was removed. The solution was then taken to dryness, the residue was dissolved twice in H_2O and once in methanol, and the solvents were removed. On recrystallization from absolute methanol–acetone, 220 mg of white deuterio-L-proline was recovered (*Anal.* Calcd for $C_5H_7DNO_2$: C, 51.7; H, 7.9; N, 12.1. Found: C, 52.0; H, 8.0; N, 12.21.): mp 220–223°, nonisotopic L-proline, mp 220–222°. There was 0.91 deuterium atom/molecule, $[\alpha]_D^{22}$ −84.70°, while $[\alpha]_D^{23}$ for nonisotopic L-proline is −85.0° (Hodgman, 1959).

Under conditions which were sensitive to 1% deuterio-D-proline, no D-proline was shown to be present when 50 mg of the deuterio-L-proline was treated with D-amino acid oxidase.

Preparation of 2,4-DNP[1] Derivatives of L- *and* D-*Proline.* The 2,4-DNP derivatives (Porter and Sanger 1948) of all samples were prepared in the following manner. The enzyme–proline solutions were taken to dryness *in vacuo* and treated with hot methanol. After the denatured protein was separated by filtration, the methanol was removed. When the 2,4-DNP derivatives were prepared using various mixtures of D- and L-proline, the derivatives isolated had the same isomer distribution.

Silicic acid columns were prepared by mixing 100 g of silicic acid powder ($SiO_2 \cdot H_2O$, 100 mesh, Mallinckrodt) and 40 ml of water. The wet silicic acid powder was suspended in water-saturated chloroform and poured into a 3.5 × 30 cm column. Each 2,4-DNP derivative (about 200 mg) was dissolved in water-saturated chloroform and introduced into a column. After washing with chloroform, a water-saturated chloroform–1% 1-butanol solution was used to elute the derivatives. The first large yellow band to be eluted with the butanol solution was the 2,4-DNP derivative of proline. In separate experiments it was shown that the silicic acid columns did not separate the optical isomers, nor did they cause racemization of the L-proline derivative. Therefore, purification on silicic acid columns did not result in loss of deuterium from the α position of proline.

Other Materials. L-Proline (hydroxy-L-proline free), hydroxy-L-proline, and allohydroxy-D-proline were obtained from the Sigma Chemical Co. D-Proline was obtained from the California Corp. for Biochemical Research and D-amino acid oxidase from Nutritional Biochemicals Corp. Tetrahydrofuroic acid was prepared by the chromate oxidation of tetrahydrofurfuryl alcohol (Hing *et al.*, 1943).

Results

Properties of Enzyme. The enzyme (fraction V) shows a single symmetrical peak in the ultracentrifuge

[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

TABLE II: Inhibition of Proline Racemase by Iodoacetate and Iodoacetamide.[a]

Incubation Mixture	Incubn Time (min)	% Inhibition +β-ME	% Inhibition −β-ME
2.3×10^{-3} M iodoacetate	5	93	4
2.3×10^{-3} M iodoacetamide	5	80	7
4.8×10^{-4} M iodoacetate	12	54	
4.8×10^{-4} M iodoacetate 2.0×10^{-2} M DL-proline	12	3.5	
4.8×10^{-4} M iodoacetate 2.0×10^{-4} M pyrrole-2-carboxylate	12	3.5	

[a] Proline racemase (25 units) was incubated with 1.2×10^{-2} M β-mercaptoethanol (+β-ME) or without β-mercaptoethanol (−β-ME) and the indicated protector for 15 min. The iodoacetate or iodoacetamide was then added and allowed to react for the times shown. One-fifth of the incubation mixture was removed and assayed by the standard procedure. If no reducing agent was added, the enzyme alone was incubated before the addition of the inhibitor. All incubations were carried out at pH 8.0 in 0.36 M Tris-HCl in a total volume of 0.05 ml.

(Figure 1). On starch gel electrophoresis (Fine and Costello, 1963) a single protein component was detected, which coincided with the location of enzymic activity. Polyacrylamide gel electrophoresis on a 5% gel at pH 8.9 (Davis, 1964) showed a major and a minor protein band. Based upon stain intensity, the minor band appears to contain 5–10% as much protein as the major band. All enzyme activity was associated with the major band.

The enzyme is extremely stable. Preparations were stored 6 months in the refrigerator without significant loss of activity. The protein is unusually heat stable. At 70°, $t_{1/2}$ for inactivation was 3.6 min. When the same heat treatment is carried out in D_2O, the protein was even more stable, $t_{1/2}$ for inactivation being 17 min.

Proline racemase is inactive in the absence of reducing agents. Addition of mercaptans, such as β-mercaptoethanol, causes rapid activation. Under standard assay conditions, the same initial velocity was observed whether the enzyme and mercaptan were incubated 15 min prior to addition of substrate or mercaptan and substrate were added simultaneously. The optimal concentration of β-mercaptoethanol is 0.02 M. Dithiothreitol is equally effective. However, 0.02 M cysteine resulted in one-fifth the activity obtained with β-mercaptoethanol at this concentration. Incubation with 0.06 M sodium borohydride gave 79% of the activity obtained with β-mercaptoethanol. These results suggest that the activation of the enzyme involves the reduction of one or more disulfide linkages.

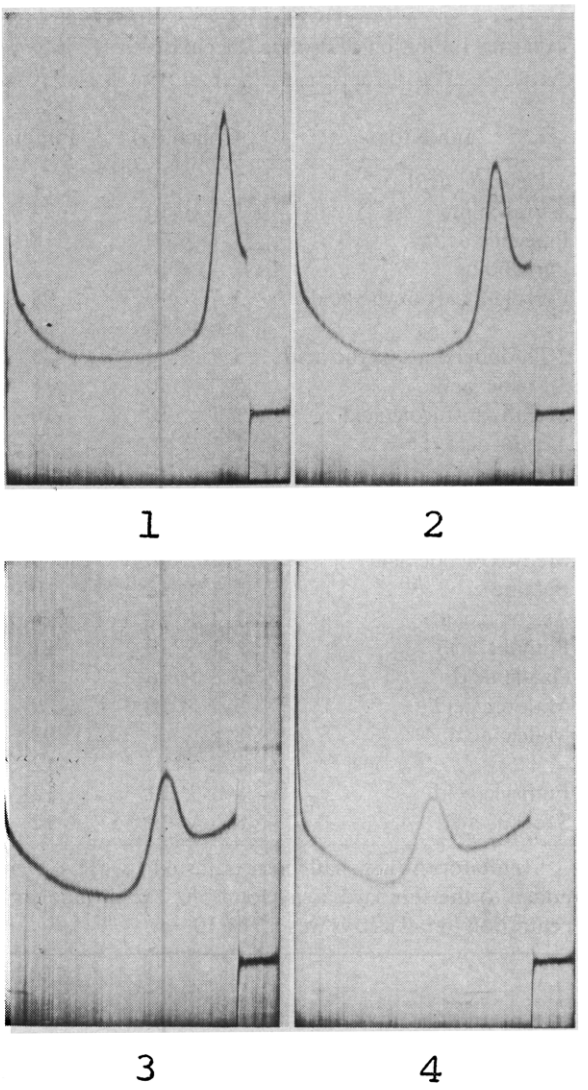

FIGURE 1: Sedimentation pattern of purified proline racemase. Fraction V (9.6 mg/ml, specific activity 600) was centrifuged at 52,640 rpm at 21.6°. The photographs shown were taken successively at 27, 43, 85, and 117 min after attainment of 52,640 rpm. $s_{20} = 3.13$ S.

The enzyme is inhibited by iodoacetate and iodoacetamide only after treatment with a reducing agent. Pertinent experimental results are summarized in Table II. Table II also shows that DL-proline and pyrrole-2-carboxylate, a potent inhibitor, can protect against iodoacetate inhibition. The SH groups of the enzyme are extremely reactive, since inactivation occurs in the presence of a tenfold excess of mercaptoethanol over iodoacetate.

Substrate Specificity. The rates of racemization of hydroxy-L-proline and allohydroxy-D-proline relative to L-proline are 2 and 5%, respectively. It is of interest to note that hydroxyproline epimerase (Adams and Norton, 1964) shows low levels of proline racemase activity. No racemization could be detected with valine and alanine.

The substrate activity of sarcosine was tested by determining whether proline racemase can catalyze the exchange of tritium from the medium into sarcosine.

TABLE III: Inhibition of Proline Racemase.[a]

Inhibitor	Concn (M)	% Inhibn
α-Picolinic acid	1.1×10^{-1}	3
α-Pipecoline	1.1×10^{-1}	24
Pipecolic acid	1.1×10^{-1}	18
Pyrrolidine	1.1×10^{-1}	3
Pyrrole-2-carboxylic acid	5.7×10^{-2}	98
	3.6×10^{-4}	50
2-Thiophenecarboxylic acid	5.7×10^{-2}	73
2-Furoic acid	5.7×10^{-2}	11
Tetrahydrofuroic acid	1.1×10^{-1}	10
Acrylic acid	1.1×10^{-1}	0
Glycine	1.1×10^{-1}	0
Alanine	1.1×10^{-1}	0
Sarcosine	1.1×10^{-1}	54
Methoxyacetic acid	1.1×10^{-1}	9
Betaine	1.1×10^{-1}	0
Aspartic acid	1.1×10^{-1}	16
Phthalic acid	1.1×10^{-1}	21
Oxalic acid	2.3×10^{-2}	6
Malonic acid	5.7×10^{-2}	9
Maleic acid	8.6×10^{-2}	93
	1.7×10^{-2}	12
Fumaric acid	8.6×10^{-2}	12
Succinic acid	8.6×10^{-2}	12

[a] Inhibitors which had been adjusted to pH 8 were added to the standard assay solution. The proline concentration for all assays was 5.7×10^{-2} M.

Sarcosine was substituted for L-proline in a tritium-enriched (7×10^3 cpm/μatom of H) standard (6 ml) assay solution which contained 159 units of proline racemase (fraction V). After incubation at 27° for 3 hr, the sarcosine was isolated on a Dowex 50 (H^+) column (Stein and Moore, 1949), and recrystallized from ethanol–ether. It had a specific activity of 14.6 cpm/μmole. The addition of pyrrole-2-carboxylate, a potent inhibitor of proline racemase, at one-tenth the concentration of the sarcosine resulted in a specific activity of 0.86 cpm/μmole. These results show that proline racemase catalyzes the exchange of the α-hydrogens of sarcosine at an extremely slow rate, and that this exchange, like the racemization of proline, is inhibited by pyrrole-2-carboxylate.

Cofactor Requirement. No evidence of cofactor requirement could be obtained. The spectrum of the enzyme showed no absorption other than that attributable to the protein. No loss of activity occurred upon treatment with charcoal, passage through Sephadex G-200, or dialysis for 15 hr against 0.1 M Tris (pH 7.5). Treatment with 0.01 M sodium borohydride, 0.01 M sodium cyanide, and 0.01 M hydroxylamine did not lead to inactivation. Addition of either DPN or pyridoxal phosphate did not increase the activity.

Inhibitors. The effects of a number of inhibitors upon the rate of racemization of proline are shown in Table III. The first group of inhibitors are heterocyclic compounds, both aromatic and nonaromatic. Of these, only five-membered-ring compounds containing a carboxyl group give significant inhibition. Pyrrole-2-carboxylic acid is the most effective inhibitor examined. The sulfur analog is less effective and the oxygen analog is a poor inhibitor. The aromatic analog, 2-furoic acid, is a better inhibitor than the saturated compound, tetrahydrofuroic acid.

The inhibitory properties of a number of amino acids were tested. The best inhibitor is sarcosine. Glycine at the same concentration as sarcosine produces no inhibition. Other nonheterocyclic compounds which were found to be inhibitory were dicarboxylic acids. Of these, maleic acid was the best inhibitor and was much more effective than fumaric or succinic acid.

Preincubation of proline racemase in 0.54 M Tris (pH 8.0) for 15 min with 1,10-phenanthroline, 1.2×10^{-2} M, resulted in 35% inhibition under standard assay conditions. If the enzyme was incubated for 10 min with 6×10^{-3} M β-mercaptoethanol before incubation with 1,10-phenanthroline the inhibition increased to 67%. Possibly, reduction of enzyme sulfhydryl groups leads to a conformational change which renders the enzyme more susceptible to the inhibitor.

Reaction Kinetics. Since the rate of reaction was measured by following changes in optical rotation, it was difficult to observe initial velocities at low substrate concentration. The lowest substrate concentration which could be used gave an initial velocity which was 60% V_{max}. The best values which could be obtained for the K_m for L-proline and D-proline were 2.3 and 3.8×10^{-3} M, respectively. The corresponding values of V_{max} at an enzyme concentration of 0.33 unit/ml were 4.4×10^{-4} mM/ml per min for L-proline and 5.8×10^{-4} mM/ml per min for D-proline.[2]

Isotope Incorporation and Kinetics in D_2O. In order to establish whether deuterium incorporation occurred, the racemixation of L-proline was carried out in 95% D_2O. Proline racemase (fraction IV, 81 units) was lyophilized, dissolved in 5 ml of D_2O, and added to 12.5 ml of 0.008 M sodium pyrophosphate buffer (pH 8.3) in D_2O, which was also 0.024 M in β-mercaptoethanol and 0.085 M in L-proline. The reaction was allowed to proceed at 37° for 8 hr at which time the optical rotation was +0.02°. The 2,4-DNP derivative was then prepared and found to contain 0.95 atom of deuterium/molecule. When deuterio-DL-proline, prepared with proline racemase, was resolved as described in the Experimental Section, the nuclear magnetic resonance spectrum of the resultant deuterio-L-proline showed that deuterium was located exclusively at the α position.

Experiments were carried out under essentially initial velocity conditions to compare the rate of isomer

[2] From K_M and V_{max}, K_{eq} = 1.2 can be calculated from the Haldane equation. The expected value is 1.0. The deviation from the expected value probably reflects errors in the determination of K_M.

TABLE IV: Comparison of the Rate of Isomer Interconversion and Deuterium Incorporation.[a]

Substrate	% Product Isomer in Reaction Mixture		% Product Isomer Remaining after D-Amino Acid Oxidase	% Proline-α-^{2}H in Total Proline[b]
	A	B		
L-Proline	5.0	5.0		5.2
			0.0	0.6
L-Proline	9.8	9.6		9.6
			0.7	1.2
D-Proline	7.2	6.6		7.4
D-Proline	12.2	13.5		13.4

[a] L- or D-proline was racemized under the conditions used in the assay procedure. Sodium pyrophosphate buffer (8×10^{-3} M, pH 8.3) was substituted for Tris buffer. At the end of the reaction the amount of product formed was determined from the optical rotation of the reaction mixture. The results are given under column A. The proline from an aliquot of the reaction mixture was converted into 2,4-DNP-proline. Its isomer composition was again determined by optical rotation. The results are given under column B. The DNP-proline so obtained was used for deuterium analysis. Another aliquot of the reaction mixture was treated with D-amino acid oxidase. After purification, the amount of D-proline remaining was determined. The proline so obtained was converted to DNP-proline and its deuterium content was determined. [b] Calculated from the deuterium content of DNP-proline on the basis of 1 atom of deuterium/molecule.

interconversion with the rate of deuterium incorporation. When L-proline was used as a substrate the reaction was allowed to proceed until 5 and 10% of the L-proline were converted into D-proline. The reaction was stopped by acidification with HCl to pH 2–3 and immersion of the reaction mixture in a boiling water bath for 60 min. The optical rotation of the neutralized solution was then measured to determine the isomer composition of the reaction mixture. The reaction mixture was then divided into two parts. Proline present in one of the aliquots was converted into the 2,4-DNP derivative which was then isolated and purified, and its deuterium content was determined. The optical rotation of the dinitrobenzene derivative was determined. These measurements showed that the isomer composition of the derivative was not significantly different from that of the proline from which it was made. The deuterium content of this derivative gave the total amount of deuterium which was incorporated both into L-proline and the product, D-proline. The other aliquot was treated with D-amino acid oxidase to remove D-proline and the remaining L-proline was isolated and purified. The amount of D-proline contaminating the purified L-proline was determined manometrically with D-amino acid oxidase. The L-proline was then converted into the 2,4-DNP derivative and its deuterium content was determined. From this analysis the amount of deuterium incorporation into L-proline was obtained. The results are summarized in Table IV. Under initial velocity conditions essentially no deuterium is incorporated into the substrate isomer and every molecule of product isomer formed contained deuterium. A similar experiment was then carried out with D-proline. The reaction was allowed to proceed until 7.2 and 12.2% of the D-proline were converted into L-proline. The total final reaction mixture was converted into the 2,4-DNP derivative. The deuterium content was determined. This gave the total deuterium incorporation into both L- and D-proline. The results are summarized in Table IV. These results show that, under initial velocity conditions, the amount of deuterium incorporated is essentially equal to the amount of product formed, as is the case when L-proline is the substrate.

The rate of conversion of L- into D-proline was measured at various pH values in H_2O and D_2O (Figure 2). The maximal rate in D_2O is approximately half the maximal rate in H_2O. It is not certain that this effect

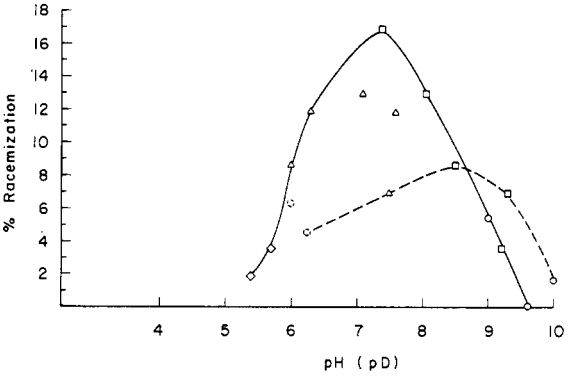

FIGURE 2: Per cent racemization as a function of pH in H_2O and D_2O. The following buffers were used: (◇) potassium acetate; (◌) potassium citrate; (△) potassium phosphate; (□) Tris-HCl; (○) glycine–NaOH; all buffers were 0.2 M. All other components and conditions as in the standard assay. Broken line indicates reaction carried out in D_2O. pH was measured with a glass electrode and converted into pD. pD = pH + 0.4.

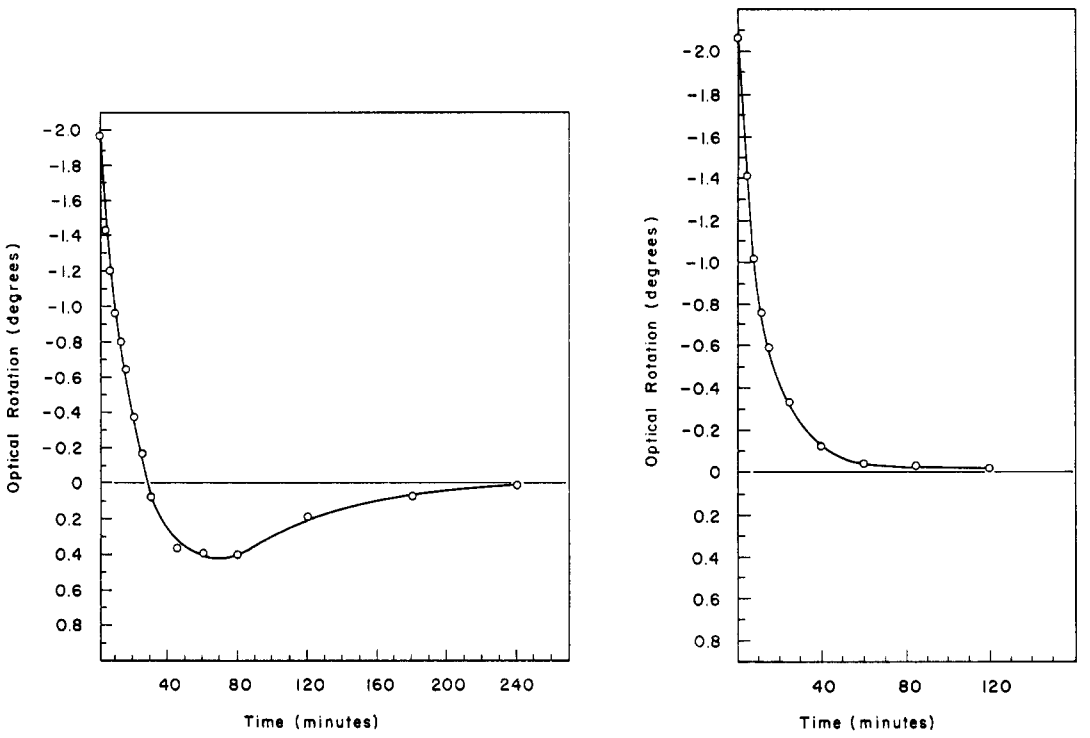

FIGURE 3: Racemization of L-proline in D_2O and H_2O. (a) Reaction in D_2O; (b) reaction in H_2O. Reaction conditions: 0.2 M Tris-HCl (pH 8.0), 0.02 M β-mercaptoethanol, 0.008 M EDTA, 0.067 M L-proline, and proline racemase (3.36 units/ml for reaction in D_2O and 2.76 units/ml in H_2O).

can be ascribed to a deuterium isotope effect; it may reflect structural changes in the protein. The effect of D_2O in increasing heat stability suggests structural changes in D_2O. The data of Figure 2 also indicate inhibition by phosphate buffer above pH 6.5. This suggests that the dianion species of phosphate is inhibitory.

In another set of experiments, the enzymic racemization was followed to completion in H_2O and D_2O (Figure 3). Surprisingly, the optical rotation is initially negative, becomes zero, then positive, and finally approaches zero again. This unusual kinetic behavior was attributed to the occurrence of a primary isotope effect. Direct evidence for such an effect was obtained. The rate of racemization of α-deuterio-L-proline was measured under standard assay conditions. The initial rate is 2.5 times lower than that of the nonisotopic compound.

Discussion

The incorporation of deuterium into the α position of proline indicates that the inversion of the configuration results from the dissociation and re-formation of the α-carbon–hydrogen bond. Racemization, therefore, involves a transposition of hydrogen from one side of the α-carbon to the other. Two general mechanisms by which this can occur were discussed earlier in this paper. These were referred to as one-hydrogen-acceptor and two-hydrogen-acceptor mechanisms. The one-hydrogen-acceptor mechanism for a reaction taking place in D_2O is represented in Figure 4A. Reaction of the substrate and enzyme leads to an intermediate (S·E–H) in which the α-hydrogen of the substrate has been transferred to the hydrogen acceptor site of the enzyme. This hydrogen is then subject to exchange with deuterium of the solvent and the intermediate is converted into S·E–D (step 3). Under the conditions of the experiment, this step is essentially irreversible. Two reaction pathways are then open to the intermediate: steps 4 and 5 to give denterio-D-proline or 6 and 7 to give deuterio-L-proline. When the reaction was carried out in D_2O with L-proline as substrate essentially all of the deuterium was incorporated into D-proline. Very little deuterio-L-proline (Table IV) was formed. In terms of the scheme in Figure 4A, this means that the intermediate S·E–D partitions so that deuterio-D-proline is formed more rapidly than deuterio-L-proline. It is therefore predicted that, if the same experiment is carried out starting with D-proline, deuterium should be incorporated into D-proline more rapidly than into the product L-proline. The total rate of deuterium incorporation into L- and D-proline should be much faster than the rate of conversion of D- into L-proline. The data in Table IV show that is not the case. Therefore, the single-hydrogen-acceptor mechanism is inconsistent with experimental results.

The two-hydrogen-acceptor mechanism for a reaction in D_2O is illustrated in Figure 4B. The substrate interacts with a form of the enzyme in which one of the hydrogen acceptor sites is not protonated (deuterated in D_2O). The α-hydrogen is then transferred to the available

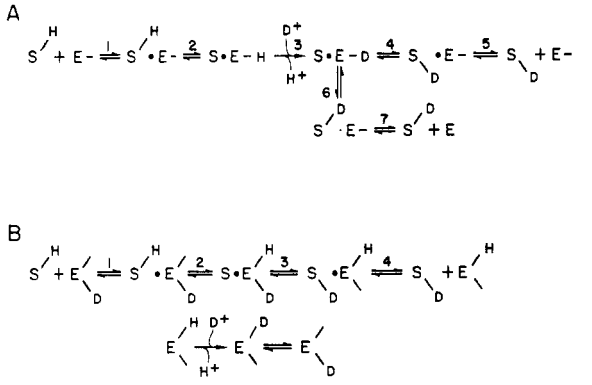

FIGURE 4: Mechanisms for racemization of proline in D_2O. (A) One-hydrogen acceptor. (B) Two-hydrogen acceptor. The following symbols are used: $S\begin{matrix}H\\ \diagup\end{matrix}$, $S\begin{matrix}\diagdown\\ H\end{matrix}$ = the two isomers of proline; $S\begin{matrix}D\\ \diagup\end{matrix}$, $S\begin{matrix}\diagdown\\ D\end{matrix}$ = the two isomers containing deuterium in the α position; E– = enzyme with a single hydrogen (deuterium) acceptor site; E< = enzyme with two hydrogen (deuterium) acceptor sites.

hydrogen acceptor site to form the intermediate represented by the symbol $S\cdot E<\begin{matrix}H\\ D\end{matrix}$. The additional assumption is now made that in the presence of the substrate the proton on the hydrogen acceptor site cannot exchange with solvent protons. The intermediate, $S\cdot E<\begin{matrix}H\\ D\end{matrix}$, can give rise to D product or re-form substrate. Since the protons of the intermediate cannot exchange with the solvent, no deuterium will be introduced into the substrate and deuterium will only be introduced into the product. The total rate of deuterium incorporation will be equal to the rate of product formation. This will be true regardless of which proline isomer is the substrate. The two-hydrogen-acceptor mechanism is therefore consistent with the experimental results.

Abstraction and readdition of the α-hydrogen could proceed through an oxidation–reduction process or through a carbanion mechanism. If oxidation–reduction is involved, it should be possible to demonstrate the participation of an appropriate cofactor or prosthetic group. Oxidation–reduction cofactors are known to participate in several reactions in which inversion of configuration of a carbon atom occurs. DPN is associated with UDPG-epimerase (Maxwell, 1956), and FAD with alanine racemase (Diven *et al.*, 1963). The apparent lack of cofactor requirement for proline racemase argues against the occurrence of an oxidation–reduction process in this reaction. The incorporation of deuterium from the solvent during the course of the racemization is consistent with a carbanion (proton transfer) mechanism.

If proton transfer is involved, part of the catalytic function of the enzyme is undoubtedly the enhancement

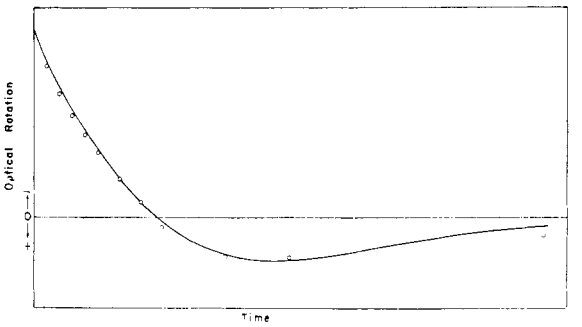

FIGURE 5: Analog computer analysis of kinetics in D_2O. The change in optical rotation was calculated for the system

$$P\_H \xrightarrow{k_1} P_+{}^D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P\_D \qquad k_1 > k_2$$

Experimentally determined K_M values were used. The solid line represents calculate values; experimental points O (data are the same as those in Figure 2).

of the acidity of the α-hydrogen. Frequently α-hydrogen atoms of amino acids are activated through interaction with pyridoxal phosphate. This does not appear to be the case for proline racemase. Furthermore, participation of a carbonyl group associated with the protein does not seem to be involved since carbonyl reagents did not inhibit. A possible mechanism of α-hydrogen activation is suggested by nonenzymatic experiments. The activation of the α-hydrogen of amino acids can be achieved by complexation with metal ions (Ikutami *et al.*, 1959; Williams and Busch, 1965). This suggests that the enzymic mechanism of α-hydrogen activation may involve interaction of the carboxyl group with either a metal ion or possibly a positive center on the enzyme other than a metal ion. No direct evidence for the involvement of a metal ion in the reaction of proline racemase is available. However, it may be significant that the structural features of the substrate and inhibitors which facilitate binding to the enzyme are also those which will favor coordination with a metal. The substrate as well as all inhibitors are potential bidentate ligands; they contain a carboxyl group and another electrogenative atom such as oxygen, nitrogen, or sulfur. Monofunctional compounds such as pyrrolidine, acrylic acid, and acetic acid do not interact with the enzyme (*i.e.*, are neither substrates nor inhibitors). $H_2PO_4^-$ does not inhibit, while HPO_4^{2-} is inhibitory. Furthermore, steric restrictions of the two binding sites, so that interaction with one center is favored, enhances inhibitory power. Of all bifunctional acids tested, maleic acid[3] in which the two carboxyl groups are restricted to a *cis* conformation is a better inhibitor than any other dicarboxylic acid which was tested. These results, although not conclusive evidence, provide suggestive evidence for the involvement of a metal ion. In view of the results obtained with model systems, this point needs to be further examined.

[3] The distance between the two carboxyl oxygens of maleic acid is very close to the distance between nitrogen and the oxygen carbonyl group of proline. Maleic acid could therefore occupy the same coordination position as proline.

Of all inhibitors tested, pyrrolecarboxylic acid was by far the best inhibitor. At a substrate to inhibitor ratio of 160:1, 50% inhibition is obtained, which suggests that the inhibitor may bind to the enzyme more tightly than the substrate. This high affinity for the enzyme could be due to the planar nature of this inhibitor. The following is suggested as a possible explanation for the high affinity of the enzyme for planar molecules: At some stage during the reaction, the α-carbon of proline must assume a planar conformation. The substrate binding site is such that the interaction with the molecule with a planar α-carbon is energetically more favorable than with the molecule with a tetrahedral α-carbon.[4] This will facilitate the conversion into the planar configuration. Therefore, due to the favorable interaction of the substrate binding site with molecules having a planar configuration, inhibitors with a planar configuration will be strongly bound.

The reaction kinetics in D_2O is unusual (Figure 3) in that the optical rotation changes sign during the course of the racemization and is zero or approaches zero twice. At first one might think that this is due to a difference in optical rotation between deuterioproline and nonisotopic proline. We have shown that the difference in rotation between deuterio-L-proline and protio-L-proline is negligible (−84.7 and −85.0°, respectively) and is not nearly enough to account for the positive rotation which was observed. We attribute this unusual kinetic behavior to the presence of two isotopic species and a kinetic isotope effect.[5] When L-proline is allowed to racemize in D_2O, the following reaction occurs.

$$P_-^H \longrightarrow P_+^D \rightleftharpoons P_-^D$$

P_-^H, P_+^D, and P_-^D represent L-proline, α-deuterio-D-proline, and α-deuterio-L-proline. The optical rotation will become zero at two points. It will be zero toward the end of the reaction when $P_-^H = 0$ and $P_+^D = P_-^D$. At this point the two proline species are at equilibrium and the rate of conversion of $P_+^D \rightarrow P_-^D$ is equal to the rate of conversion of $P_-^D \rightarrow P_+^D$. The optical rotation will also be zero at some earlier stage when $P_-^H + P_-^D = P_+^D$. At this point the total amount of L-proline ($P_-^H + P_-^D$) is also equal to the D-proline, but the L-proline molecules are made up of two isotopic species. P_-^H will react more rapidly than the P_-^D due to a kinetic isotope effect. P_+^D will therefore be formed more rapidly than it would be if all of the L-proline were P_-^D. Consequently, the rate of formation of P_+^D will exceed its rate of conversion into P_-^D, P_+^D will accumulate, and the optical rotation will increase, *i.e.*, it will go from zero to a positive value. After isotope exchange is complete so that $P_-^H = 0$, P_+^D and P_-^D will come to equilibrium and the optical rotation will reach its final zero value.

[4] This effect has been discussed by Jencks (1966) and referred to as catalysis by strain.

[5] Dr. Q. Gibson has examined this reaction with an analog computer. By introducing an isotope effect, *i.e.*, the deuterated isomer reacts more slowly than the nondeuterated species, our experimental curve was reproduced (Figure 5).

References

Adams, E., and Norton, I. J. (1964), *J. Biol. Chem. 239*, 1524.

Argranoff, B. W., Eggerer, H., Henning, V., and Lynen, F. (1960), *J. Biol. Chem. 235*, 326.

Bergmann, M. (1935), *J. Biol. Chem. 110*, 471.

Chinard, F. P. (1952), *J. Biol. Chem. 199*, 91.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Diven, W. F., Johnston, R. B., and Scholz, J. J. (1963), *Biochim. Biophys. Acta 67*, 161.

Fine, I. H., and Costello, L. (1963), *Methods Enzymol. 6*, 958.

Hing, A., Meyer, G., and Schücking, G. (1943), *Ber. 76B*, 676.

Hodgman, C. D., Ed. (1959), *in* Handbook of Chemistry and Physics, Cleveland, Ohio, Chemical Rubber, p 1754.

Ikutami, Y., Okuda, T., Sato, M., and Akabori, S. (1959), *Bull. Chem. Soc. Japan 32*, 203.

Jencks, W. P. (1966), *in* Current Aspects of Biochemical Energetics, Kaplan, N. O., and Kennedy, E. P., Ed., New York, N. Y., Academic, p 273.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1957), *J. Biol. Chem. 193*, 265.

Massey, V. (1960), *Biochim. Biophys. Acta 37*, 310.

Maxwell, E. S. (1956), *J. Am. Chem. Soc. 78*, 1074.

Porter, R. R., and Sanger, F. (1948), *Biochem. J. 42*, 287.

Rose, I. A. (1966), *Ann. Rev. Biochem. 35*, 23.

Shah, D. H., Cleland, W. W., and Porter, J. W. (1965), *J. Biol. Chem. 240*, 1946.

Stadtman, T. C., and Elliot, P. (1957), *J. Biol. Chem. 228*, 983.

Stein, W. H., and Moore, S. (1949), *Cold Spring Harbor Symp. Quant. Biol. 14*, 179.

Stetten, M. R., and Schoenheimer, R. (1944), *J. Biol. Chem. 153*, 113.

Swingle, S. M., and Tiselius, A. (1951), *Biochem. J. 48*, 171.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Williams, D. H., and Busch, D. H. (1965), *J. Am. Chem. Soc. 87*, 4644.

Wright, B. E., and Stadtman, T. C. (1956), *J. Biol. Chem. 219*, 863.