Reaction Mechanism and Structure of the Active Site of Proline Racemase†

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ABSTRACT: Proline racemase catalyzes the interconversion of D- and L-proline. Previous studies in this laboratory have established that the reaction proceeds by means of a two-base mechanism in which one base on the enzyme removes the substrate α-hydrogen as a proton and the conjugate acid of another base donates a proton to the opposite side of the α-carbon (Cardinale, G. J., and Abeles, R. H., (1968), *Biochemistry 7*, 3970). An assumption of the proposed mechanism was that no proton exchange occurs from the enzyme–substrate complex. In the present study, we have shown that the rate of ^3H release from DL-[α-^3H]proline, in the presence of proline racemase, decreases with increasing proline concentrations. These results establish that release of the substrate derived proton from the enzyme occurs largely, possibly exclusively, after release of the product. Under initial velocity conditions, the rate of ^3H release from L-[α-^3H]proline is not reduced with increasing L-proline concentrations. Thus, the enzyme-bound proton derived from one isomer can only be "captured" by the other isomer. We conclude that there are two forms of the enzyme; one binds L-proline and the other D-proline. Release of the substrate derived proton from the enzyme is more rapid than the interconversion of these two forms. These results are consistent with the previously proposed mechanism. Proline racemase is composed of similar subunits of mol wt 38,000 as determined by gel electrophoresis in the presence of sodium dodecyl sulfate. Equilibrium dialysis experiments detect only one substrate binding site for every two subunits. When the oxidized form of the enzyme, which is inactive and cannot bind substrate, is reduced by thiol to yield active enzyme, two cysteine sulfhydryl groups per dimer become available to react with iodoacetate. Inactivation of the enzyme occurs upon modification of one of these cysteines. All iodoacetate incorporation occurs at the same point in the primary sequence of the enzyme, and can be prevented by the presence of proline or pyrrole-2-carboxylate, a substrate analog. A model is proposed in which a single active site is formed by elements of two identical subunits. Although the data are consistent with this model, another interpretation, in which half of the subunits are nonfunctional, cannot be ruled out.

Figure 1 illustrates a mechanism of action of proline racemase which we have proposed (Cardinale and Abeles, 1968). This mechanism involves two bases at the active site which function as proton donor and acceptor. One of these bases removes the α-hydrogen of proline as a proton and the conjugate acid of the other base protonates proline from the opposite side of the α-carbon. An assumption of this mechanism is that the proton removed from proline is protected from solvent and does not exchange until product has dissociated. The mechanism also implies that there are two forms of the protonated enzyme (I and IV, Figure 1), one of which binds L-proline and the other D-proline. These two forms can only equilibrate by exchange of enzyme-bound protons with solvent. Experiments are reported here which bear on these two points. Previous results suggested the presence of SH groups at the active site which could function as proton donor and acceptor. We now report experiments which provide further support for this proposal.

Experimental Procedure

Purification of Enzyme. Proline racemase was purified using the previously published procedure (Cardinale and Abeles, 1968) with the following modifications. The supernatant fluid from the protamine sulfate step was brought to 70% saturation with 490 g of solid ammonium sulfate per liter of solution. The resultant precipitate was extracted with 400 ml of 0.01 *M* Tris-Cl (pH 8.7) plus 140 g of ammonium sulfate for 16 hr at 4°. The crude enzyme which was extracted by this procedure was precipitated by addition of 264 g of solid ammonium sulfate per liter of solution. The gradient elution of the DEAE column (4 × 4.5 cm) was changed to a linear gradient of 2 l. total volume, from 0.1 to 0.3 *M* Tris-Cl (pH 7.5). The calcium phosphate column (4.5 × 4.5 cm) was eluted with a linear gradient of 600 ml total volume, from 0.01 to 0.06 *M* potassium phosphate (pH 6.8). After calcium phosphate chromatography, the enzyme (20–30 mg) was electrophoresed in a pH 7 Tris-barbital–Tris-Cl discontinuous gel system (Williams and Reisfeld, 1964) using a 7.5% polyacrylamide gel cast in a Canalco preparative gel electrophoresis apparatus using a PD 2/150 upper column. Fractions containing enzyme activity were concentrated using an Amicon UM-10 ultrafilter.

Synthesis of L-[α-^3H]- and DL-[α-^3H]Proline. The previously published procedure (Cardinale and Abeles, 1968) for synthesis of L-[α-^2H]proline was used with [^3H]water instead of [^2H]water. Proline racemase (0.5 mg), 5 g of L-proline, 10 μl of 2-mercaptoethanol, and 1 ml of H_2O con-

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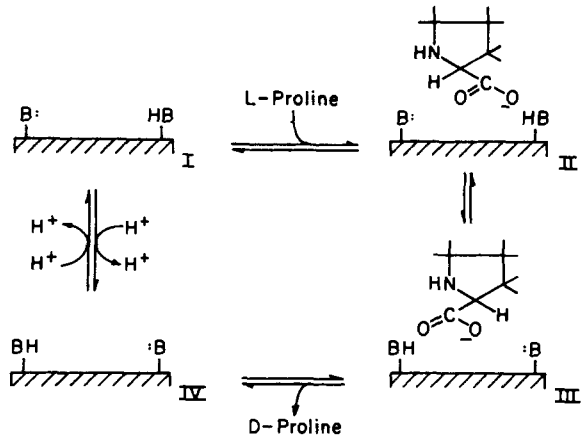

FIGURE 1: Mechanism of action of proline racemase.

taining 1.0 Ci of ^3H were incubated with 3.5 ml of 0.02 *M* sodium pyrophosphate buffer (pH 8.2) (total volume ~5 ml) for 18 hr at 37°. Another 0.5 mg of enzyme was added at this time and the reaction was allowed to proceed at 37° for 22 hr. Solvent was removed by bulb-to-bulb distillation and the residue was dissolved in 4 ml of hot methanol and filtered. Proline precipitated upon addition of acetone and was recrystallized from methanol–acetone. The product DL-[α-^3H]proline had a specific radioactivity of 6.4×10^4 cpm/μmol and had no optical rotation at 366 nm. It was radiochemically pure upon paper electrophoresis at pH 1.9 followed by paper chromatography in 1-butanol–acetone–diethylamine–water (10:10:2:5, v/v), a system which separates proline from the other amino acids. All of the radioactivity could be released by proline racemase.

L-[α-^3H]Proline was prepared as follows. The reaction mixture consisted of 100 mg of DL-[α-^3H]proline, 450 mg of L-proline, 3.5 units of D-amino acid oxidase, and 6 ml of 0.1 *M* sodium pyrophosphate buffer (pH 8.5). The reactants were incubated at 37° for 65 hr in the dark in a 40-ml flask filled with 100% oxygen. At this time less than 30 μg of D-proline remained as determined by assaying the reaction mixture with an oxygen electrode using added D-amino acid oxidase. The enzyme was precipitated from the reaction mixture by the addition of 1 ml of 20% trichloroacetic acid and the supernatant fluid was evaporated to dryness. The residue was dissolved in methanol and after decolorization with charcoal, acetone was added to precipitate the proline. The slightly pink powder was washed with acetone and dried overnight in a desiccator. The proline was redissolved in absolute ethanol and crystallized by addition of ether. The product had a specific activity of 10.5×10^9 cpm/mol and contained less than 0.5% D-proline as determined by measuring oxygen uptake with an oxygen electrode in the presence of D-amino acid oxidase. It was radiochemically pure upon paper electrophoresis at pH 1.9 and all of the radioactivity could be released by proline racemase.

Preparation of [^{14}C]*Pyrrole-2-carboxylic Acid.* Pyrryl-Grignard was prepared according to the procedure of McCay and Schmidt (1926) from 0.32 g of magnesium turnings, 0.76 ml of methyl iodide, and 0.87 ml of pyrrole. After allowing the mixture to separate into two phases, 0.5 ml of the lower phase was withdrawn for carboxylation with $^{14}CO_2$. The pyrryl-Grignard was placed in a flask connected with glass tubing to another flask containing, in three lobes, 0.5 ml of sulfuric acid, 3.52 mg (1 mCi) of barium [^{14}C]carbonate, and 12 mg of unlabeled barium carbonate. The pyrryl-Grignard was cooled with liquid nitrogen and the sulfuric acid was added first to the barium [^{14}C]carbonate and then to the nonradioactive carbonate. The mixture was warmed at room temperature and after 10 min the reaction was terminated with water and enough sodium hydroxide to bring the pH above 10. The aqueous phase was washed twice with ether and then heated with charcoal. The filtrate was applied to a 0.5 × 5.0 cm column of Bio-Rad AG 1-X8 (OH^-). Ammonium carbonate (0.2 *M*) was used to elute the pyrrole-2-carboxylate which corresponds to the peak of radioactivity with an A_{256}/A_{230} greater than 2.1. The specific activity was 3.33×10^7 cpm/μmol and was constant through the peak. The product was radiochemically pure on five chromatographic systems: paper chromatography in 2-butanol–acetic acid–water (12:3:5) (R_f 0.88) and isoamyl alcohol–pyridine–water (7:7:6) (R_f 0.62), paper electrophoresis at pH 1.9, chromatography on Dowex 1 as described above, and silicic acid chromatography according to the method of Varner (1957) using 2% butanol in chloroform. A nonradioactive trial synthesis on a larger scale gave a product with an infrared spectrum identical with that of authentic pyrrole-2-carboxylic acid.

Proline Racemase Assay. The assay is based on the fact that the enzyme catalyzes the exchange of the α-hydrogen of proline with the solvent (Cardinale and Abeles, 1968). Proline racemase (0.01–0.2 unit) is added to 0.1 ml of 0.24 *M* Tris (pH 8.0) containing 0.01 *M* EDTA, 0.08 *M* L-proline, 0.02 *M* DL-[α-^3H]proline, and 0.024 *M* 2-mercaptoethanol. After 15 min at 37°, the reaction mixture is then applied to a 0.5 × 2.5 cm column of Bio-Rad AG 50-X2 (H^+) and eluted with 2 ml of water. The ion exchange column retains proline. The effluent is collected in a scintillation vial containing 10 ml of counting fluid (Bray, 1960) and counted. The release of radioactivity from proline is linear with time under these conditions and the rate of release is proportional to the amount of enzyme added.

Rates of Conversion of L-[^{14}C]*Proline to* D-*Proline.* Reaction conditions are described in the legend of Figure 3. After stopping the reaction with trichloroacetic acid, carrier DL-proline was added to a final concentration of 0.43 *M* in 0.2 ml. The mixtures were applied to a 0.5 × 2.5 cm column of Bio-Rad AG 50-X2 (H^+) and washed with 2 ml of water, and the proline was then eluted with 4 *N* HCl. The eluate was taken to dryness and redissolved in 1 ml of 0.1 *M* sodium pyrophosphate buffer (pH 8.5). The pH was again adjusted to 8.5 by addition of NaOH and 4.7 units of D-amino acid oxidase and 79 units of catalase were added. The reaction mixture was incubated for 16 hr at 37° in the dark and deproteinized by addition of 0.6 ml of 20% trichloroacetic acid and centrifugation. Upon addition of 2 ml of a 5 mg/ml solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl at 70°, and standing overnight at room temperature, the dinitrophenylhydrazone of 2-keto-5-aminopentanoic acid crystallized out of solution (Krebs, 1939). The crystals were washed and dried and 2–3 mg from each reaction mixture was weighed out on an electrobalance and solubilized in a few drops of 1.4 *M* KOH in methanol. The solutions were diluted to 1 ml with water and an aliquot was counted in Bray's counting fluid using an internal standard of [^{14}C]-toluene to correct for quenching. From the specific activity of the dinitrophenylhydrazone (which was formed from the D-proline in the reaction mixture) the amount of ^{14}C in D-proline was calculated.

Equilibrium Dialysis. Equilibrium dialysis was carried

out in an apparatus with four cells, each of which was separated by dialysis tubing into two chambers of about 150 μl each. The solutions were stirred by gentle rotation; agitation was effected by fire-polished fragments of glass helices which were enclosed in each side of the cell. At 4°, pyrrole-2-carboxylate equilibrates in this apparatus within 5 hr. In each experiment, 43 μg of enzyme and [^{14}C]pyrrole-2-carboxylate were added to one side of the chamber in a volume of 12–20 μl and 10 μl of 0.875 *N* 2-mercaptoethanol was added to the other side. The chambers were then filled with 0.01 *M* Tris-Cl (pH 7.5) and sealed with Parafilm. After rotating for 5 hr at 4°, the chambers were emptied and the amount of pyrrole-2-carboxylate in each side was determined by radioactivity measurements and the enzyme concentration was determined by assaying its activity. The concentration of bound pyrrole-2-carboxylate was calculated and the molar ratio of bound pyrrole-2-carboxylate to total enzyme (*r*) was determined.

Rates of Inactivation. The inactivation mixtures contained 1.9 units of proline racemase in 1 ml of 0.24 *M* Tris-Cl (pH 7.5) containing 0.014 *M* 2-mercaptoethanol. This mixture was placed in a constant temperature bath regulated at 10°. A sample of 0.1 ml of this mixture was removed and added to 10 μl of 0.176 *M* DL-[α-^{3}H]proline (6.4×10^4 cpm/μmol) in 50% 2-mercaptoethanol (v/v). This sample was incubated at 37° for 15 min. The reaction was stopped with 0.1 ml of 20% trichloroacetic acid. The amount of tritium released into the reaction medium was measured as described above (see proline racemase assay). After removal of the first aliquot, an addition of iodoacetic acid, bromoacetic acid, or iodoacetamide was made to the inactivation mixture. Aliquots were then removed for assay as above at 1, 2, 5, 10, and 20 min after addition of inactivator. The natural logarithm of the remaining activity was plotted as a function of time and the pseudo-first-order rate constant for inactivation (*k*) was determined by the method of least-squares analysis.

Determination of Specific Activity of Iodoacetic Acid. A neutral solution of 0.07 mg of [1-^{14}C]iodoacetic acid (0.38 μmol) (obtained from New England Nuclear, Boston, Mass.; lot No. 613-173) in 0.01 ml was incubated with 2 mg of cysteine (16.5 μmol) and 0.05 ml of 0.6 *M* Tris-Cl (pH 7.5) at 70° for 2 hr. The mixture was applied to a 1 × 52 cm column of Bio-Rad AG 50-X8 minus 400 mesh (H^+) and eluted first with 4 ml of water and then with 1.5 *N* HCl. The fractions of the HCl effluent containing radioactivity were dried down in a vacuum desiccator over KOH and redissolved in 1 ml of water. Each fraction was assayed for carboxymethylcysteine by the ninhydrin method of Stein and Moore (1954) and counted. The specific radioactivity of the peak fractions was 16×10^6 cpm per μmol.

Alkylation of Proline Racemase and Isolation of [^{14}C]*Peptide.* Proline racemase (156 units) in 0.2 ml of 0.06 *M* Tris-Cl buffer (pH 7.5) containing 12 m*M* 2-mercaptoethanol and 1.9 m*M* [1-^{14}C]iodoacetate (16×10^6 cpm per μmol) was incubated at 37° for 2 hr in the dark. At this time the enzyme was completely inactivated and 20 μl of 2-mercaptoethanol was added. The mixture was then dialyzed against 500 ml of 50 m*M* Tris-Cl buffer (pH 7.0). After two more dialyses against 1 m*M* HCl, the dialysate was assayed for protein (Lowry et al., 1951) and counted. Enzyme (2 mg) which had been inactivated with [^{14}C]iodoacetate was reduced, denatured, and carboxymethylated by the procedure of Crestfield et al. (1963) and then dialyzed against 1 l. of 0.1 *M* ammonium formate–0.01 *M* $CaCl_2$ (pH 8.1). Worthington Tos-PheCH_2Cl[1] trypsin was added (10% by weight relative to protein) and the mixture was incubated overnight at 37°. The resultant peptide mixture was applied to a 1 × 55 cm column of Sephadex G-25 fine and eluted with 1 *M* acetic acid. The elution pattern (Figure 2) shows a small peak at the void volume which is heterogeneous upon paper electrophoresis at pH 8.9 and corresponds to about one-fourth of the radioactivity. This is followed by a second peak of radioactivity which contains only one radioactive component. This second peak was concentrated to dryness and redissolved in 0.2 *M* pyridine acetate buffer (pH 3.1) and chromatographed on Dowex 50 according to Schroeder (1967a). The sample was applied to a 0.6 × 60 cm column of Bio-Rad AG 50-X2 in the pyridine form at 37° and eluted with a linear gradient of 160 ml total volume formed by the addition of 80 ml of 2.0 *M* pyridine acetate buffer (pH 5.0) (64.5 ml of pyridine, 57.3 ml of acetic acid, and water to 400 ml) to 80 ml of 0.2 *M* pyridine acetate buffer (pH 3.1) (6.45 ml of pyridine, 111.4 ml of glacial acetic acid, and water to 400 ml). Fractions containing radioactivity were pooled and concentrated to dryness and redissolved in pH 8.4 buffer (6 ml of *N*-ethylmorpholine, 8 ml of α-picoline, 4 ml of pyridine, and approximately 0.3 ml of acetic acid to give a final pH of 8.4 when diluted to 400 ml with water) for chromatography on Dowex 1 (Schroeder, 1967b). The sample was adjusted to pH 10 with NaOH and applied to a 0.6 × 60 cm column of Bio-Rad AG 1-X2 acetate at 37°. The column was developed with a gradient created by the addition of 60 ml of pH

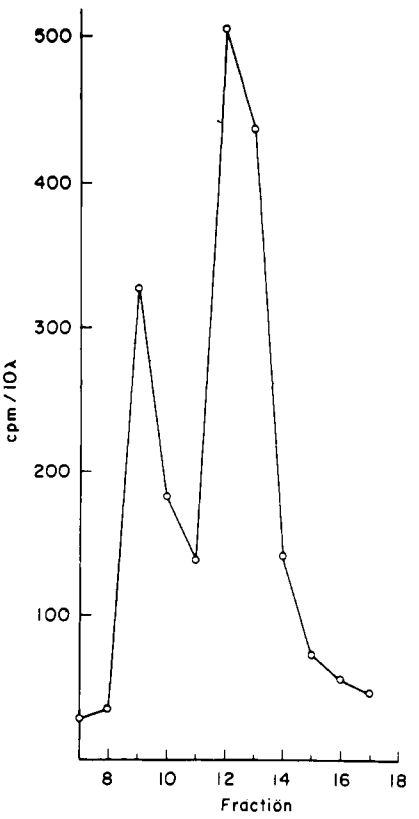

FIGURE 2: Gel filtration of tryptic digest. Enzyme inactivated with [^{14}C]iodoacetate was digested with trypsin and chromatographed on Sephadex G-25 as described under Experimental Procedure.

[1] Abbreviations used are: Tos-PheCH_2Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; dansyl, 8-dimethylamino-1-naphthalenesulfonate.

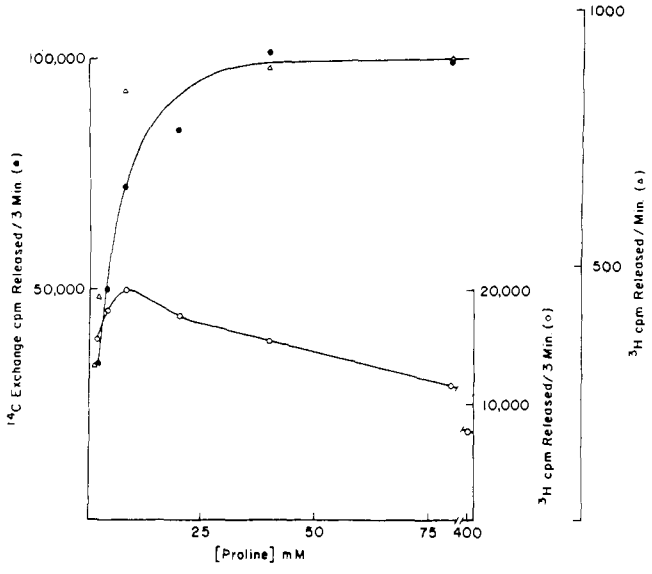

FIGURE 3: Patterns of tritium release and ^{14}C exchange at equilibrium. Rates of tritium release were measured in reaction mixtures of 0.1 ml of 0.24 *M* Tris-Cl (pH 8.0) containing 24 m*M* 2-mercaptoethanol, 10 m*M* EDTA, and DL-[α-^{3}H]proline (6.4×10^4 cpm/μmol) or L-[α-^{3}H]proline (1.05×10^4 cpm/μmol). Reactions were started by addition of 0.25 unit of proline racemase in 5 μl, incubated for various times at 37°, and stopped by addition of 0.1 ml of 20% trichloroacetic acid. Tritium release was determined as in the enzyme assay. Rates of tritium release are initial velocities and represent release of less than 20% of the total radioactivity: (○) tritium release from L-[α-^{3}H]proline; (△) tritium release from L-[α-^{3}H]proline. Proline concentrations are given as the concentration of the L isomer. Rates of [U-^{14}C]proline exchange were measured in reaction mixtures identical with those described above with the exception that DL-[^{14}C]proline labeled in the L isomer (0.2 Ci/mol) was substituted for [^{3}H]proline. Initial rates of ^{14}C exchange were calculated from the time course of isotope equilibration which was a first-order process. In all cases less than 50% equilibration had occurred before the first time point was taken. The appearance of radioactivity in D-proline was measured as described under Experimental Procedure: (●) initial rates of ^{14}C appearance in D-proline.

6.4 buffer (same quantities as for the 8.4 buffer except that about 3.7 ml of acetic acid is required) added to 40 ml of the pH 8.4 buffer in a constant volume mixing chamber Fractions containing radioactivity were evaporated and redissolved in 1 *M* acetic acid and rechromatographed on Sephadex G-25 fine. Solvent was removed and the radioactive peptide was redissolved in 50 μl of 50% pyridine. The yield determined by radioactivity was 4–10 nmol of peptide which was radiochemically pure on paper electrophoresis at pH 8.9 and paper chromatography in 1-butanol–pyridine–acetic acid–water (90:60:18:72, v/v), R_f 0.15. The purified peptide was partially sequenced by a modification of the dansyl-Edman[1] procedure described by Gray (1967). The peptide solution in 50–100 μl of 50% pyridine was transferred to a 5 × 60 mm test tube. A suitably sized sample was removed for dansylation (Gray, 1967) and radioactivity determination and 5 μl of phenyl isothiocyanate was added to the remainder. The test tube was then flushed with nitrogen and covered with Parafilm. Coupling was carried out for 30 min at 50°, with agitation every 5 min. Solvent and excess reagent were then removed by heating at 60° in a vacuum desiccator for 10–20 min. The dried residue was dissolved in 50 μl of anhydrous trifluoroacetic acid, flushed with nitrogen, covered with Parafilm, and incubated at 50° for 5 min. Removal of trifluoroacetic acid was achieved by heating in a vacuum desiccator until the residue was completely dry. The shortened peptide was then redissolved in 50 μl of water and extracted twice with 100 μl of water-saturated butyl acetate. The aqueous phase was then dried down in a heated vacuum desiccator and redissolved in 50 μl of 50% pyridine. Dansylation and hydrolysis were carried out on samples from each Edman cycle (Gray, 1967) and the resulting dansyl amino acids were identified using the chromatographic procedure described by Weiner et al. (1972).

Materials. [1-^{14}C]Iodoacetic acid, [^{3}H]water, and L-[U-^{14}C]proline were obtained from New England Nuclear. Barium [^{14}C]carbonate was obtained from ICN. Iodoacetic acid was recrystallized before use from petroleum ether. Urea was recrystallized before use from 95% ethanol. All other chemicals were of reagent grade obtained from commercial sources and used without further purification.

Results

Patterns of Exchange of the α-Proton of Proline. When a molecule is isomerized by proline racemase, its α-hydrogen is removed and released into the solvent (Cardinale and Abeles, 1968). The previously proposed mechanism for proline racemase assumed that no exchange of proline α-hydrogens with solvent protons occurs from the enzyme–proline complex. Therefore, release of an α-hydrogen from proline would occur only after the product has dissociated from the enzyme. In order to test this assumption, the rate of tritium release from DL-[α-^{3}H]proline was measured as a function of proline concentration. The results in Figure 3 show that the rate of tritium release is inhibited at high proline concentrations. Under the same conditions, the rate of exchange of L-[^{14}C]proline into D-proline follows normal saturation behavior indicating that no substrate inhibition is occurring at high proline concentrations. These results demonstrate that the proton removed from proline is retained by the enzyme until after product release (Silverstein and Boyer, 1964). Also shown in Figure 3 is the rate of tritium release from L-[α-^{3}H]proline. No inhibition is seen at high concentrations. This indicates that the product isomer is responsible for the inhibition. Only when both isomers are present is inhibition seen.

Subunit Molecular Weight and Number of Binding Sites. The purified enzyme gives a single major band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, with impurities accounting for less than 5% of the total protein. When proteins of known molecular weight were compared with samples of proline racemase according to the procedure of Weber and Osborne (1969) the relative mobility of the enzyme corresponded to a molecular weight of 38,500 g/mol. Values from several experiments ranged from 37,000 to 40,000 g/mol. The amino acid composition (Table I) is also consistent with a molecular weight of 38,000–39,000 g/mol. Since there are 33 lysines and 9 arginines in the protein, 43 tryptic peptides are expected. Peptide maps of tryptic digests (Figure 4) resolve 40 to 44 peptides, indicating that the enzyme is composed of only one type of polypeptide chain.

To determine the number of binding sites per subunit, the binding of pyrrole-2-carboxylate, a competitive inhibitor of the enzyme, was measured by equilibrium dialysis. The results, shown in Figure 5, indicate 0.98 ± 0.036 binding site per two subunits, or 77,000 daltons. This suggests that the enzyme is present in solution as a dimer. The dissociation constant calculated for pyrrole-2-carboxylate by this method is 0.4×10^{-5} *M*. This agrees with the K_I for pyrrole-2-carboxylate measured by competitive inhibition (1.8×10^{-5}

Table I: Results of Amino Acid Analysis.[f]

Amino Acid	Amount Rel. to Leu	No. of Residues	Integral No.	Approximate Mol Wt
Lysine	1.344	33.36	33 ± 5	4224
Aspartic acid	1.171	29.06	29 ± 3	3335
Glutamic acid	1.150	28.54	29 ± 2	3741
Proline	0.557	13.82	14 ± 3	1358
Glycine	1.379	34.23	34 ± 4	1938
Alanine	0.970	24.07	24 ± 3	1704
Cystine	0.292	7.24	7 ± 2	1442
Cysteic acid[a]	0.170	29.04	29 ± 5	
Cysteine[b]	0.586	14.60	15 ± 7	1545
Valine[e]	1.006	24.97	25 ± 4	2125
Methionine	0.436	10.82	11 ± 1	1441
Isoleucine[e]	1.134	28.14	28 ± 5	3164
Leucine	1.000	24.82	25 ± 4	2825
Tyrosine	0.423	10.50	11 ± 1	1793
Phenylalanine	0.574	14.25	14 ± 2	2058
Arginine	0.365	9.06	9 ± 1	1404
Serine[c]	0.674	16.72	17 ± 2	1479
Threonine[c]	0.768	19.06	19 ± 1	1919
Histidine[d]	0.414	10.23	10 ± 2	1370
Total				38,865

[a] From performate oxidation. [b] Cysteic acid minus cystine. [c] Extrapolated to zero hydrolysis from 24, 48, and 72 hr. [d] Resolved from lysine on DuPont curve resolver. [e] From 72-hr hydrolysis. [f] Protein samples were dialyzed exhaustively against distilled water, then lyophilized and hydrolyzed with constant boiling HCl for 24, 48, or 72 hr. Another sample was oxidized with performic acid according to the method of Hirs (1967) and hydrolyzed in constant boiling HCl for 24 hr. HCl was removed in vacuo and samples were dissolved in 0.4 ml of 0.2 *M* sodium citrate (pH 2.2). Amino acid analyses were performed according to the method of Spackman et al. (1958). Data are results from duplicate or triplicate analyses.

M). Although data are not shown, it is noteworthy that no binding of pyrrole-2-carboxylate was detected in the absence of 2-mercaptoethanol.

Inactivation of Proline Racemase by Iodoacetate. Proline racemase is rapidly and completely inactivated by iodoacetate. For example, at 0.4 m*M* iodoacetate (pH 7.0, 37°) the enzyme is inactivated 90% in less than 4 min. This inactivation occurs in the presence of an excess (10 m*M*) of 2-mercaptoethanol. The initial rate of the inactivation follows pseudo-first-order kinetics. The first-order rate constant so obtained (k) is initially proportional to iodoacetate but becomes zero order in iodoacetate at high concentrations. These kinetic results are consistent with a mechanism in which iodoacetate combines reversibly with the enzyme prior to inactivation. From these data a dissociation constant (K_I) of 1.3 m*M* was calculated for iodoacetate. The maximum rate of inactivation (k_{max}) was determined to be 0.16 min^{-1}.

Proline and pyrrole-2-carboxylate can protect the enzyme from inactivation by iodoacetate (Cardinale and Abeles, 1968). From these protection experiments, a dissociation constant for pyrrole-2-carboxylate of 0.7×10^{-5} *M* can be calculated. This is comparable to the dissociation constant as measured by competitive inhibition and binding experiments.

Iodoacetamide and bromoacetate also inactivate proline racemase, and show similar kinetic behavior. For iodoacetamide K_I = 1.6 m*M* and k_{max} = 0.14 min^{-1}. For bromoacetate K_I = 0.22 m*M* and k_{max} = 0.025 min^{-1}. The fact that iodoacetate and iodoacetamide have very similar binding constants indicates that the carboxylate group does not play a major role in binding the inactivator.

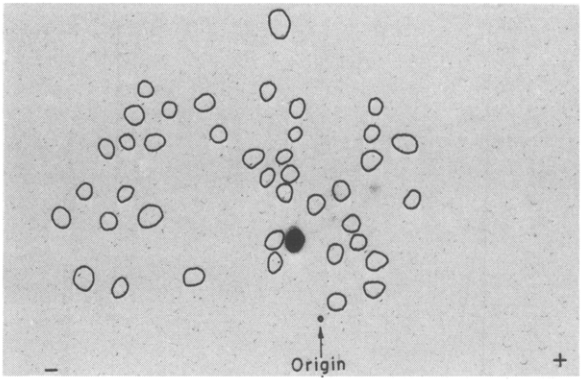

FIGURE 4: Peptide map of tryptic hydrolysate of proline racemase. Proline racemase was reduced, carboxymethylated, and digested with trypsin as described under Experimental Procedure. The peptide mixture (50–100 μg) was applied to a 20 × 20 cm plate of silica gel G (Analtech Uniplate 250 μm) and chromatographed in the first dimension with 1-propanol–ammonia (7:3). Electrophoresis in the second dimension was performed in pyridine acetate buffer (1.25 *M* pyridine, acetic acid to pH 6.5) at 400–600 V for 100 min. The plates were visualized under ultraviolet light after spraying with a 0.03% solution of fluorescamine in acetone. When enzyme inactivated with [1-^{14}C]iodoacetate was subjected to the above procedure and the chromatogram was exposed for 1 week to Kodak Blue Brand X-ray film, the pattern shown here superimposed over the spots was observed.

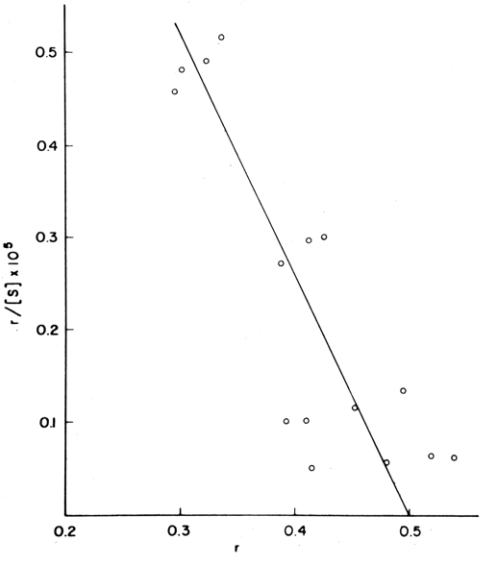

FIGURE 5: Scatchard plot of pyrrole-2-carboxylate binding to proline racemase. Equilibrium dialysis experiments were carried out as described under Experimental Procedure. [S] is the concentration of pyrrole-2-carboxylate and r is the molar ratio of bound pyrrole-2-carboxylate to enzyme. A molecular weight of 38,500 was used. Least-squares analysis of the data gives an intercept on the abcissa of 0.49 ± 0.018 mol/mol of the 38,500-dalton subunit.

When proline racemase is inactivated with iodoacetate, about 1 mol of iodoacetate is incorporated covalently per 38,500 g of protein. Values obtained with three different enzyme preparations were 0.95, 0.97, and 1.15 mol/38,500 g of protein. In the absence of mercaptoethanol, about 2% of this incorporation occurs. Since 1 mol of substrate binds per 77,000 g, this incorporation corresponds to two iodoacetate molecules per binding site.

When enzyme inactivated with [^{14}C]iodoacetate is hydrolyzed in 6 *N* HCl for 24 hr at 110°, 82% of the radioactivity is recovered as *S*-carboxymethylcysteine. Carrier *S*-carboxymethylcysteine was recovered to the same extent. This product was identified by co-chromatography with au-

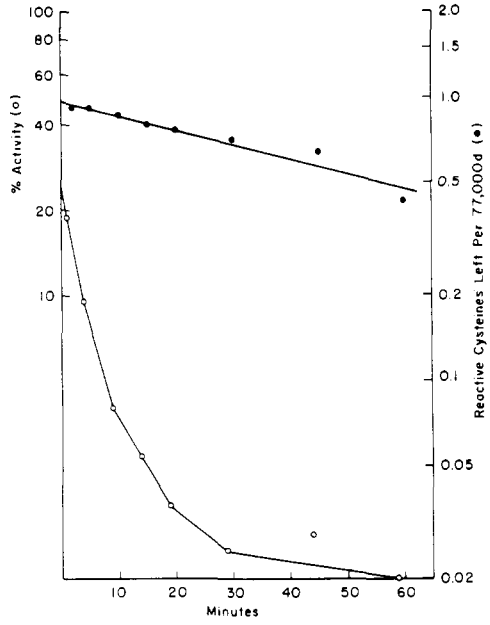

FIGURE 6: Time course of inactivation by iodoacetate and modification of cysteine residues. Reaction mixtures consisted of 52 units of enzyme in 50 μl of 60 m*M* Tris-Cl (pH 7.0) containing 10 m*M* 2-mercaptoethanol. After 5 min at 37°, 2 μl of a 9.4 m*M* solution of [^{14}C]iodoacetate was added. At various times, 1-μl portions were removed for enzyme assay and 4-μl portions were diluted into 1 ml of ice-cold 5% trichloroacetic acid and filtered on Millipore HAWP filters. The filters were washed with 5 ml of 5% trichloroacetic acid, dried, and counted in scintillation vials containing 10 ml of counting fluid (Bray, 1960). The amount of remaining reactive cysteine residues was determined at each time point from the amount of iodoacetate incorporated.

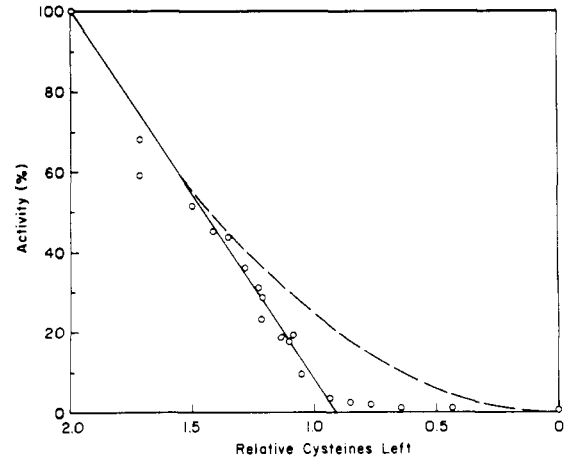

FIGURE 7: Loss of activity vs. modification by iodoacetate: (O) data from Figure 6 and similar experiments at different temperatures; (- - -) theoretical line for two essential cysteines reacting at the same rate (see text).

thentic compound in two paper chromatography systems, 1-butanol–acetic acid–water (4:1:1) (R_f 0.25) and methyl ethyl ketone–propionic acid–water (15:5:6) (R_f 0.24), and ion exchange chromatography on Bio-Rad AG 50-X8 eluted with 2.5 *N* HCl. The specific radioactivity of the carboxymethylcysteine was unchanged after co-chromatography with cold carrier on AG 50. In order to test for the presence of a thioether linkage, the product was oxidized with hydrogen peroxide by the procedure of Goodman et al. (1958). After this treatment, which does not affect oxygen ethers, the chromatographic behavior of the product in methyl ethyl ketone–propionic acid–water was changed, consistent with its oxidation to the sulfone.

The time course of [1-^{14}C]iodoacetate incorporation and inactivation of enzyme activity is shown in Figure 6. There is a rapid reaction of one cysteine per binding site during which most of the activity is lost, followed by a slower reaction of a second cysteine residue. The loss of activity, initially a first-order process, deviates from first-order behavior as the reaction proceeds. This may reflect decreasing concentration of iodoacetate due to its reaction with 2-mercaptoethanol. In Figure 7 the results of Figure 6 and similar experiments are plotted according to the method of Tsou (1962). It can be seen that, as the first cysteine residue reacts, the enzyme loses almost all of its activity. The incorporation of iodoacetate continues until a total of two cysteines per active site are labeled. These results are expected if there is one essential sulfhydryl group per dimer. It is also consistent with two essential sulfhydryl groups which react with iodoacetate at different rates. Also shown in Figure 7 (dashed line) is the expected behavior if two essential cysteines reacted at the same rate (Tsou, 1962).

In order to determine the site of iodoacetate labeling, enzyme which had been inactivated with [1-^{14}C]iodoacetate was digested with trypsin and the resulting peptides were chromatographed. The peptide mixture was applied to silica gel thin-layer plates and separated by two-dimensional chromatography and electrophoresis. When the peptides were visualized with fluorescamine, it was observed that most of the radioactivity of the hydrolysate was present in a single peptide (Figure 4). The remainder was distributed among a series of minor spots, none of which corresponded to any peptide visualized by fluorescamine. Similar results were obtained when the peptide mixture was subjected to gel filtration on Sephadex G-25 (Figure 2). The first peak of radioactivity, eluted from the column at the void volume, corresponded to 25% of the total radioactivity and was extremely heterogeneous on electrophoresis at pH 8.9. This peak probably corresponds to the minor spots seen on autoradiography of the peptide map. A second peak, which emerged about half-way between the void volume and the small molecules, contained about 75% of the radioactivity of the peptide mixture. The radioactivity in this peak moved as a single component on electrophoresis at pH 8.9, paper chromatography in 1-butanol–acetic acid–pyridine–water (90:60:18:72) (R_f 0.15), and ion exchange chromatography on Dowex 50 and Dowex 1, as described under Experimental Procedure. The relative amounts of radioactivity in the two peaks did not depend on the extent of iodoacetate incorporation into the enzyme. The second peak was purified to homogeneity by ion exchange chromatography and gel filtration as described under Experimental Procedure. At no step of the purification after the first G-25 column was more than one peak of radioactivity observed. The purified peptide contained a single N-terminal residue, serine, as determined with dansyl chloride (Weiner et al., 1972).

The purified radioactive peptide was degraded sequentially by the Edman procedure and gave a single N-terminal amino acid at each step as determined with dansyl chloride (Gray, 1967; Weiner et al., 1972). At each step, the radioactivity remaining in the peptide was measured and is shown in Figure 8. Most of the radioactivity is lost with the third amino acid, showing that the peptide is labeled in only one position. This result, taken together with the facts that one cysteine per subunit reacts with iodoacetate and one tryptic peptide is modified in the reaction, indicates that each subunit reacts with iodoacetate at the same point in its

primary sequence. The N-terminal sequence of the peptide determined by the dansyl-Edman technique is Ser-Pro-S-CH_2CO_2H-Cys-Gly-Thr-.

Discussion

The release of tritium to the solvent from DL-[α-^{3}H]proline is depressed as the DL-proline concentration is increased (Figure 3). This is a consequence of an ordered product release in which proline is released prior to the proton. Consider intermediate IV in Figure 1 after release of D-proline. That intermediate can either release $^+$H to give I or bind proline to give III. The rate of conversion IV $\rightarrow$ III will depend on proline concentration. Thus, at infinitely high concentration of proline IV $\rightarrow$ I, i.e., $^+$H release, should be totally suppressed if no release of $^+$H prior to product release can occur. In the experiment of Figure 3, release of $^+$H ($^3H^+$) was suppressed to 40% of maximum. This indicates that in most turnovers, possibly all turnovers, proline is released prior to the proton from the enzyme–proline–proton complex. These results support one of the assumptions made in the proposed mechanism shown in Figure 1.

Suppression of ^{3}H release does not occur when L-[α-^{3}H]proline is incubated with the enzyme and L-proline concentration is increased. Therefore, to capture the enzyme-bound proton derived from L-proline, D-proline must be present. Two species of EH^+ must exist, one derived from L-proline, which can only react with D-proline, and one derived from D-proline, which can only react with L-proline. Interconversion of the two species cannot occur without exchange of the proton with the solvent. The existence of two forms of EH^+ is part of the proposed mechanism. The two forms of EH^+ are represented by I and IV in Figure 1.

What bases at the active site function as proton donor and acceptor? The nature of the reaction catalyzed, as well as the similarity of kinetic constants (K_m, V_{max}) in the forward and reverse reactions, suggest that two identical groups are at the active site. The evidence available so far implicates sulfhydryl groups. When L-proline reacted with the enzyme in D_2O, under conditions where reversal of the overall reaction is negligible, essentially no ^{2}H was incorporated into L-proline (Cardinale and Abeles, 1968). This lack of ^{2}H incorporation shows that the basic group at the active site probably is a base with a single proton. If it were a base with more than one proton, at least some ^{2}H would be incorporated into L-proline. Similar results were obtained with D-proline. Therefore, amino groups can be excluded as active-site proton acceptors or donors. Alkylation of a cysteine residue inactivates proline racemase. This inactivation can be prevented by substrate or competitive inhibitors. The pH dependence of V_{max} is consistent with the presence of thiol and thiolate in the active form of the enzyme (Cardinale and Abeles, 1968). Moreover, a reducing agent is required for catalytic activity, as well as for reaction of the enzyme with iodoacetate. These observations suggest that a disulfide in the inactive enzyme becomes reduced to two sulfhydryls, and the sulfhydryl groups then function as proton donor and acceptor in the catalytic process. Although two sulfhydryl groups per binding site (one sulfhydryl group per subunit) are alkylated by iodoacetate, reaction of a single sulfhydryl group leads to loss of activity. This would be expected for a mechanism requiring two sulfhydryl groups. Furthermore, one sulfhydryl group reacts more rapidly with iodoacetate than the other. If both sulfhydryl groups are at the active site, reaction of one could easily reduce the reactivity of the other. A single active site in which two sulfhydryl groups function as proton donor and acceptor is, therefore, consistent with the available data. Others have concluded that sulfhydryl groups function as proton donor and acceptor in hydroxyproline epimerase (Finlay and Adams, 1970), an enzyme which resembles proline racemase in many respects.

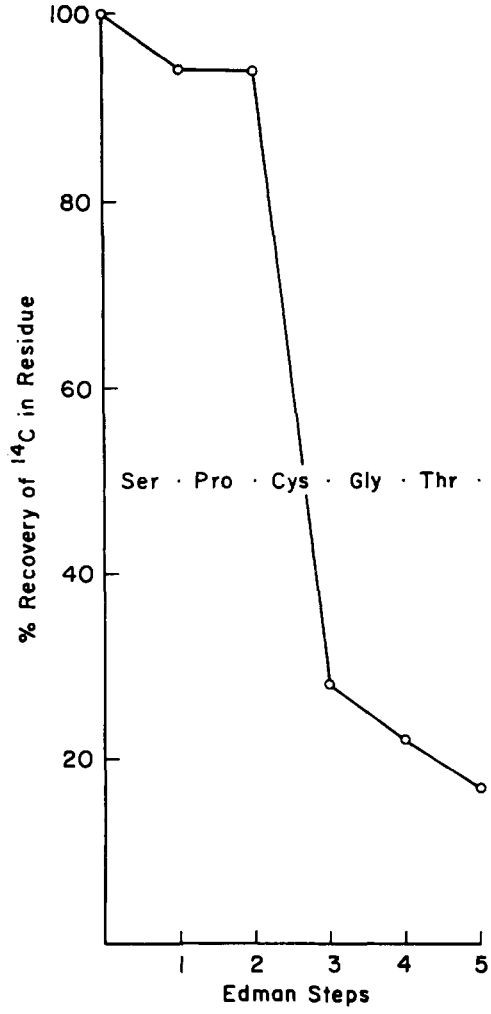

FIGURE 8: Edman degradation of active-site peptide. Purified [^{14}C]peptide from enzyme inactivated with [^{14}C]iodoacetate was subjected to Edman degradation as described under Experimental Procedure. Before each step of the degradation, the N-terminal amino acid was determined using dansyl chloride and after each step the residual radioactivity remaining in the peptide was determined.

The two sulfhydryl groups which are labeled by iodoacetate are found in an identical amino acid sequence. Therefore, it is unlikely that they are in the same polypeptide chain and are probably located on different subunits. This fact, together with the observation that there is only one binding site per two subunits, has led us to propose that the active site of proline racemase is located at the interface of two identical, or nearly identical, subunits. Each of these subunits furnishes one of the two active-site sulfhydryl groups.

It is possible, with additional assumptions, to propose other models. For instance, there could be an active site on each subunit of the dimeric enzyme, with two nonequivalent sulfhydryl groups, one of which is significantly more reactive toward iodoacetate. If that were the case, a high degree of negative cooperativity (Stallcup and Koshland, 1973) is

required, so that binding of a substrate molecule at one site prevents reaction at the other. Also, reaction of iodoacetate at one site must reduce catalytic activity and reactivity toward iodoacetate at the other site. Possibly other models could be proposed. At this time, we prefer the model in which the active site is made up of two subunits. As far as we know, an active site of that type has not been conclusively established for any enzyme. Obviously, additional evidence is required to establish this type of active site for proline racemase.

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On the Role of Sulfhydryl Groups in the Structure and Function of the *Azotobacter vinelandii* RNA Polymerase†

Joseph S. Krakow

ABSTRACT: Exposure of sulfhydryl groups as indicated by titration kinetics is decreased under conditions where RNA polymerase exists as a dimer or higher aggregate (low salt), in the presence of Mn^{2+}, or when bound to d(A-T). Incubation of phenylmercurisulfonate with RNA polymerase above pH 9.0 results in loss of d(A-T) binding ability. Poly(U) binding is more sensitive to sulfhydryl modification and is lost at pH's above 8.0. The presence of 4 m*M* Mn^{2+} has an obvious effect in stabilizing the polymerase–poly(U) complex when incubated with 10 μM phenylmercurisulfonate + 1 *M* urea. Incubation of the enzyme with the mercurial and urea results in disaggregation to subprotomeric forms and release of the α subunit. Similar treatment in the presence of 4 m*M* $MnSO_4$ stabilizes the protomeric structure of the enzyme. During chain elongation the enzyme exists as a ternary $d(A\text{-}T)_n$–enzyme–$r(U\text{-}A)_n$ complex in which the bound $d(A\text{-}T)_n$ is refractory to the destabilizing effect of the mercurial; however, further phosphodiester bond formation is inhibited. The results are defined in terms of a role which reflects the involvement of polymerase sulfhydryl groups in the various conformations necessary for subunit–subunit interaction, tight template binding, and catalytic activity.

RNA synthesis by RNA polymerase is a result of a complex sequence of several substeps carried out by a complex enzyme consisting of several subunits (von Hippel and McGhee, 1972; Chamberlin, 1974). Although it is known that RNA polymerase is sensitive to a variety of sulfhydryl inhibitors, the large number of cysteinyl residues, 30–35, and their distribution among the enzyme subunits (β', β, $\alpha \pm \sigma$) makes the assignment of a specific functional role difficult. The following are affected by sulfhydryl modification: subunit–subunit interaction (Ishihama, 1972; Ito and Ishihama, 1973), template binding (Ishihama and Hurwitz, 1969), tight template binding (Smith et al., 1971; Krakow, 1972a), template recognition and specific initiation (Yarbrough and Wu, 1974), and phosphodiester bond formation (Krakow, 1966; Lee-Huang and Warner, 1969; Ishihama and Hurwitz, 1969; Sumegi et al., 1971; Smith et al., 1971; Harding and Beychok, 1973; Nicholson and King, 1973; Yarbrough and Wu, 1974). The published data and that to be presented in this paper do not exclude a direct involvement of sulfhydryl groups in the catalytic role of RNA polymerase but are also compatible with their involvement

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