Oxidation of L-Proline and L-3,4-Dehydroproline by D-Amino Acid Oxidase*

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Highly purified preparations of D-amino acid oxidase catalyze the oxidation of L-proline to Δ^1-pyrroline-2-carboxylic acid and of L-3,4-dehydroproline to pyrrole-2-carboxylic acid. These reactions are strongly inhibited by benzoate, a known inhibitor of D-amino acid oxidase. The oxidation of L-3,4-dehydroproline is inhibited by D-alanine but not by L-alanine. The apparent K_m for L-proline is about 0.15 M. L-Proline is oxidized about 3000–4000 times more slowly than D-proline.

The D-amino acid oxidase of mammalian kidney has been very useful to the biochemist for the detection of D-amino acids present in natural materials or produced by various reactions. This enzyme has long been thought to be absolutely specific for amino acids of the D configuration. However, Neims and Hellerman (1962) have recently reported convincing evidence that glycine is oxidized by D-amino acid oxidase. Glycine, therefore, represents the first example of a substrate for D-amino acid oxidase which does not possess an asymmetric carbon atom of the D configuration. Although glycine is optically inactive, it may be considered in a formal sense to belong to both the D and the L series of amino acids. Glycine, however, is not oxidized at a measurable rate by snake venom L-amino acid oxidase (Zeller and Maritz, 1944; Singer and Kearney, 1950; Scannone *et al.*, 1964). Indications that some amino acids of the L configuration are oxidized by D-amino acid oxidase were obtained through the use of a sensitive new assay method based on the principle of catalytic amplification (Scannone *et al.*, 1964). Attempts to obtain more direct evidence for these reactions and to identify their products were therefore carried out. In this report, evidence is presented that D-amino acid oxidase catalyzes the oxidation of L-proline to Δ^1-pyrroline-2-carboxylic acid and the oxidation of L-3,4-dehydroproline to pyrrole-2-carboxylic acid.

Experimental

Materials.—The enzymes used and the definitions of enzyme units are the same as in the accompanying paper (Scannone *et al.*, 1964). Several D-amino acid oxidase preparations, with specific activities ranging from 38 to 190 units per mg of protein, were used. They all had about the same ratio of activities toward D-alanine and L-proline. L-3,4-Dehydroproline and DL-3,4-dehydroproline were synthesized according to Robertson and Witkop (1962). Samples of these amino acids generously donated by Dr. Bernhard Witkop were also used and gave similar results. L-Allo-4-hydroxyproline was synthesized by the method of Patchett and Witkop (1957). Other amino acids were twice-recrystallized commercial preparations. *o*-Aminobenzaldehyde was prepared freshly as needed from *o*-nitrobenzaldehyde (Matheson, Coleman and Bell) according to Smith and Opie (1955). Pyrrole-2-carboxylic acid, synthesized according to Radhakrishnan and Meister (1957) was a gift from Dr. Alton Meister. *p*-Dimethylaminobenzaldehyde was obtained from Distillation Products Industries. L-Thiazolidine-4-carboxylic acid was synthesized as described by Greenstein and Winitz (1961).

Methods.—All enzymatic reactions were carried out with shaking at 37° in an atmosphere of air, unless otherwise indicated. Reaction mixtures were deproteinized by the addition of 0.1 volume of 50% trichloroacetic acid, followed by centrifugation.

Deproteinized reaction mixtures were assayed for pyrrole-2-carboxylic acid as follows. A 0.3-ml aliquot of the solution was added to 3.7 ml of 1 N H_2SO_4, heated to 70° for 10 minutes, and cooled to 25°. One ml of 5% *p*-dimethylaminobenzaldehyde in 1-propanol was then added, and after 10 minutes the color was measured in a Weston colorimeter with a 550-mμ filter. A freshly prepared solution of pyrrole-2-carboxylic acid was used as standard (Corrigan *et al.*, 1963).

Δ^1-Pyrroline-2-carboxylic acid was made enzymatically by incubating DL-proline with D-amino acid oxidase at *p*H 8.3 in the presence of catalase (Krebs, 1939; Meister, 1954). The amount of proline oxidized was determined manometrically. Δ^1-Pyrroline-5-carboxylic acid was made enzymatically by incubating L-proline with a freshly prepared rat liver homogenate as described by Johnson and Strecker (1962).

Both Δ^1-pyrroline-2-carboxylic acid and Δ^1-pyrroline-5-carboxylic acid form a deep yellow complex with *o*-aminobenzaldehyde at acid *p*H. The amount of complex formed was measured colorimetrically at 445 mμ, 1 hour after the addition of a 0.2-ml aliquot of the deproteinized reaction mixture to a tube containing 1.8 ml of 6% aqueous trichloroacetic acid and 2 ml of 0.5% *o*-aminobenzaldehyde in ethanol (Strecker, 1957).

The 2,4-dinitrophenylhydrazone of Δ^1-pyrroline-2-carboxylic acid (α-keto-δ-aminovaleric acid) was determined as follows: A 0.2-ml aliquot of the deproteinized reaction mixture was added to 1.5 ml of a 0.033% solution of 2,4-dinitrophenylhydrazine in 0.67 N HCl. After 1 hour, 2.5 ml of 2.5 N NaOH was added, and the color was measured 10 minutes later with a 520-mμ filter (Ikawa *et al.*, 1960). Δ^1-Pyrroline-5-carboxylic acid gave no color under these conditions. Since the high concentration of L-proline (1 M) present in some of the reaction mixtures decreased the color yield in this reaction, the same amount of L-proline was added in the standards.

Electrophoretic Separation of the o-Aminobenzaldehyde Derivatives of Δ^1-Pyrroline-2-carboxylic Acid and Δ^1-Pyrroline-5-carboxylic Acid.—The deproteinized reaction mixture was extracted three times with 5 volumes

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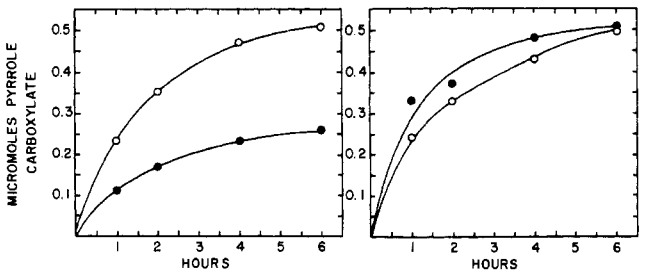

FIG. 1.—Enzymatic oxidation of 3,4-dehydroproline. The reaction mixtures consisted of 3,4-dehydroproline (0.5 μmole), amino acid oxidase, flavin adenine dinucleotide (12.5 μg), catalase (400 units), and Tris-HCl buffer (100 μmoles, *p*H 7.8) in a final volume of 2.5 ml. Open circles (○), L-3,4-dehydroproline; solid circles (●), DL-3,4-dehydroproline. (a, left): L-amino acid oxidase (45,000 units). (b, right): D-amino acid oxidase (325 units).

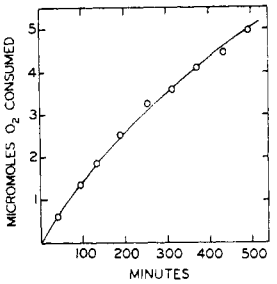

FIG. 2.—Enzymatic oxidation of L-proline. The reaction mixture consisted of L-proline (1 mmole), D-amino acid oxidase (325 units), flavin adenine dinucleotide (12.5 μg), and sodium pyrophosphate buffer (40 μmoles, *p*H 8.3) in a final volume of 1 ml.

of ether to remove trichloroacetic acid, and then evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of water, and an aliquot containing about 0.2 μmole of the derivative was spotted on Whatman 3MM paper. The paper was wet with 6.7% aqueous formic acid, and a potential of 60 v/cm was applied for 15 minutes in a Savant apparatus. Under these conditions the derivative of Δ^1-pyrroline-5-carboxylic acid moved 12 cm, while that of Δ^1-pyrroline-2-carboxylic acid moved 9 cm toward the cathode. When a large amount of L-proline (10–20 μmoles) was spotted simultaneously, the mobilities were changed but the separation was still good. Under these conditions the derivatives moved 10 cm and 7.5 cm, respectively.

RESULTS

Enzymatic Oxidation of 3,4-Dehydroproline.—Weissbach *et al.* (1960) have shown that L-amino acid oxidase catalyzes the oxidation of 3,4-dehydroproline to pyrrole-2-carboxylic acid. We have confirmed this finding and have shown that only the L isomer of the amino acid is susceptible to oxidation by that enzyme. Thus, as shown in Figure 1, L-amino acid oxidase oxidized L-3,4-dehydroproline completely, and the racemic modification only to the extent of 50%. D-Amino acid oxidase, on the other hand, oxidized both DL- and L-dehydroproline completely to pyrrole-2-carboxylic acid (Figure 1). This reaction could be detected easily by the very sensitive color test for pyrrole (obtained by decarboxylation of pyrrole-2-carboxylic acid) with Ehrlich's reagent. The reaction could also be followed manometrically in the Warburg apparatus. However, because of the very powerful inhibitory effect of the product, the reaction becomes very slow and difficult to measure as soon as an appreciable amount of substrate has been oxidized. Tne K_i of pyrrole-2-carboxylic acid has been reported by Parikh *et al.* (1958), to be 9.9×10^{-6} M. For this reason, it was not possible to obtain an accurate value of the K_m for this reaction. However, under the conditions of Table I, the approximate concentration of L-3,4-dehydroproline required to give half-maximal velocity with D-amino acid oxidase was found to be about 10^{-3} M.

Although a highly purified preparation of D-amino acid oxidase was used, it was desirable to obtain additional evidence that the oxidation of L-3,4-dehydroproline was not owing to a contaminating enzyme. Table I shows that benzoate, a specific inhibitor of D-amino acid oxidase, completely inhibited the oxidation of L-3,4-dehydroproline. It was also shown (Table I) that D-alanine, a good substrate of D-amino acid oxidase, inhibited the reaction significantly under conditions where L-alanine caused no appreciable inhibition.

TABLE I
INHIBITORS OF L-3,4-DEHYDROPROLINE OXIDATION

Inhibitor Added	Pyrrole-2-carboxylic Acid Formed (μmoles)	Inhibition (%)
None[a]	0.040	
Sodium benzoate (0.02 M)	0.000	100
D-Alanine (0.02 M)	0.009	77
L-Alanine (0.02 M)	0.039	3

[a] The reaction mixture consisted of L-3,4-dehydroproline (0.5 μmole), D-amino acid oxidase (6.5 units), flavin adenine dinucleotide (2.5 μg), catalase (10 units), and Tris-HCl buffer (20 μmoles, *p*H 8.0) in a final volume of 0.5 ml. The incubation time was 60 minutes.

The possibility that racemization (enzymatic or nonenzymatic) might produce D-3,4-dehydroproline which would be subsequently oxidized by D-amino acid oxidase was tested as follows: L-3,4-dehydroproline was incubated with D-amino acid oxidase under anaerobic conditions. After the D-amino acid oxidase was inactivated by heating the reaction mixture to 80° for 5 minutes, the configuration of the amino acid was examined by testing its susceptibility to oxidation by L-amino acid oxidase as shown in Figure 1. No significant racemization was observed.

Enzymatic Oxidation of L-Proline.—The oxidation of L-proline in the presence of D-amino acid oxidase is shown in Figure 2. Since the product of this reaction is not markedly inhibitory at low concentrations, the reaction could be followed manometrically for many hours. A high concentration of both enzyme and substrate is necessary, since the reaction is slow and the K_m for L-proline is high. From the effect of substrate concentration on the velocity of the reaction (Fig. 3), the apparent K_m for L-proline was estimated to be about 0.15 M. L-Proline which had been extensively pretreated with D-amino acid oxidase to remove traces of D-proline (Scannone *et al.*, 1964) and then recrystallized gave results similar to those obtained with L-proline which had been only recrystallized. Some proline samples gave lower oxidation rates before recrystallization, presumably because of the presence of inhibitory impurities. Additional evidence that the oxidation observed was not owing to traces of D-proline was that, when 1 μmole of D-proline was added to 1 mmole of L-proline (other conditions as in Fig. 2), the oxygen uptake became very rapid for about 10 minutes (until the D-proline was oxidized) and then continued at the

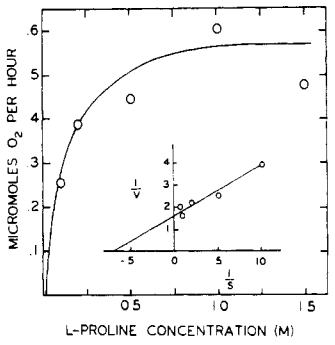

FIG. 3.—Effect of L-proline concentration on rate of enzymatic oxidation. Conditions as in Figure 2.

same rate as shown in Figure 2. D-Proline is oxidized about 3000–4000 times faster than L-proline.

Sodium benzoate, at a concentration of 0.001 M, decreased the rate of oxidation of L-proline by 86% under the conditions of Figure 2. A decrease in the rate of oxygen uptake was also observed when catalase was added, indicating that hydrogen peroxide is a product of the reaction.

In order to see if racemization of L-proline, followed by the oxidation of the D isomer, was taking place, the following experiment was carried out. A reaction mixture containing D-amino acid oxidase and L-proline (conditions as in Fig. 2) was incubated at 37° for 18 hours under an atmosphere of nitrogen in a Warburg flask. After that time, O_2 saturated with water at 37° was passed through the flask for 1 minute, and pressure measurements were started immediately. The rate of oxygen uptake was found to be of the same order of magnitude as in the experiment of Figure 2, and no burst of oxidation, which would have been expected had racemization taken place, was observed. The formation of 1 μmole of D-proline would have been readily detected.

Since D-allo-4-hydroxyproline is oxidized by D-amino acid oxidase to Δ^1-pyrroline-4-hydroxy-2-carboxylic acid, and this compound is readily converted to pyrrole-2-carboxylic acid at low *p*H (Radhakrishnan and Meister, 1957), the same assay conditions which are used for the measurement of 3,4-dehydroproline oxidation (Fig. 1) may also be used as a sensitive measurement of 4-hydroxyproline oxidation (Corrigan *et al.*, 1963). L-4-Hydroxyproline and L-allo-4-hydroxyproline were tested under these conditions, both with D-amino acid oxidase and with L-amino acid oxidase, but no reaction was detected. The following amino acids were also tested as substrates for D-amino acid oxidase by the manometric procedure, under conditions similar to those of Figure 2: L-4-hydroxyproline, L-allo-4-hydroxyproline, L-alanine, and L-serine, each at a concentration of 1 M. None of these exhibited any activity measurable by this technique. However, L-thiazolidine-4-carboxylic acid, at a concentration of 0.225 M, was oxidized at about 25% of the rate of L-proline at the same concentration.

The product of oxidation of L-proline by D-amino acid oxidase was identified as Δ^1-pyrroline-2-carboxylic acid by its reaction with *o*-aminobenzaldehyde, by its reaction with 2,4-dinitrophenylhydrazine, and by the electrophoretic mobility of its *o*-aminobenzaldehyde derivative. In the presence of catalase, the number of μmoles of product formed was about twice the number of μmoles of O_2 taken up (Table II).

TABLE II
STOICHIOMETRY OF Δ^1-PYRROLINE-2-CARBOXYLIC ACID FORMATION FROM L-PROLINE[a]

L-Proline Oxidized (μmoles)	Δ^1-Pyrroline-2-carboxylic Acid Formed (μmoles)
9.1[b]	10.3[c]
	9.8[d]

[a] The reaction flask contained L-proline (1 mmole), D-amino acid oxidase (800 units), flavin adenine dinucleotide (12.5 μg), catalase (40 units), and sodium pyrophosphate buffer (40 μmoles, *p*H 8.3) in a total volume of 1 ml. The incubation time was 300 minutes. [b] Calculated from O_2 uptake. [c] Calculated from absorbancy at 445 mμ after treatment of an aliquot with *o*-aminobenzaldehyde. [d] Calculated from absorbancy at 520 mμ after treatment of an aliquot with 2,4-dinitrophenylhydrazine followed by alkali.

DISCUSSION

Evidence has been presented that highly purified preparations of D-amino acid oxidase catalyze the oxidation of L-proline and L-3,4-dehydroproline. The finding that these reactions are inhibited by benzoate, a specific D-amino acid oxidase inhibitor, and that no racemization took place in the absence of oxygen supports the concept of a direct oxidation of these L-amino acids by D-amino acid oxidase. The possibility that an oxygen-dependent racemization takes place has not been excluded but does not appear very likely. The product of oxidation of L-proline by D-amino acid oxidase has been shown to be the same as the product of oxidation of D-proline, namely, Δ^1-pyrroline-2-carboxylic acid. Thus in both cases the same pair of hydrogen atoms is removed from the substrate. The oxidation observed could not have been owing to contamination of the enzyme with proline oxidase, since the latter enzyme oxidizes L-proline to Δ^1-pyrroline-5-carboxylic acid (Johnson and Strecker, 1962). The formation of hydrogen peroxide was shown by the decrease in rate of oxygen uptake when catalase was added during the oxidation of L-proline, and by the inhibition of catalase in the presence of 3-amino-1,2,4-triazole (Scannone *et al.*, 1964).

It is of interest that the addition of a hydroxyl group to the 4- position of L-proline, on either side of the ring, abolishes the activity, and that L-alanine and L-serine, which do not possess rings, are not oxidized at a measurable rate by D-amino acid oxidase. It is also noteworthy that, whereas L-3,4-dehydroproline is a substrate of L-amino acid oxidase, L-proline, L-4-hydroxyproline, and L-allo-4-hydroxyproline are not oxidized appreciably. It appears possible, therefore, that a relatively planar ring may play an important part in binding the substrate to the enzyme. The double bond in 3,4-dehydroproline would be expected to enhance the planarity of the ring, while addition of a hydroxyl function would be expected to make the ring less planar. These observations also support the hypothesis that D-amino acid oxidase and L-amino acid oxidase have a similar mechanism of action, since these two enzymes have at least one substrate (L-3,4-dehydroproline) in common.

Of the L-amino acids tested in this study, only L-proline and L-3,4-dehydroproline were found to be oxidized by D-amino acid oxidase at a sufficiently high rate to be measured by the manometric procedure. With the more sensitive catalase inhibition assay (Scannone *et al.*, 1964), evidence suggesting that L-alanine may be a substrate of D-amino acid oxidase was also obtained. It is possible that if more sensitive methods were available the oxidation of other L-amino acids could be demonstrated. Because of the very great difference in the rate of attack of the D and L isomers of amino acids, D-amino acid oxidase nevertheless remains

a useful enzyme for the detection or selective destruction of D-amino acids. Thus, although L-proline is oxidized at a significant rate, it was still possible to detect one part of D-proline in 15,000 parts of L-proline (Scannone *et al.*, 1964).

In recent years a number of other purified enzymes have been found to catalyze reactions at a rate which is several orders of magnitude lower than that of the reactions which they were previously known to catalyze. These include the formation of 2-pyrrolidone-5 carboxylic acid by glutamine synthetase (Krishnaswamy *et al.*, 1962), a number of transamination reactions catalyzed by aspartate β-decarboxylase (Novogrodsky and Meister, 1964a) and by glutamate aspartate transaminase (Novogrodsky and Meister, 1964b), the hydrolysis of acetylglycine ethyl ester by trypsin (Inagami and Murachi, 1963), the oxidation of α-aminovaleric acid and other amino acids by glutamic dehydrogenase (Bässler and Hammar, 1958; Struck and Sizer, 1960), and the hydrolysis of polyadenylic acid by pancreatic ribonuclease (Beers, 1960). These examples, as well as the present findings, indicate the necessity for caution in assigning "absolute specificity" to an enzyme. Although these "minor" activities may be of little physiological importance, they are significant in the understanding of the mechanism of the enzymatic reaction.

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Acetylcholinesterase: Trimethylammonium-Ion Inhibition of Deacetylation

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The noncompetitive inhibition of acetylcholinesterase by trimethylammonium ion is shown to result from binding of an inhibitor molecule to the acetyl enzyme intermediate, which is formed from the enzyme-substrate complex. The evidence for this is independent of any initial assumption that an acetyl enzyme is formed, and comes from studies of the inhibition of hydrolysis of a number of acetylcholine analogs, all acetyl esters, some hydrolyzed as rapidly as acetylcholine and others more slowly. The observed direct proportionality between the experimental noncompetitive inhibition constant and the maximum velocity indicates that the inhibitor adds to a common intermediate in the hydrolysis of these substrates. The only intermediate likely to be formed during the enzymic hydrolysis of all the acetyl esters is an acetyl enzyme. Other evidence shows that trimethylammonium ion can completely block deacetylation of the acetyl enzyme, and that the inhibition is reversible. The inhibitor is bound at the anionic site in the active center, as are other substituted ammonium ions. The experiments also suggest that deacetylation limits the rate of hydrolysis of acetylcholine, acetylthiocholine, and phenyl acetate.

Characteristic substrates of acetylcholinesterase (AChE)[1] contain a methyl-substituted ammonium ion, which probably becomes bound at the anionic site in the active center of the enzyme. Substituted ammonium ions should therefore compete with substrates for the enzyme and should inhibit in a purely competitive manner. Surprisingly, some compounds of this type

[1] Abbreviations used in this work: AChE, acetylcholinesterase; AcCh, acetylcholine; ES, enzyme-substrate complex; EA, acetyl enzyme.