HYDROXY-L-PROLINE AS A SUBSTRATE FOR HOG KIDNEY D-AMINO ACID OXIDASE

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Received January 14, 1974

Summary: In contrast to earlier findings, hydroxy-L-proline is oxidized by hog kidney D-amino acid oxidase, with a V_{max} comparable to that of L-proline but with a somewhat higher K_m. Kinetic constants and pH-dependency data are reported both for hydroxy-L-proline and allohydroxy-D-proline.

Because of their high specificity for the D-antipodes of common amino acids, mammalian D-amino acid oxidases have been widely used both analytically and preparatively (1). Nevertheless, certain L-amino acids are slowly oxidized by the hog kidney enzyme, as was first described for L-proline and L-3,4-dehydroproline by Wellner and Scannone (2), and confirmed for L-proline by Yagi and Nishikimi (3).

Wellner and Scannone (2) reported no demonstrable substrate activity for either hydroxy-L-proline or allohydroxy-L-proline even with a sensitive assay method (detection of pyrrole-2-carboxylate) which sufficed to detect oxidation of L-3,4-dehydroproline. Our interest in this question arose from investigating the source of the small quantities of pyrrole-2-carboxylate in mammalian urine. Our own studies (4) and a subsequent report (5) have indicated that the oxidation of hydroxy-L-proline by kidney L-amino acid oxidase is a likely source of excreted pyrrole-2-carboxylate.

In considering the possible additional role of D-amino acid oxidase in hydroxy-L-proline oxidation, we have observed seemingly unambiguous oxidation of this compound by the commercial hog-kidney enzyme to yield the expected product, Δ^1-pyrroline-4-hydroxy-2-carboxylate (6), detected and measured by its conversion to pyrrole-2-carboxylate. We report here the evidence for this conclusion, together with kinetic constants both for hydroxy-L-proline and allohydroxy-D-proline. The latter compound was first shown to produce pyrrole-2-carboxylate after oxidation by D-amino acid oxidase (6), and has been used for the sensitive detection of this enzyme (7). However, systematic kinetic data with this substrate have not, to our knowledge, been reported previously.

MATERIALS AND METHODS

D-Amino acid oxidase (hog-kidney, crystalline, 15 units per mg) was pur-

chased from Sigma, as was hydroxy-L-proline, allohydroxy-D-proline and L-proline ("hydroxyproline-free"). Beef liver catalase was obtained from Worthington. Hydroxy-L-proline was recrystallized twice from water-ethanol. The D-amino acid oxidase preparation, supplied in 3.2 M $(NH_4)_2SO_4$, was dialyzed against 0.01 M sodium pyrophosphate, pH 8.3, prior to incubations with hydroxy-L-proline (dialysis conditions: 5 mg enzyme per liter of buffer at 4° for 2 - 4 hr). Dialysis was omitted when much smaller amounts of enzyme were used with better substrates; the rate with 0.1 M D-alanine was uninfluenced by dialysis.

Incubation conditions with D-amino acid oxidase were as follows: 37°, air, with shaking, 0.02 - 0.03 M sodium pyrophosphate, pH 8.3 (except where noted), 50 units of catalase, total volume 1.6 ml. Pyrrole-2-carboxylate was measured by color formation with p-dimethylaminobenzaldehyde essentially as described earlier (8). With D-alanine as substrate, pyruvate formation was measured by a modification of the 2,4-dinitrophenylhydrazine assay described earlier (9).

Pretreatment of hydroxy-L-proline with D-amino acid oxidase was carried out as follows: 2 g of hydroxy-L-proline plus 3.7×10^7 dpm of ^3H (G)-allohydroxy-D-proline (the preparation and purification of this are described separately (10)) were incubated in air with 4 ml of 0.1 M sodium pyrophosphate, pH 8.3, 8000 units of catalase and 100 units of D-amino acid oxidase in a 10 ml volume at 37°. After 12 hr incubation with shaking, a further 50 units of the oxidase were added and the incubation continued for an additional 4 hr. The reaction was stopped by heating at 100° for 10 min. Centrifuged samples of the incubation mixture were applied to a Dowex 50 H^+ column (1.1 x 25cm, X-8, 100-200 mesh); the column was washed with water and hydroxyproline was eluted with 1 N NH_4OH. Less than 5% of the added radioactivity was eluted with hydroxyproline; after crystallization of hydroxyproline from the reaction mixture by ethanol addition, only 0.5% of the added radioactivity was recovered with hydroxyproline.

RESULTS AND DISCUSSION

Incubation of twice-recrystallized hydroxy-L-proline with D-amino acid oxidase led to the continued production of the pyrroline oxidation product, measured as pyrrole-2-carboxylate in acidified samples of the reaction mixture. The latter product was identified by thin-layer chromatography in several solvents (10) and by its U.V. spectrum (6). The rate of product-formation was linear with time over a 4-hr period, the initial rate being about 30 nmoles per hr in the standard reaction mixture (see METHODS) containing 0.25 M hydroxy-L-proline and 7.9 units of D-amino acid oxidase. Over a 5-hr period the total rate in such a reaction mixture was not linear with enzyme, deviating from strict proportionality by a drop of about 30% over a range from 3.5 to 40 units;

this deviation from linearity is attributable to the inhibitory effect of increasing concentrations of the pyrrole product (11).

Alternatives to the conclusion that this slow reaction represented oxidation of hydroxy-L-proline by D-amino acid oxidase were the possible contamination of the substrate by a D-isomer of hydroxyproline (6), or contamination of the D-amino acid oxidase by the L-specific oxidase of kidney (12); the latter possibility is not likely on the basis of failure to detect significant L-specific activity in hog kidney (12).

Significant contamination by a D-hydroxyproline isomer (or any active substrate capable of producing a pyrrole-reactive product on oxidation) was excluded by the finding that the reaction with hydroxy-L-proline proceeded much farther and much more slowly than that attributable to a trace of added allohydroxy-D-proline (Fig. 1). Furthermore, the time-course of the reaction with

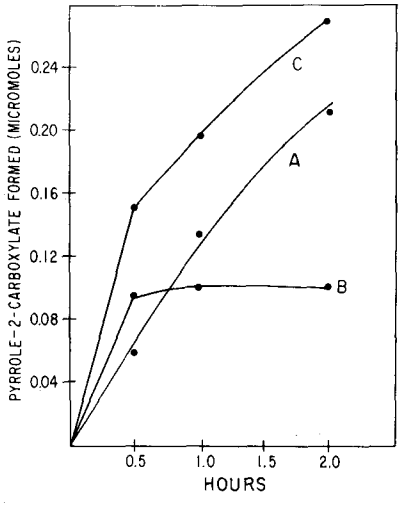

Fig. 1 Time-course of oxidation of hydroxy-L-proline and allohydroxy-D-proline by D-amino acid oxidase. Reaction conditions as in text, with 9 units of oxidase. A, 1000 μmoles of hydroxy-L-proline; B, 0.1 μmole of allohydroxy-D-proline; C, 1000 μmoles of hydroxy-L-proline plus 0.1 μmole of allohydroxy-D-proline.

hydroxy-L-proline was unchanged by pretreatment of the latter with D-amino acid oxidase and its recrystallization, although these procedures were shown to remove essentially all of an added trace of labeled allohydroxy-D-proline.

That the oxidation of hydroxy-L-proline was not attributable to a contaminating L-specific oxidase was inferred from the comparative inhibitory effect of D- and L-alanine on the reaction. D- or L-alanine (0.0625 M) was incubated

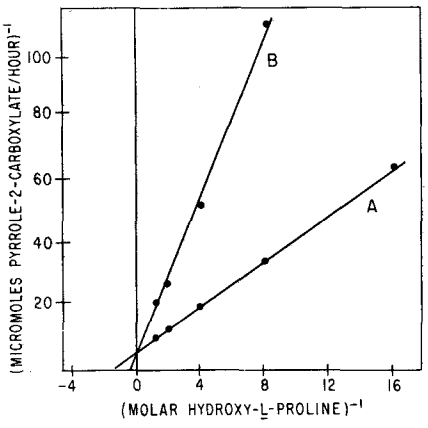

Fig. 2 Evaluation of kinetic constants for hydroxy-L-proline as substrate and proline as inhibitor. Reaction mixtures contained 0.03 M sodium pyrophosphate, pH 9.0, 7.3 units of D-amino acid oxidase and varying concentrations of hydroxy-L-proline; incubation period was 5 hr; other incubation conditions as described in the text. A, No L-proline; B, 0.25 M L-proline.

with 0.25 M hydroxy-L-proline and 10 units of the oxidase for 5 hr. D-Alanine completely inhibited the formation of pyrrole-2-carboxylate while L-alanine inhibited only 19%. Furthermore, a 5-hr incubation of D-amino acid oxidase (1 mg) with L-leucine (6.25 mM) at pH 7.9 produced no detectable keto-acid product. Under the same conditions, incubation with hydroxy-L-proline (180 mM) produced 0.15 μmole of pyrrole-2-carboxylate. From the ratio of rates of oxidation of L-leucine and hydroxy-L-proline under these conditions by the partly-purified rat-kidney L-amino acid oxidase (10), L-leucine should have yielded 0.75 μmole of α-ketoisocaproate if the oxidation of hydroxy-L-proline had been catalyzed by the L-specific enzyme. These data indicated that less than 6% of the oxidation of hydroxy-L-proline observed with D-amino acid oxidase could be due to a contaminating L-specific oxidase, assuming it resembled the rat-kidney enzyme in substrate specificity.

No reaction with hydroxy-L-proline and boiled oxidase was observed, nor did incubation in the dark with active enzyme alter the rate of oxidation. Thus, there was no support for the possibility of a significant non-enzymatic or photo-sensitized oxidation by FAD under the usual conditions of the enzyme-catalyzed reaction. Free FAD (2.8×10^{-5} M) incubated with 0.625 M hydroxy-L-proline for 5 hours, however, did lead to detectable pyrrole-2-carboxylate formation, estimated at 0.01% of the added hydroxyproline. Comparable incubations with FMN produced the pyrrole at a 5-fold faster rate (10).

Table 1 Kinetic constants for D-alanine and D- and L-isomers of proline and hydroxyproline. Conditions were pH 8.3 and 37° except as noted (values from the data of Fig. 2 for hydroxy-L-proline were based on pH 9.0)

Substrate	K_m[a]	V_{max}[b]
	mM	μmoles/mg enzyme/min
D-Alanine	1.8	19.6
D-Proline	1.7	60.0
Allohydroxy-D-proline[c]	1.0	8.5
L-Proline	150	0.0063
Hydroxy-L-proline	720	0.0033

[a] Values for D-alanine and D-proline from reference (13), based on 25° and pH 8.5; value for L-proline from (2); value for hydroxy-L-proline from Fig 2; value for allohydroxy-D-proline from data (not shown) like those of Fig 2.

[b] Value for D-alanine estimated by rate with 0.1 M substrate; value for D-proline based on V_{max} ratio for D-alanine/D-proline (13) at 25°; value for L-proline calculated from (2), correcting to the units/mg of our preparation of D-amino acid oxidase; values for allohydroxy-D-proline and hydroxy-L-proline, as for footnote a.

[c] Incubation in presence of 1.4×10^{-5} M FAD; rate of oxidation of allohydroxy-D-proline only was decreased 3-fold without FAD. Oxidation rates with D-alanine (0.1 M) or with hydroxy-L-proline were uninfluenced by FAD addition.

The competitive inhibition by L-proline of the oxidation of hydroxy-L-proline is shown in Fig. 2. The K_i for L-proline (0.1 M) is in general agreement with its K_m as substrate, estimated as 0.15 M (2) or 0.06 - 0.08 M (3), or its K_i as an inhibitor of D-alanine oxidation, 0.1 M (3).

Table 1 summarizes kinetic constants available both from other sources and from present data for the D- and L-isomers of proline and hydroxyproline, with those for D-alanine included for comparison. Fig. 3 shows the difference in pH dependency for oxidation rates with the L- and D-epimers of hydroxyproline. We are not aware of such data comparing D- and L-proline, although Dixon and Kleppe's detailed study (14) indicates rather different pH-dependency curves for different D-amino acid substrates.

The data presented here show that hydroxy-L-proline is a substrate for

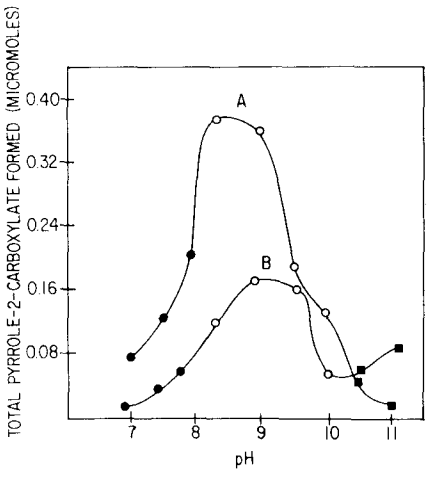

Fig. 3 pH-Dependency of the oxidation of hydroxyproline epimers. Reaction mixtures with allohydroxy-D-proline (A) contained 0.5 μmole of substrate, 0.31 unit of D-amino acid oxidase and were incubated 1 hr. Reaction mixtures with hydroxy-L-proline contained 200 μmoles of substrate, 8.3 units of oxidase and were incubated 4.5 hr; other incubation conditions as in text. Buffers were 0.062 M sodium phosphate (●), 0.062 M sodium pyrophosphate (○), 0.062 M triethylammonium chloride (■). All pH values were determined after incubation.

D-amino acid oxidase, although a somewhat poorer substrate than is L-proline, both with respect to K_m and V_{max}. Relative to their corresponding D-forms L-proline has a lower V_{max} (and lower K_m) than does hydroxy-L-proline. Data presented in detail elsewhere (10) indicate that, in the rat, D-amino acid oxidase probably contributes little, compared with L-amino acid oxidase, to the production of urinary pyrrole-2-carboxylate from hydroxy-L-proline. In the human and guinea-pig, however, while low levels of pyrrole-2-carboxylate are measurable in urine (10), there is little or no detectable kidney L-amino acid oxidase (10,12). In these and possibly other species, D-amino acid oxidase should be considered as a possible source for the formation of pyrrole-2-carboxylate from hydroxy-L-proline.

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