Stereospecificity of Enzymatic Formation and Oxidation of Glycine*

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ABSTRACT: Asymmetrically labeled glycine-2-*t* has been prepared by three methods: (1) by enzymatic transamination between glyoxylate and L-aspartate in tritiated water, (2) from L-serine, in a reaction catalyzed by serine transhydroxymethylase in tritiated water, and (3) by an incorporation of tritium from water into glycine catalyzed by serine transhydroxymethylase. When all three glycine samples were converted into glyoxylate in the presence of D-amino acid oxidase, almost all of the tritium in the glycine prepared by methods 2 and 3 and very little tritium in the glycine prepared by method 1 was released into water. When racemic glycine-2-*t* was oxidized by D-amino acid oxidase, half of the tritium was released into water. It is concluded that glycine-2-*t* prepared by method 1 has the *R* configuration, and that methods 2 and 3 yield (*S*)-glycine-2-*t*.

The D-amino acid oxidase reaction affords a method for elucidating the stereospecificity of glycine formation in other enzymatic reactions.

Both D-amino acid oxidase (Neims and Hellerman, 1962) and L-amino acid oxidase (Lichtenberg and Wellner, 1968) have been shown to catalyze the oxidation of glycine, although in both cases the reaction is very slow. In view of the stereospecificity of these enzymes toward other amino acids, it may be expected that in each case the reaction would involve the removal of a specific α-hydrogen atom of glycine. This report presents evidence that D-amino acid oxidase selectively removes the α-hydrogen atom of glycine which corresponds in configuration to that of D-alanine. It is also shown that the hydrogen atom removed by D-amino acid oxidase is the same as the one introduced into glycine by serine transhydroxymethylase and is not the one introduced by an L-specific transaminase. The D-amino acid oxidase reaction thus provides a method for elucidating the stereospecificity of glycine formation in various enzymatic reactions.

Materials and Methods

D-Amino acid oxidase was prepared from hog kidney by the method of Massey *et al.* (1961) and, for some of the experiments, was made benzoate free by repeated treatments with D-alanine followed by ammonium sulfate precipitation. The transaminase preparation was obtained from rat liver according to Meister *et al.* (1952). Serine transhydroxymethylase was purified from rabbit liver by the procedure of Schirch and Mason (1963). Tetrahydrofolic acid was obtained from the Sigma Chemical Co. It was dissolved just before use in 0.001 M dithiothreitol which was found to stabilize it against oxidation. Its concentration was determined from the absorbancy at 298 mμ (Blakley, 1960). Tritium-labeled water (100 mCi/ml) and (*RS*)-glycine-2-*t* were obtained from New England Nuclear Corp. Amino acid solutions were desalted and freed of exchangeable tritium as follows: the solution was applied to a small (9 × 20 mm) column of Dowex 50 (H^+), the column was washed with 20 ml of water, the amino acids were then eluted with 5 ml of 2 N NH_4OH, and the eluate was evaporated to dryness under reduced pressure at 37°. Glycine was purified by high-voltage paper electrophoresis (2500 V, 2 hr on 90-cm lengths of Whatman No. 3MM paper, 7% formic acid, eluted with water) or by ion-exchange chromatography using a Beckman Model 120C amino acid analyzer. Glycine concentration was measured by the ninhydrin procedure (Moore and Stein, 1954). Radioactivity was measured by scintillation counting in Bray's (1960) solution. Tritium released by oxidation of glycine was estimated by freeze drying the reaction mixture and collecting the ice quantitatively into specially designed traps (Stone, 1962; Stone and Meister, 1962). The radioactivity of the water was then determined. The amount of glycine oxidized was obtained by measuring the oxygen uptake manometrically (assuming 2 moles of glycine oxidized per mole of O_2 consumed in the presence of an excess of catalase) and by measuring the glycine remaining at the end of the reaction.

Glycine Labeled by Enzymatic Transamination. A reaction mixture containing L-aspartate (10 μmoles), sodium glyoxylate (20 μmoles), enzyme (40 mg), barbital buffer (5 μmoles, pH 8.0), and tritiated water (10 mCi) in a final volume of 1 ml was incubated at 37° for 1 hr. The solution was deproteinized by the addition of 0.2 ml of 50% trichloroacetic acid followed by centrifugation. The amino acid fraction was isolated from the supernatant solution, freed of exchangeable tritium, and the glycine was then purified by high-voltage paper electrophoresis.

Glycine Labeled by Nonenzymatic Transamination. Transamination between glyoxylate and ornithine catalyzed by aluminum ions was carried out according to Cabello *et al.* (1963). A mixture containing sodium glyoxylate (20 μmoles), DL-ornithine (10 μmoles), aluminum potassium

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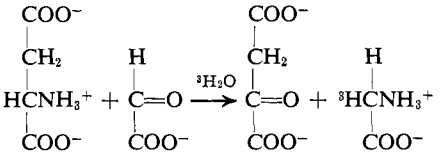
sulfate (1 μ mole), and tritiated water (10 mCi) in a final volume of 1 ml was sealed in a Pyrex tube and heated at 97° for 2 hr. The glycine formed was isolated as above.

Labeled Glycine Formed in the Presence of Serine Transhydroxymethylase. The reaction mixture contained L-serine (2 μmoles), pyridoxal phosphate (2 μmoles), tetrahydrofolic acid (1.8 μmoles), dithiothreitol (5 μmoles), potassium phosphate buffer (20 μmoles, final pH 7.3), ethylenediaminetetraacetate (1 μmole), tritiated water (10 mCi), and enzyme in a final volume of 1.2 ml. Incubation was allowed to proceed for 1 hr at 25°. The amount of enzyme was sufficient to convert 47% of the serine into glycine under these conditions. No glycine was found in a control experiment lacking enzyme. The solution was deproteinized by the addition of 4 ml of 95% ethanol and 0.2 ml of 2 N acetic acid. After centrifugation, the amino acid fraction was freed of exchangeable tritium and the glycine was isolated by ion-exchange chromatography. Labeled glycine was also prepared by a proton-exchange reaction catalyzed by serine transhydroxymethylase (Schirch and Jenkins, 1964). In this case an identical reaction mixture was used, except that serine was replaced by glycine. In all cases, unlabeled carrier glycine was added before the reaction with D-amino acid oxidase.

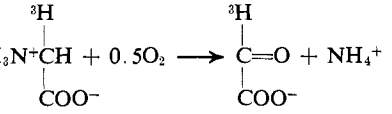
Results

In order to test the specificity of D-amino acid oxidase toward the α-hydrogen atoms of glycine it was necessary to prepare glycine asymmetrically labeled with deuterium or tritium. One way in which this could be accomplished was by enzymatic transamination in tritiated water. If an L-specific transaminase is used, the expected configuration of the resulting labeled glycine is *R* (see eq 1).[1] Upon incuba-



(1)

tion of such labeled glycine with D-amino acid oxidase in the presence of catalase, it may be expected that the tritium would remain on the glyoxylate (eq 2). On the other hand,

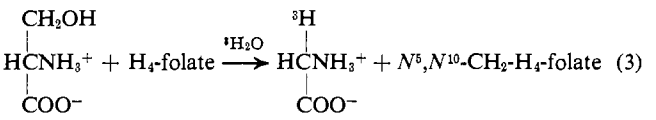


(2)

labeled glycine resulting from nonenzymatic transamination in a system containing no asymmetric elements must be racemic and tritium equivalent to 50% of the glycine oxidized would be expected to be released into water by D-amino acid oxidase. The results of such an experiment are shown in Table I.

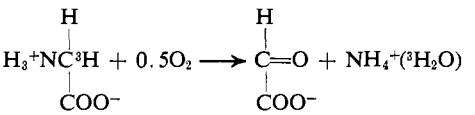
The fact that only 41% of the tritium from the oxidized glycine obtained by nonenzymatic transamination was released by D-amino acid oxidase indicates isotope discrimination in the oxidation reaction. However, only about 9% of the tritium was released from the oxidized glycine obtained by enzymatic transamination. This suggests that this glycine was predominantly of the *R* configuration and that D-amino acid oxidase released mainly, if not exclusively, the α-hydrogen atom opposite the one introduced by the transaminase. The apparent lack of complete specificity is attributable, at least in part, to the occurrence of some nonenzymatic transamination in the course of preparation of the glycine. Nonenzymatic transamination between glyoxylate and amino acids is known to proceed readily under mild conditions (Nakada and Weinhouse, 1953; Fleming and Crosbie, 1960; Cabello *et al.*, 1963).

Asymmetrically labeled glycine was also prepared from L-serine with serine transhydroxymethylase, a reaction in which the hydroxymethyl group of serine is replaced by a proton from the medium. If this reaction occurs with retention of configuration, the glycine formed in tritiated water will have the *S* configuration (eq 3). When this glycine is oxidized



(3)

by D-amino acid oxidase, the tritium may be expected to be released into water as shown by eq 4.



(4)

Since it was reported by Schirch and Jenkins (1964) that serine transhydroxymethylase catalyzes the incorporation of tritium from water into glycine and that this reaction was stimulated by tetrahydrofolate, labeled glycine was also prepared by such an exchange reaction. The result of treating these glycine preparations with D-amino acid oxidase is shown in Table II. In order to minimize isotope rate effects, the reaction was carried as close to completion as possible. It may be seen that close to the theoretical amount of tritium (49%) was released from racemic glycine-2-*t* in a control experiment. Also, both samples of glycine labeled in the presence of serine transhydroxymethylase released more than 90% of the tritium incorporated upon treatment with D-amino acid oxidase. This finding demonstrates a high degree of stereospecificity in the reactions catalyzed by both serine transhydroxymethylase and D-amino acid oxidase.

Discussion

The absolute configuration of a compound may not, in general, be deduced unequivocally from its susceptibility to the action of any one enzyme. Thus, it could not be excluded, *a priori*, that the specificity of D-amino acid oxidase toward glycine might be different from that toward other amino acids, particularly in view of the fact that the reaction is very slow and that D-amino acid oxidase has been shown to act on L-proline and L-3,4-dehydroproline (Wellner and Scannone, 1964). In addition, isotope discrimination effects may complicate the interpretation of the data. However, since the results reported above are those expected from the

[1] The Fischer convention is used to represent configurations.

TABLE I: Oxidation by D-Amino Acid Oxidase of Glycine from Transamination Reaction.[a]

Source of Gly	Total Gly (μmoles)	Total cpm	Gly Oxidized (μmoles)	cpm Released in H_2O	% cpm in H_2O: % Gly Oxidized
Enzymatic transamination	57.6	66,000	36.4 (63.2%)	3,920 (5.9%)	0.09
Nonenzymatic transamination	57.2	12,200	34.8 (60.8%)	3,070 (25.1%)	0.41

[a] The glycine was oxidized for 7.25 hr at 37° in a reaction mixture containing D-amino acid oxidase (1 mg), FAD (12.5 μg), catalase (1000 units), and sodium pyrophosphate buffer (140 μmoles, pH 8.3) in a final volume of 2.5 ml.

TABLE II: Oxidation by D-Amino Acid Oxidase of Glycine from Serine Transhydroxymethylase Reaction.[a]

Source of Gly	Total Gly (μmoles)	Total cpm	Gly Oxidized (μmoles)	cpm Released in H_2O	% cpm in H_2O: % Gly Oxidized
From serine (enzymatic)	51	8,700	48.3 (94.5%)	7,580 (87%)	0.92
From glycine (enzymatic exchange)	51	13,130	48.2 (94.5%)	11,960 (91%)	0.96
Synthetic (nonenzymatic)	52	15,580	50.3 (96.7%)	7,370 (47%)	0.49

[a] The incubation mixture contained glycine, FAD (5 μg), catalase (500 units), sodium pyrophosphate buffer (200 μmoles, pH 8.3), and benzoate-free D-amino acid oxidase (20 mg) in a final volume of 2.6 ml. The incubation was carried out at 37° for a total of 22 hr, and the enzyme was added in portions at 3–12-hr intervals.

known stereospecificity of three different enzymes, it may be concluded that the glycine-2-*t* formed from glyoxylate in tritiated water in the presence of liver transaminase has the *R* configuration and that the glycine-2-*t* formed in tritiated water in the presence of serine transhydroxymethylase has the *S* configuration. Although the stereospecificity of D-amino acid oxidase toward glycine may not be absolute, the data show that at least 90% of the hydrogen liberated in the course of oxidation corresponds in configuration to that of D-amino acids. The enzyme therefore provides a tool for elucidating the stereospecificity of other reactions in which glycine is formed. Thus, Palekar *et al.* (1970) have established the stereospecificity of enzymatic decarboxylation of aminomalonic acid to glycine using this procedure.

Although L-amino acid oxidase also acts on glycine, preliminary experiments have shown that the reaction is slower and the isotope discrimination considerably more pronounced than in the case of D-amino acid oxidase. The preparation of asymmetrically labeled glycine has also been described in preliminary reports by Akhtar and Jordan (1968, 1969); their results are in accord with the findings made in this laboratory. Wilson and Snell (1962) and Schirch and Mason (1963) have presented convincing evidence that the serine transhydroxymethylase reaction proceeds with retention of configuration about the α-carbon atom of serine or α-methylserine. The present results confirm their conclusion.[2]

[2] The same conclusion is reported (without data) in a recent abstract by Besmer and Arigoni (1968).

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Inhibition of Aspartate β-Decarboxylase by Aminomalonate. Stereospecific Decarboxylation of Aminomalonate to Glycine*

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ABSTRACT: L-Aspartate β-decarboxylase of *Alcaligenes faecalis* catalyzes the stereospecific decarboxylation of aminomalonate to glycine. When the reaction is carried out in H_2O-*t*, (*S*)-glycine-*t* is formed; on the other hand, (*R*)-glycine-*t* is produced by transamination in H_2O-*t* between the pyridoxamine 5′-phosphate form of the enzyme and glyoxylate. The decarboxylation of aminomalonate is accompanied by a slower reaction in which the enzyme becomes irreversibly inactivated, and in which aminomalonate becomes tightly bound to the enzyme.

L-Aspartate β-decarboxylase of *Alcaligenes faecalis*, is a multifunctional enzyme, which can catalyze, in addition to the β decarboxylation of L-aspartate, desulfination of L-cysteinesulfinate (Soda *et al.*, 1964); transamination between several pairs of L-amino acids and α-keto acids (Novogrodsky and Meister, 1964); and a β-elimination reaction with β-chloro-L-alanine leading to the formation of pyruvate, Cl^-, and NH_3 (Tate *et al.*, 1969). We now report that this enzyme can catalyze the stereospecific decarboxylation of aminomalonate to glycine. This reaction is analogous to the reactions catalyzed by the amino acid α-decarboxylases; however, L-aspartate β-decarboxylase acts selectively on the carboxyl group of aminomalonate which corresponds to the β-carboxyl group of L-aspartate.

The decarboxylation of aminomalonate is accompanied by a slower reaction in which the enzyme becomes irreversibly inactivated. Such inactivation, which was first noted by Novogrodsky and Meister (1964), is accompanied by the firm attachment of aminomalonate to the enzyme.

Experimental Section

Materials. L-Aspartate β-decarboxylase was isolated from *A. faecalis*, strain N (ATCC 25094), as described by Tate and Meister (1968). Aminomalonic acid was synthesized from unlabeled malonic acid, malonic acid-1-^{14}C, and malonic acid-2-^{14}C *via* the corresponding monobromomalonic acid derivative according to the procedure of Matthew and Neuberger (1963). The preparations of aminomalonic acid used in these studies did not contain detectable amounts of glycine as determined by thin-layer chromatography carried out as described below. The malonic acids-^{14}C and $NaBH_4$-*t* were obtained from the New England Nuclear Corp.

We are indebted to Dr. Daniel Wellner for a generous supply of hog kidney D-amino acid oxidase.

Methods. Thin-layer chromatography was carried out on silica gel G using a solvent consisting of chloroform–methanol–17% ammonium hydroxide (2:2:1). After chromatography the plates were sprayed with ninhydrin. Under these conditions the R_F values for glycine and aminomalonic acid were, respectively, 0.72 and 0.44. Glycine was identified in reaction mixtures containing aminomalonic acid and enzyme as follows. A reaction mixture containing 5 mM aminomalonic acid, 0.2 M sodium acetate buffer (pH 5.5), and enzyme (1 mg/ml) was incubated at 37° for 15 min. The mixture was treated with five volumes of ethanol saturated with ammonia gas; after centrifugation of the precipitated protein, the supernatant solution was lyophilized. The dried residue was dissolved in the chromatography solvent and subjected to thin-layer chromatography. Glycine was the only amino acid product found after spraying with ninhydrin. A similar result was obtained when eluates obtained from the Dowex 1-acetate columns (see below) were chromatographed.

The formation of radioactive glycine was determined as follows. Aliquots of the reaction mixture were applied to small columns of Dowex 1-acetate prepared in pasteur pipets. Glycine-^{14}C was eluted from the column with 1 ml of water; an aliquot of the eluate was mixed with 10 ml of liquid scintillation medium and counted in a scintillation counter. Total glycine was determined by the ninhydrin method (Rosen, 1957).

L-Cysteinesulfinate desulfinase activity was determined by measuring formation of sulfite as previously described (Soda *et al.*, 1964; Tate and Meister, 1968). Protein was determined as described by Lowry *et al.* (1951).

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