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

Anil G. Palekar, Suresh S. Tate, and Alton Meister

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 pyridox-

amine 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.

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). Aminomalononic 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 aminomalononic 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 aminomalononic acid were, respectively, 0.72 and 0.44. Glycine was identified in reaction mixtures containing aminomalononic acid and enzyme as follows. A reaction mixture containing 5 mM aminomalononic 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).

* From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. Received January 29, 1970. Supported in part by grants from the National Institutes of Health, Public Health Service, and the National Science Foundation.

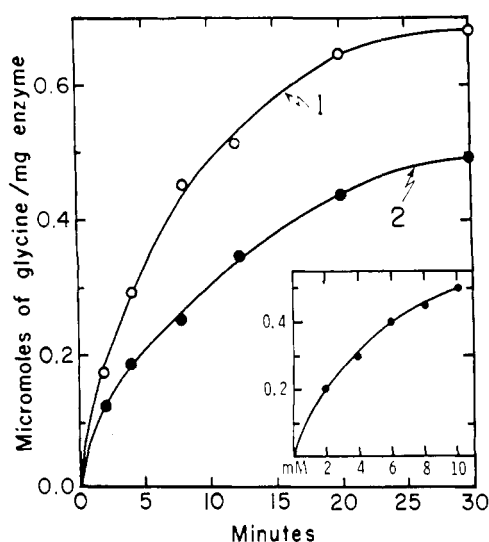


FIGURE 1: Decarboxylation of aminomalonate-2- ^{14}C by L-aspartate β -decarboxylase. The reaction mixtures contained 0.2 M sodium acetate buffer (pH 5.5), 5 mM aminomalonate- ^{14}C (105,000 cpm/ μmole), and enzyme (1 mg/ml); 37°. Curve 1: 1 mM sodium α -ketoglutarate added; curve 2: no α -ketoglutarate added. The inset describes experiments carried out in the presence of 1 mM α -ketoglutarate and the indicated concentrations of aminomalonate; incubated for 5 min at 37°.

Results

Decarboxylation of Aminomalonate by L-Aspartate β -Decarboxylase. When aspartate β -decarboxylase was incubated with aminomalonate-2- ^{14}C at pH 5.5 there was significant formation of glycine- ^{14}C (Table I and Figure 1). As indicated in the experiment described in Table I, decarboxylation in the absence of enzyme was less than 1% of that found in its presence. Under these conditions but with heat-inactivated enzyme there was approximately 8% as much decarboxylation as with active enzyme; a similar value was obtained in an experiment carried out with an equivalent amount of pyridoxal 5'-phosphate in the absence of enzyme. The initial rate of glycine formation was estimated to be about 0.2% of that

TABLE I: Decarboxylation of Aminomalononic Acid.^a

Expt	Reaction Mixtures	Gly Formed (m $\mu\text{moles/ml}$)
1	Complete	347
2	Enzyme omitted	2.9
3	With heat-inactivated enzyme ^b	28.2
4	With pyridoxal 5'-phosphate ^c in place of the enzyme	27.4

^a The reaction mixtures contained 0.2 M sodium acetate buffer (pH 5.5), enzyme (1 mg/ml), 5 mM aminomalonate-2- ^{14}C , and 1 mM sodium α -ketoglutarate. They were incubated at 37° for 5 min. Glycine-2- ^{14}C was determined as described under Methods. ^b The enzyme was placed at 100° for 1 min prior to the experiment. ^c 17 μM .

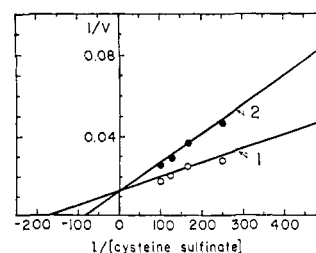


FIGURE 2: Effect of aminomalononic acid on the L-cysteinesulfinate desulfinate activity of L-aspartate β -decarboxylase. The concentration of L-cysteinesulfinate was varied from 2 to 10 mM in the absence (curve 1) and in the presence (curve 2) of 10 mM aminomalononic acid; the reaction mixtures also contained 0.2 M sodium acetate buffer (pH 5.5), 1 mM α -ketoglutarate, and 10 μg of enzyme; final volume, 1 ml; 37°.

of L-aspartate decarboxylation. Although the addition of α -ketoglutarate increased the rate of decarboxylation, the reaction was not linear with time (in the presence or absence of added α -keto acid) and it tended to level off when only a small proportion of the aminomalonate present was decarboxylated. The amount of glycine formed in 5 min under these conditions increased with increasing concentrations of aminomalonate; it was estimated that a concentration of about 3 mM is required for the half-maximal rate of glycine

TABLE II: Studies on the Stability of Aminomalonate- ^{14}C -Enzyme.

Expt	Procedure	^{14}C Bound (moles)/60,000 g of Enzyme
1 ^a	Dialysis vs. 0.05 M acetate buffer	0.92
2	Solution from expt 1 dialyzed vs. 1 M acetate buffer	0.87
3	Solution from expt 1 + 10 mM mercaptoethanol (15 min); dialyzed vs. 0.05 M acetate buffer	0.98
4	Solution from expt 1 + $(\text{NH}_4)_2\text{SO}_4$ (60% of saturation); protein dissolved in 0.05 M acetate buffer	0.93
5	Solution from expt 4 + trichloroacetic acid (5%); protein dissolved in ethanolic KOH	0.17
6	Solution from expt 4 adjusted to pH 8 + five volumes of ethanol; 0°, protein dissolved in ethanolic KOH	0.19

^a The enzyme (2.8 mg/ml) was incubated for 30 min at 37° in a solution containing 10 mM aminomalonate-2- ^{14}C , 0.2 M sodium acetate buffer (pH 5.5), and 1 mM α -ketoglutarate. The solution was then dialyzed for 18 hr at 5° against 0.05 M sodium acetate buffer (pH 5.5) (expt 1). All dialyses were carried out against 1000 volumes for 18 hr at 5°.

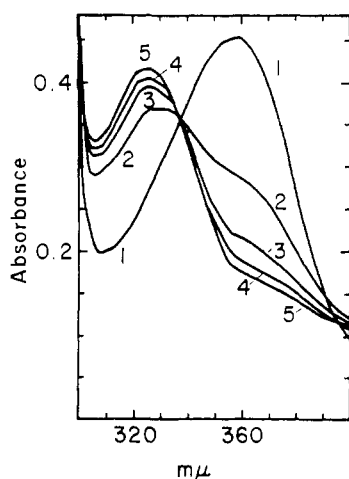


FIGURE 3: Effect of aminomalonic acid on the spectrum of L-aspartate β -decarboxylase. Curve 1: holoenzyme (2.8 mg/ml), 0.2 M sodium acetate buffer (pH 5.5), and 1 mM α -ketoglutarate; 37°. Curves 1, 2, 3, 4, and 5 were obtained at 0, 1, 4, 7, and 10 min, respectively, after addition of aminomalonic acid (final concentration 10 mM).

formation under these conditions (Figure 1, inset). The decarboxylation reaction was studied over the pH range 4.5–8 (pH 4.5–5.5, 0.2 M sodium acetate buffers; pH 6–8, 0.2 M imidazole-HCl buffers) in the presence of 1 mM α -ketoglutarate. The optimal range was 5.0–6.0; the reaction proceeded about 60% as rapidly at pH 4.5 and 6.5.

Inhibition of Aspartate β -Decarboxylase by Aminomalonic Acid. The tendency of the rate of aminomalonic acid decarboxylation to decrease with time suggested that this amino acid inhibits the enzyme. Additional evidence of such inhibition was obtained in experiments in which the effect of amino-

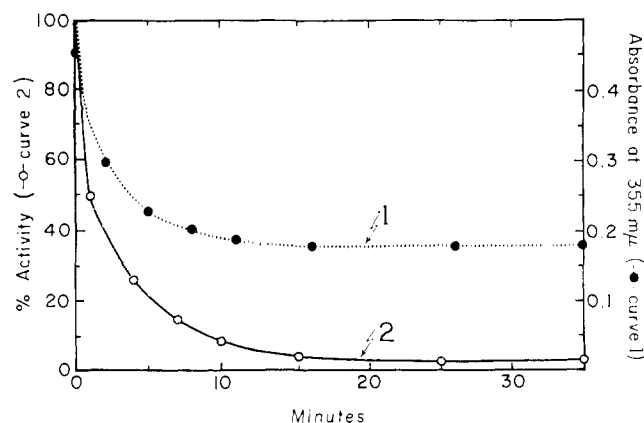


FIGURE 4: Inactivation of L-aspartate β -decarboxylase by aminomalonic acid. The reaction mixture contained 1 mM α -ketoglutarate, 10 mM aminomalonic acid, 0.2 M sodium acetate buffer (pH 5.5), and holoenzyme (2.8 mg/ml); 37°. The decrease in the absorbance at 355 m μ was followed with time (curve 1). Samples (2 μ l) were withdrawn at the indicated time intervals and added to 1 ml of a reaction mixture containing 1 mM α -ketoglutarate, 10 mM L-cysteine-sulfinate, and 0.2 M sodium acetate buffer (pH 5.5); these solutions were incubated for 1 min at 37°. The reaction was stopped by adding 0.2 ml of 2 N acetic acid, after which the sulfite formed was determined.

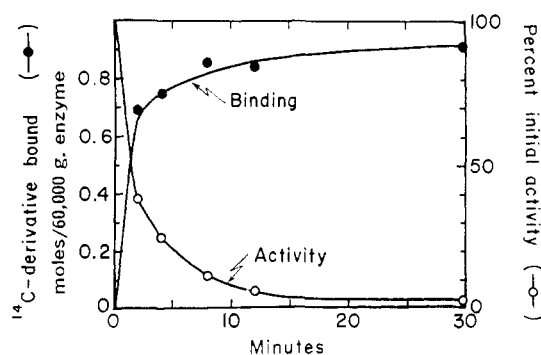


FIGURE 5: Binding of aminomalonic acid to the enzyme. The enzyme (2.8 mg/ml) was incubated in a solution containing 10 mM aminomalonic acid-2- 14 C, and 0.2 M sodium acetate buffer (pH 5.5) at 37°. At the time intervals indicated, aliquots of the solution were applied to a column (1 \times 30 cm) of Sephadex G-25. The enzyme was eluted with 0.05 M sodium acetate buffer (pH 5.5). The fractions containing the enzyme were combined and dialyzed for 18 hr against 1000 volumes of 0.05 M sodium acetate buffer. Following dialysis, determinations of protein, cysteinesulfinate desulfinate activity, and of radioactivity were performed.

malonic acid was determined on the rate of desulfination of cysteinesulfonic acid (Figure 2). The data indicate that aminomalonic acid acts as a competitive inhibitor; the K_i value calculated from these data is 7×10^{-3} .

A marked spectral change was observed when the enzyme was incubated with aminomalonic acid; thus, the absorbance maximum of the holoenzyme at about 355 m μ shifts to about 325 m μ (Figure 3). It is notable that this change in absorbance was not affected by the presence of added α -ketoglutarate. As indicated by the data given in Figure 4 the decrease in absorbance at 355 m μ was closely correlated with loss of cysteinesulfinate desulfinate activity. Again, there was no significant difference in the results when 1 mM α -ketoglutarate was present.

TABLE III: Treatment of the Aminomalonic Acid-Enzyme, Holoenzyme, and Apoenzyme with Sodium Borohydride- t .^a

Expt	Enzyme Form	Tritium cpm/ mg of Protein
1	Aminomalonic acid-enzyme	16,700
2	Holoenzyme	15,300
3	Apoenzyme	5,030

^a The reaction mixtures contained 0.2 M sodium acetate buffer (pH 5.5), 1 mM α -ketoglutarate, 10 mM aminomalonic acid, and enzyme (2.7 mg/ml). After incubation at 37° for 1 hr, the enzyme was dialyzed for 18 hr at 5° against 0.05 M sodium acetate buffer (pH 5.5). Separate experiments were carried out in which aminomalonic acid was omitted (expt 2) and in which the apoenzyme was substituted for the holoenzyme (expt 3). After dialysis the solutions were treated with 2 mg of sodium borohydride at 0°. After 1 hr the solutions were dialyzed against 0.05 M sodium acetate buffer (pH 5.5) for 48 hr. The dialyzed solutions were analyzed for radioactivity and protein as described under Methods.

TABLE IV: Incorporation of Tritium from $\text{H}_2\text{O}-t$ into Glycine Formed by Decarboxylation of Aminomalonate and by Transamination of Glyoxylate.

Expt	Procedure ^a	Total Gly Formed (μmoles)	Incorp of Tritium into Gly		Gly Taken for DOX Reaction ^b (μmoles)	Glyoxylate Formed (μmoles)	H ₂ O- <i>t</i> Formed	
			cpm/ μmole	$\mu\text{atoms of Tritium}/\mu\text{mole}$			cpm	% Tritium Released ^c
1	Nonenzymatic decarboxylation of aminomalonate	5.0	93,700	0.70	2.0	1.6	79,500	53
2	Enzymatic decarboxylation of aminomalonate	1.2	19,500	0.15	0.48	0.33	6,480	101
3	Enzymatic transamination of glyoxylate	0.06 ^d	49,200		1.4	1.3	1,510	4.9

^a Expt 1: Aminomalonate (10 μmoles) was dissolved in 0.2 ml of 0.25 M acetic acid containing 12.5 mCi of $\text{H}_2\text{O}-t$. The solution was placed at 60° for 1 hr and then adjusted to pH 5.0 by addition of 1 N NaOH. It was then applied to a small column of Dowex 1-acetate prepared in a pasteur pipet. Glycine was eluted with 5 ml of water and the eluate was adjusted to pH 2.0 by adding glacial acetic acid. It was then applied to a column of Dowex 50 (H^+) which was washed with 10 ml of water; the glycine was then eluted with 5 ml of 2 N NH_4OH . The eluate was lyophilized and the dried residue was dissolved in 1 ml of 1 N acetic acid. This solution was lyophilized and the dried residue was dissolved in 0.5 ml of water; aliquots of this solution were analyzed for glycine (Rosen, 1957). Glycine was further identified in this experiment (and in expts 2 and 3) by thin-layer chromatography (see Methods). Expt 2: The reaction mixture contained enzyme (2.78 mg), α -ketoglutarate (1 μmole), aminomalonate (5 μmoles), sodium acetate buffer (pH 5.5; 200 μmoles), and 62.5 mCi of $\text{H}_2\text{O}-t/\text{ml}$. After incubation at 37° for 30 min, the solution was applied to a column of Dowex 1-acetate and processed as described for expt 1. Expt 3: The reaction mixture contained apoenzyme (3.6 mg), pyridoxamine 5'-phosphate (0.1 μmole), glyoxylate (1 μmole), and 313 mCi of $\text{H}_2\text{O}-t$. After incubation for 20 min at 37°, 0.3 ml of 10% trichloroacetic acid was added together with 2 μmoles of unlabeled carrier glycine. After removal of the protein by centrifugation, the supernatant solution was applied to a column of Dowex 50 (H^+) and the column was washed with 10 ml of water. The glycine was eluted with 5 ml of 2 N ammonium hydroxide. The eluate was lyophilized and the glycine was determined as described above. ^b The conditions used for the oxidation of glycine by D-amino acid oxidase were as follows. A sample (0.2 ml) containing glycine-*t* was treated with 0.2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3), 0.05 ml of FAD (5 μg), 0.04 ml of D-amino acid oxidase (1 mg), and 5 μl of catalase (3450 units); the mixture was then incubated at 37° for 6 hr in a stoppered tube. The solution was then lyophilized and the distillate was quantitatively trapped in a Dry Ice-acetone bath. The volume of the condensate was measured and an aliquot was taken for determination of radioactivity. The lyophilized residue was dissolved in water and an aliquot was taken for the determination of glyoxylate (McFadden and Howes, 1960). ^c Calculated from the amount of glycine oxidized to glyoxylate by D-amino acid oxidase (DOX). ^d Calculated from the number of coenzyme binding sites on the apoenzyme, and based on the assumption that all of the enzyme-bound pyridoxamine 5'-phosphate is utilized in the transamination reaction.

It was readily demonstrated that aminomalonate acid is bound to the enzyme under these conditions; thus, after incubation of the enzyme with aminomalonate-2-¹⁴C acid, labeled enzyme was recovered on subjecting the enzyme to gel filtration. In the experiment described in Figure 5, parallel determinations of cysteinesulfinate desulfinate and of amino malonate binding were carried out; the data indicate close correlation between loss of enzymatic activity and binding. The amount of aminomalonate-¹⁴C bound approached a value of 1 mole/60,000 g of enzyme in these studies and in identical studies with aminomalonate-1-¹⁴C.

When the inactivated labeled enzyme was extensively dialyzed under various conditions there was no loss of radioactivity from the enzyme (Table II). Precipitation of the enzyme by addition of ammonium sulfate failed to liberate the bound radioactivity. However, most of the radioactivity was released when the enzyme was precipitated by adding trichloroacetic acid or ethanol.

Table III describes studies in which the enzyme was inacti-

vated by incubation with unlabeled aminomalonate acid and then reduced by treatment with sodium borotritide. After extensive dialysis substantial amounts of tritium remained on the enzyme. A control experiment in which the holoenzyme was treated with sodium borotritide gave a similar value, which was about three times greater than that obtained with the apoenzyme.

Stereospecificity of the Decarboxylation of Aminomalonate Acid. When aminomalonate acid-1-¹⁴C was incubated with the enzyme the glycine-¹⁴C formed had, as expected, about 50% of the specific activity of the aminomalonate used. Thus, in an experiment in which the reaction mixture contained 0.2 M sodium acetate buffer (pH 5.5), aminomalonate-1-¹⁴C (297,000 cpm/ μmole), 1 mM α -ketoglutarate, and enzyme (1 mg/ml), the specific activity of the glycine formed was found (from determinations of glycine and radioactivity) to be 163,000, 151,000, 140,000, 126,000, and 138,000 cpm per μmole after 2, 8, 12, 20, and 30 min, respectively, at 37°. However, when the decarboxylation of unlabeled

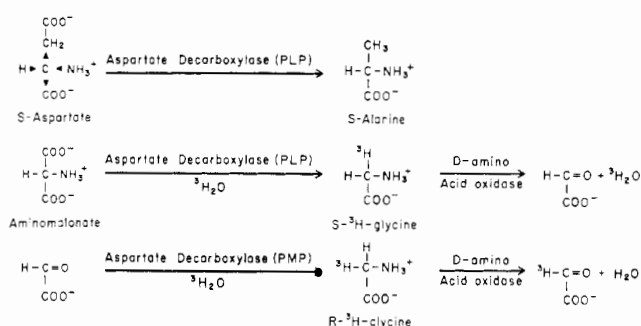


FIGURE 6: Synthesis of both optical isomers of mono(tritio)glycine catalyzed by L-aspartate β -decarboxylase (see the text).

aminomalononic acid was carried out in tritiated water, the reaction was shown to be stereospecific. In expt 2 (Table IV) the enzyme was incubated with aminomalononic acid in the presence of tritiated water and the glycine formed was then isolated and incubated with D-amino acid oxidase, which has been shown to remove a specific α -hydrogen atom from glycine (Wellner, 1967, 1970; see also Meister, 1965). As indicated in Table IV virtually all of the label present in the glycine derived from the decarboxylation was liberated by D-amino acid oxidase. In the control nonenzymatic decarboxylation reaction (expt 1) only about one-half of the tritium of the glycine formed by decarboxylation was released by D-amino acid oxidase. In expt 3 (Table IV) glycine was formed enzymatically by incubating glyoxylate with the pyridoxamine form of aspartate β -decarboxylase in the presence of tritiated water. On treatment of the isolated glycine with D-amino acid oxidase, only a very small fraction of the tritium present in glycine was liberated.

Discussion

These studies indicate that the decarboxylation of aminomalonate to glycine by L-aspartate β -decarboxylase is stereospecific, *i.e.*, that a specific carboxyl group is lost and is replaced by a hydrogen atom, which is specifically removed from glycine by D-amino acid oxidase. On the other hand, the hydrogen atom which is introduced into glycine in the course of transamination of glyoxylate catalyzed by L-aspartate β -decarboxylase, is not removed by D-amino acid oxidase (Figure 6). The very small amount of tritium released by D-amino acid oxidase from the monotrityglycine synthesized by transamination may probably be ascribed to the occurrence of some nonenzymatic transamination under the experimental conditions employed. Wellner (1967, 1970; see also Meister, 1965) has shown that D-amino acid oxidase specifically releases one of the α -hydrogen atoms of glycine and that the other α -hydrogen atom is stereospecifically introduced into glycine by L-specific transaminase. Wellner has also shown that the enzymatic conversion of serine into glycine catalyzed by serine hydroxymethylase is accompanied by stereospecific introduction of a hydrogen atom into glycine; this hydrogen atom is released by D-amino acid oxidase. The present findings may be readily integrated with those of Wellner. Thus, the L-specific transaminase activities of both aspartate β -decarboxylase and the transaminase catalyze the conversion (in H_2O -*t*) of glyoxylate

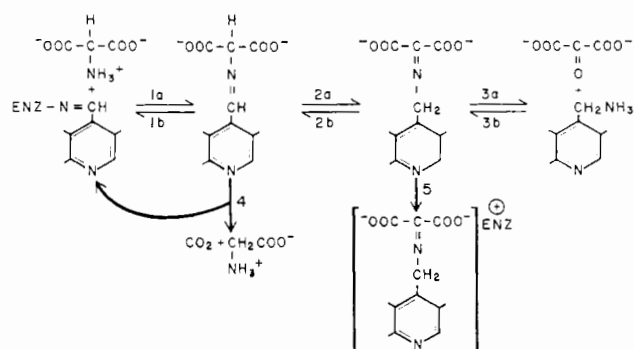


FIGURE 7: Postulated steps in the interaction of aminomalonate with L-aspartate β -decarboxylase (see the text).

into (*R*)-glycine-*t*. (*S*)-Glycine-*t* is formed in the reaction catalyzed by serine hydroxymethylase and in the decarboxylation of aminomalonate; in the latter reaction the carboxyl group that is lost may be considered to be in a position analogous to the CH_2OH group of L-serine and to the CH_2COO^- moiety of L-aspartate. It seems possible, although less likely, that the carboxyl group of aminomalonate in the position analogous to that of the α -carboxyl group of aspartate is lost and that addition of a proton is accompanied by inversion of configuration.

The decarboxylation of aminomalonate to glycine has previously been observed with enzyme preparations obtained from the posterior silk glands of silkworms and from rat liver (Shimura *et al.*, 1956; Thanassi and Fruton, 1962), and there is evidence that the liver enzyme utilizes pyridoxal 5'-phosphate as a coenzyme (Thanassi and Fruton, 1962). Highly purified preparations of this activity have not yet been obtained from animal tissues. The possibility that this activity is a function of one or more of the known vitamin B_6 -enzymes must be considered. However, the question as to whether the decarboxylation of aminomalonate has physiological significance cannot yet be definitely answered.

The studies on the stereospecificity of the decarboxylation of aminomalonate support the conclusion that the aldimine Schiff base is decarboxylated (reaction 4; Figure 7). Thus, α decarboxylation comparable with that catalyzed by typical amino acid decarboxylases may now be added to the growing list of enzymatic activities exhibited by L-aspartate β -decarboxylase. Presumably, decarboxylation of the ketimine Schiff base would lead to the introduction of two hydrogen atoms from the medium. Although direct transfer of hydrogen from the methylene group of pyridoxamine 5'-phosphate to the amino acid α -carbon atom is conceivable, the studies of Ayling *et al.* (1968) on pyridoxamine-pyruvate transaminase suggest that the extent of this type of reaction would be greatly reduced by exchange with hydrogen of the medium.¹

The nature of the inhibitory aminomalonate-enzyme complex requires further study. However, the data indicate that all three of the carbon atoms of aminomalonate are involved.

¹ It is notable (Table IV, expt 2) that glycine-*t* formed from decarboxylation of aminomalonate in H_2O -*t* contained only 0.15 μ atom of tritium/mole of glycine. Such isotope effects were also observed by Dunathan *et al.* (1968), in the transamination of deuterated pyridoxamine catalyzed by apoglutamate-oxaloacetate transaminase.

The spectral shift to a maximum at about 325 m μ is consistent with a formation of a ketimine derivative, which is also in accord with the uptake of tritium after treatment with sodium borotritide (Table III). However, the ketimine derivative would be expected to exist in rapid equilibrium with the aldimine form (reaction 2; Figure 7), and indeed the observed activation of the aspartate decarboxylase activity by ketomalonnate (Novogrodsky and Meister, 1964) indicates that reactions 3b, 2b, and 1b can occur rapidly. We therefore tentatively postulate that the inhibitory complex is a ketimine form which interacts with a group (or groups) on the enzyme in a reaction (reaction 5; Figure 7) which is relatively slow compared with the other transformations which occur on the enzyme. It seems possible that the strong negative charge on the ketimine might promote a conformational change in the enzyme. It is of interest that Matthew and Neuberger (1963) observed that aminomalonnate strongly inhibits δ -aminolevulinatase synthetase and serine hydroxymethyl transferase, but does not inhibit tyrosine decarboxylase or glutamate-aspartate transaminase. Further work is in progress in this laboratory in an effort to elucidate the mechanisms of inhibition of L-aspartate β -decarboxylase by aminomalonnate and other substrate analogs.

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