Role Reversal for Substrates and Inhibitors

SLOW INACTIVATION OF D-AMINO ACID TRANSAMINASE BY ITS NORMAL SUBSTRATES AND PROTECTION BY INHIBITORS*

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D-Amino acid transaminase, which catalyzes the synthesis of D-alanine and D-glutamate for the bacterial cell wall, is a candidate for the design of specific inhibitors that could be novel antimicrobial agents. Under the experimental conditions usually employed for enzyme assays, kinetic parameters for its substrates were determined for short incubation periods, when intermediates and products do not accumulate and the enzyme activity is linear with time. Such kinetic analyses indicate that the enzyme accepts most D-amino acids but D-aspartate and D-glutamate are the best substrates. Under a different type of experimental conditions when the enzyme is exposed to D-alanine, intermediates, and products for periods of hours, it slowly becomes inactivated (Martinez del Pozo, A., Yoshimura, T., Bhatia, M. B., Futaki, S., and Manning, J. M. (1992) Biochemistry 31, 6018–6023). We now report that D-aspartate, D-glutamate, and L-alanine also lead to slow inactivation. Methylation or amidation of the α-COOH group of D-alanine prevents inactivation, indicating that decarboxylation is required for inactivation; the slow release of CO₂ from substrate is demonstrated. The α-methyl analog of D-alanine, D-aspartate, and D-glutamate do not lead to inactivation, showing that the α-hydrogen of the substrate is required, i.e. that some processing is required. Lys145, which binds pyridoxal 5'-phosphate in the wild-type enzyme, is not involved in the inactivation since two active site mutant enzymes, K145Q and K145N, are also inactivated. Reactivation of the inactive enzyme at acidic pH is accompanied by the release of ammonia corresponding to 1 mol/mol of dimeric enzyme. Competitive inhibitors, amine-containing buffers, and thiols effectively impede the inactivation. This reversal in the roles of substrates and inhibitors, i.e. when a substrate can be an inactivator and an inhibitor can act as a protector, occurs during a time period not usually used to measure steady-state kinetics or initial velocities of enzyme reactions and could have physiological relevance in cells.

D-Amino acid transaminase catalyzes the reversible transfer of the amino group of a D-amino acid to the keto acid analog of a different D-amino acid (1–5). For example, this enzyme catalyzes the synthesis of D-alanine and D-glutamate (Equation 1), which are present in most, if not all, bacterial cell walls.

\[ \text{D-Alanine} + \alpha\text{-ketoglutarate} \rightarrow \text{pyruvate} + \text{D-glutamate} \]  

(Eq. 1)

Depletion of these two D-amino acids would be expected to prevent synthesis of the bacterial cell wall. Hence, specific inhibitors of this enzyme may be novel antimicrobial agents. D-Amino acid transaminase utilizes pyridoxal 5'-phosphate (PLP) as the coenzyme, which shuttles the amino group of the substrate between the aldehyde (PLP) and the amine (pyridoxamine 5'-phosphate, PMP) forms during the reaction. The coenzyme is anchored to Lys145 at the active site of the enzyme as a Schiff's base, but little else is known about the important residues at the active site, i.e. those involved in the binding of substrates or in catalysis. In this paper, we describe the effectiveness of several D-amino acids and their structural analogs of varying chain length in their interaction with D-amino acid transaminase, and we compare our results with corresponding studies for L-aspartate transaminase.

The information obtained from studies with alternate substrate and inhibitors has been used to probe the mechanism of the substrate-induced slow inactivation of the enzyme (6), which required that substrates, intermediates, and products be present in order for inactivation to occur. By employing D-[13C]-alanine isotopically labeled at specific positions, it was determined that a 2-carbon fragment derived from D-alanine was formed during the process. At slightly acidic pH, enzyme activity was restored with formation of the PLP form of the enzyme. However, not all the events surrounding the inactivation were studied nor were the substrate requirements investigated. In this paper, we present further studies on the products as well as a possible mechanism for the substrate-induced inactivation of D-amino acid transaminase. We also evaluate the effects of various additives on the inactivation.

EXPERIMENTAL PROCEDURES

Materials—Some amino acids and their analogs as well as rabbit muscle L-lactic dehydrogenase (EC 1.1.1.27), mitochondrial porcine heart malic dehydrogenase (EC 1.1.1.37), and bovine liver L-glutamic dehydrogenase (EC 1.4.1.3) were obtained from Sigma. D-Alanine methyl ester and D-alanine amide were purchased from Bachem. [1-15N]Ketoglutarate was a product of Amersham Corp. D-[1-15C]Ketoglutarate was a product of Amersham Corp.

The abbreviations used are: PLP, pyridoxal 5'-phosphate; K145N and K145Q, D-amino acid transaminase in which Lys145 has been mutated to asparagine and glutamine, respectively; PMP, pyridoxamine 5'-phosphate.

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alanine and d-[1-¹⁴C]alanine were from ICN. Cation exchange resin AG50W-X2 (Bio-Rad) in disposable polystyrone chromatography columns (Pierce Chemical Co.) were used to separate amino acids from keto acids.

**General Procedures**—D-Amino acid transaminase was purified as described previously (7-9). All kinetic experiments for the determination of $K_M$, $V_{max}$, and $K_i$ were conducted in 50 mM bis-Tris-HCl, pH 7.2, at 25 °C. Data for the determination of $K_M$ and $V_{max}$ were fit to lines with the program HYPER (10). For the determination of $K_i$ values for inhibitors, the data were fit to the programs COMP, UNCOMP, and NONCOMP (10).

**Kinetic Studies with Substrates and Analogs**—To test d-amino acids and their analogs as potential substrates, an assay method that measured the time-dependent conversion of radiolabeled α-ketoglutarate was employed (11). A 100-μl reaction mixture containing the amino acid at 100 μM, either 100 μM L-DNH or L-DNH analogs, 50 μM α-ketoglutarate and radiolabeled [α-¹⁴C]ketoglutarate (200,000 counts/min) were incubated with the appropriate amount of enzyme (0.1-10 μg) in 50 mM bis-Tris-HCl, pH 7.2 at 25 °C. After 5-10 min, the reaction was terminated by addition of 10% trichloroacetic acid (final concentration) and applied to a disposable column (0.4 x 4 cm) described above. The keto acid was eluted with water and the amino acid with 2 ml of 2 N HCl in 200-μl volumes. These samples were neutralized with 10 N NaOH, mixed with 5 ml of Ready Safe (Beckman) scintillation fluid and counted for 2 min in a LKB model 1218 liquid scintillation counter.

Assays—D-Amino acid transaminase activity was assayed by coupling the pyruvate produced from d-alanine to the NADH-dependent lactate dehydrogenase-catalyzed reaction. Assays were performed at 25 °C on a Varian Cary 2200 spectrophotometer at 340 nm. A 1-ml reaction mixture contained 5 mM α-ketoglutarate, 200 mM D-alanine, 100 μM D-NH analogs, and 0.4 mg/ml α-ketoglutaric acid dehydrogenase in 50 mM bis-Tris-HCl, pH 7.2 at 25 °C (9). The reaction was initiated by addition of 3 μg of d-amino acid transaminase. One unit of activity was defined as the amount of enzyme needed to convert 1 μmol of NADH to NAD/min at 25 °C.

In the experiments designed to investigate the inhibition of d-amino acid transaminase by mono- and dicarboxylic acids, the concentration of one substrate was varied between 0.25 to 4 times its $K_M$ value, and the other was kept constant at a saturating concentration; the other was kept constant at a saturating concentration; and malic dehydrogenase was substituted for lactic acid dehydrogenase (2-3 mg/ml) was incubated with concentrations of each inhibitor was determined from the negative intercept on the y-axis. The protection constant ($K_p$) for each inhibitor was calculated as the amount of enzyme needed to convert 1 pmol of NADH to NAD/min at 25 °C.

For the determination of $K_I$ values for the inhibitors, the data were fit to the programs COMP, UNCOMP, and NONCOMP (10).

**RESULTS**

**Kinetic Parameters for Substrates**—Previous steady state kinetic analysis indicated a rather high $K_M$ for d-alanine (48 mM) and low second-order rate constant of 2.6 × 10⁴ M⁻¹ s⁻¹ (11). However, d-glutamate, the substrate for the reverse reaction, as indicated in Equation 1, has a substantially lower $K_M$ of 1.2 mM (Table I). These results suggested that the distal carboxyl group of d-glutamate played an important role in substrate binding. Previous results (11) had shown that the $K_M$ for an amino acid substrate for d-amino acid transaminase is a reasonable estimate of its binding constant ($K_i = k_{cat}/V_{max}$). Thus, in this study, $K_M$ values for alternate substrates are employed to estimate binding affinities of amino acids.

The $K_M$ for d-aspartate was determined to be 0.4 mM and its $V_{max}$ to be 32 units/mg (Table I); its $k_{cat}/K_M$ was calculated to be 3.8 × 10⁴ M⁻¹ s⁻¹. D-Asparagine and D-glutamic acid were also found to be substrates for D-amino acid transaminase, but their $K_M$ values were about twice of their corresponding $K_M$ values. The same was observed for all the other amino acids tested, and the order of magnitude of the $K_M$ values for D-amino acid transaminase was determined to be 10⁻³ M for all the amino acids tested.

**Inhibition by Mono- and Dicarboxylic Acids**—Dicarboxylic acids such as maleic acid and succinic acid are known to be competitive inhibitors of mitochondrial L-aspartate transaminase as described by Michuda and Martinez-Carrion (13). In the crystal structure of the succinate complex of L-aspartate transaminase at 2.8 Å, these dicarboxylic acids mimic substrate binding and the two carboxyl groups of succinate interact with Arg⁹⁰ and Arg⁹⁰ (14). We find that maleic acid...
and succinic acid had the lowest $K_I$ values as competitive inhibitors for D-amino acid transaminase (Table II); perhaps they bind two analogous positively charged residues in the active site of D-amino acid transaminase. They were uncompetitive inhibitors versus $\alpha$-ketoglutarate, suggesting that the dicarboxylic acids mimic amino acid binding; they have an affinity for the E-PLP form of the enzyme. This conclusion is similar to that reported by Michuda and Martinez-Carrion (13) for L-aspartate transaminase. The 3-carbon dicarboxylic acid malonic acid was also a good inhibitor of D-amino acid transaminase. The absence of a carboxylate moiety, as in succinic acid, led to a 50-fold increase in the $K_I$ value; blocking of both carboxylates, as in succinamide, led to a very poor inhibitor (Table II). Dicarboxylic acids with longer carbon chains, such as glutaric acid (5-carbon) and adipic acid (6-carbon) were found to have increased $K_I$ values.

Monocarboxylic acids were also tested as potential inhibitors of the enzyme (Table II). Acetic acid was a poor competitive inhibitor versus either D-aspartate or D-alanine; it was uncompetitive versus $\alpha$-ketoglutarate. Propionic acid was a better inhibitor than acetic acid. In duplicate experiments, acetic acid and malonic acid were found to be non-competitive inhibitors versus D-alanine.

*Inactivation of D-Amino Acid Transaminase by Normal Substrates*—We have reported that there was slow inactivation of the enzyme in the presence of D-alanine, intermediates, and the product of the single turnover, pyruvate, when these were permitted to remain at equilibrium with the enzyme for a prolonged period (6). We have now extended that observation to include other D-amino acids. As shown in Fig. 1, D-glutamate and D-aspartate, which are substrates (Table I), also inactivated the enzyme. L-Alanine, which is a poor substrate (11, 15) but forms the same product as does D-alanine, i.e. pyruvate, also inactivated the enzyme but at a slower rate than D-alanine. $\alpha$-Aminoisobutyric acid, the $\alpha$-methyl analog of alanine, as well as $\alpha$-methyl-D-aspartate and $\alpha$-methyl D-
Thus, this result indicates that the presence of a free carboxyl
CO\textsubscript{2} release are reported below.

| Competitive inhibitor | \( K_{\text{i}} \) \( \text{mM} \) |
|-----------------------|-----------------|
| Acetic acid           | 29 ± 3.5        |
| Propionic acid        | 3.5 ± 0.3       |
| Malonic acid          | 1.3 ± 0.1       |
| Maleic acid           | 1.5 ± 0.1       |
| Succinic acid         | 2.3 ± 0.4       |
| Succinimide           | 28.4 ± 2.4      |
| Succinamic acid       | >10             |
| Glutaric acid         | 10.7 ± 0.8      |
| Adipic acid           | 43 ± 4          |

*Inhibition studies with D-aspartate or \( \alpha \)-ketoglutarate as the varied substrate were carried out employing the malic acid dehydrogenase spectroscopic assay described in the text. All of the carboxylic acids tested were competitive inhibitors versus D-aspartate and uncompetitive versus \( \alpha \)-ketoglutarate.

*Inhibition studies with D-alanine were measured with the lactic acid dehydrogenase assay as described in the text. Acetic acid and malonic acid were non-competitive versus D-alanine. The others listed were competitive inhibitors.

Previously, we had determined that the inactivated enzyme

\[ \text{D-Ala ester}, \text{D-Ala amide}, \text{Me-Ala, Me-Glu = Me-Ala, Me-Glu} \]

and glutamate did not inactivate the enzyme (Fig. 1), indicating that turnover of substrate was necessary in order for inactivation to occur.

Previously, we had determined that the inactivated enzyme contained a 2-carbon fragment derived from D-alanine and that D-alanine labeled in the \( \alpha \)-COOH group did not lead to incorporation of radioactivity into the inactivated enzyme (6).

It was not ascertained in that study whether \( \alpha \)-decarboxylation was a prerequisite for inactivation. D-Alanine amide and D-alanine methyl ester, both of which are substrates (Table I), although poor ones, do not inactivate the enzyme (Fig. 1).

Under these experimental conditions, some inactivation might have been observed over a 6-h period, if it had occurred.

Thus, this result indicates that the presence of a free carboxyl moiety is necessary for inactivation. Direct measurements of \( \text{CO}_2 \) release are reported below.

**Pyruvate Formation**—Each dimer of D-amino acid trans-
aminase was functional (15) in producing 2 mol of pyruvate in single turnover experiments with D-alanine alone (Table III). The maximum amount of pyruvate, 2.1 ± 0.3 mol of pyruvate/mol of enzyme dimer, was formed at the earliest times (~0.01 h); the same amount of pyruvate was found at all concentrations of D-alanine tested from 0.5 to 5.6 mM, representing a 5- to over 50-fold excess of D-alanine over enzyme. These results indicate that pyruvate and the E-PMP form of the enzyme are formed rapidly; at this early time there is no detectable inactivation of the enzyme. After the inactivation was complete (4 h), the pyruvate initially formed had decreased to about half of its initial value (Table III).

This result is consistent with the other data in Table III, which show that the products formed amount to about 1 mol/mol of enzyme dimer.

It is possible that pyruvate bound to E-PMP or some intermediate formed from this complex loses \( \text{CO}_2 \) to form the inactivating species. Inactivation resulting from a spontaneous decarboxylation of D-alanine alone is highly unlikely.

We have also determined that the incubation of 100 mM D-
alanine with the E-PMP form of the enzyme in 100 mM HEPES, pH 7.5 at 25 °C, does not lead to any loss in enzyme activity over a period of 6 h.

**\( \text{CO}_2 \) Formation**—The formation of \( \text{CO}_2 \) in the reaction mixture was also determined by direct analyses. After 4 h of exposure of enzyme to uniformly labeled D-[\( ^{14} \text{C} \)]alanine, the enzyme was about 90% inactivated (Table III). The release of radiolabeled \( \text{CO}_2 \) was found to increase as a function of incubation time. After 4 h, the amount of \( ^{14} \text{C} \)CO\textsubscript{2} released from D-[\( ^{14} \text{C} \)]alanine was about 0.7 mol/subunit or about 1.4 ± 0.3 mol/dimer (Table III). This determination, which has a precision of 20%, suffers mainly from the error due to the continuous non-enzyme release of \( \text{CO}_2 \) from pyruvate because of its slow spontaneous breakdown. For example, in a separate experiment with [\( ^{14} \text{C} \)]pyruvate alone, the spontaneous non-enzyme release of \( \text{CO}_2 \) was found to amount to 20% of the value released in the presence of enzyme. Nevertheless, the amount of \( \text{CO}_2 \) released is close to the amount calculated from the inactivation of one subunit. These results, as well as the value found for the amount of ammonia released (see below and Table III), are consistent with the amount of acetaldehyde, the amount of D-[\( ^{14} \text{C} \)]alanine incorporated, and the 50% coenzyme fluorescence remaining in the inactive enzyme; they support the conclusion that inactivation in one subunit leads to inactivation of the complete enzyme (6).

**NH\textsubscript{3} Formation**—Previously, it was reported that treatment of the inactivated enzyme at pH 5 resulted in restoration of activity with formation of the PLP enzyme and the release of a 2-carbon fragment which was identified as acetaldehyde by assay with aldehyde dehydrogenase (6). However, the fate of the nitrogen originating from D-alanine was not established. To test the possibility that the amine moiety originally derived from D-alanine and transferred to the coenzyme to form E-PMP may be lost as ammonia, a spectroscopic procedure

| Product | Time for formation \( \text{h} \) | Inactivation \% | Moles formed/mole enzyme dimer |
|---------|----------------|---------------|-------------------------------|
| Pyruvate| ~0.01          | 0             | 2.1 ± 0.2                     |
| Pyruvate| 4              | 90            | 1.0 ± 0.1                     |
| CO\textsubscript{2} | 4              | 90            | 1.4 ± 0.3                     |
| NH\textsubscript{3} | 4              | 90            | 0.84 ± 0.08                   |
using L-glutamic dehydrogenase was employed, as described above. The results indicate that the amine moiety of D-alanine is indeed lost as ammonia; the reaction mixture contains 0.84 mol of ammonia/mol of D-amino acid transaminase dimer (Table III). This result is consistent with the inactivation of the complete enzyme upon derivatization of coenzyme in one of the two subunits.

**Inactivation of D-Amino Acid Transaminase Mutant Enzymes** — To investigate the possibility that the mechanism of inactivation involved the amino group of Lys145, studies were carried out on two mutant D-amino acid transaminases, K145Q (9) and K145N (16) in which Lys145 had been mutated to glutamine or asparagine, respectively. The activity of these attenuated mutant enzymes is intrinsic and each contains the full complement of coenzyme PLP bound through non-covalent linkages (9, 11, 16). As shown in Figs. 2 and 3, both enzymes were inactivated by D-alanine although at slower rates. The rate constants for the inactivation for the mutant enzymes were 0.05 h⁻¹ (K145Q) and 0.08 h⁻¹ (K145N), compared with 0.6 h⁻¹ for the wild-type enzyme. The findings agree with the postulated mechanism in Scheme I (see below) which shows that Lys145 that binds PLP in the wild-type enzyme is not involved in the inactivation, a feature that distinguishes this mechanism from other systems (17–22), as described more fully under “Discussion.”

**Effect of Mono- and Dicarboxylate Inhibitors on Substrate-induced Inactivation** — The inactivation of the enzyme by D-amino acids could occur by a species generated at the active site or free in solution. These possibilities were tested by conducting the substrate-induced inactivation in the presence of some of the competitive inhibitors whose properties are described above. As shown in Fig. 2, maleate protected D-amino acid transaminase from inactivation induced by D-alanine. The protection was greater with increasing concentrations of maleate; a secondary plot of slopes versus concentration of maleate gave a protection constant (KP) of 1.5 mM (Table IV). Similar experiments with other mono- and dicarboxylate acids clearly indicated that all of these competitive inhibitors protected against inactivation by D-alanine (Table IV). There was a reasonably good correlation between the protection constant, KP, for a given carboxylic acid inhibitor listed in Table IV and its Kp value as a competitive inhibitor versus D-aspartate (Table II). Protection by the mono- and dicarboxylate compounds would occur by preventing the reactive intermediate from binding at the active site. Compounds such as n-propyl alcohol and isobutanol, which lack the carboxyl moiety and are not competitive inhibitors of D-amino acid transaminase, did not protect the enzyme from a substrate-induced inactivation (KP > 1 mM).

**Protection by Thiol Compounds** — As shown in Table IV, thiols protected D-amino acid transaminase from D-alanine-induced inactivation. L-Cysteine was a strong protecting agent with a considerably low KP = 0.6 mM (Table IV). L-Cysteic acid, which lacks the mercapto group, offers very poor protection against inactivation. Mercaptoethamine, dithioerythritol, dithiothreitol, and glutathione GSH each provided protection against inactivation to varying degrees as indicated by the KP values shown in Table IV. These compounds could exert their protective effect either by competitive binding with substrate at the active site or by reacting with the inactivating intermediate in the solvent environment. Since none of the above mentioned compounds are active-site-directed, the second possibility appears more likely although this remains to be established. The oxidized form of glutathione does not protect against the inactivation of D-amino acid transaminase (Table IV).

**Protection by Buffers against Inactivation** — The substrate-induced inactivation is dependent on the buffer employed, i.e. it is rapid in HEPES, pH 7.5, slower in bis-Tris-HCl, pH 7.5, and slowest in Tris-HCl buffer at the same pH (Fig. 4). There is a correlation between the rate of inactivation in a given buffer and its degree of nucleophilicity. Therefore, it is likely that such nucleophilic buffers prevent the inactivation by reacting with and removing a reactive intermediate formed upon decarboxylation. These buffers do not protect against the inactivation by binding and forming a Schiff's base complex with the coenzyme since in separate experiments it was determined that bis-Tris-HCl and Tris-HCl do not form Schiff bases with the coenzyme either in the K145N or in the K145Q mutant D-amino acid transaminases (9, 16). At a fixed pH of 7.5 in Tris-HCl buffer, there was a dependence of protection against inactivation with increasing concentration of Tris-HCl (Fig. 5).
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Scheme I. Proposed pathway for the inactivation of D-aminoo acid transaminase by substrates and reactivation at acidic pH.

Table IV
Protection of D-aminoo acid transaminase from D-alanine induced inactivation by carboxylic acids and thiols

| Carboxylic acid      | $K_p$ (mM) |
|----------------------|------------|
| Malonic acid         | 0.8        |
| Maleic acid          | 1.5        |
| Succinic acid        | 2.0        |
| Glutaric acid        | 3.5        |
| Propionic acid       | 5.8        |
| Acetic acid          | 30.0       |
| Thiol or related compounds |         |
| L-Cysteine           | 0.6        |
| Mercaptoproethyamine | 1.8        |
| Dithioerythritol     | 18.3       |
| GSH                  | 25.0       |
| Dithiothreitol       | 32.0       |
| L-Cysteic acid       | 44.0       |
| GSSG                 | >250.0     |

**DISCUSSION**

A major goal of our studies on bacterial D-aminoo acid transaminase has been to obtain basic information on its mode of action in order to design an efficient inhibitor that might be a useful antimicrobial agent. The specific inactivation of this enzyme, which occurs in bacterial but not mammalian cells, has been shown to confer antimicrobial activity on compounds such as β-chloro-D-alanine (23) and gabaculine (24). In an effort to obtain more selectivity in a suicide substrate, studies using three different approaches have been undertaken to elucidate the mechanism of action of D-aminoo acid transaminase. These approaches include studies on its catalytic mechanism through site-directed mutagenesis (9, 16), investigation into the kinetic parameters for this enzyme (11), and studies on the mode of the substrate-induced inactivation described initially by Martinez del Pozo et al. (6), and also in the present paper.

It is interesting to compare the relative specificities of the transaminases for D- and L-amino acids; L-aspartate transaminase is a very selective enzyme with L-aspartate and L-
of the reaction indicate that the inactivation event likely for inactivation to occur. Studies with competitive inhibitors providing further evidence that a decarboxylation was necessary for the enzyme. Release of glutamate as its best substrates, whereas L-alanine and other amino acids are extremely poor substrates (25–28). In contrast, D-amino acid transaminase can catalyze the transamination of most D-amino acids suggesting a more “open” active site for the D-amino acid-specific enzyme. The results presented here on the kinetic parameters for substrates and substrate analogs of D-amino acid transaminase have provided information on the efficiency of their interactions with the enzyme. The data have defined the optimal length of the carbon chain of a substrate to be 4; experiments with dicarboxylic acids used as competitive inhibitors are also consistent with this conclusion.

The $K_M$ for D-alanine decreased 30-fold in the presence of formate ion with no effect on the $V_{max}$ value (Table I). It is likely that formate mimics the function of the carboxyl moiety of D-aspartate and binds to a positively charged residue in the active site to trigger a conformational change that allows for improved binding of D-alanine, and thus the lowering of its $K_M$. Similar studies have been conducted on L-aspartate transaminase (29, 30). However, in this enzyme the formate ion addition resulted in a 20-fold increase in $V_{max}$, with no effect on the $K_M$ value. Morino et al. (29, 30) have proposed that the formate ion interacts with the distal positively charged residue. In the case of L-aspartate transaminase, this positively charged residue is Arg29 (31). Thus, there is considerable support for the proposal that a positively charged residue exists in the active site of D-amino acid transaminase.

The studies described herein reinforce the conclusion that the substrate-induced inactivation of D-amino acid transaminase results from a slow decarboxylation of D-alanine since D-alanine methyl ester and D-alanine amide did not inactivate the enzyme. Release of CO$_2$ was also measured directly, providing further evidence that a decarboxylation was necessary for inactivation to occur. Studies with competitive inhibitors of the reaction indicate that the inactivation event likely occurs at the active site rather than by some non-enzymic event, as indicated by the correlation found for the $K_I$ of a given competitive inhibitor with respect to D-aspartic acid (Table II) and the ability of that inhibitor to protect against the inactivation ($K_I$ value in Table IV).

There are several examples of side reactions catalyzed by various PLP-dependent enzymes, where a secondary pyridoxal-catalyzed reaction affects enzyme activity. A minor transamination reaction catalyzed by L-aspartate β-decarboxylase eventually led to complete loss in its activity as described by Novogrodsky and Meister (20). This was shown to be due to the formation of the PMP form of the enzyme which dissociates since it is not tightly bound to that particular protein; activity could be restored by added PLP. Other transamination-dependent side reactions were later shown to follow this pathway (19). However, such a pathway was shown not to be operative for the substrate-induced inactivation of D-amino acid transaminase (6). Attempts to demonstrate an intermediate of the type described by Likos et al. (18) for L-glutamate decarboxylase and the suicide substrate L-serine O-sulfate did not provide any evidence that the inactivation of D-amino acid transaminase involved this mechanism. Thus, the mode of inactivation does not involve Lys185, which binds coenzyme PLP in the wild-type enzyme since both the K145N as well as the K145Q resistant enzymes are inactivated by D-alanine. The available data suggest a pathway more similar to that described by O’Leary and Baughn (21) and by Sukhareva and Braunstein (22) for the decarboxylation-dependent transamination of L-glutamate by glutamate decarboxylase. These pathways have been carefully evaluated by John (32). The pathway described here is also different from those described by Morrison and Walsh (33).

A tentative working model for the mechanism of inactivation is shown in Scheme I. First, the presence of a free α-COOH group and the α-proton of substrate is required for the inactivation as shown by the results in this study. Second, almost immediately after D-alanine is added, the absorption maximum at 420 nm due to the external aldimine is converted to E-PMP or ketimine form absorbing at 330 nm; the process of inactivation then occurs slowly. A small amount of a quinonoid intermediate absorbing at 483 nm is also formed and slowly disappears as the enzyme is inactivated. Scheme I is consistent with the observation that substrates, intermediates, and products must be present at equilibrium in order for the inactivation to occur. These results suggest that the inactivation occurs through some intermediate formed after the quinonoid and is accompanied by decarboxylation. Thus, $E_{inact}$ of Scheme I is equivalent to component C described previously (6). The inactive enzyme can be reactivated at pH 5. During this process, the enzyme is converted to the active PLP form which absorbs at 420 nm; this is component B described previously (6). The amino group from the original D-amino acid is released as ammonia in an amount that corresponds to nearly 1 mol/mol of dimeric enzyme. This result also is consistent with the original observation that the inactivation of one subunit renders the entire enzyme molecule inactive.

The reversal of the usual roles for substrates and inhibitors under the different experimental conditions described here could have some physiological relevance. Hence, in the cell where enzymes, substrates, and products can exist together for an extended period, slow loss of enzyme activity could occur. However, depending upon which metabolites, i.e. thiols, amines, and dicarboxylic acids, are present such metabolites could play an important protective function in the cell.

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