Control of Pyridoxal Phosphate Enzyme Reaction Specificity Studied with α-Dialkylamino Acid Transaminase*

Gordon B. Bailey,† Orathai Chotamangsa, and Kreurvul Vuttivej‡

ABSTRACT: Aspects of the substrate and reaction specificity of α-dialkylamino acid transaminase have been investigated to test the hypothesis that the first α-carbon bond cleaved is directed to a single activated conformation in the catalytic center by site-specific binding of one of the groups attached to the substrate α-carbon. During transamination this enzyme catalyzes removal of the α-carboxylate of α-aminoisobutyrate and the α-hydrogen of L-alanine. From this observed reactivity, the above model predicts that α-dialkylamino acid transaminase should also catalyze transamination of D-alanine, initiated by removal of the α-carboxylate, and that the active site contains two distinct subsites capable of accepting an α-carboxylate ion. Substrate conformation must be controlled by binding of the amino acid side chain. When excess D-[1-^{14}C]alanine was reacted with pyruvate and enzyme, transamination occurred exclusively *via* α-carboxylate cleavage. Acetaldehyde and alanine were detected as the other products.

During exhaustive exchange transamination between L-[1-^{14}C]alanine and pyruvate under the same conditions, only minor amounts of [^{14}C]CO_2 were released, possibly as a result of D-alanine generation from pyruvate and subsequent decarboxylation. α-Methyl α-aminomalonate, prepared by bromination of α-methyl malonate followed by ammonolysis, was transaminated by the enzyme, indicating acceptance by the active center of two α-carboxylate ions simultaneously. Substrate analogs lacking an α-methyl or α-methylene group were unreactive. Analogous to the reactivity of both enantiomers of alanine, the enzyme was competitively inhibited by both D- (K_I, 8 mM) and L-cycloserine (K_I, 2.5 μM). The lower K_I found for the L form may reflect conformational activation and cleavage of the inhibitor α-hydrogen. In agreement with the model tested, the findings indicate that there is a unique conformation for each substrate (and competitive inhibitor) in the active center of α-dialkylamino acid transaminase. This conformation is determined by site-specific binding of the amino acid side chain and places the bond to be cleaved from the α-carbon of all substrates in a common subsite, presumably in the plane perpendicular to that of the cofactor ring.

Over the full range of PLP[1] enzymes there may be found examples which catalyze cleavage of each of the bonds to an amino acid α-carbon (see, for example, Braunstein, 1963), yet individual enzymes display fairly strict specificity with regard to the type and configuration of the group first removed from the α-carbon. Dunathan (1966) explained this element of PLP enzyme specificity in a model postulating that the conformation of the groups about the substrate α-carbon to nitrogen bond in the initial amino acid–PLP aldimine is such that the bond to be cleaved is oriented in an activated position in a plane perpendicular to the plane of the PLP ring. He suggested that this orientation could be directed by site-specific binding of the substrate α-carboxylate in the active site. Although the observed reaction specificity of nearly all PLP enzymes can be satisfactorily explained by this hypothesis, an existing reaction system which would allow experimental testing of the model was not obvious.

More recently (Bailey and Dempsey, 1967), we described the purification and some reaction properties of a bacterial transaminase which catalyzed amino transfer from α-dialkylamino acids (*e.g.*, AIB) and L-alanine to pyruvate. Reaction of AIB involved cleavage of the substrate α-carboxylate (decarboxylation-dependent transamination, as named by Kalyankar and Snell, 1962), whereas L-alanine transamination proceeded, within the limits of experimental error, exclusively *via* α-hydrogen cleavage from the amino acid. (Herein, we will refer to the former mechanism as decarboxylation transamination and to the latter as deprotonation transamination.) The observed reaction specificity of this PLP-requiring enzyme, referred to as α-dialkylamino acid transaminase (α-DAT), is not predicted by the Dunathan model if α-carboxylate binding controls substrate geometry. If this were the case, the bond cleaved from at least one of the above substrates would be directed outside the plane perpendicular to the cofactor ring, as shown in Scheme I. α-DAT reaction specificity, however, is consistent with the model if one assumes that site-specific binding of the amino acid side chain directs the conformation of the substrate bonds relative to the cofactor. This is illustrated in Scheme II. When AIB was substrate, attachment of the appropriate side-chain methyl at site (a) would place the α-carboxylate in the activated position (b) for scission; with L-alanine as substrate, binding of the α-methyl at site (a) would direct the α-hydrogen to the cleavage position. Site

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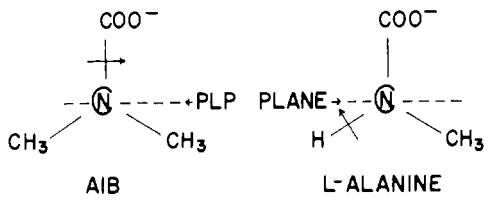
[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; AIB, α-aminoisobutyrate; α-DAT, α-dialkylamino acid transaminase.

SCHEME I



(c) must be considered relatively nonspecific, accepting either the second α-methyl of AIB or the α-carboxyl of L-alanine.

Certain predictions of the substrate and reaction specificity of α-DAT follow from such a view. Testing of these should help to establish the tenability of the basic principle of Dunathan's hypothesis, namely, that site-specific binding of one of the α-carbon substituents holds the bond to be cleaved in a single activated orientation. For example, if site (c) is indeed nonspecific, one predicts specific activation and hence cleavage of the α-carboxylate of D-alanine directed by α-methyl binding at site (a). If this is so, two distinct positions within the α-DAT active site capable of accepting α-carboxylate groups are indicated. Thus, the dicarboxylic hybrid of D- and L-alanine, α-methyl α-aminomalonate, should fit in the active site and undergo decarboxylation transamination. The predicted conformations and reactivities of these test compounds are illustrated in Scheme II. Substrate analogs lacking an α-methyl, such as glycine and α-aminomalonate, may or may not be reactive, depending on the requirement of the side chain for binding or reaction.

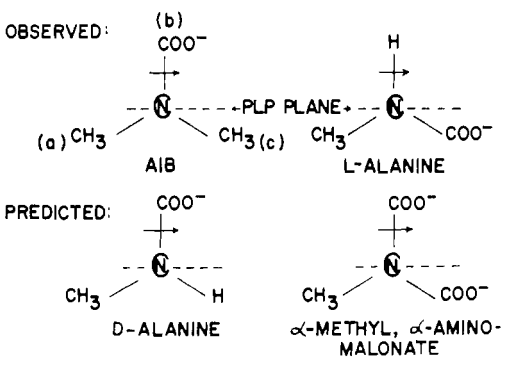
Dempsey and Bailey (1968) demonstrated that α-DAT is inhibited by D-cycloserine. Since the conformation of this compound resembles that of alanine, reactivity of both stereomers of alanine should be paralleled by inhibition by L- as well as D-cycloserine.

Here we describe experiments which confirm the above predictions.

Experimental Procedures

Materials. α-DAT was purified from *Pseudomonas* sp. as described by Bailey and Dempsey (1967) with minor modifications. The final preparation appeared essentially homogeneous when analyzed by polyacrylamide gel disc electrophoresis and analytical ultracentrifugation. Radioactive compounds were purchased from New England Nuclear. PLP was obtained both from Calbiochem and Sigma, and PMP from Calbiochem. α-Methylmalonic acid, diethyl aminomalonate, and DL-cycloserine were purchased from Aldrich Chemical Co.; D-cycloserine came from Sigma.

SCHEME II



Synthesis of α-Methyl α-Aminomalonate, and α-Aminomalonate. α-Methyl α-aminomalonate was prepared from α-methylmalonic acid by bromination followed by ammonolysis. Insofar as possible, the total preparation was carried out at 0–5° to reduce decarboxylation of intermediates and the product. The bromination procedure was similar to that described by Greenstein and Winitz (1961) for α-*n*-butylacetoacetic ester. Liquid bromine (145 mmoles) was added slowly to a stirred solution of α-methylmalonate (145 mmoles) in 240 ml of 2 N NaOH. When the bromine color disappeared, the pH was adjusted to 1.8 (glass electrode) with concentrated HCl and the solution extracted with an equal volume of ether. Readjustment of the pH and ether extraction were repeated 4 times, after which the extracts were combined and the ether removed under vacuum. The clear oily residue was dissolved in 250 ml of 33% NH_4OH and warmed at 45° for 72 hr in a sealed flask. Following ammonolysis, the solvent and excess ammonia were removed by lyophilization and the remaining white powder was dissolved in a minimum volume of water and filtered. α-Methyl α-aminomalonic acid was isolated free of detectable impurities by chromatography of the filtered solution on a 2 × 30 cm column of Dowex 50W-X4 (H^+). The product was detected as a ninhydrin-positive peak which was retarded by the resin but eluted with 200–300 ml of distilled water. The ninhydrin-positive fractions were lyophilized and the product crystallized from aqueous solution by the addition of acetone.

α-Aminomalonate was prepared from diethyl aminomalonate by hydrolysis in 50% KOH and purified by chromatography on Dowex 50 as described above.

The structures of the two compounds were confirmed by decarboxylation of samples in hot, dilute HCl. α-Methyl α-aminomalonate was converted quantitatively into alanine and α-aminomalonate to glycine, as revealed by thin-layer chromatography with standards. Both amino acids were converted into ammonium salts by lyophilization from 33% NH_4OH, and stored dry at −20°. Fresh solutions were prepared for each experiment and checked for decarboxylation products by thin-layer chromatography. α-Methyl α-aminomalonate was stable for several days in neutral, buffered solution, whereas α-aminomalonate under similar conditions decayed slowly to glycine.

Transamination Assays. Several techniques were employed to detect transamination of the various amino acids tested. The details of these procedures have been described (Bailey and Dempsey, 1967) and are merely outlined here. Pyruvate was used as the α-keto acid substrate in all assays, and ^{14}C was quantitated by liquid scintillation spectrometry using the fluid described by Bray (1960).

The extent of decarboxylation transamination of D- and L-alanine was determined by measuring the $[^{14}C]CO_2$ released from acidified reaction mixtures in which either D- or L-[1-^{14}C]-alanine had been used as substrate. The extent of deprotonation transamination in the same reactions was then determined by ether extraction of any [1-^{14}C]pyruvate which had been

formed. Decarboxylation transamination of AIB was quantitated by spectrophotometric measurement of the 2,4-dinitrophenylhydrazone of the acetone produced. Transamination of nonradioactive amino acids was detected by using [^{14}C]-pyruvate as the α-keto acid substrate and by separating the [^{14}C]alanine produced on Dowex 50W columns.

Results

Transamination of D- *and* L-*Alanine.* When equimolar amounts of D-[1-^{14}C]alanine and pyruvate were incubated with α-DAT and PLP, little reaction could be detected (Bailey and Dempsey, 1967). However, when the D-alanine to pyruvate ratio was increased as shown in Figure 1, decarboxylation occurred slowly but ultimately to an extent equivalent to over 90% of the original pyruvate concentration. Deprotonization transamination of D-alanine to pyruvate could not be detected. Presumably, the overall reaction was

$$\text{D-alanine} + \text{pyruvate} \longrightarrow CO_2 + \text{acetaldehyde} + \text{L-alanine} \quad (1)$$

and proceeded until pyruvate was exhausted. This stoichiometry was verified in separate but identical reactions. A carbonyl product was formed, which could be isolated as its 2,4-dinitrophenylhydrazone, using the procedure described for acetone (Experimental Procedures). The derivative was crystallized and identified as acetaldehyde 2,4-dinitrophenylhydrazone from its melting point and by thin-layer chromatography. Furthermore, when [1-^{14}C]pyruvate and nonradioactive D-alanine were used as substrates, [^{14}C]alanine was produced. Thus, the mechanism of transamination of D-alanine is directly analogous to that with AIB and proceeds exclusively *via* release of the α-carboxylate group.

We have previously demonstrated that when equimolar amounts of L-[1-^{14}C]alanine and pyruvate were incubated with PLP and α-DAT, the principal radioactive product was [^{14}C]pyruvate (Bailey and Dempsey, 1967); insignificant amounts of the alanine carboxylate were released. In those experiments isotopic equilibrium was not attained before the reaction was stopped. In the present study the reaction was repeated under the same conditions which allowed essentially quantitative decarboxylation transamination of D-alanine. After 30 min, when the first measurement was taken, the specific activity of pyruvate was approximately 80% that of the L-[1-^{14}C]alanine added, indicating that isotopic equilibrium had been reached (Figure 1). At this time negligible amounts of [^{14}C]CO_2 could be detected. After longer incubation, the [^{14}C]pyruvate concentration remained constant within experimental error, but increasing amounts of [^{14}C]CO_2 were released.

The appearance of [^{14}C]CO_2 after equilibrium of the exchange transamination between L-[1-^{14}C]alanine and pyruvate suggested that decarboxylation was subsequent to and, therefore, dependent upon that reaction. Itoh and Dempsey (1969) have noted a similar release of [^{14}C]CO_2 during transamination of AIB with [1-^{14}C]pyruvate, catalyzed by an α-DAT isolated from another bacterium. Although the possibility of direct cleavage of the α-carboxyl of L-alanine has not been excluded, a more likely explanation is that rarely during the second half-reaction of transamination, the stereochemistry of proton addition to the α-carbon of pyruvate was opposite to that which normally occurred. This lead to the formation of the

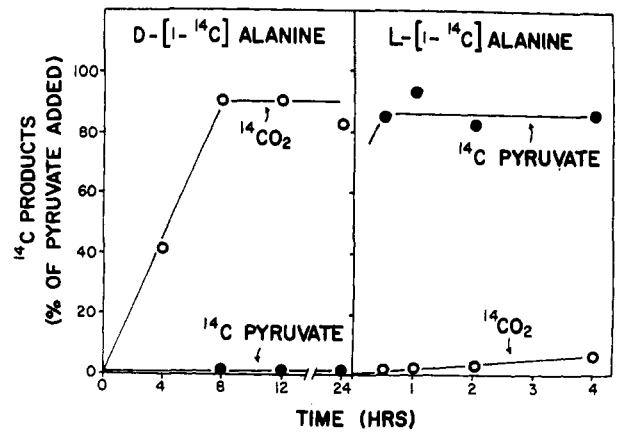


FIGURE 1: Reaction of D- and L-alanine with pyruvate catalyzed by α-DAT. Each reaction mixture contained 10 μmoles of amino acid (3×10^4 cpm/μmole of D-[1-^{14}C]alanine or 2.5×10^4 cpm/μmole of L-[1-^{14}C]alanine), 2 μmoles of pyruvate, 50 mμmoles of PLP, and 50 μg of enzyme in 0.5 ml of 50 mM potassium phosphate, pH 7.5. Separate reactions were stopped at the times indicated and analyzed for radioactive products as described in the Experimental Section.

aldimine of D- rather than L-alanine with the carboxylate group now positioned in the bond cleavage site (b, Scheme II). Reaction 1 could then occur, freeing [^{14}C]CO_2. Whether or not this explanation is correct, it is clear that α-DAT exhibits strong preference for cleavage of the α-hydrogen of L-alanine.

A precise comparison of the kinetics of transamination of D- and L-alanine was not conducted. However, it is clear from Figure 1 and our previous results (Bailey and Dempsey, 1967) that reaction with the less common stereomer is much slower than with L-alanine and certainly of no physiological significance.

Transamination of Analogs of D- *and* L-*Alanine.* The ability of α-DAT to catalyze transamination of amino acids which may be considered to be structural hybrids of D- and L-alanine was tested using nonradioactive amino acids as substrates with [^{14}C]pyruvate. The compounds tested and the amount of ^{14}C converted in each reaction into a form which could be retained by the cation-exchange resin is shown in Table I.

Only α-methyl α-aminomalonate stimulated enzyme-catalyzed [^{14}C]pyruvate transamination. The radioactive product in this reaction was verified as [^{14}C]alanine by thin-layer chromatography of reaction mixtures after incubation. A radioactive, ninhydrin-sensitive spot with the color and R_F of alanine appeared only on chromatograms of the α-methyl α-aminomalonate plus enzyme reaction.

Under the conditions of these assays both α-aminomalonate and α-methyl α-aminomalonate reacted nonenzymatically with PLP. This was first evidenced by bleaching of the yellow color of the added cofactor in reactions containing these amino acids. Furthermore, ninhydrin-sensitive spots, with the color and R_F value of PMP, appeared on the chromatograms of all reactions containing either of the aminomalonates, and glycine was evident on chromatograms of the α-aminomalonate-containing solutions. There was, however, no evidence of nonenzymatic decarboxylation of α-methyl α-aminomalonate to alanine. When spectral changes of solutions of either α-aminomalonate or α-methyl α-aminomalonate (50 mM) and PLP (0.2 mM) in potassium phosphate

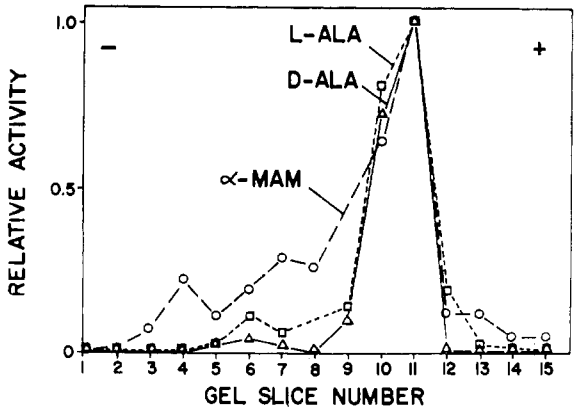


FIGURE 2: Localization of transaminase activity after electrophoresis in polyacrylamide gels. Disc electrophoresis was accomplished at pH 9.0 using the method of Clark (1964). Samples containing 100 μg of 75% purified α-DAT in 0.2 ml of 10% sucrose were applied to the top of replicate gels and subjected to a current of 5 mA/gel until the tracking dye reached the anodic end of each gel. For enzyme assays gels were cut into 1-mm slices, and these added directly to appropriate incubation mixtures. Conversion of L-alanine into pyruvate and release of CO_2 from D-alanine was measured as described in Figure 1. Transamination of α-methyl α-aminomalonate was determined as described in Table I. Several unsliced gels were stained to reveal the protein bands.

(50 mM, pH 7.5) were followed at room temperature, the PLP absorption at 390 nm disappeared completely and was replaced by a peak at approximately 325 nm. These spectral changes and the chromatogram patterns suggested some nonenzymatic decarboxylation-dependent transamination of PLP to PMP by the reactive dicarboxylic amino acids.[2] A similar nonenzymatic reaction was observed by Kalyankar and Snell (1962) to occur between pyridoxal and AIB.

The generation of PMP in the reaction of α-methyl α-aminomalonate with PLP introduced the possibility that the enzyme-catalyzed transamination of [^{14}C]pyruvate (Table I) arose *via* nonenzymatic transamination of PLP by α-methyl α-aminomalonate followed by enzyme-mediated transamination of pyruvate by PMP—a sequence not requiring binding of the dicarboxylicamino acid in the enzyme active site. We have shown (Bailey *et al.*, 1970) that α-DAT will catalyze transamination of free PLP to PMP in the presence of a suitable amino donor. This reaction, however, appeared to be essentially irreversible since additions of high levels of pyruvate to reaction solutions containing PMP and α-DAT caused no change in cofactor absorption (Vuttivej, 1969). Furthermore, when PMP was used as amino substrate with [^{14}C]pyruvate following the conditions of Table I no [^{14}C]alanine production was detected. It was concluded that α-DAT-catalyzed conversion of [^{14}C]pyruvate into [^{14}C]alanine by α-methyl α-aminomalonate required transamination of the cofactor to the amine form within the enzyme active site. The complete stoichiometry of the enzyme-catalyzed transamination of α-methyl α-aminomalonate was not determined; however, the reaction must have involved decarboxylation of the amino acid yielding CO_2 and pyruvate as the carbon products.

Slightly less than 2% of the radioactive pyruvate was converted into a form bound by Dowex 50 in both the nonenzymatic and enzymatic reactions with α-aminomalonate. No attempt was made to determine the chemical nature of this product(s), since the reaction was apparently irrelevant to α-DAT catalysis.

Finally, as seen in Table I, there was no evidence of α-DAT-catalyzed transamination of [^{14}C]pyruvate when either glycine, lacking an alkyl side chain, or isopropylamine, lacking an α-carboxyl group, were tested as amino donors.

Coincidence of Enzyme Activity in Polyacrylamide Gels after Electrophoresis. The rate of transamination of D-alanine and α-methyl α-aminomalonate by the purified enzyme preparation was slow compared to the rate with either AIB or L-alanine. For this reason we considered it important to confirm that α-dialkylamino acid transaminase, and not a trace protein contaminant, was the catalyst of each of these reactions. This was accomplished by disc electrophoresis of a 75% purified enzyme preparation through polyacrylamide and localization of the transaminase activity with each amino acid substrate in sliced gels. After the run, replicate gels were

TABLE I: Transamination of Analogs of D- and L-Alanine with [^{14}C]Pyruvate Catalyzed by α-DAT.[a]

Amino Donor	^{14}C Retained by Dowex 50 (net cpm)	[^{14}C]Alanine Detected by Thin-Layer Chromatography
α-Methyl α-aminomalonate		
+ Native enzyme	3145	+
+ Heat-denatured enzyme	25	
α-Aminomalonate		
+ Enzyme	520	
− Enzyme	495	
Glycine		
+ Enzyme	25	
Isopropylamine		
+ Enzyme	30	

[a] Each reaction mixture contained the amino donor indicated (10 μmoles), [1-^{14}C]pyruvate (2 μmoles, 1.5 × 10^4 cpm/μmole), PLP (0.1 μmole) and, when indicated, α-DAT (60 μg) in 0.13 ml of 50 mM potassium phosphate, pH 7.5. After 4-hr incubation at 30°, each reaction was added to the top of a 1 × 2 cm column of Dowex 50 W-X4 (H^+). Unbound ^{14}C was washed from the resin with 10 ml of water; bound ^{14}C was then eluted directly into a liquid scintillation vial with 4 ml of 5 M NH_4OH. The solvent was evaporated at 100°, and the residue dissolved in 0.1 ml of water followed by 10 ml of scintillation fluid. For thin-layer chromatography, 25-μl samples of separate, identical reactions were used. Developed plates were either sprayed with ninhydrin or analyzed for radioactivity by the method of Snyder (1964).

[2] Thanassi (1970) has studied the nonenzymatic reactions of α-aminomalonate with deoxypyridoxal and PLP under different conditions than used here. A condensation product, "β-5-deoxypyridoxylserine," predominated in the reaction with deoxypyridoxal, and an analogous substance was formed in the reaction with PLP. Only traces of PMP were detected. The condensation reaction proposed by this author would not be possible with α-methyl α-aminomalonate, which lacks an α-hydrogen.

sliced into 1-mm segments and these used directly as a source of enzyme in incubation mixtures. The peaks of transaminase activity with all substrates coincided with each other (Figure 2) and with the major protein band in stained gels.

Competitive Inhibition of α-DAT by D- *and* L-*Cycloserine.* We predicted that reactivity of both D- and L-alanine should be paralleled by inhibition of α-DAT by both stereomers of the conformationally related compound, cycloserine. In preliminary experiments, a Technical grade of L-cycloserine was employed, and was found to inhibit α-DAT more effectively than does D-cycloserine. To eliminate the possibility that this strong inhibition was due to an impurity, a purer grade of DL-cycloserine was also tested. If the difference observed with the resolved stereomers was real, inhibition by D-cycloserine, in the low concentration of the racemate used, could be ignored. DL-Cycloserine showed the same strong inhibition as the resolved L form. Double-reciprocal plots of the kinetics of α-DAT-catalyzed transamination of AIB with pyruvate, using D- and DL-cycloserine as inhibitors are shown in Figure 3. It is noteworthy that although, in agreement with the prediction, both enantiomers were competitive inhibitors of AIB binding in the α-DAT active site, the K_I for L-cycloserine, calculated from its concentration in the racemic mixture, was 2.5 μM, whereas that for D-cycloserine was 8 mM.

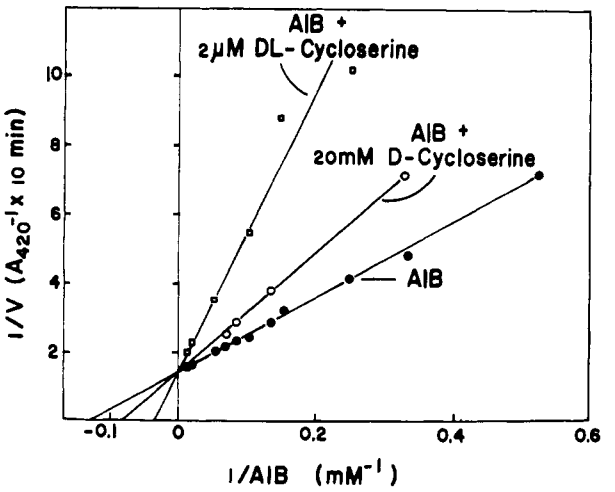


FIGURE 3: Kinetics of inhibition of α-DAT by D- and DL-cycloserine. Reactions contained 20 mM pyruvate, 0.1 mM PLP, 50 mM potassium phosphate, 10 μg/ml of α-DAT and AIB, or AIB plus cycloserine, as indicated. Acetone production was measured as described by Bailey and Dempsey (1967).

Discussion

The results reported here support the conclusion that there is but a single orientation within the α-DAT active center in which bonds to the substrate α-carbon are activated for cleavage. If it were otherwise, some loss of the D-alanine α-hydrogen or more significant loss of the L-alanine α-carboxylate should have occurred during reaction of these substrates. When D-alanine is substrate, the α-carboxylate is oriented in this subsite, and decarboxylation precedes transamination; when L-alanine reacts, its α-hydrogen is located in this position and is removed. As pointed out earlier, the assumption of a single cleavage site insists that there be two distinct regions within the α-DAT active center which may be occupied by α-carboxylate groups. The reactivity observed with α-methyl α-aminomalonate supports this.

Bailey *et al.* (1970) have recently demonstrated that the stereospecificity of proton addition to the 4′-carbon of PLP during α-DAT-catalyzed transamination is the same whether reaction proceeds *via* decarboxylation or removal of the substrate α-hydrogen. Although not restrictive, this finding is consistent with orientation of the labilized bond in the same relative position for all substrates.

Positioning of the substrate amino group is fixed by aldimine formation with PLP. The geometry about the α-carbon to nitrogen bond in this complex must be directed by site-specific binding of one or more of the remaining groups attached to the α-carbon. Since our results argue that the α-carboxylate and α-hydrogen of the alanine enantiomers occupy opposite sites when these substrates are bound, the conformation directing role must be fulfilled by the side-chain methyl. Only this group would be oriented in the same relative position when either substrate reacted (Scheme II). Furthermore, all other amino acids which have been found to be substrates for α-DAT possess a methyl or methylene group in this position (Bailey and Dempsey, 1967; Bailey *et al.*, 1968). Glycine and α-aminomalonate were unreactive; thus it appears that the carbon side chain not only directs substrate conformation during binding but is essential for subsequent reaction. This raises interesting, deeper questions concerning the conformational control of PLP enzyme reactivity, but these cannot yet be answered.

The finding of competitive inhibition by both enantiomers of cycloserine agrees with our view of substrate binding to α-DAT. Furthermore, the remarkable difference in the ability of D- and L-cycloserine to inhibit can also be related to conformational control of reactivity. After aldimine formation, site-specific binding of the ring methylene would direct the α-hydrogen of L-cycloserine to the activated position. Similar binding of D-cycloserine would place the carbonyl of the isoxazolidinone ring in this position. The free α-hydrogen, but not the ring-bound carbonyl, could undergo cleavage. Such further reaction by L-cycloserine may have produced a more stable cofactor–inhibitor complex within the active site and thereby enhanced inhibition. Khomutov *et al.* (1968) observed a similar difference in the ability of cycloserine enantiomers to inhibit an L-alanine–oxaloacetate transaminase. This they related to the stereospecificity of the enzyme but not to a possible conformational effect on the reactivity of the more potent L-cycloserine.

In conclusion, our findings agree with the hypothesis that site-specific binding of one of the substituents attached to the substrate α-carbon directs the bond to be cleaved during initiation of a PLP enzyme catalyzed reaction to a single, activated position. To this extent the proposals of Dunathan (1966) are supported. In α-DAT the directing group appears to be the amino acid side chain. The results do not establish that the activated orientation is in a plane perpendicular to the cofactor ring plane, as suggested by Dunathan, but the obvious energetic advantages of this conformation make it the most probable.

Acknowledgment

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Copper(II) and Manganese(II) Effects on Ribonuclease A Activity*

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ABSTRACT: This work attempts to delineate mechanisms for the metal activation and inhibition observed in enzyme–substrate hydrolysis by two approaches: (1) direct investigation of the effects of metal ions on the ribonuclease A activity utilizing the substrates, ribonucleic acid and cyclic 2′–3′-cytidine monophosphate; and (2) binding studies of both metal–enzyme and metal–substrate interactions. The assay indicates that metal activation is substrate dependent (activation with RNA, none with cyclic 2′–3′-CMP) while the binding studies show the strongest metal binding sites are the phosphate groups on tRNA. Such data indicate that metal activation of the hydrolysis is a substrate phenomenon occurring only when binding of the metal ion to the phosphate groups on the substrate is pronounced. In contrast, metal inhibition of the reaction appears to be an enzyme phenomenon occurring when metal concentrations are sufficient to bind to the active sites of the enzyme. Thus, in the case of the hydrolysis of ribonucleic acid by ribonuclease, metal ions first activate by binding to the phosphates on RNA; and then with increasing metal concentration, inhibit as the active sites of RNase A are blocked. With cyclic 2′–3′-CMP, no activation is observed since the metal binds weakly with the single phosphate; but inhibition is observed for both Cu^{2+} and Mn^{2+} ions. Manganese(II) concentrations necessary for inhibition are about 100 times larger than copper(II) concentrations as a result of similar variations in their binding constants to ribonuclease A.

The general inhibition of ribonuclease A by divalent metal ions is well established (Anfinsen and White, 1961; Crestfield *et al.*, 1963; Ukita *et al.*, 1964; Glitz and Dekker, 1964; Takahashi *et al.*, 1967; Eichhorn *et al.*, 1969) and has been ascribed to the binding of metal ions to the essential histidine-12 and -119. However, there are contradictions in the literature regarding which divalent metal ions inhibit and whether or not activation also occurs. The resolution of such contradictions is difficult because the varying substrates, metal ions, experimental conditions, and reactions used by the various authors make cross-correlation of their work impossible. To demonstrate this, we will abstract the results of two recent papers. Takahashi *et al.* (1967) demonstrated that Cu^{2+}, Zn^{2+}, and Hg^{2+} ions had an inhibiting effect on the ribonuclease A hydrolysis while Mg^{2+}, Ca^{2+}, and Mn^{2+} were found to have little effect. No activation was observed. The substrates used were cyclic 2′–3′-cytidine monophosphate (cyclic 2′–3′-CMP) and benzylcytidine 3′-phosphate, while the experimental conditions were pH 7.0, $\mu = 0.1$, $T = 37°$, and metal concentrations from 10^{-4} to 10^{-3} M. On the other hand, when RNA was used as the substrate, Eichhorn *et al.* (1969) demonstrated that metal concentrations of about 10^{-4} M activated the hydrolysis at pH 5. The metal ions used were Cu^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+},

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