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Chiral Discrimination among Aminotransferases: Inactivation by 4-Amino-4,5-dihydrothiophenecarboxylic Acid

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ABSTRACT: Mechanism-based inhibitors such as cycloserine and gabaculine can inactivate aminotransferases via reactions of the compounds with the pyridoxal phosphate cofactor forming an irreversible adduct. The reaction is chirally specific in that any one enzyme usually only recognizes one enantiomer of the inactivator. For instance, L-aspartate aminotransferase (L-AspAT) is inactivated by 4-amino-4,5-dihydro-2-thiophenecarboxylic acid (ADTA), however, only by the *S*-isomer. We have now shown that D-amino acid aminotransferase (D-a-AT) is irreversibly inactivated by the *R*-isomer of the same compound. The X-ray crystal structure (PDB code: 3LQS) of the inactivated enzyme shows that in the product the enzyme no longer makes a Schiff base linkage to the pyridoxal 5′-phosphate (PLP) cofactor, and instead the compound has formed a derivative of the cofactor. The adduct is similar to that formed between D-cycloserine and D-a-AT or alanine racemase (Ala-Rac) in that the thiophene ring of *R*-ADTA is intact and seems to be aromatic. The plane of the ring is rotated by nearly 90° with respect to the plane of the pyridine ring of the cofactor, in comparison with the enzyme inactivated by cycloserine. Based on the structure of the product, the mechanism of inactivation most probably involves a transamination followed by aromatization to form an aromatic thiophene ring.

Amino acid aminotransferases are pyridoxal 5′-phosphate (PLP)[1] dependent enzymes responsible for the conversion of a variety of amino acids to the corresponding keto acids. In order to regenerate the pyridoxal form of the enzyme, the reverse reaction utilizes keto acids and produces the equivalent amino acids. In general, all aminotransferases are known to discriminate between L- or D-amino acids as substrates in one direction or as products in the other direction. Structurally they are distinctive in that those that are specific for L-amino acids usually belong to fold type I, and those that are specific for D-amino acids belong to fold type IV (B. Lepore, Thesis, 2004). An exception is the fold type III alanine racemase (Ala-Rac), where transamination is observed as a side reaction, leading to inactivation in the presence of the inhibitor cycloserine. An exception to the chirality rule is a remarkable set of structures belonging to two families of aminotransferases that have the same fold type (IV), but one is specific for D-amino acids (D-amino acid aminotransferase; D-a-AT) (*1*, *2*) and the other for L-amino acids (branched chain aminotransferase; BCAT) (*3*).

A number of naturally occurring chiral inhibitors are known to inactivate transaminases, although they are not highly specific. For instance, gabaculine is a dihydrobenzene antibiotic produced by various *Streptomyces* species that is known to inactivate γ-aminobutyrate aminotransferase (GABA-AT) (*4*). Possibly because of its lack of specificity, it is too toxic to be useful as a pharmaceutical (*5*). Cycloserine is a dihydroisoxazole amino acid that is also produced by various *Streptomyces* species (*6*). It has also been shown to inactivate a number of PLP-dependent enzymes. Cycloserine is used to treat schizophrenic patients and infection by *Mycobacterium tuberculosis*. However, patients can experience toxic side effects (*7*). Nonetheless, gabaculine and cycloserine are effective enzyme inhibitors, so they are templates for development of compounds for the inactivation of specific PLP-containing enzymes.

Originally, inactivation by gabaculine or cycloserine was expected to occur by derivatization of a group on the enzyme after formation of an external aldimine with the cofactor and ring opening. Mechanistic studies and crystal structures have shown that gabaculine reacts with γ-aminobutyrate aminotransferase (GABA-AT) (*8*, *9*) and ornithine aminotransferase (O-AT) (*10*); cycloserine reacts with GABA-AT (*11*), D-a-AT (*12*), and alanine racemase (*13*). Both compounds inactivate these PLP-dependent enzymes by a mechanism that involves the first steps in transamination but does not involve reaction with a side chain group on the enzyme (Table 1, Figures 1 and 2). For instance, D-a-AT from *Bacillus stearothermophilus* is rapidly inactivated by D-cycloserine as a result of formation of a stable dihydroisoxazole adduct with the cofactor after tautomerization, followed by deprotonation to form an aromatic ring (*14*) (Figure 2). Cycloserine is thus a mechanism-based inactivator and can be considered a paradigm for development of similar compounds to inactivate PLP-dependent enzymes, particularly if specificity can be incorporated into the structure of the compound.

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[1]Abbreviations: CS, cycloserine; *R*-ADTA, (*R*)-4-amino-4,5-dihydro-2-thiophenecarboxylic acid; *S*-ADFA, (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid; PLP, pyridoxal 5′-phosphate; GABA-AT, γ-aminobutyric acid aminotransferase; D-a-AT, D-amino acid aminotransferase; L-AspAT, L-aspartate aminotransferase; Ala-Rac, alanine racemase; BCAT, branched chain aminotransferase; O-AT, ornithine aminotransferase; PPDA, *N*-(5′-phosphopyridoxyl)-D-alanine.

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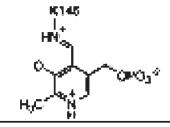
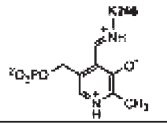
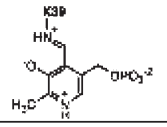
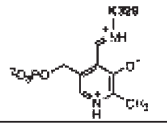
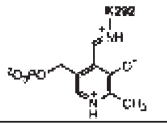
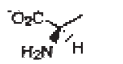
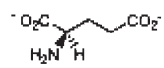
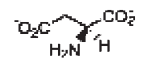
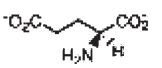
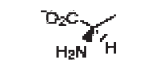
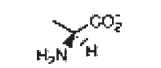
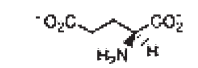
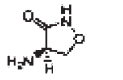
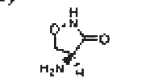
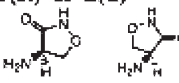
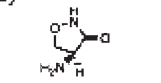
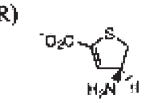
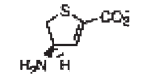
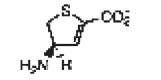
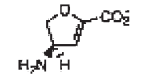
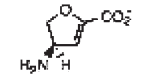
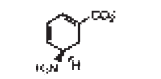
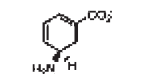
Table 1: Stereochemistry of Inactivators of PLP-Dependent Enzymes[a]

Enzyme	D-a-AT	L-Asp-AT	Ala-Rac	GABA-AT	O-AT
					
Amino acids Substrates	D(R)-ala & D(R)-glu  & 	L(S)-asp & L(S)-glu  & 	D(R)-ala & L(S)-ala  & 	GABA & L(S)-glu  & 	L(S)-orn & L(S)-glu  & 
CS Isomers	D(R) 	L(S) 	D(R) & L(S) 	L(S) 	
Product	PLP-adduct[a]	NA	PLP-adduct[b]	PLP-adduct[c]	NA
ADTA Isomers	(R) 	(S) 		(S) 	
Product	PLP-adduct	K246-adduct & PLP-adduct[d]	NA	PLP-adduct[e]	NA
ADFA Isomers		(S) 		(S) 	
Product	NA	K246-adduct[f]	NA	PLP-adduct[f]	NA
Gabaculine Isomers				(S) 	(S) 
Product	NA	NA	NA	PLP-Product[g]	PLP-Product[h]

[a]The cofactor is drawn as the internal aldimine from the perspective of the substrate coming into the active site of the enzyme. The substrate/inactivator is drawn from a view representing the expected direction in which it would interact with the cofactor on the enzyme in order to optimize the proton abstraction catalyzed by the active site lysine during the aldimine−ketimine rearrangement. These conformations are taken from structures of known complexes with inhibitors. The substrates for the forward and reverse reaction are given. For the inactivators, the isomer and inactivation product (when known) are given. References: (a) Peisach et al., 1998b; (b) Fenn et al., 2003; (c) Olson et al., 1998; (d) Liu et al., 2007a; (e) Fu et al., 1999; (f) private communication; (g) Rando, 1977; (h) Shah et al., 1997; (i) Fu and Silverman, 2004.

The compound 4-amino-4,5-dihydro-2-thiophenecarboxylic acid (ADTA) is such an analogue of cycloserine, in which the dihydroisoxazole ring has been replaced by a dihydrothiophene ring (*15*). Because of their chemical similarity, it was expected that ADTA could also use the tautomerization and deprotonation mechanism observed with cycloserine to become covalently attached to the cofactor of the enzyme. The *S*-isomer was shown to inactivate GABA-AT by forming a ring-closed, aromatic cofactor derivative on the enzyme (*15*, *16*). The reaction actually forms at least two products: 0.7 equiv of enzyme undergoes normal transamination for every equivalent of inactivation. It has been shown structurally with the *Escherichia coli* aspartate aminotransferase (L-AspAT) that inactivation by *S*-ADTA can form two types of aromatic adducts, one with the cofactor and one with the active site lysine, which are mutually exclusive (*17*) (Figure 3). Although a mixture of both products is observed at all pH values tried (range from pH 6 to pH 8) (*17*), the PLP adduct is dominant at low pH, and the lysine adduct is dominant at higher pH. Therefore, if ADTA reacts the same way with D-a-AT as it does with GABA-AT and L-AspAT, the product of inactivation of D-a-AT may involve two adducts depending on the pH at which the inactivation occurs.

Based on the chiral preference for substrates, it is expected that the reactivity of the ADTA molecule for an enzyme that is specific for D-amino acids, such as D-aAT, will be specific for only one enantiomer of the inactivator. D-a-AT is the enzyme responsible for the conversion of a variety of α-keto acids into the D-amino acids (mainly aspartate, glutamate, and alanine) required for bacterial cell wall synthesis. The chirality of an enantiomer of ADTA can be related to that of the amino acid substrate that is recognized by any one enzyme. Thus, since the *S*-isomer is specific for enzymes that recognize L-amino acids, the *R*-isomer should be specific for an enzyme that recognizes D-amino acids. The same is true for cycloserine, where the L-(*S*)-isomer is specific

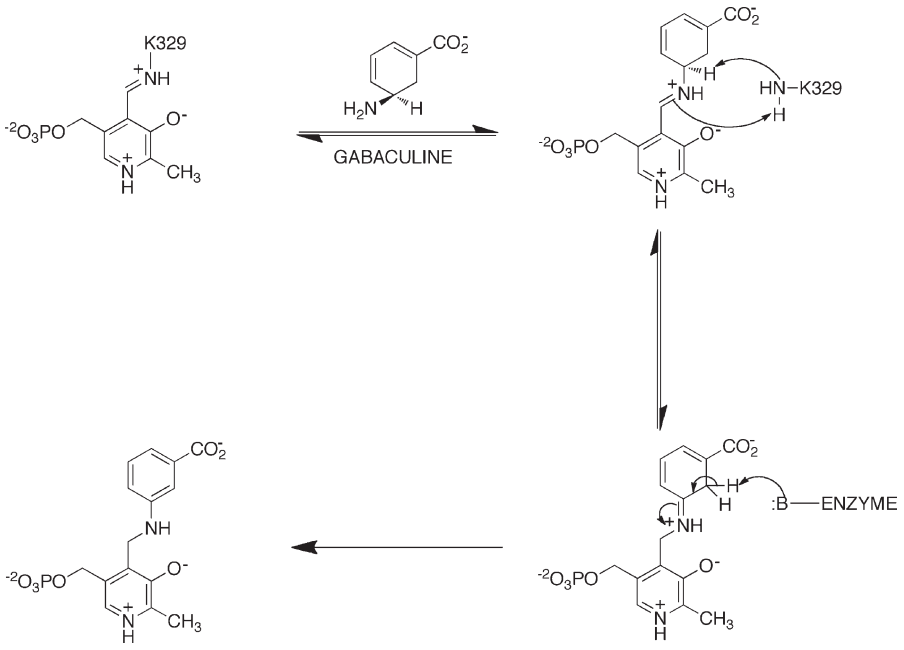


FIGURE 1: Mechanism of inactivation of GABA-AT by gabaculine (Fu and Silverman, 1999; Shah et al., 1997).

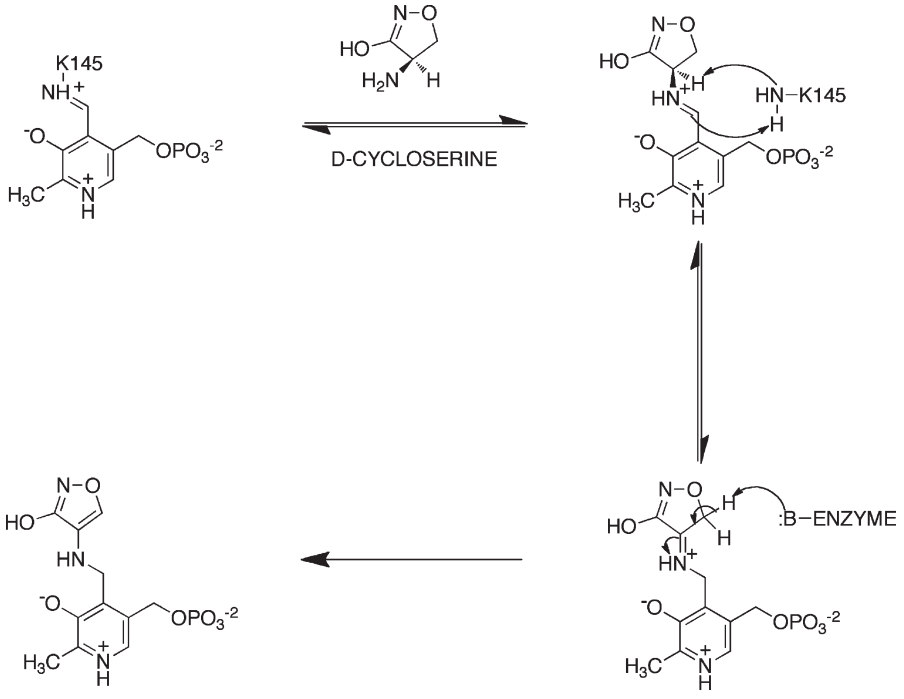


FIGURE 2: Mechanism of inactivation of D-a-AT by cycloserine (Peisach et al., 1998b).

for enzymes recognizing L-amino acids and the D-(*R*)-isomer is specific for D-a-AT (Table 1). So far, this principle has been true for ADTA. GABA-AT is inactivated irreversibly by the natural product gabaculine (*R*-isomer) (*4*, *16*) and by *S*-ADTA (*9*, *18*) both in vitro and in vivo but not by *R*-ADTA. The chirality of *R*-gabaculine and *S*-ADTA can be related to the L-(*S*)-isomer of glutamate, a substrate for the enzyme. The same is true of cycloserine where the *S*-isomer (related to L-glutamate) inactivates GABA-AT (*11*). By comparison, compounds related to D-(usually *R*)-isomers of amino acids should inactivate PLP-dependent enzymes that are specific for these amino acid isomers. One example is the inactivation of D-a-AT by D-cycloserine (*12*). It is therefore expected that *R*-ADTA, which is stereochemically related to the D-isomer of most amino acids and D-cycloserine, would inactivate D-a-AT specifically and irreversibly. The crystal structure of the product, reported here, shows that this expectation has been met, although only one product is observed: the product of inactivation of D-a-AT by *R*-ADTA is the aromatized cofactor adduct of *R*-ADTA and PLP.

MATERIALS AND METHODS

Purification, Crystallization, and Data Collection. Wild-type D-amino acid aminotransferase was purified as described (*19*). Crystals were grown by the hanging drop method based on the protocol previously described (*20*). The enzyme was dialyzed in 0.2 M potassium phosphate buffer, pH 7.2, concentrated

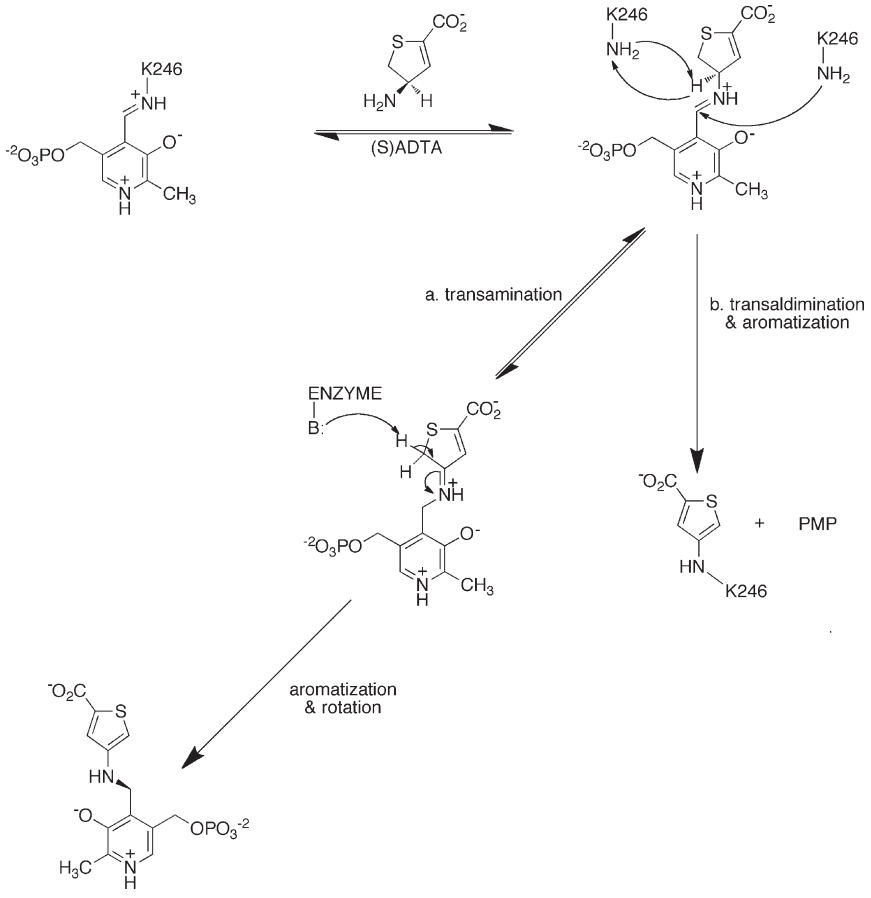


FIGURE 3: Mechanism of inactivation of Asp-AT by *S*-ADTA (Liu et al., 2007).

to 40 mg/mL, and incubated with an *R*-ADTA:protein molecular ratio of 200:1 prior to crystallization. The enzyme solution was mixed with crystallization buffer at pH 8.5, incubated initially at 30−40 °C, and then allowed to return to room temperature. Pale yellow crystals were formed within 1 day and were allowed to grow 2−3 days longer to a size of 0.3 × 0.3 × 0.1 mm. The crystal used for data collection had the symmetry of the orthorhombic space group *P*212121 (a = 77.0 Å, b = 90.7 Å, c = 88.9 Å) and had two molecules in the asymmetric unit, giving a solvent content of 51.8% and a Matthews coefficient of 2.55 $Å^3$ Da^{-1} (Table 2). Data were collected at 4 °C with a scan width of 1 deg per frame and an exposure time of 30 min per frame on a RAXIS-IV image plate detector mounted on a Rigaku RU-300B X-ray generator operating at 36 kV and 26 mA. The data were collected from a single crystal and were 97.5% complete to 1.9 Å resolution.

Solution of the Crystal Structure. Data frames were integrated with Denzo and scaled with Scalepack (*21*). The structure was solved by applying phases from a crystal structure of D-a-AT solved from a crystal with *P*212121 symmetry directly to the data (PDB ID 3DAA (*14*)). Strict 2-fold noncrystallographic symmetry (NCS) was applied, resulting in *R* factors that dropped to nearly 30% after rigid body minimization with data extending to 3.0 Å resolution.

Electron density maps (sigma-A weighted $2mF_o - DF_c$) calculated with the model including the PLP cofactor showed clearly that a cofactor derivative not covalently attached to the protein was contained in the crystal. The electron density observed around the region of the pyridinium ring of the cofactor extends beyond carbon C4A, which is the location where amino acids are known to form a Schiff base linkage with the cofactor and thus was continuous from the pyridinium ring to the added derivative. Both the pyridinium ring and the added derivative due to the inactivation are planar, indicating an aromatic product resulted from the inactivation. In addition, the plane formed by the pyridinium ring was angled approximately 15 deg outward from the position of the internal aldimine (in the native enzyme), toward the solvent face of the active site. Further refinement included simulated annealing, energy minimization, and restrained *B*-factor (grouped and individual) minimization, interspersed with manual refitting of the model into electron density maps using O (*22*). The process was repeated with the 2-fold NCS constraints released. At this stage, various cofactor derivatives were built and evaluated based on how well they fitted into electron density maps that were calculated with the modeled derivatives included in the calculation. The best fit and lowest *R*factor value were obtained with the simplest cofactor derivative model. After placement of 10−20 waters, weak difference electron density was still evident that could be modeled as an acetate moiety in the carboxylate binding pocket of the active site, and a model of acetate was included in refinement. Finally, more waters were placed into difference electron density maps contoured at the 2σ level, refined using CNS, and evaluated based on difference electron density quality with DDQ (*23*). The final model had an *R*factor of 20% and *R*free of 25% (Table 2).

Enzyme Activity. D-Amino acid aminotransferase activity was measured using a coupled assay as described previously (*19*). Inhibitor and enzyme were incubated in a ratio of 20:1, and aliquots of the incubation mixture were assayed over time at room temperature. α-Ketoglutarate was not included in the inactivation mixture. The reaction mixture contained 10 units

of lactate dehydrogenase, 0.2 M D-alanine, 0.1 M NADH, and 25 mM α-ketoglutarate in 0.1 M K-EPPS buffer at pH 8.1. The loss of absorbance of NADH was monitored at 338 nm as lactate is produced from pyruvate (derived from alanine). One unit of D-a-AT activity is defined as the amount needed for conversion of 1 μmol of NADH to NAD^+ per minute.

Enzyme Inactivation. Inactivation of the enzyme was followed by monitoring the decrease in absorbance at 420 nm, indicative of loss of the internal aldimine, and by measuring residual enzymatic activity. The loss of the absorbance intensity of D-a-AT at 420 nm (and appearance of an absorbance signal at 330 nm) occurs over approximately 30 min when the enzyme is incubated with 10 mM *R*-ADTA at room temperature. This change in absorbance and shift in absorbance maximum are also observed when D-cycloserine reacts with the enzyme and are typical for the conversion of the PLP form to the PMP or ketimine forms of the enzyme. The decrease of absorbance at 420 nm is initially faster than inactivation (not shown), indicating that *R*-ADTA may be turned over by the enzyme as well as inactivating it. The active form of the enzyme may then have been regenerated from the PMP form of the enzyme by the α-ketoglutarate of the assay buffer.

Table 2: Data Collection and Refinement Statistics

PDB code	3LQS
space group	*P*212121
unit cell parameters *a*, *b*, *c* (Å)	77.0, 90.7, 88.9
resolution range (highest resolution shell) (Å)	63.5−1.9
no. of reflections (unique)	46241
no. of reflections (test)	3762 (7.6%)
$(I)/\sigma(I)$ (overall/1.97−1.90 Å resolution shell)	8.5/1.3
completeness (%) (overall/highest resolution shell)	97/95.0
average multiplicity	7.0
*R*merge[a] (%) (overall/highest resolution shell)	8.6/56.0
*R*factor (*R*free)[b] (%)	16.2 (19.6)
no. of protein residues	560
no. of cofactor derivative atoms	48
no. of water molecules	330
average *B*-factor	23.1
rmsd bond (Å)	0.014
rmsd angle (deg)	1.8

[a] $R\text{merge} = \sum|I\text{obs} - I\text{avg}|/\sum I\text{avg}$. [b] $R\text{factor} = \sum|F_o - F_c|/\sum F_c$.

RESULTS AND DISCUSSION

Inactivation of Aminotransferases by ADTA. The 4-amino-4,5-dihydro-2-thiophenecarboxylic acid (ADTA) was designed to take advantage of the similarity of the compound to gabaculine or cycloserine to inactivate aminotransferases. It has previously been shown that the *S*-isomer inactivates GABA-AT (*18*) and L-AspAT (*17*). The *R*-isomer, which does not inactivate these two enzymes, does inactivate D-a-AT. The difference in reactivity between the two isomers is related to the chiral preference of the enzymes for amino acid substrates.

Both GABA-AT and L-AspAT are specific for L-amino acids and only react with the *S*-isomer of the inactivator (Table 1). For instance, GABA-AT was shown chemically to be inactivated by formation of an aromatic thiophene adduct of the cofactor (*9*). Since complete inactivation required 1.7 equiv of inactivator, the interpretation was that the ketimine adduct of the inactivator, before tautomerization, can partition to form PMP by hydrolysis. Inactivation of L-AspAT by *S*-ADTA is more complicated. Crystallographic evidence shows that the inactivator produces two types of adducts (*17*). Both are aromatic adducts, but in one case the adduct forms with the cofactor; in the other, the adduct forms with the side chain of the active site lysine. The distribution of the two adducts is pH dependent, with the lysine adduct being favored at high pH (pH 8) and the PLP adduct being favored at low pH (pH 6). The two adducts also show a remarkable difference in recognition for the enzyme: the carboxylate group

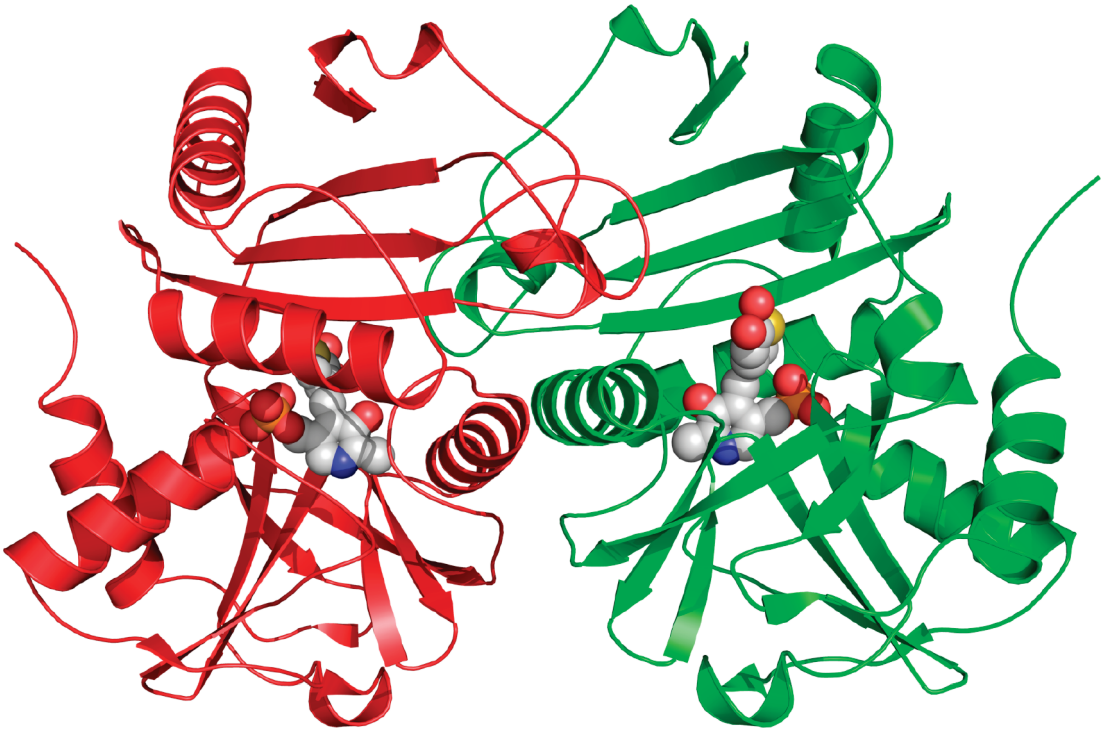


FIGURE 4: Overall structure of D-a-AT. The active site is located between two subunits of the dimer such that each active site has residues from both. The PLP−*R*-ADTA adduct is shown in space-filled form in each active site.

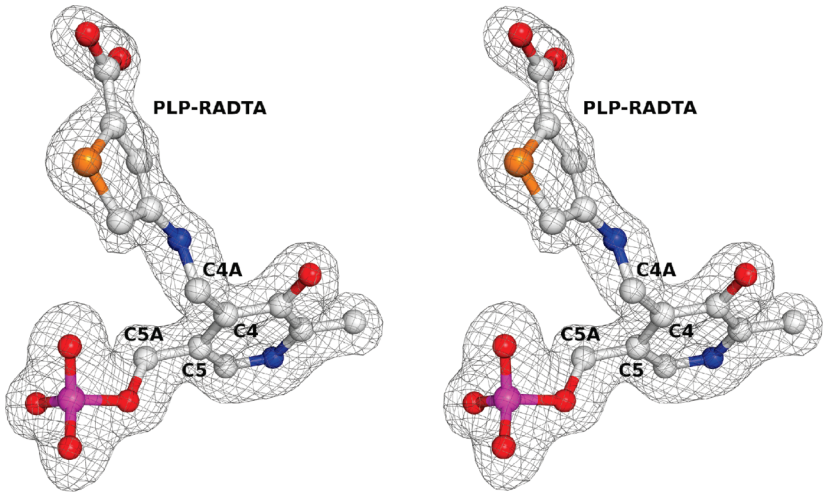


FIGURE 5: Stereoview of electron density map (with coefficients $2F_o - F_c$, drawn at the 1σ level) in the active site showing the *R*-ADTA adduct to the cofactor of D-a-AT.

of the thiophene moiety of the PLP adduct interacts with the arginine that normally interacts with the α-carboxylate of the substrate; the carboxylate group of the thiophene moiety of the lysine adduct interacts with the arginine that normally interacts with the side chain carboxylate of the substrate.

D-a-AT, the enzyme responsible for the conversion of a variety of α-keto acids into the D-amino acids (mainly aspartate, glutamate, and alanine) required for bacterial cell wall synthesis, reacts with the *R*-isomer of the inactivator. The enzyme is inactivated with a second-order rate constant of 5.0×10^{-3} M^{-1} min^{-1}, measured as change in activity. The second-order constant k was calculated via the equation $R_{inactivation} = k$[enyme][inhibitior]. Inactivation is not as efficient as is observed with the reaction of L-AspAT with *S*-ADTA (1.5×10^{-2} M^{-1} min^{-1}, measured as change in activity). It should be noted that L-AspAT is a more efficient enzyme than D-a-AT based on second-order rate constants for the substrate in the forward direction at pH 7.5 (k_{cat}/K_m = 9.1×10^4 M^{-1} s^{-1} for L-aspartate (*24*) and 2.7×10^3 M^{-1} s^{-1} for D-alanine (*25*), respectively). The implication is that the active site residues can orient the substrate in the active site such that catalysis can occur more readily in the L-AspAT case than the D-a-AT case and that inactivation can occur more readily for L-AspAT than for D-a-AT. This analysis could also provide an argument that the active site lysine is in a better position for the transaldimination of an intermediate on the pathway leading to L-AspAT inactivation, giving a mixture of the PLP and K258 adducts (Figure 3) (Liu et al., 2007). Since the lysine adduct is not observed with D-a-AT (see below), it would seem this side reaction does not occur as readily. Both enzymes carry out the same reaction albeit on opposite isomers of their respective substrates. It is possible that the difference in observed products is related to detailed differences in the inactivation mechanisms of these two enzymes (Figures 3 and 9).

Overall structure of the inactivated enzyme. The overall structure of the dimeric D-a-AT protein is the same as that determined previously in the presence or absence of inhibitors (Figure 4). The rms deviation of the protein from the native enzyme is 0.47 Å [2], with most differences occurring in the active site region. There are two molecules of the enzyme in the asymmetric unit, essentially identical to each other (rmsd 0.31 Å^2).

The active site of the enzyme is located near the interface between subunits, composed mainly of residues from one subunit, with two residues contributed from the other subunit. In the native enzyme the cofactor is anchored covalently to the large domain of one subunit, in the form of a Schiff base linkage, the internal aldimine, between the cofactor aldehyde and lysine 145. In addition, a number of interactions between the protein and the cofactor provide the anchors that keep the pyridoxal phosphate moiety in place, especially in the reduced (PMP) form, when the covalent attachment is broken (*2*, *14*). Two residues from the other subunit, *H100 and *R98, provide the interactions with the carboxylate of the amino acid substrate, directing its position in the active site such that the *si* face of the cofactor (*14*) is directed toward both the protein and the active site lysine responsible for proton transfer during the transamination reaction.

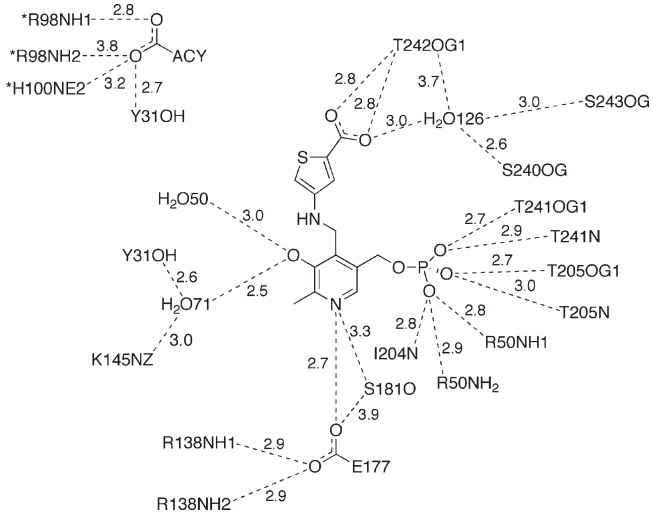


FIGURE 6: Schematic of the active site of D-a-AT inactivated with *R*-ADTA. Only subunit b has an acetate bound as shown. It is very likely that *H100 is rotated so that the interaction is with NE2 rather than CE1. Shown are the residues that interact with the carboxylate of the adduct, thought to be the site of interaction of the side chain carboxylate of α-ketoglutarate in the D-glutamate formation direction of the reaction.

In the structure of the inactivated enzyme, the active sites of both subunits show electron density consistent with a derivative of pyridoxal phosphate (Figures 5 and 6), and the cofactor is no longer attached to lysine 145. Compared to the unreacted native enzyme, the plane of the pyridine ring of the cofactor is tilted outward by 25 deg and away from K145. This movement is

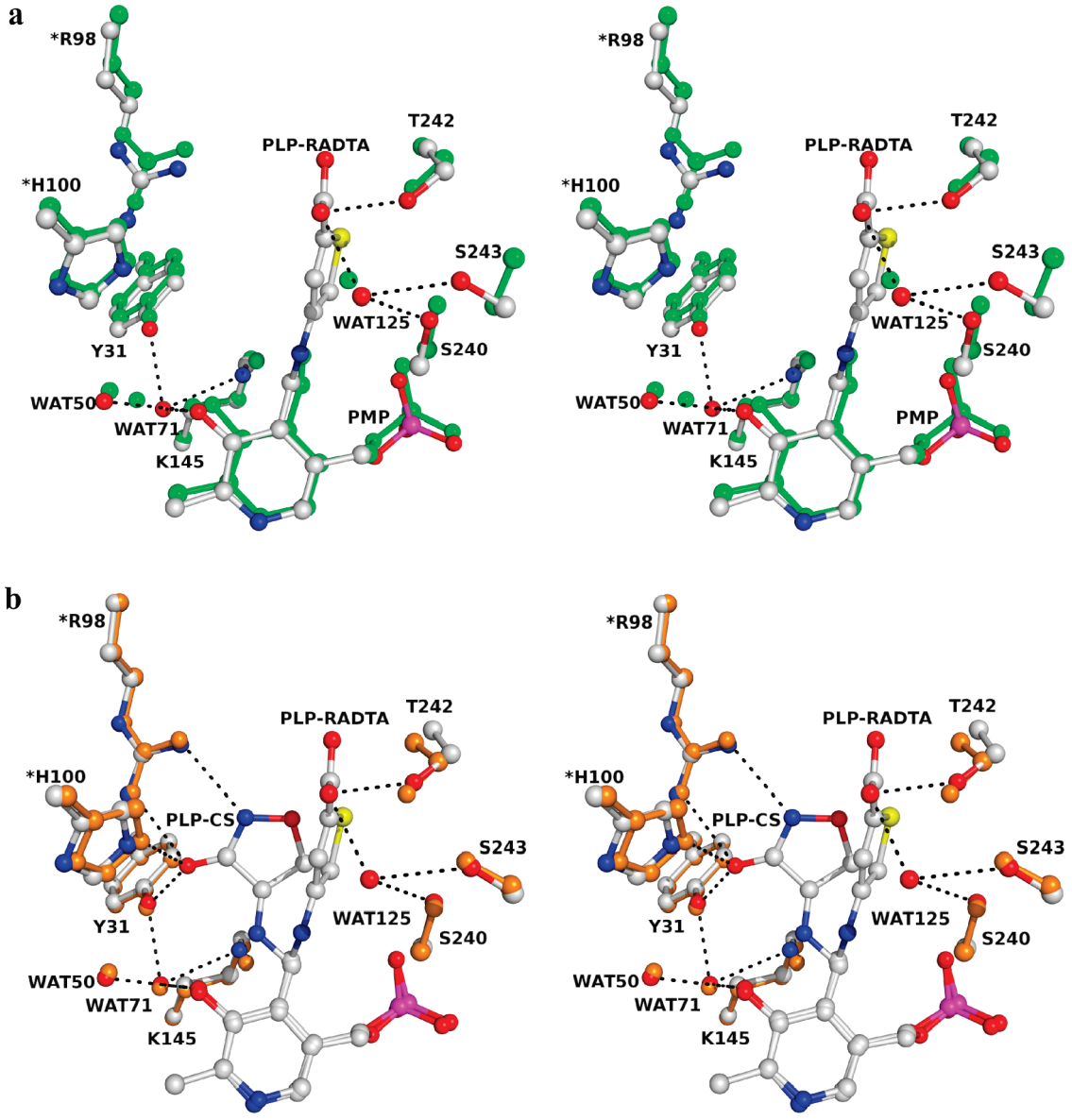


FIGURE 7: (a) Overlay of native (PMP form; green; PDB 1daa) and *R*-ADTA inactivated enzymes in the region of the active site. The pyridine ring of the cofactor is tilted toward solvent in both cases relative to the PLP form of the enzyme. (b) Overlay of the cycloserine inactivated enzyme (orange; PDB 2daa) and the *R*-ADTA inactivated enzyme (shown in atom colors) in the region of the active site. The pyridoxal−inactivator derivatives for both structures are shown in atom colors (C, white; N, blue; O, red; S, yellow). The pyridine rings of the cofactors overlay almost precisely. The isoxazole ring derived from cycloserine and the thiophene ring derived from *R*-ADTA do not overlap at all and are at right angles to each other.

tracked by a loop composed of residues 178−182 that helps to keep the cofactor positioned in the protein by forming a barrier to further tilt of the pyridine ring. This outward tilt is observed in many PLP-dependent enzymes when the native PLP form, in which the cofactor is covalently attached to the active site lysine, is compared to a PMP form of the enzyme in which this covalent bond is broken (*2*, *10*, *12*, *17*). Despite this tilt, the interactions between the cofactor-derived part of the derivative and the protein have not changed significantly relative to the native enzyme, with all of the dominant interactions that hold the cofactor into the protein still being intact (Figure 7a).

The derivative fits into an electron density feature that can only be modeled as a cyclic group that seems to be planar and is derived from the thiophene moiety of the inactivator (Figure 5). In this model, the thiophene ring of the derivative is positioned in the electron density based on a bulge of electron density, modeled as the sulfur atom of the ring. Due to the greater scattering mass of second row elements, the position of this atom is clearly identifiable. Because the electron density in this region appears planar, the ring structure must be aromatic. The C4A and C5A atoms of the derivative are modeled as sp^3 hybridized based on the geometry of the electron density at these positions (Figure 5). The plane of the thiophene ring is rotated about the C4−C4A bond of the cofactor by 90 deg relative to the plane of the pyridine ring of the cofactor. The carboxylate oxygens of this ring make hydrogen-bonding interactions with T242 and, indirectly through a water molecule, with residues T242, S243, and S240 (Figures 6 and 7). These residues are in the same loop from which one of the residues that interacts with the phosphate group of PLP arises (T241).

Identification of Substrate Interaction Sites. A number of structures of D-a-AT, both in the native form and with ligands bound, have been used to attempt to map out the substrate recognition sites on the enzyme. The substrate in the forward direction is alanine; therefore, a site for recognition of a

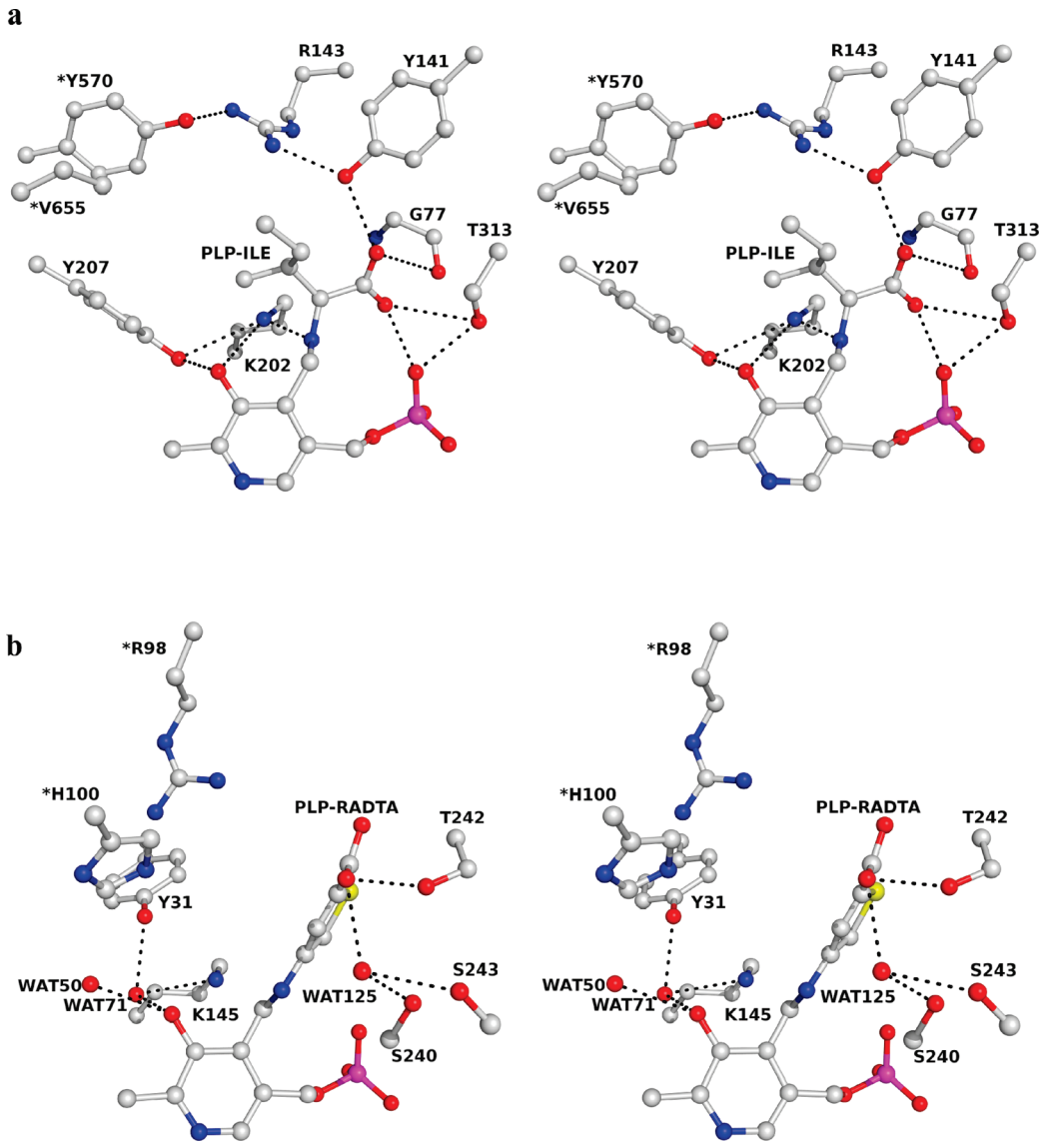


FIGURE 8: (a) The active site region of the BCAT−leucine complex (PDB 1ekf). (b) The active site region of the D-a-AT−*R*-ADTA complex. The comparison shows the interactions between the carboxylate of L-leucine in the BCAT structure and the carboxylate of the adduct from *R*-ADTA in the D-a-AT structure. Both active sites are viewed from the same perspective.

carboxylate is expected to be important for positioning of the substrate. Observations of anions bound in the active site, and the direction in which inhibitors are bound, would therefore be indicative of substrate orientation.

The native structure of D-a-AT in the PLP form has a sulfate ion bound at the active site. This ion interacts with *R98 and *H100 from the other subunit and with the hydroxyl of Y31 (Figures 6 and 7) (*14*). The initial assumption was that these residues form the carboxylate recognition site of the substrate alanine. This assumption was supported by the structure of D-a-AT complexed with PPDA (the reduced PLP-alanine external aldimine) in which the carboxylate of the alanine moiety interacts with these same residues (*14*). Further support for this assumption came from the inactivation of D-a-AT with cycloserine. D-Cycloserine forms an aromatic adduct with PLP. The carbonyl oxygen and ring nitrogen of the isoxazole ring interact with these same residues, orienting the ring of the inactivator in a direction facing the other subunit (*12*).

There is weak electron density for an acetate molecule in subunit B of the structure reported here, with correspondingly high *B*-factors for the model, presumably derived from the crystallization medium. The acetate is in the position that would be occupied by the α-carboxylate of the substrate, making a bidentate salt bridge with the side chain of *R98. There is no indication of such an interaction in subunit A, and the side chains of the R98's have distinctly different configurations in the two subunits. The thiophene carboxylate is nowhere near this position, due to the rotation of the ring, so that the carboxylate of the inhibitor interacts with residues not expected to interact with the α-carboxylate of the substrate (Figures 6 and 7).

None of the earlier structures indicate where the side chain carboxylate of the D-glutamate, that is the product of the reverse reaction from α-ketoglutarate, interacts with the enzyme. The new structure of the D-a-AT/*R*-ADTA adduct indicates where this interaction might occur. The configuration of the thiophene ring is almost at right angles to that of the isoxazole ring of the cycloserine adduct, with the carboxylate group pointing in a completely different direction from the putative alanine substrate recognition site (Figure 7b). In a comparison of the thiophene adduct with the PPDA complex, the thiophene ring overlaps with the methyl of the PPDA group, indicating a different direction of binding (not shown). Consequently, the

carboxylate of the D-a-AT/*R*-ADTA adduct interacts with an unusual pocket derived from three side chain hydroxyl groups (S240 directly, T242 and S243 through a water molecule; Figures 6 and 7). A model of glutamate bound to PLP in a manner similar to the conformation of alanine in PPDA could place the side chain carboxylate into this pocket (not shown), indicating that this pocket could be the specificity site for the side chain carboxylate of the α-ketoglutarate substrate of the reverse reaction.

One possible way to identify the specificity pocket for the α-carboxylate of ketoglutarate is to assume that this carboxylate interacts with the same region of the enzyme as the α-carboxylate of a branched chain amino acid in branched chain aminotransferase (BCAT). This enzyme has the same fold as D-a-AT, binds PLP in the same mode as D-a-AT (Figure 7), but is specific for L-amino acids (*3*). Therefore, the amino acid substrate must bind in the opposite direction to that of the D-a-AT substrate in order for the active site lysine (K145 in D-a-AT or K202 in BCAT, respectively) to catalyze the proton transfer that is the hallmark of the transamination reaction. Comparison of the binding mode of the carboxylate of the thiophene adduct in D-a-AT with that of a PLP−leucine adduct in BCAT (*3*) shows that the former interacts with T242 (Figure 8) and the latter interacts with T313 (among others). These two residues are conserved between the two enzymes and are found in the same location structurally, relative to the PLP cofactor. Therefore, it would seem that the two enzymes have evolved to recognize a carboxylate group in the same binding regions, but with a preference for the direction in which an amino acid binds dependent on the stereochemistry of the substrate.

Mechanism of Inactivation of D-a-AT by R-ADTA. The mechanism of inactivation of PLP-dependent enzymes by cyclic compounds such as cycloserine and ADTA is expected to be governed by two primary factors: chirality at the α carbon position and the susceptibility of the ring structure toward aromatization in the presence of other nucleophiles present in the active site of the enzyme that can react with any potential intermediates. Chirality should play a role in orienting the inactivator in the active site so that transaldimination can occur and so that the base required for proton shuttling in the aldimine to ketimine step, lysine 145, is in the correct position. Therefore, in the first step of the inactivation mechanism, *R*-ADTA should form an external aldimine with D-a-AT in an orientation similar to the orientation of the D-cycloserine adduct. This is the only orientation in which the active site lysine can act as catalytic base to transfer a proton in the aldimine to ketimine transformation on the *si* face of the complex. It has been suggested that the same lysine could also be the base that promotes tautomerization and deprotonation to form the aromatic ring (Figure 8), requiring the same orientation. If the inactivation mechanism proceeds in this way, then the reorientation of the thiophene ring of the product must occur after the reaction is complete.

Inactivation of D-a-AT by R-ADTA Is Slow Relative to Inactivation by D-Cycloserine. There are a number of possible explanations for this observation, including lower affinity of *R*-ADTA for the enzyme, incorrect positioning in the active site, and lower reactivity of the intermediates in any one of the steps leading to the final product. For instance, the steric bulk of the carboxylate group could prevent the inhibitor from binding as precisely as D-cycloserine can, leading to inefficient catalysis, suggesting that the enzyme is not flexible enough to accommodate an extra carboxylate group in the cycloserine orientation.

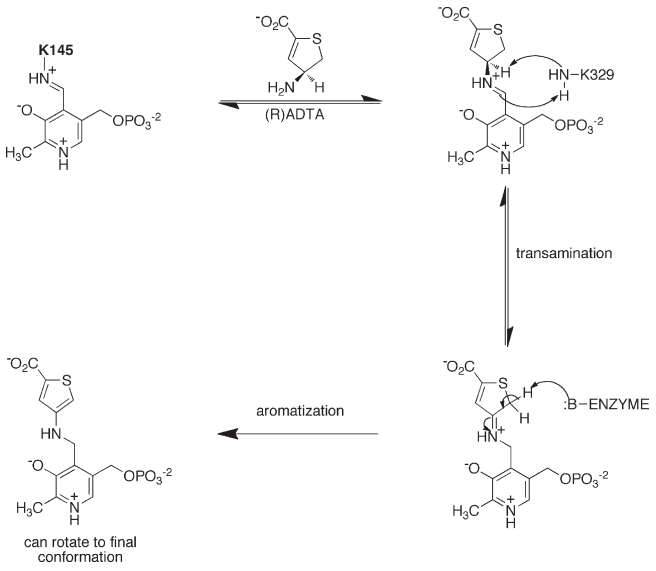


FIGURE 9: Mechanism of inactivation of D-a-AT by *R*-ADTA.

Model building suggests that such an explanation is possible. If we assume that the mechanism in Figure 9 is likely to occur in the formation of the final inactivated product, the inactivator must bind with the carboxylate interacting with the interaction site composed of residues *R98, *H100, and Y31, as does substrate, followed by rotation after transamination has occurred. This would imply that deprotonation is not catalyzed by a residue on the protein and could therefore be considerably slower than in the case of cycloserine where the lysine is still positioned to catalyze the aromatization. Alternatively, aromatization could have been catalyzed by K145 and could have occurred prior to the adduct rotating toward the putative side chain specificity site. Rotation could then have occurred to relieve steric stress or as a result of competition with acetate for the carboxylate binding site.

SUMMARY

Inactivation of D-a-AT by ADTA is chirally specific and forms an aromatic adduct with the PLP cofactor. Only the *R*-isomer, related to D-alanine, reacts with the enzyme. Despite the chemical possibility of other adducts and alternative reactions, as has been observed for inactivation of other aminotransferases by ADTA's, only one product is observed. Comparison of the conformation of the inactivated complex with a substrate complex of the homologous enzyme, BCAT, specific for the opposite isomers of amino acids, shows that the binding pocket that interacts with the carboxylate group of the inhibitor is most likely to be the interaction site for the substrate side chain of α-ketoglutarate of the reverse reaction in transamination (leading to D-glutamate). Because the reaction leading to inactivation requires that the inactivator bind in the normal direction for a substrate, a rotation must occur after inactivation in order for the product to be rotated into the observed configuration. The implication is that the specificity of the enzyme for substrate is dictated more by the interactions of an α-carboxylate group with specificity sites rather than by interactions of a side chain.

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