D-Cycloserine Inactivation of D-Amino Acid Aminotransferase Leads to a Stable Noncovalent Protein Complex with an Aromatic Cycloserine-PLP Derivative

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Abstract: D-amino acid aminotransferase (D-aAT, EC 2.6.1.21) is a pyridoxal-phosphate (PLP) dependent enzyme that specifically transaminates D-amino acids. D-aAT provides one of the routes for the biosynthesis of D-alanine and/or D-glutamate, which are essential constituents of the bacterial cell wall peptidoglycan, thereby making this enzyme a potential antimicrobial target. One agent that inhibits this enzyme is D-cycloserine, believed to react with the cofactor and subsequently form a covalent link to the protein. We have recently reported the high-resolution crystal structure of D-aAT from a thermophilic Bacillus species (Sugio et al. *Biochemistry* **1995**, *34*, 9961−9969). We now report the crystal structure (PDB accession code 2DAA) of this enzyme inactivated by D-cycloserine. Contrary to expectations, cycloserine is not covalently attached to the protein but rather forms a stable aromatic species attached to the cofactor and held in place by many noncovalent interactions. The chemical nature of the complex between D-aAT and cycloserine was confirmed by infrared and nuclear magnetic resonance spectroscopy. This observation sheds light not only on the mechanism of inhibition of PLP-dependent aminotransferases by cycloserine in general but also on the nature of substrate recognition by D-aAT.

Introduction

D-Amino acid aminotransferase (D-aAT, EC 2.6.1.21) is a eubacterial pyridoxal-phosphate (PLP) dependent enzyme that specifically transaminates D-amino acids and provides one of the routes for the biosynthesis of D-alanine and/or D-glutamate, essential constituents of the bacterial cell wall peptidoglycan. D-Cycloserine is a naturally occurring antibacterial compound that inhibits, among other processes, peptidoglycan synthesis. In the course of a search for a substrate analogue whose complex with D-aAT might illuminate the nature of the substrate recognition site, we examined the crystal structure of D-aAT complexed with D-cycloserine (4-amino-3-isoxazolidone, compound A in Scheme 1).

Cycloserine derivatives are cyclic analogues of serine and react essentially irreversibly with many transaminases and other PLP dependent enzymes.[1] This reaction has been studied extensively with L-aspartic acid aminotransferase (L-AspAT).[2] The inhibited enzymes show typical absorbance maxima near 330−340 nm, indicative of a pyridoxal derivative with a saturated C4A carbon atom. The presumed reaction mechanism involves a process which includes the initial steps of the normal transamination reaction as follows (Scheme 1). Formation of an external aldimine between the amino group and the bound cofactor is followed by a 1,3 prototropic shift, catalyzed by the active site lysine, to yield a ketimine. This intermediate, which in the case of a normal substrate would hydrolyze to form a ketoacid and the PMP form of the enzyme, was assumed to undergo further reaction leading to formation of a covalent bond to the protein. However, the nature of the product remains controversial, and no covalent protein derivative has yet been clearly characterized.

We report here the crystal structure of the complex formed between D-cycloserine and D-aAT. Unexpectedly, the complex appears not to involve any covalent interaction between the inhibitor and the protein. Instead, the inhibited enzyme contains a stable derivative of the coenzyme and cycloserine, with the cycloserine ring intact. The cycloserine-PLP derivative probably arises from an intermediate on the accepted catalytic pathway for transamination which tautomerizes into a stable aromatic species. It is held on the enzyme by many noncovalent interactions, some of which make up the recognition site for the substrate α-carboxyl group. Thus, the structure of the complex sheds light both on the mechanism of inhibition by cycloserine derivatives in general and on the nature of substrate recognition by D-aAT.

Methods

Crystallization. D-aAT (4 mg, 2 mg/mL) purified as previously described[3] was concentrated to 30 mg/mL in 100 mM potassium phosphate buffer pH 7.6 containing 0.05 mM PLP and 0.01 mM β-mercaptoethanol. The protein was

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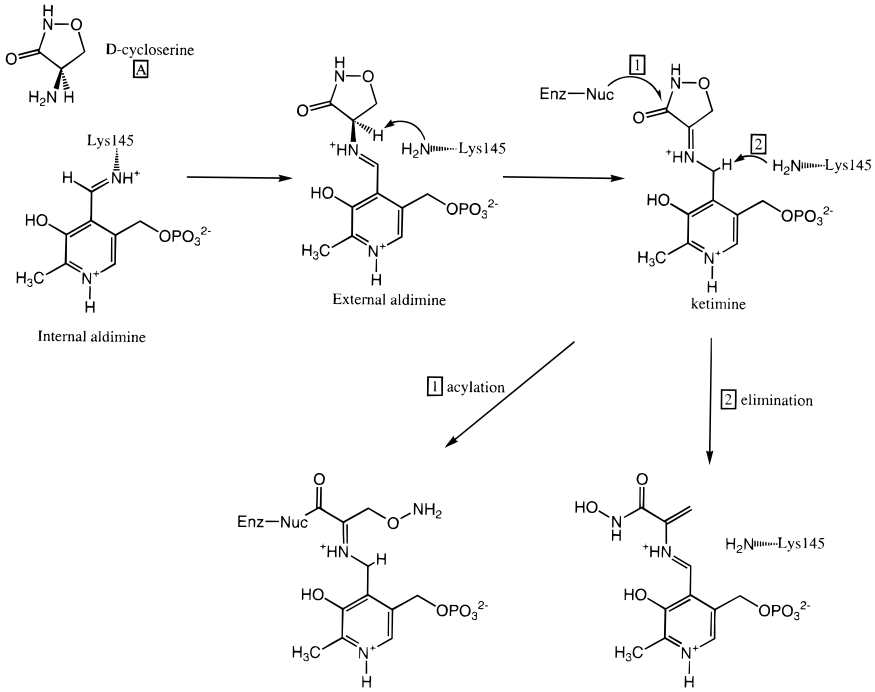
Scheme 1. Putative Mechanism of Inactivation for PLP-Dependent Enzymes by D-Cycloserine as Given by Braunstein[2]



crystallized by the hanging drop method with 25 mM D-cycloserine, 26% poly(ethylene glycol) 3350 (Sigma Chemical Co., St. Louis), 200−300 mM sodium acetate, and 100 mM Tris-Cl buffer pH 8.5. Addition of D-cycloserine causes the protein solution to lose its characteristic yellow color. Two μL of protein solution was mixed with 2 μL of the above crystallization solution and suspended at room temperature over 0.5 mL of crystallization solution. Plate-shaped crystals (colorless because of derivatization of the bound PLP) first appeared after 2 days and continued to grow for a week. Most crystals grew in bundles, which were unsuitable for diffraction methods, but some crystals gave clean diffraction patterns. One such crystal had dimensions $0.85 \times 0.5 \times 0.25$ mm^3 and was used for data collection. The crystals have the symmetry of the orthorhombic space group $P2_12_12_1$. The unit cell parameters are $a = 77.3$ Å, $b = 91.0$ Å, and $c = 89.7$ Å, which corresponds to a unit cell volume of 631 000 Å^3. The calculated volume per unit mass (V_m) of 2.5 Å^3/Da is also in the expected range assuming one dimer of D-aAT in the crystallographic asymmetric unit (Table 1).

Solution of the Crystal Structure. Data were collected at 4 °C with a scan width of 1° per frame and an exposure time of 15 min per frame on a RAXIS IIC image plate system mounted on a Rigaku RU-200B X-ray generator running at 45 kV and 140 mA. The data set was collected from a single crystal and was 99.1% complete to 2.1 Å resolution. Frames were integrated and scaled together using the HKL package (DENZO and SCALEPACK) from Molecular Simulations Corporation.

The structure was solved by the method of molecular replacement[4] using the coordinates of the native monoclinic structure of D-aAT (PDB accession code 1DAA)[3] as search probe in the program AMORE,[5] which is part of the CCP4 package.[6] Coordinates for all protein atoms but not for the cofactor or water molecules were included in the search model. The program XPLOR[7] was then used for all remaining refinement. Rigid body refinement (of each monomer in the asymmetric unit) was first performed to optimize the initial

Table 1. CS-D-aAT Data Collection and Refinement

Crystal Data	
protein	D-aAT + cycloserine
space group	$P2_12_12_1$
unit cell parameters	
a (Å)	77.3
b (Å)	91.0
c (Å)	89.7
Data Collection	
reflections, observed	83705
reflections, unique	36340
R_{merge} (% on I)[a]	5.5
resolution (Å)	30−2.0
$I/\sigma(I)$ cutoff	>1.0
completeness, overall (%)	97.9
highest resolution shell (Å)	2.2−2.0
completeness, highest resolution (%)	99.1
Refinement	
resolution (Å)	30−2.1
$I/\sigma(I)$ cutoff	>1.0
reflections	34719
R_{factor}[b]	18.5
R_{free}	23.9
protein atoms	4466
cofactor atoms	44
water molecules	200
B-factor model	individual
restraints (rms observed)	
bond length (Å)	0.007
bond angles (deg)	1.3
improper angles (deg)	1.2
dihedral angles (deg)	24.5

[a] $R_{merge} = \Sigma|I_{obs} - I_{avg}|/\Sigma(I_{avg})$. [b] $R_{factor} = \Sigma|F_o - F_c|/\Sigma(F_o)$.

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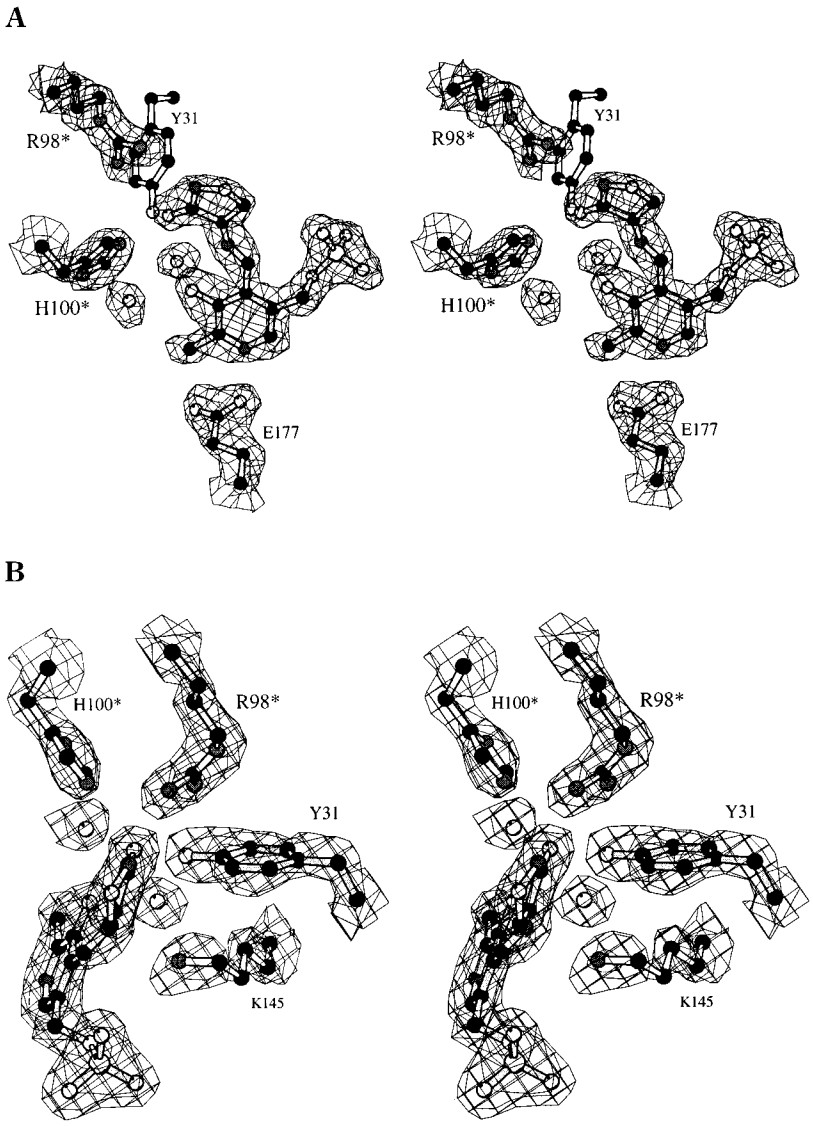

Figure 1. Two views of electron density in the active site of D-cycloserine inactivated D-aAT. Electron density shown is an unbiased simulated annealing $2F_o$-F_c omit map of the active site at a 0.75 σ contour. All atoms drawn were left out of the model for this map. (A) shows the view seen from the solvent. Electron density around tyrosine 31 has been left out for clarity. (B) shows the view from above the PLP cofactor, roughly orthogonal to the view in (A) and with electron density for tyrosine 31 and lysine 145 drawn.

location. A solvent mask was calculated to approximate low resolution data better. This was followed by several cycles of positional refinement, group temperature factor (B-factor) refinement and placement of water molecules. Water molecules were placed with PEAKMAX and WATPEAK from the CCP4 package.

After 60 water molecules were added, it was clear that electron density at the active site could only be modeled by a cycloserine derivative covalently attached to the PLP but not covalently bound to the protein. The two views of the active site in Figure 1 show that there is a disk of electron density extending from the pyridoxal C4A carbon, with no connectivity to any protein residue electron densities (see Figure 2 for atom nomenclature). The electron density is planar and appropriate for a small ring with a single atom projecting from it. Initial coordinates for D-cycloserine were obtained from the Cambridge Structural Database of small molecule crystal structures. These coordinates were modeled to the electron density by eye together with the atoms of PLP up to and including carbon C4A. Several other possible derivatives of cycloserine with PLP (as shown in Scheme 1) were tested with models built and minimized in QUANTA version 4.1 (from Molecular Simulations Corp.). The program XPLO2D was run with the coordinates of all of these models to generate initial XPLOR topology and parameter files.[8] Cycles of XPLOR refinement were then resumed. Only the ring-closed derivative successfully modeled the electron density and was used in all further stages of refinement. After 200 water molecules were added, individual B-factor refinement reduced the R-factor for the entire dimer, cycloserine-PLP, and waters to 18.5% with data from 30 to 2.1 Å resolution and a one σ cutoff. The final model is good, with small deviations from ideal geometry (Table 1).

Activity Assay. The activity of D-aAT was followed spectrophotometrically with the coupled assay using lactate dehydrogenase to follow the production of pyruvate from D-alanine (200 mM) in the presence of α-ketoglutarate (20 mM) at pH 7.5 and 37 °C.[9]

Spectroscopic Analysis. Native D-aAT was passed through a 2.7 × 10 cm Sephadex G25 column equilibrated and eluted with potassium phosphate buffer at pH 8.0 to remove excess

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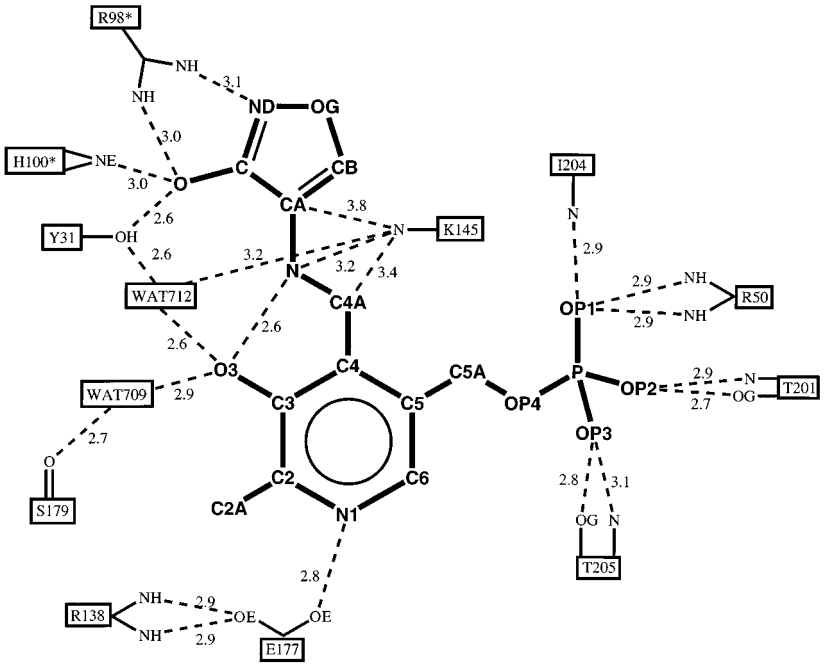

Figure 2. Schematic of all polar interactions less than 3.5 Å between cycloserine-PLP adduct and residues in the active site of D-aAT. Protons are left out because they are not observed in the electron density. Atoms are named according to crystallographic convention.

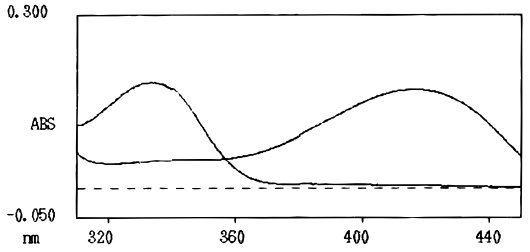

Figure 3. Optical spectra of D-aAT before and after addition of D-cycloserine. A spectrum was recorded for D-aAT stripped of excess PLP (λ_{max} 415 nm). Ten μM D-cycloserine was added and a spectrum recorded within 1 min (λ_{max} 330 nm). The color of the solution changed from yellow, indicative of an internal aldimine, to clear.

PLP. The protein was diluted in 100 mM Tris-Cl pH 8.5 to a concentration of approximately 1 mg/mL and scanned in a 1 cm quartz cuvette on a Hitachi U-2000 spectrophotometer. D-Cycloserine was then added (10 μM), and the resultant mixture scanned again in less than 15 s (see Figure 3).

The solution prepared above still had residual activity in the coupled enzyme assay. Since the assay mixture contains α-ketoglutarate, the residual activity probably arises from a small fraction of the D-aAT preparation being in the PMP form, which does not react with D-cycloserine. Addition of α-ketoglutarate to the protein/D-cycloserine mixture converts the remaining PMP form of the enzyme into the PLP form, leading to a transient yellow color. This is quickly bleached as it reacts with D-cycloserine. Following this treatment the enzyme has less than 0.01% residual activity.

Synthesis of D,L-[3-^{13}C]Cycloserine. D,L-[3-^{13}C]Cycloserine (35 mg) was synthesized from 250 mg of D,L-[3-^{13}C]serine (Cambridge Isotope Labs) by the method of Plattner et al.,[10] with the following changes: D,L serine methyl ester was synthesized according to the method of Rachele,[11] and the final product purified by the method of Olson et al.[12]

NMR Experiment. All ^{1}H−^{13}C heteronuclear multiple quantum correction (HMQC) experiments were recorded at 298 K on a Bruker AMX-500 spectrometer equipped with a three-channel Bruker Acustar pulsed field gradient amplifier and x,y,z-gradient triple resonance inverse detection probe.[13] The spectrometer operates at 500.13 and 125.77 MHz for ^{1}H and ^{13}C, respectively. Time proportional phase incrementation (TPPI) was used to achieve quadrature determination in w_1. Spectral widths of 7042 Hz and 27 669 Hz were used for ^{1}H and ^{13}C, respectively. Two hundred fifty-six fids were averaged for each of 128 values of t_1. Each fid was acquired using 2048 complex points.

Data were processed using FELIX 95.0 from Biosym. A convolution difference window function was applied prior to Fourier transformation in t_2, and a 70° shifted sine bell applied prior to zero-filling and transformation in t_1. All spectra were referenced to 3-(trimethylsilyl)-1-propanesulfonic acid (TPS) in H_2O.

Two hundred microliters of 25 mg/mL (1.6×10^{-7} mol, 1 equiv) D-aAT was mixed with 30 μL D_2O for deuterium lock, and an ^{1}H−^{13}C HMQC experiment acquired. Ten microliters of 48 mM D,L-[3-^{13}C] cycloserine (2.5 mg taken up in 500 μL H_2O, 4.8×10^{-7} mol, 3 equiv.) was then added to the protein, and another HMQC experiment acquired (Figure 4). Three new peaks appeared. Two large peaks were observed at 75 ppm in the ^{13}C dimension and 4.08 and 4.47 ppm in the ^{1}H dimension corresponding to unbound labeled cycloserine. A third peak appeared at 145 ppm in the ^{13}C dimension and 7.8 ppm in the ^{1}H dimension. As a control, the second NMR experiment was repeated with unlabeled cycloserine to confirm that the 145 ppm peak arises only from bound cycloserine.

Results and Discussion

Crystal Structure of D-aAT-Cycloserine Complex. D-aAT is inhibited by D-cycloserine in a manner similar to other PLP-dependent enzymes, although it can be reactivated slowly by

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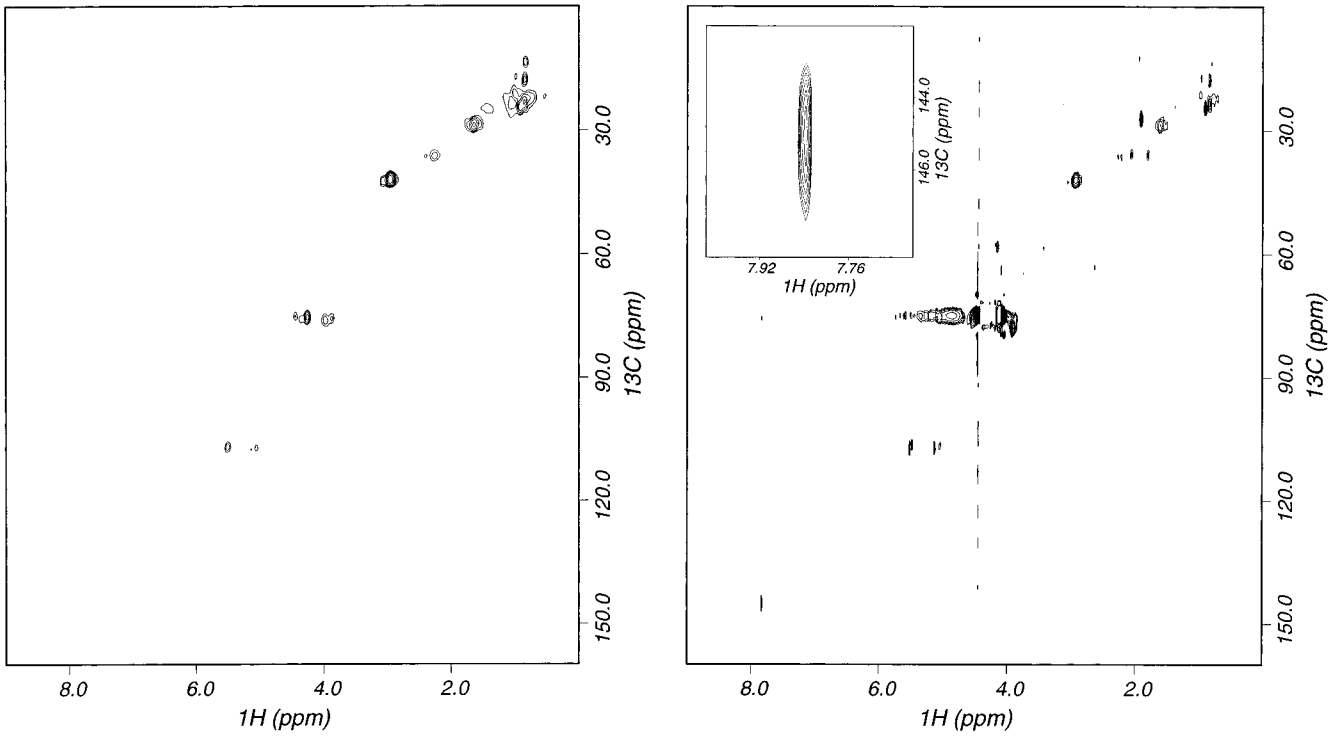

Figure 4. Two-dimensional $^{1}H-^{13}C$ HMQC NMR spectrum of D-aAT alone and inactivated with [3-^{13}C] cycloserine. The peak at 145 ppm in the ^{13}C dimension and 7.8 ppm in the ^{1}H dimension is shown enlarged in the insert. This peak is typical for the C5 position of an aromatic isoxazole.

dialysis against pyridoxal phosphate at pH 7 or below.[1] We have solved the structure of D-aAT complexed with D-cycloserine in hopes that it would shed light on the positioning of substrates and substrate analogues in the active site as well as on the nature of the inactivation process.

The overall structure of the protein inactivated by D-cycloserine is almost identical with that of the native enzyme in the PMP form, despite the fact that it crystallized in a different space group.[3] Neither the relative positions of the two domains in a subunit nor the monomer−monomer interactions in the dimer change on formation of the derivative (rms deviation for α carbons is 0.52 Å). There are small differences in surface loops, but none of these are relevant to the chemical properties of the active site. The pyridine ring and the phosphate of the cofactor are in very similar positions in the two structures.

However, we were surprised to find a well defined region of electron density connected to the cofactor, clearly separated from any protein residues. Figure 1 shows two views of the electron density, which accommodates beautifully a derivative of the closed form of cycloserine. We attempted to build both ring-closed and ring-opened forms of cycloserine into this electron density. No open form could be fitted to our crystallographic data because any such form would have atoms lying outside the observed electron density. In addition, such a model does not account for the electron density into which we have placed oxygen atom OG of the cyclic form. The low-temperature factors (<25 Å^2) refined for the derivatized cofactor indicate that it is tightly held in place.

Several interactions account for this tight binding (Figure 2). Arginine 98* (* indicates that the residue comes from a loop reaching into the active site from the neighboring monomer) forms a bidentate interaction with the exocyclic oxygen (O) and ring nitrogen (ND). In addition, tyrosine 31 and histidine 100* form hydrogen bonds to this oxygen (O). We suggest that these three residues form the site for interaction with the α-carboxyl of an amino acid substrate. It is important to note that two of the three residues forming this tight "carboxyl trap" are provided by a loop from the neighboring monomer in the dimeric enzyme. This loop thus determines stereospecificity of the enzyme for D-amino acids; only a D-amino acid with its carboxyl bound in the "carboxyl trap" would have the proton on its α-carbon pointed toward the catalytic base, lysine 145.

The coenzyme part of the derivative is held in place by all the usual interactions observed in PLP-dependent enzymes. The phosphate is anchored by several polar interactions with the protein. The N1 atom of the pyridine ring is within ion pairing distance of the carboxylate of glutamate 177. Lysine 145 lies closest to atom N (3.2 Å) and is nearly equidistant from atoms C4A and CA (3.4 and 3.8 Å, respectively). This geometry is perfect for catalysis of the tautomerization step of an amino acid substrate.

There are no protein residues close enough to interact directly with the phenolic oxygen atom (O3) of the pyridine ring. In L-aspartic acid aminotransferase, the hydroxyl of tyrosine 225 is within hydrogen bonding distance of this oxygen[14] and plays an important role in the stabilization of intermediates during the reaction.[15] Other polar residues have been seen to interact with the O3 hydroxyl of PLP in other PLP dependent enzymes.[16,17] In D-aAT, on the other hand, two tightly bound water molecules are the only groups within hydrogen bonding distance of the phenolic oxygen. One of these (WAT712) is in close contact with tyrosine 31 and lysine 145. The other (WAT709) is hydrogen bonded to additional water molecules and to the backbone oxygen of serine 179.

The structure of the cycloserine portion of the derivative was refined without bond angle and dihedral restraints, and using

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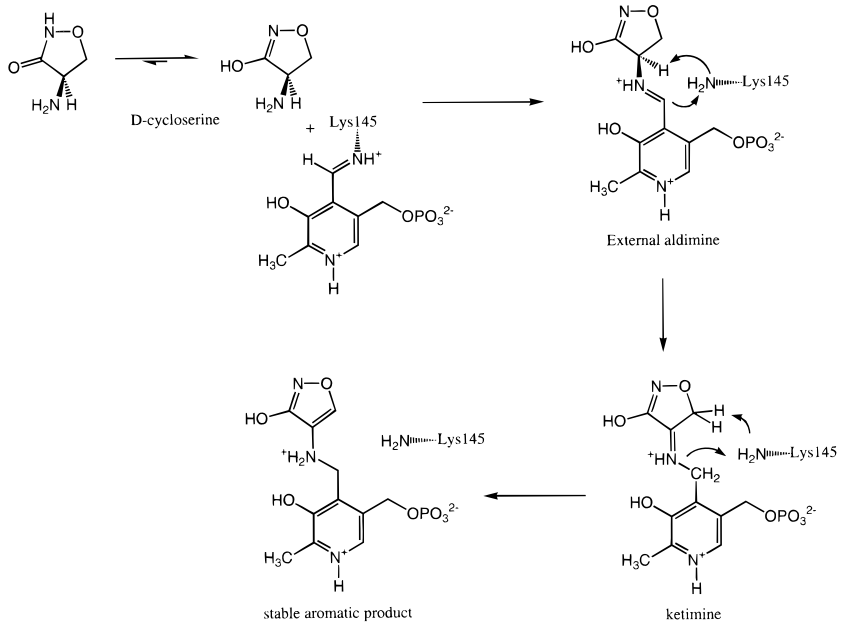
Scheme 2. Proposed Mechanism for Inactivation by Cycloserine



weak bond length restraints, in order not to bias the outcome of the structure to a particular chemical interpretation. The resulting structure has some interesting features. The five-membered ring is essentially planar, with atom OG less than 0.3 Å out of the plane of the other four atoms. The dihedral angle C3−C4−C4A−N is 0°, even though it was not constrained during refinement in any way. This may be due to the hydrogen bond interaction between atoms N and O3, which are 2.7 Å apart. The C4−C4A−N−CA dihedral is 165° and the C4A−N−CA−CB dihedral is 0° so the plane of the cycloserine is tilted with respect to the pyridine ring. The geometry of the complex is thus in accord with sp^3 hybridization at C4A, which is implied by the shift observed in the ultraviolet spectrum of the complex (Figure 3). The geometry is compatible with saturation of the N−CA bond, although at the resolution of this structure, a double bond at N−CA cannot be ruled out.

Chemical Nature of the Interaction of D-aAT with Cycloserine. Previous reports suggested that cycloserine (or its substituted derivatives) reacts with pyridoxal phosphate-dependent enzymes by initial formation of an external aldimine, tautomerization to the ketimine and nucleophilic attack on the cycloserine ring by a protein residue to form a covalent derivative (Scheme 1, reaction 1). This scheme was based on spectroscopic evidence, including the disappearance of the absorbance maximum at 415−430 nm and the appearance of a new maximum at 330−340 nm, and on the apparent irreversibility of the inhibition. One could also imagine other ring-opening reactions such as an elimination (Scheme 1, reaction 2) followed by Michael addition to the β-carbon. It should be noted, however, that attempts to isolate covalent derivatives of amino acids after denaturation of the cycloserine inhibited proteins were never successful.[2]

Needless to say, our crystallographic structure is not in accord with the accepted mechanism for inactivation by cycloserine. Although the structure unambiguously shows a noncovalently bound complex consistent in geometry with a PLP-cycloserine ketimine or some tautomer of it, we reexamined the chemical nature of the complex. Figure 3 shows the optical spectral change as cycloserine is added to D-aAT. Within seconds, the absorbance at 415 nm characteristic of the internal aldimine is abolished. The shift of the maximum from 415 to 330 nm is indicative of conversion of this aldimine to a derivative that is saturated (sp^3 hybridization) at position C4A.[18] This could be a form of the PLP-cycloserine adduct in which the N−CA bond is unsaturated, equivalent to a ketimine such as shown in Scheme 1.

If the cycloserine-PLP derivative is in the ketimine form, then reactivation should be possible though hydrolysis, through reversal back to the aldimine form followed by transaldimination with lysine 145, or through exchange of the cofactor. It has been shown that complete reactivation can be obtained within 1 day by dialysis of the inhibited protein, but only in the presence of pyridoxal phosphate.[1] This observation is explained by our structure since there are no covalent bonds between the cofactor derivative and the protein. This observation suggests that reversible exchange of the derivatized pyridoxal moiety (possibly accompanied by breakdown of the complex in solution) could be responsible for reactivation.

What remains to be explained is why this cofactor derivative is chemically stable to hydrolysis. Our crystal structure of D-aAT inactivated with D-cycloserine leads us to believe that the final product is a tautomer of the cycloserine-PLP adduct in which the ring is aromatized to the 3-hydroxyisoxazole shown in Scheme 2. The spectral data that were originally interpreted in terms of a ring-opened product are consistent with such a compound.

NMR Experiment. An identifying characteristic of such an aromatic derivative is that the CB atom of the original D-cycloserine is now sp^2 hybridized. To establish hybridization of this carbon atom, we synthesized D,L-cycloserine specifically enriched with ^{13}C at the β carbon. Two-dimensional HMQC spectra of the enzyme and the enzyme inactivated with ^{13}C labeled cycloserine are shown in Figure 4. The only peak which is not observed in the spectrum of the protein alone, of cycloserine alone (not shown), or of the protein inactivated with unlabeled cycloserine (not shown) occurs at 145 ppm in the carbon dimension and 7.85 ppm in the proton dimension. These

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are typical chemical shifts for the C5 position of isoxazoles.[19] It has also recently been shown that the same derivative of PLP that we propose here can be isolated by denaturation from γ-aminobutyric acid aminotransferase (GABA) inactivated with ^{14}C labeled L-cycloserine.[12] A similar formation of an aromatic derivative of PLP was suggested for the reaction of the neurotoxin gabaculine with GABA.[20] A crystal structure of ornithine aminotransferase complexed with gabaculine has also been interpreted as an aromatic derivative of PLP.[21]

Mechanism of Inactivation of D-aAT by D-Cycloserine. Given the accepted mechanism for transamination by D-aAT, it is easy to see how an aromatic derivative could arise (Scheme 2). The infrared spectrum of cycloserine indicates that it exists in solution primarily as the lactim rather than the lactam tautomer.[22] If we assume that cycloserine reacts with PLP as the lactim, then a ketimine intermediate is formed by the enzyme's usual reaction pathway.[3] The β protons of cycloserine are now sufficiently acidic that the ketimine double bond can tautomerize, forming an aromatic ring system. Because of the thermodynamic stability of an aromatic ring, this tautomer is expected to be stable, and its formation irreversible.

Conclusions

Reaction of D-cycloserine with D-aAT represents an interesting form of enzyme-promoted inhibition, akin to suicide inhibition. The enzyme is inhibited because of the tight, but not covalent, binding of the cycloserine derivative of PLP, involving all the potential interactions of the enzyme with both coenzyme and substrates. Formation of this derivative is catalyzed by the enzyme, apparently using the normal sequence of enzymatic steps. It is not formed spontaneously at a significant rate when cycloserine and PLP are mixed without enzyme at neutral pH. Because of the particular structure of cycloserine, an additional tautomerization possibly catalyzed by the active site lysine leads to a stable aromatic isoxazole, which the enzyme cannot process further at a significant rate. It appears likely that this mechanism explains the inhibition of other PLP-dependent enzymes by appropriate cycloserine derivatives.

The conformation of the cycloserine adduct of PLP in the active site of D-aAT also gives insight about substrate recognition by the enzyme and about the mechanism by which this enzyme reacts with physiological substrates. The substrate is recognized by three residues (Arg 98*, His 100*, and Tyr 31) that can form four potential hydrogen bonds to the α-carboxyl group. The NZ of lysine 145 is close enough to atoms C4A and CA to catalyze the prototropic shift required for transamination. The location of this carboxyl recognition site and the catalytic lysine 145 relative to the bound cofactor determine the stereospecificity of the enzyme for D-amino acids.

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