Mechanistic Crystallography. Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase by (1*R*,3*S*,4*S*)-3-Amino-4-fluorocyclopentane-1-carboxylic Acid As Elucidated by Crystallography†

Paola Storici,‡,§ Jian Qiu,||,⊥ Tilman Schirmer,*,‡ and Richard B. Silverman*,||

Department of Chemistry, Department of Biochemistry, Molecular Biology, and Cell Biology, and Drug Discovery Program, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113, and Division of Structural Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Received June 21, 2004; Revised Manuscript Received August 25, 2004

ABSTRACT: (1*R*,3*S*,4*S*)-3-Amino-4-fluorocyclopentane-1-carboxylic acid (**7**) was previously shown to be a mechanism-based inactivator of γ-aminobutyric acid aminotransferase (GABA-AT) [Qiu, J. and Silverman, R. B. (2000) *J. Med. Chem. 43,* 706−720]. Two mechanisms were considered as reasonable possibilities, a Michael addition mechanism and an enamine mechanism. On the basis of a variety of chemical studies, including tedious radiolabeling experiments, it was concluded that inactivation by **7** proceeds by a Michael addition mechanism. Here, a crystal structure of **7** bound to pig liver GABA-AT is reported, which clearly demonstrates that the adduct formed is derived from an enamine mechanism. This represents another example of how crystallography is an important tool for elucidation of inactivation mechanisms.

Mechanism-based enzyme inactivators are unreactive compounds that have a structure similar to that of the substrate for a specific enzyme and are converted by the normal mechanism of action of the enzyme to a species that inactivates it (*1*). These compounds have been shown to be very useful for the elucidation of enzyme mechanisms because they require the catalytic action of the enzyme for inactivation. Many approaches have been used to investigate the mechanism by which the target enzyme converts the inactivator into the activated species (*2*). In general, a variety of isotopically labeled analogues of the inactivator are synthesized and are incubated with the enzyme. If the isotope is radioactive, then the radioactive products are isolated and characterized; either the radiolabeled enzyme is degraded and the modified peptide characterized or chemical reactions are carried out on the modified enzyme in an attempt to determine the structure of the covalently bound adduct. Site-directed mutagenesis has been used to identify the nucleophilic residue involved in inactivation, but this does not give mechanistic information (*3*). Mass spectrometry of modified peptide adducts is a convenient tool, but it only gives mass, not complete structure information, and therefore generally is not important for mechanism elucidation (*4*). It is rare for the exact structure of the enzyme-bound adduct to be elucidated, and often more than one mechanism can rationalize a proposed inactivation reaction. The use of X-ray crystallography, however, could readily depict the exact structure of the enzyme adduct without the necessity of radioactivity or degradation of the enzyme. Often alternative inactivation mechanisms produce different potential adduct structures (*5*); in these cases, the crystal structure could be used to differentiate these alternative mechanisms directly.

The crystal structure of γ-aminobutyric acid aminotransferase (GABA-AT)[1] has been determined (*6*), and the elucidation of adduct structures with this enzyme allowed the differentiation of alternative inactivation mechanisms for γ-vinyl-GABA and γ-ethynyl-GABA directly (*7*). We refer to the use of X-ray crystallography to support mechanistic rationalizations as "mechanistic crystallography".

GABA-AT is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the conversion of the inhibitory neurotransmitter GABA to succinic semialdehyde concomitant with the conversion of α-ketoglutarate to the excitatory neurotransmitter L-glutamate (Scheme 1; the protonated base that donates a proton to the coenzyme may also be Lys-329). Inhibition of this enzyme, which raises the GABA levels in the brain, is one therapy for the treatment of epilepsy (*8*). Several years ago we designed 4-amino-5-fluoropentanoic acid (**1**) as a mechanism-based inactivator of GABA-AT (*9*). Initially, it was proposed that its mechanism of inactivation proceeded by a Michael addition pathway (Scheme 2, pathway a) (*9*), but later it was demonstrated to proceed via an enamine mechanism (Scheme 2, pathway b) (*10*) by isolation and characterization of the modified PLP coenzyme **6**. The noncovalent complex **6** was isolated after denaturation of GABA-AT that had been inactivated by **1**.

† We are grateful for financial support from the National Institutes of Health (Grant GM66132).

* To whom correspondence should be addressed. Phone (847) 491-5653 (R.B.S.). Fax: (847) 491-7713 (R.B.S.). E-mail: Agman@chem.northwestern.edu (R.B.S.).

‡ University of Basel.

§ Current address: Department of Biology, Discovery Research Oncology, Pharmacia Corp., Viale Pasteur 10, 20014 Nerviano, Italy.

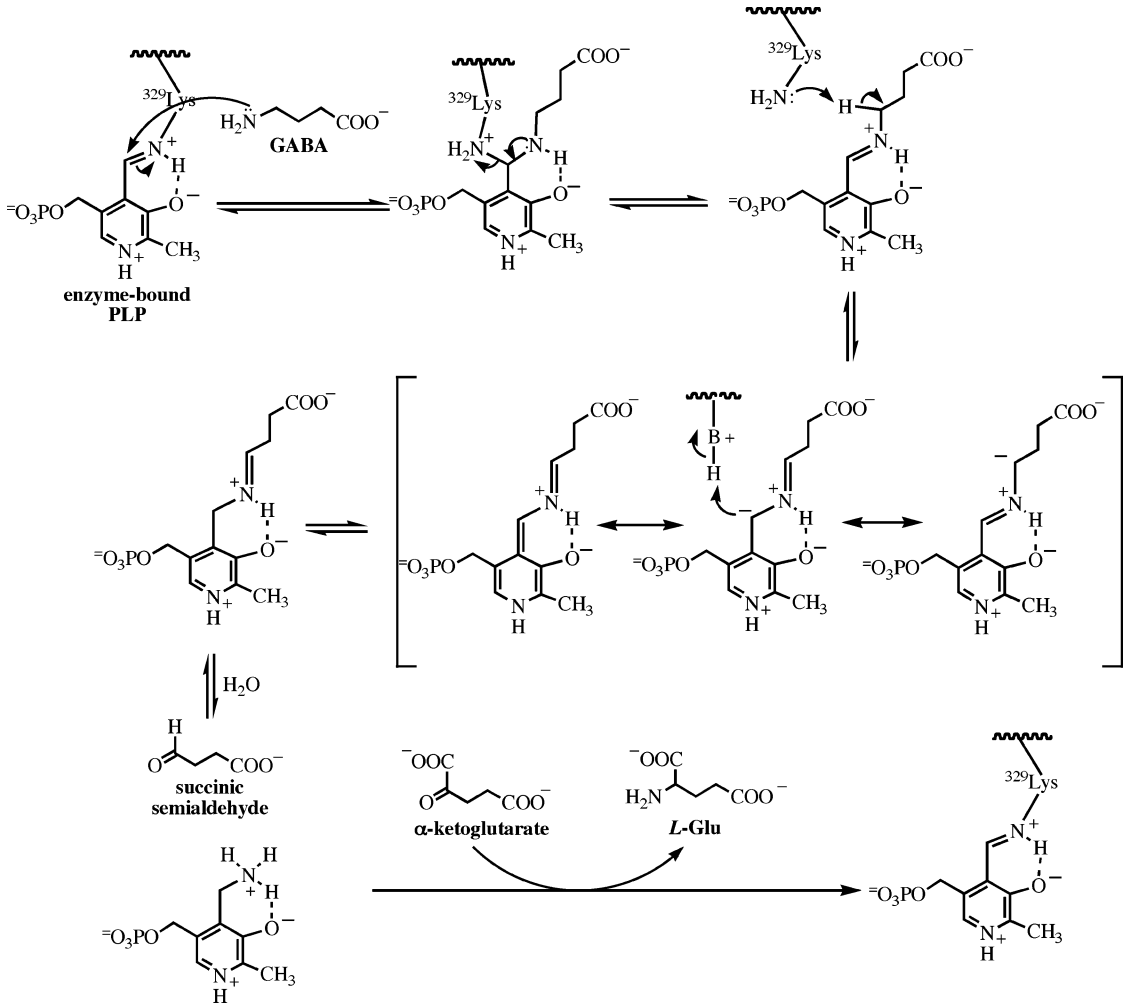
|| Northwestern University.

⊥ Department of Medicinal Chemistry, Replidyne, Inc., 1450 Infinite Dr., Louisville, CO 80027.

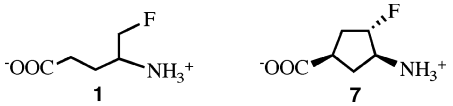
[1] Abbreviations: GABA, γ-aminobutyric acid; GABA-AT, γ-aminobutyric acid aminotransferase; PLP, pyridoxal 5′-phosphate.

10.1021/bi0487185 CCC: $27.50
Published on Web 10/16/2004

Scheme 1

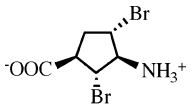


Apparently, upon denaturation of GABA-AT inactivated by **1**, the covalent adduct **5** decomposes to the noncovalent adduct **6**.



1 **7**

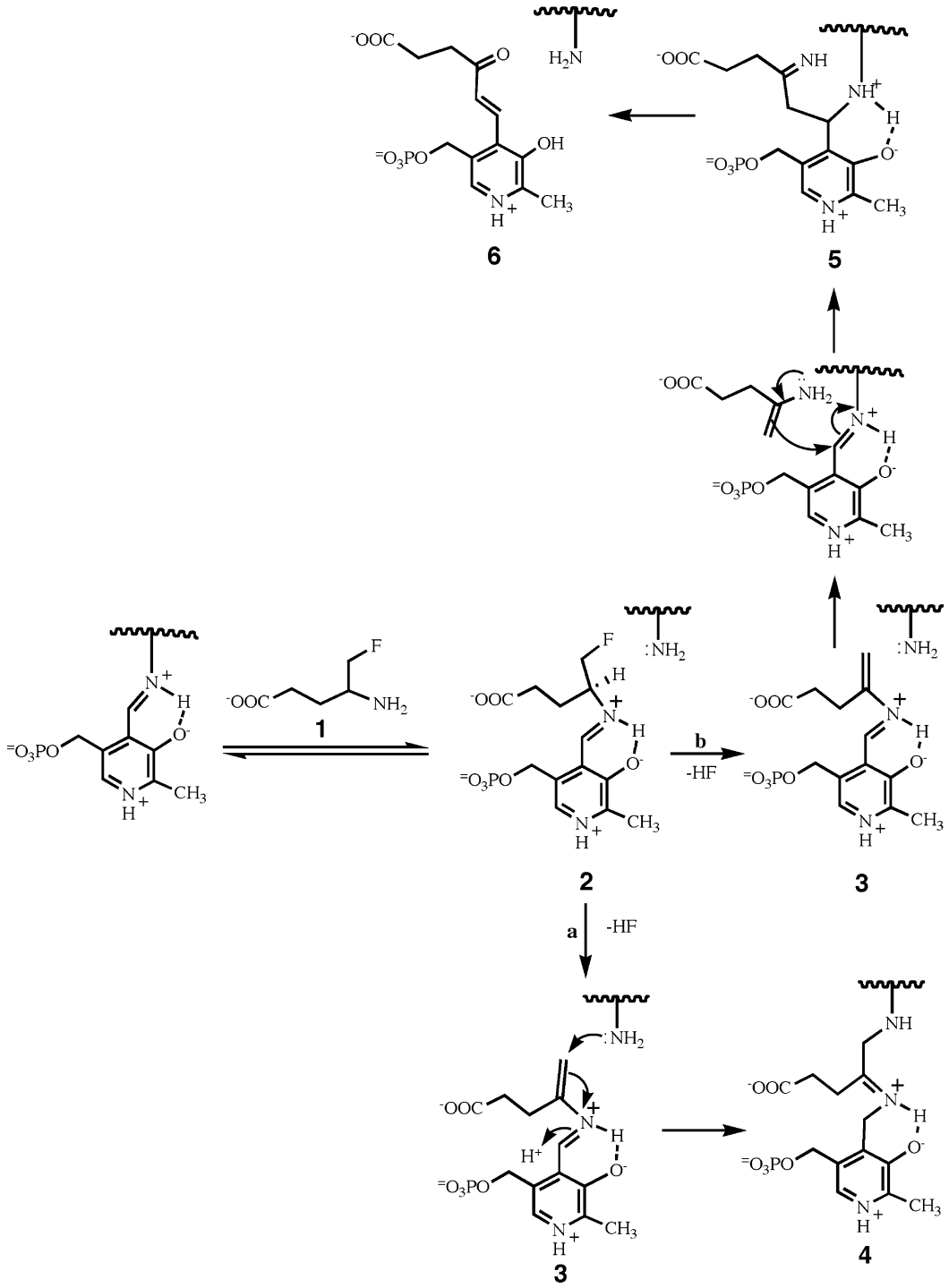
On the basis of the structure of **1**, a series of halogenated 4-aminocyclopentanecarboxylic acid derivatives were designed as potential, more lipophilic, inactivators of GABA-AT (*11*). The most potent of these cyclopentane analogues was (1*R*,3*S*,4*S*)-3-amino-4-fluorocyclopentane-1-carboxylic acid (**7**), a time-dependent, irreversible inactivator. On the basis of the inactivation mechanism for **1** (Scheme 2, pathway b) and the previously obtained mechanistic results described below for **7** (*11*), two inactivation mechanisms for **7** were proposed, a Michael addition mechanism (Scheme 3, pathway a) and an enamine mechanism (Scheme 3, pathway b), a mechanism originally proposed for the inactivation of L-aspartate aminotransferase by serine sulfate (*12*). Cleavage of the C−F bond was shown not to be rate determining; 3-C−H bond cleavage is the rate-determining step in the inactivation mechanism. The rate of inactivation of GABA-AT by **7** was found to be 1/15 that of inactivation by the corresponding open-chain analogue **1**. Whereas inactivation by **1** releases only one fluoride ion, inactivation by **7** releases 148 fluoride ions (i.e., 148 molecules of **7** are turned over), accounting for the less efficient inactivation rate. Evidence using a tritium-labeled dibromo analogue (**13**) indicated that, unlike **1**, the cyclic analogue appears to inactivate GABA-AT by covalent attachment to the protein rather than to the PLP; 1 equiv of radioactivity was apparently covalently attached to the enzyme after inactivation and urea denaturation (*11*).



13

The mechanism of inactivation of GABA-AT by **7**, therefore, was proposed to occur via a Michael addition mechanism (Scheme 3, pathway a) rather than by the enamine mechanism (Scheme 3, pathway b) previously proposed for **1**. Both mechanisms are initiated by Schiff base formation (**8**) followed by rate-determining deprotonation and then elimination of fluoride ion to **9**. The Michael addition mechanism (pathway a) would produce adduct **10**, and the enamine mechanism (pathway b) would generate modified coenzyme **12**. Although the chemical evidence supports a Michael addition mechanism (the adduct formed by inactivation with dibromo analogue **13** is apparently covalently attached to the protein) over an enamine mechanism (the

Scheme 2

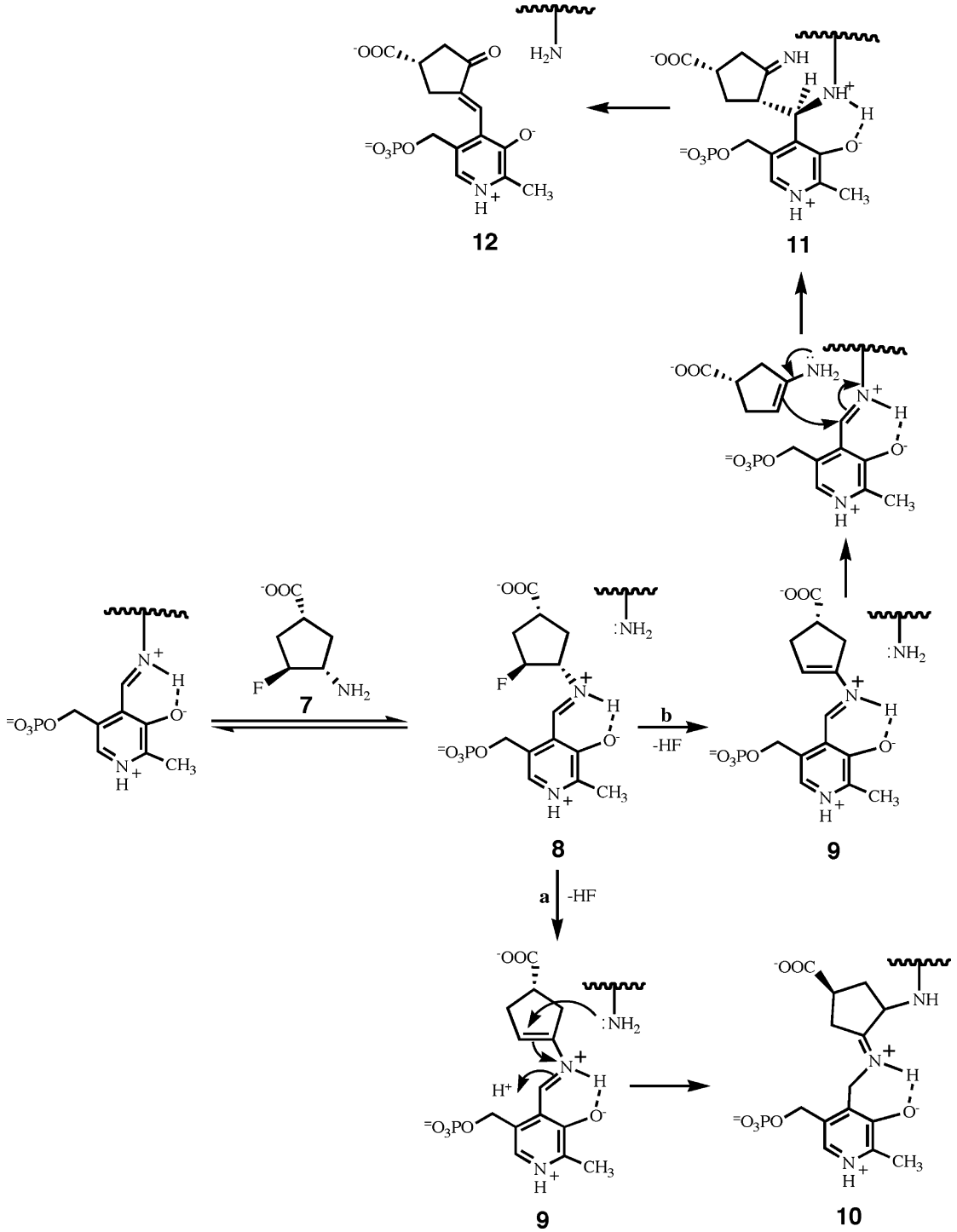


adduct would not be attached to the protein), a crystal structure of **7** bound to GABA-AT could more definitively differentiate these mechanisms. Here we report the use of X-ray crystallography to elucidate the structure of the adduct produced by inactivation of GABA-AT by **7**, which provides evidence for its mechanism of inactivation.

MATERIALS AND METHODS

Crystallography. Pig liver GABA-AT was purified as described previously (*13*). Crystals of the enzyme inactivated by **7** were grown at room temperature in sitting drops by vapor diffusion and microseeding. The protein solution (10−14 mg/mL in 40 mM sodium acetate buffer, pH 5.4, containing 100 mM **7**) was mixed in a 1:1 ratio with a precipitant solution containing 16−18% PEG 4000, 0−10% glycerol, and 50 mM sodium cacodylate buffer, pH 6.0, and 1 mM DTT. Diffraction data were obtained from a single flash-cooled crystal using 10% ethylene glycol as cryoprotectant. Data were collected on MAR image plates, processed with MOSFLM, and scaled with SCALA from the CCP4 suite (*14*). The models were refined with REFMAC5 including TLS refinement (*15*). Strong NCS restraints were imposed on the positional and thermal parameters of the four monomers in the asymmetric unit. In the initial rounds of refinement, the cofactor and Lys-329 were set as dummies. This was followed by cyclic averaging using DM (*16*) to

Scheme 3



obtain the best unbiased map for interpretation of the adducts. After model building, refinement of the final model was performed without torsional angle restraints on the linkages among Lys-329, cofactor, and the inhibitor moiety. Crystallographic data are given in Table 1.

RESULTS AND DISCUSSION

GABA-AT was incubated with **7** and allowed to crystallize in the presence of **7**, and then the structure was solved and refined to 1.9 Å resolution (Figure 1 and Table 1). There is little difference in the native protein structure and the structure with **7** bound (Figure 2). Figure 1 shows the electron density of the active site with the superimposed atomic model. It can be clearly seen that a ternary adduct is formed among the active site Lys-329, the PLP cofactor, and **7**. The C-4′ atom of the cofactor shows tetrahedral geometry with an *R*-configuration. Lys-329 was shown previously to attach to vigabatrin and γ-ethynyl-GABA, not to the cofactor, supporting a Michael addition mechanism in those cases (*7*). Arg-192 forms a salt bridge with the carboxylate of **7** (Figure 1), as was also observed in the other complex structures (*7*). In the reaction with GABA, this electrostatic interaction is expected to be important for substrate binding (*6*).

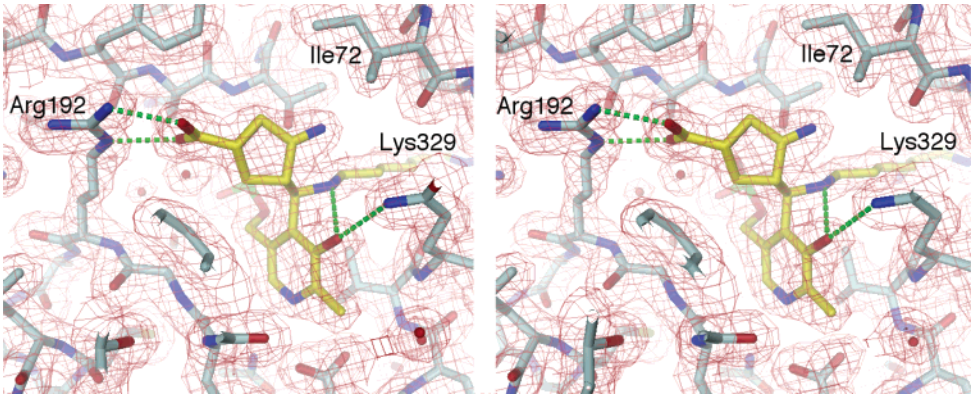


FIGURE 1: Stereographic projection of the active site of GABA-AT after chemical modification with **7**. The final model is shown together with the $2F_o - F_c$ map (contour level 1.2σ). The ternary adduct has been modeled according to structure **11** of Scheme 3.

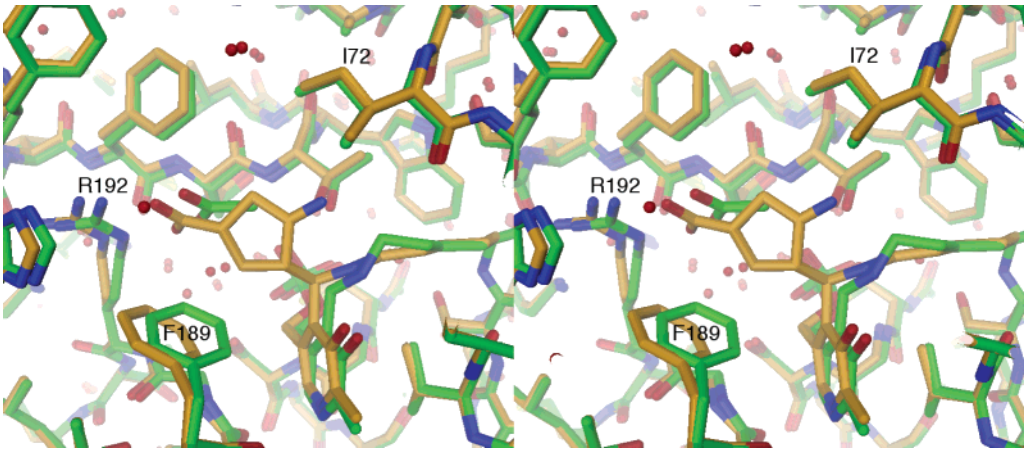


FIGURE 2: Comparison of the active site of native GABA-AT (green) (*7*) and the **7**-modified protein (orange). In the native stucture an acetate molecule is bound to R192, an interaction that most probably also occurs with the carboxylate moiety of the natural GABA substrate. Both protein structures are virtually identical. F189, however, is shifted to make room for the cyclopentane moiety in the inactivated structure. Similarily, R192 is slightly moved out of the substrate binding site.

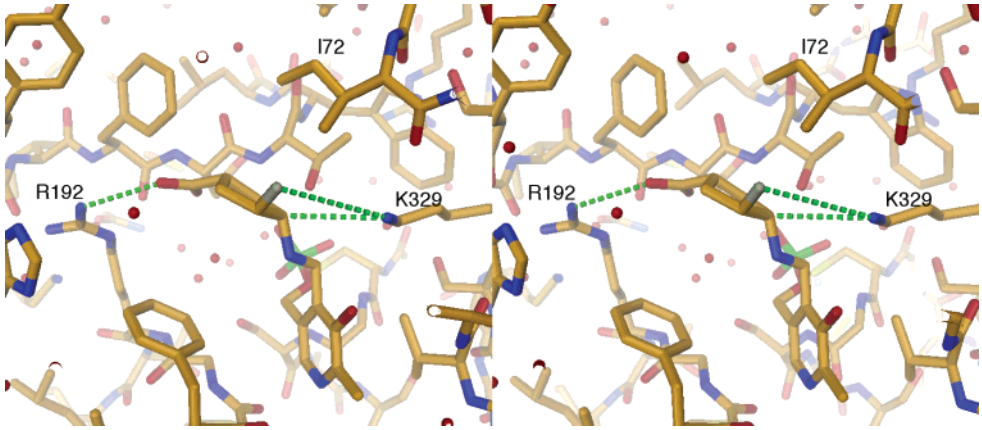


FIGURE 3: Three-dimensional model of the external aldimine of **7** with the PLP cofactor of GABA-AT (structure **8** of Scheme 3). The model has been built manually at the interactive graphic display. Correct stereochemistry has been enforced on the ligand. The torsion angle around the aldimine linkage is 180°. Although the protein structure, which is very similar to that of the cyclopentane inactivated structure, has not been adopted, only a few close contacts occur. Note that K329 is well poised to abstract the proton from C-4 and to donate it subsequently to the leaving fluoride ion (**8** → **9**, Scheme 3).

Unexpectedly, the electron density is entirely consistent with adduct **11** (Scheme 3, pathway b), implying that the reaction with **7** had proceeded along the enamine pathway, not the Michael addition pathway (Scheme 3, pathway a) suggested by the covalent protein adduct produced by the radioactive dibromo analogue **13** described above. Therefore, the observation that **13** becomes covalently attached to the protein, even after denaturation, suggests that this analogue undergoes a mechanism of inactivation different from that of **7** or at least forms a complex with the enzyme that is stable to denaturation.

To gain further insight into the stereochemical requirements of the reaction with **7**, a three-dimensional model of the external aldimine (**8**, Scheme 3) was built (Figure 3). This model resembles the proposed structure of the external aldimine with GABA (*6*) and shows that the additional atoms

Table 1: Data Collection and Refinement Statistics[a]

X-ray source; λ (Å)	ESRF, Grenoble; 0.984	
a, b, c (Å); β (deg)	69.3, 227.0, 71.1; 108.8	
resolution range (Å)	55−1.9	2.0−1.9
no. of reflns	150426	19892
R_{sym}[b] (%)	7.2	29
$\langle I\rangle/\sigma(I)$	3.6	2.1
completeness (%)	93	84
multiplicity	1.9	1.8
R_{factor}[c] (%)	18.6	25.7
R_{free}[d] (%)	20.4	28.0
no. of protein atoms	14683	
no. of cofactor/adduct atoms	24	
no. of FeS cluster atoms	8	
no. of water molecules	910	
mean overall B factor[e] (Å^2)	10.8	
rms deviation for bond lengths[f] (Å)	0.012	
rms deviation for bond angles[f] (deg)	1.2	

[a] The GABA-AT crystals are of space group $P2_1$ and contain two dimers in the asymmetric unit. [b] $R_{sym} = \sum_{hkl}\sum_i(|I(hkl) - \langle I(hkl)\rangle|)/\sum_{hkl}\sum_i\langle I(hkl)\rangle$. [c] R_{factor} is the conventional R factor. [d] R_{free} is the R factor calculated with 5% of the data that were not used for refinement. [e] Residual overall B factor after TLS refinement. [f] Rms deviation from ideal stereochemistry.

of the inactivator can easily be adopted in the binding pocket provided the amino group is rotated out of the pyridoxal plane. Lys-329 is well poised to abstract the proton from the C-3 atom and to donate it to the leaving fluoride ion. Subsequent transimination between the amino group of Lys-329 and the aldimine linkage (**9**) would liberate the modified inactivator that still would stay bound to the active site via the electrostatic interaction of its carboxylate moiety with Arg-192. An approximate 90° rotation of the modified inactivator about the long axis of the molecule and with the carboxylate as pivot would allow attack of the cyclopentene double bond at the PLP−Lys-329 aldimine linkage, resulting in observed adduct **11**.

Michael addition of Lys-329 to the cyclopentene double bond (**9** to **10** in Scheme 3**)** appears not to be feasible because the electrophilic double bond cannot be brought into proximity to the lysine amino group; furthermore, a high-energy rotation would be required as shown in Scheme 3, pathway a. For this same reason, the reactions with the conformationally restricted analogues of vigabatrin previously studied do not proceed via a Michael addition mechanism but by an enamine mechanism (*17*).

In conclusion, despite the tedious mechanistic studies that were carried out for the inactivation of GABA-AT by **7**, ambiguous results were obtained (*11*). The mechanistic quandary was resolved directly from the X-ray crystal structure of the inactivated enzyme as having proceeded by an enamine mechanism (Scheme 3, pathway b). This is the same mechanism hypothesized for the acyclic analogue **1** (*10*); the Michael addition mechanism, however, is the major pathway for γ-ethynyl-GABA and for the epilepsy drug vigabatrin (*5*). It must be noted, however, that the conclusions made for the mechanism of inactivation of GABA-AT by vigabatrin solely on the basis of its X-ray crystal structure bound to GABA-AT were incomplete (*7*). The major (Michael addition) product was readily identified, but the product of the minor pathway characterized chemically (the enamine product) was not observed, because either its relative occupancy was too low or the respective protein−adduct complex molecules were not incorporated into the crystal. In the case of **7**, however, the mechanistic studies led to an incorrect conclusion, which was rectified by an X-ray crystal structure.

REFERENCES

1. Silverman, R. B. (1995) Mechanism-Based Enzyme Inactivators, *Methods Enzymol. 249*, 240−283.
2. Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, Vols. I and II, CRC Press: Boca Raton, FL, 1987.
3. (a) Kent, U. M., Jushchyshyn, M. I., and Hollenberg, P. F. (2001) Mechanism-based inactivators as probes of cytochrome P450 structure and function, *Curr. Drug Metab. 2*, 215−243. (b) Yang, Y., Rasmussen, B. A., and Shlaes, D. M. (1999) Class A β-lactamases−enzyme-inhibitor interactions and resistance, *Pharmacol. Ther. 83*, 141−151. (c) Littlepage, L. E., Wu, H., Andresson, T., Deanehan, J. K., Amundadottir, L. T., and Ruderman, J. V. (2002) Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora-A, *Proc. Natl. Acad. Sci. U.S.A. 99*, 15440−15445.
4. (a) Dietz, E. C., Schaefer, A., Omichinski, J. G., and Nelson, S. D. (1997) Inactivation of glyceraldehyde-3-phosphate dehydrogenase by a reactive metabolite of acetaminophen and mass spectral characterization of an arylated active site peptide, *Chem. Res. Toxicol. 10*, 1097−1103. (b) Zechel, D. L., He, S., Dupont, C., and Withers, S. G. (1998) Identification of Glu-120 as the catalytic nucleophile in Streptomyces lividans endoglucanase CelB, *Biochem. J. 336*, 139−145. (c) Doerge, D. R., Divi, R. L., Hutton, T., and Lowes, S. (1995) Electrospray mass spectrometric analysis of resorcinol suicide substrate binding to cytochrome c peroxidase, *J. Mass Spectrom. Rapid Commun.* S136−S142.
5. (a) Burke, J. R., and Silverman, R. B. (1991) Mechanism of inactivation of γ-aminobutyric acid aminotransferase by 4-amino-5-hexynoic acid (γ-ethynyl GABA), *J. Am. Chem. Soc. 113*, 9329−9340. (b) Nanavati, S. M., and Silverman, R. B. (1991) Mechanisms of inactivation of γ-aminobutyric acid aminotransferase by the antiepilepsy drug γ-vinyl GABA (vigabatrin), *J. Am. Chem. Soc. 113*, 9341−9349.
6. Storici, P., Capitani, G., De Biase, D., Moser, M., John, R. A., Jansonius, J. N., and Schirmer, T. (1999) Crystal structure of GABA-aminotransferase, a target for antiepileptic drug therapy, *Biochemistry 38*, 8628−8634.
7. Storici, P., De Biase, D., Bossa, F., Bruno, S., Mozzarelli, A., Peneff, C., Silverman, R. B., and Schirmer, T. (2004) Structures of GABA aminotransferase, a pyridoxal 5′-phosphate and [2Fe-2S] cluster containing enzyme, complexed with the antiepilepsy drug vigabatrin and with γ-ethynyl GABA, *J. Biol. Chem. 279*, 363−373.
8. (a) Mumford, J. P., and Cannon, D. J. (1994) Vigabatrin, *Epilepsia 35* (Suppl. 5), 525−528. (b) Moshe, S. L. (2000) Mechanisms of action of anticonvulsant agents, *Neurology 55* (5, Suppl. 1), S32−40. (c) Kuzniecky, R., Hetherington, H., Ho, S., Pan, J., Martin, R., Gilliam, F., Hugg, J., and Faught, E. (1998) Topiramate increases brain GABA in healthy adults, *Neurology 51*, 627−629. (d) Petroff, O. A., and Rothman, D. L. (1998) Measuring human brain GABA in vivo: effects of GABA-transaminase inhibition with vigabatrin, *Mol. Neurobiol. 16*, 97−121.
9. Silverman, R. B., and Levy, M. A. (1981) Mechanism of Inactivation of γ-Aminobutyric acid-α-ketoglutaric acid aminotransferase by 4-amino-5-halopentanoic acids, *Biochemistry 20*, 1197−1203.
10. Silverman, R. B., and Invergo, B. J. (1986) Mechanism of inactivation of γ-aminobutyrate aminotransferase by 4-amino-5-fluoropentanoic acid. First example of an enamine mechanism for a γ-amino acid with a partition ratio of 0, *Biochemistry 25*, 6817−6820.
11. Qiu, J., and Silverman, R. B. (2000) A new class of conformationally-rigid analogues of 4-amino-5-halopentanoic acids, potent inactivators of γ-aminobutyric acid aminotransferase, *J. Med. Chem. 43*, 706−720.
12. (a) Likos, J. J., Ueno, H., Feldhaus, R. W., and Metzler, D. E. (1982) A novel reaction of the coenzyme of glutamate decarboxylase with l-serine O-sulfate. *Biochemistry 21*, 4377−4386. (b) Ueno, H., Likos, J. J., and Metzler, D. E. (1982) Chemistry

of the inactivation of cytosolic aspartate aminotransferase by serine O-sulfate. *Biochemistry 21*, 4387−4393.
13. De Biase, D., Barra, D., Bossa, F., Pucci, P., and John, R. A. (1991) Chemistry of the inactivation of 4-aminobutyrate aminotransferase by the antiepileptic drug vigabatrin. *J. Biol. Chem. 266*, 20056−61.
14. Collaborative computational project number 4. 1994. The CCP4 suite: programs for protein crystallography, *Acta Crystallogr., Sect. D: Biol. Crystallogr. 50*, 760−763.
15. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 57*, 122−33.
16. Cowtan, K. (1994) *Joint CCP4 ESF-EACBM Newsl. Protein Crystallogr. 31*, 34−38.
17. Choi, S., Storici, P., Schirmer, T., and Silverman, R. B. (2002) Design of a conformationally-restricted analogue of the antiepilepsy drug vigabatrin that directs its mechanism of inactivation of γ-aminobutyric acid aminotransferase, *J. Am. Chem. Soc. 124*, 1620−1624.

BI0487185