Stereochemistry in Inactivation of Carboxypeptidase A. Structural Analysis of the Inactivated Carboxypeptidase A by an Enantiomeric Pair of 2-Benzyl-3,4-epoxybutanoic Acids

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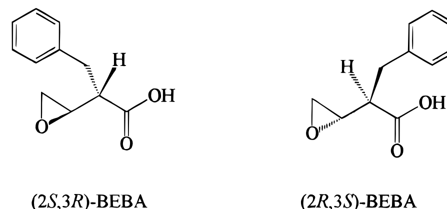
Abstract: The X-ray crystal structure of inactivated carboxypeptidase A by (2*R*,3*S*)-2-benzyl-3,4-epoxybutanoic acid, a pseudomechanism-based inactivator, was determined to show that the carboxylate of Glu-270 at the active site of the enzyme is covalently modified: the inhibitor is tethered to the carboxylate forming an ester linkage which is brought about by the attack of the carboxylate on the oxirane ring of the inhibitor. Examination of the crystal structure in comparison with that inactivated by its enantiomer, (2*S*,3*R*)-2-benzyl-3,4-epoxybutanoic acid, shows that the two inhibitors are bound covalently to the enzyme in a symmetrical fashion. An explanation for the lack of inactivating activity found previously with (2*R*,3*R*)- as well as (2*S*,3*S*)-2-benzyl-3,4-epoxybutanoic acids was offered.

Introduction

Carboxypeptidase A (CPA, EC 3.4.17.1) is a well studied zinc containing proteolytic enzyme[1] and has received much attention as it represents a large family of zinc containing metalloenzymes of physiological importance.[2] More importantly, CPA serves as a model enzyme in the development of inhibitor design principles which can be useful for designing inhibitors of therapeutic potential.[3−5] Recently, we have reported that 2-benzyl-3,4-epoxybutanoic acid (BEBA) is a pseudomechanism-based inactivator of CPA designed rationally making use of the unique property of the active site zinc ion: as for being a Lewis acid, the zinc ion activates the scissile amide bond of substrate for catalytic nucleophilic attack.[5] Kinetic analysis of the inactivation of CPA by BEBA revealed that both enantiomers having configurations of (2*S*,3*R*) and (2*R*,3*S*) are highly efficient and fast acting inactivators, and the remaining two stereomers do not exhibit irreversible inhibitory activity.[5d] The X-ray crystal structure analysis of the inactivated enzyme obtained by cocrystallizing the enzyme with the active racemic BEBA showed that the carboxyl group of Glu-270 is covalently modified by (2*S*,3*R*)-BEBA with formation of an ester linkage.[5b] The *S*-configuration at the 2-position of the inhibitor surprised us, because it corresponds to that of D-Phe which is unacceptable as the P_1' substrate residue for the enzyme. We have now obtained the X-ray crystal structure of CPA which is inactivated using optically active BEBA of (2*R*,3*S*)-configuration. In this paper we describe the X-ray crystal structure of the inactivated CPA and address the origin of the stereochemistry associated with the CPA inactivation by BEBA. Understanding of stereochemistry in enzyme inhibition



is extremely important especially in connection with the development of enzyme inhibitors having therapeutic potential. In general, stereoisomers of chiral inhibitors manifest differing biological responses, sometimes of opposite property.[8] Since CPA serves as a prototypical enzyme, the stereochemical knowledge learned from

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Table 1. Summary of the Crystallographic Data

space group	$P1$
unit cell	
a, b, c (Å)	49.43, 70.42, 48.49
α, β, γ (deg)	90.45, 110.63, 73.48
resolution range (Å)	8.0−2.2
number of unique reflcns	28298
number of atoms	5169
overall completeness (8.0−2.2 Å) (%)	90.6
completeness (2.5−2.2 Å) (%)	88.0
R-merge[a] (%)	9.0
R-value[b] (%), $F > 4\sigma(F)$	18.2
rms deviations	
bonds (Å)	0.011
angles (deg)	2.75
dihedrals (deg)	25.53
impropers (deg)	1.15

[a] R-merge $= \sum|\langle I\rangle - I|/\sum I$. [b] R-value $= \sum||F_o| - |F_c||/\sum|F_o|$.

the inactivation of the enzyme bears a special value in connection with designing inactivators for other zinc-containing enzymes.

Experimental Section

Crystallization. Carboxypeptidase A (CPA) was purchased from Sigma and used without further purification. The (2*R*,3*S*)-BEBA was prepared as described previously.[5d] The covalent complex of CPA and (2*R*,3*S*)-BEBA was prepared by incubating the mixture of the protein and the inhibitor in 1:10 molar ratio overnight at 4 °C. Crystals were grown by stepwise microdialysis of the CPA•(2*R*,3*S*)-BEBA complex in 1.0 M LiCl solution against lower concentration of LiCl solutions. Rod shaped crystals appeared after 2 days when the complex was equilibrated with 0.2 M LiCl solution at 4 °C. Even though these crystals grew as twinned, we were able to isolate single crystals by microtools.

Data Collection and Processing. The single crystals obtained as above had the typical dimension of 0.15 mm × 0.08 mm × 0.03 mm. The space group of these crystals is $P1$, and cell parameters are a = 49.43 Å, b = 70.42 Å, c = 48.49 Å, α = 90.45°, β = 110.63° and γ = 73.48°. From the unit cell and the partial specific volume of the enzyme, it is seen that the asymmetric unit contains two complexes of CPA•(2*R*,3*S*)-BEBA, and the solvent content is 44%. A data set with 90.6% completeness to 2.2 Å resolution was collected from one crystal by using a Rigaku RU300 rotating anode X-ray generator operating at 40 kV × 100 mA and R-Axis IIc imaging plate detector system. The R-Axis IIc data processing software was used for data processing. A summary of the crystallographic data is presented in Table 1. A self-rotation function calculated from the data showed that there is a 2-fold axis at ψ = 95.0, ϕ = 70.0, κ = 180.0. This diad is close to the c-axis. However, the unit cell translations do not satisfy the requirement of a monoclinic cell.

Structure Determination and Refinement. The three-dimensional structure was determined by molecular replacement using X-PLOR. The native CPA (pdb code: 5cpa) was used as a search model for the cross-rotation and translation searches. The cross-rotation search followed by Patterson correlation (PC) refinement gave two clear solutions at θ_1 = 330.492, θ_2 = 102.27, θ_3 = 43.70 with a correlation value of 0.397 and θ_1 = 287.81, θ_2 = 116.91, θ_3 = 205.77 with the same correlation value. All other peaks had a correlation value below 0.100. These two solutions are related by the diad found by the self-rotation function. The relative translation search between the two molecules in asymmetric unit gave a clear solution of 22σ above the mean at a = 0.743, b = 0.629, c = 0.286 in a fractional coordinates system. The next unrelated translation peak had a height of 4σ above the mean. The rigid body refinement with the rotation and translation function solution treating the two CPA molecules as different rigid groups gave a crystallographic R-value of 27.9% for 8.0−3.5 Å data. $2F_o - F_c$ maps calculated after the rigid body refinement clearly showed an extra density for the inhibitor. After the zinc atom was added in the model, a round of positional refinement with 8.0−2.2 Å data yielded the R-value of 26.1%. At this stage, the inhibitor was added into the extra density. Several rounds of positional refinement with individual B values and manual rebuildings decreased the R-value to 21.5%. The R-value of the final model with 5169 atoms including two molecules of the CPA•inhibitor complex and 267 H_2O molecules is 18.2%. The rms deviations from bond and angle idealities are 0.011 Å and 2.75°, respectively.

Results and Discussion

When CPA was cocrystallized with racemic BEBA of (2*R*,3*S*)- and (2*S*,3*R*)-configurations, CPA inactivated by (2*S*,3*R*)-BEBA was preferentially crystallized.[5b] This is in agreement with the result of kinetic study[5d] which revealed that (2*S*,3*R*)-BEBA is a more effective inactivator having the k_{incat}/K_i value of 139.5 M^{-1} s^{-1} than (2*R*,3*S*)-BEBA, the corresponding kinetic value of which is 53.9 M^{-1} s^{-1}. In the present study, therefore, we used optically active (2*R*,3*S*)-BEBA which was prepared as described previously.[5d] The inactivated CPA crystals were grown by stepwise microdialysis of CPA and (2*R*,3*S*)-BEBA mixture in 1.0 M LiCl solution against lower concentration of LiCl solutions. Rod shaped enzyme crystals were obtained. Figure 1 shows a stereoview of the active site electron density map of the inactivated CPA. As observed in the CPA inactivated by (2*S*,3*R*)-BEBA,[5b] the electron density map of the (2*R*,3*S*)-BEBA inactivated CPA also shows the presence of a continuous electron density to which a covalent bond can be modeled between one of oxygens of the Glu-270 carboxyl group and the ring cleaved BEBA (Figure 1). The average bond distance of the new covalent bond in the two CPA•BEBA complexes in the asymmetric unit is 1.43 Å (1.42 Å for one and 1.44 Å for the other) after a restrained refinement with a target value of 1.42 Å. The covalent bond between the Glu-270 carboxyl oxygen and the C_4 atom of BEBA indicates that the carboxyl of Glu-270 is again involved in the inactivation chemistry, attacking the oxirane ring of the bound (2*R*,3*S*)-BEBA at the 4-position with the generation of a hydroxyl group (Figure 1).[7] The newly generated hydroxyl at the C_3 of the inhibitor is coordinated to Zn^{2+} having the average bond length of 1.96 Å and the C−O−Zn^{2+} angle of 114.9°. Other structural data are listed in Table 2. The water molecule that is bound to the Zn^{2+} in the native CPA is not seen in the BEBA inactivated CPA.

In order to gain knowledge on this seemingly inexplicable stereochemistry associated with the inhibition of CPA by each of the enantiomeric pair of BEBA, the active site structure of covalently modified CPA by (2*R*,3*S*)-BEBA was superimposed with that of CPA which was inactivated by its enantiomer, (2*S*,3*R*)-BEBA (Figure 2). This direct comparison reveals that there are only very minor differences between the two structures except Tyr-248 and Ile-247. In the case of the (2*S*,3*R*)-BEBA inactivated CPA, the aromatic ring of Tyr-248 is noticeably pushed outward compared with that of the (2*R*,3*S*)-BEBA inactivated CPA. Apparently, this conformational reorganization was brought about in order for the substrate recognition pocket at the subsite S_1' to accommodate the benzyl group of (2*S*,3*R*)-BEBA whose stereochemistry at the 2-position corresponds to the "D" series. The substrate recognition pocket is constructed for the hydrophobic side chain of the P_1' amino

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(7) It may be worthy noting that in the inactivation of CPA by BEBA, the epoxide ring cleavage occurs exclusively *via* the S_N2 type mechanism rather than the S_N1 which is thought to be the preferred process for the Lewis acid catalyzed ring cleavage: Lewars, E. G. In *Comprehensive Heterocyclic Chemistry*; Lwowski, W., Ed.; Pergamon Press: Oxford, 1984; Vol. 7, pp 108−109.

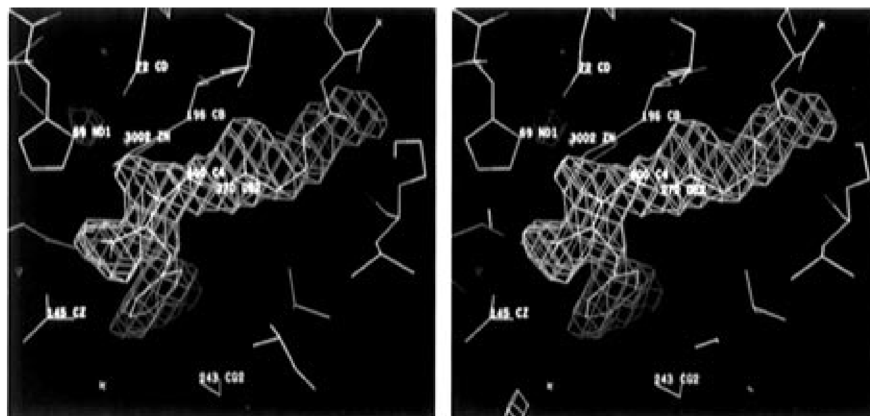

Figure 1. Stereoview of the electron density map at the active site region of the inactivated CPA by (2*R*,3*S*)-BEBA. The final model of the CPA·(2*R*,3*S*)-BEBA is superposed with the $F_o - F_c$ electron density map drawn in stereo at a contour level of 2.8σ. The $F_o - F_c$ map was generated by omitting Glu-270 and BEBA from the map calculation. The covalent bond between the C_4 atom of BEBA (labeled as 600 C4) and the OE2 of Glu-270 carboxyl group is evident.

Table 2. Interactions of BEBA with CPA

	(2*R*,3*S*)-BEBA			(2*S*,3*R*)-BEBA[a]		
	mol I[b]	mol II	average	mol I	mol II	average
angle (Zn−BEBA O3−C3) (deg)	118.5	111.2	114.9	115.8	101.7	108.8
distance (Zn−BEBA O3) (Å)	1.91	2.01	1.96	1.95	2.19	2.06
distance (Tyr 248 OH−BEBA C2) (Å)	4.04	3.87	3.96	3.74	3.60	3.67
distance (Arg 145 NH1−BEBA OE1) (Å)	2.79	2.77	2.78	2.99	2.87	2.93
distance (Arg 145 NH2−BEBA OE2) (Å)	2.76	2.78	2.92	2.92	3.13	3.03

[a] Values listed for (2*S*,3*R*)-BEBA were obtained from ref 5b. [b] Mol I and II represent the CPA:BEBA complexes I and II in the asymmetric unit of the CPA:BEBA crystals, respectively.

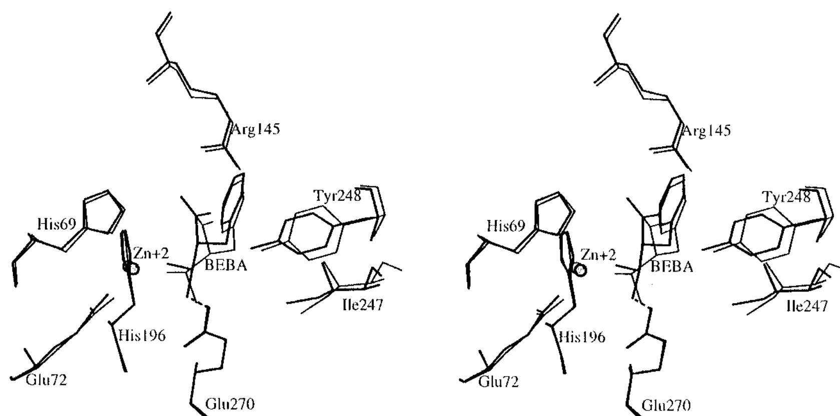

Figure 2. Comparative stereoview of the active site of the inactivated CPA by the enantiomeric pair of BEBA. Thick line represents CPA inactivated by (2*R*,3*S*)-BEBA and thin line that inactivated by (2*S*,3*R*)-BEBA. Dashed line denotes the C−O covalent bond that is formed as a result of the inactivation.

acid residue having the "L" configuration. It has been well documented that the side chain of Tyr-248 plays a pivotal role in binding of a ligand to CPA.[8] It is known to function as a lid, closing the mouth of the occupied S_1' subsite pocket by moving downwards from the surface of the enzyme to the mouth of the pocket when a ligand anchors at the active site.[8] Thus, the aromatic side chain of the bound substrate is held in the pocket until the catalytic action is over. In the case of inhibitor, the hydrophobic side chain anchored in the pocket is retained there by the "down" positioned side chain of Tyr-248. Expectedly, in binding of (2*S*,3*R*)-BEBA, the benzyl group cannot be fitted in the pocket perfectly. As a consequence, the aromatic ring of Tyr-248 cannot approach the mouth of the pocket as closely as it can in closing the pocket and is shown as if it is pushed outward compared with that of (2*R*,3*S*)-BEBA bound CPA. It is interesting to observe that the two inhibitors bind the enzyme in a symmetrical fashion, that is, they are tethered to the Glu-270 carboxyl having a mirror image relationship to each other along an imaginary plane consisting of the covalently modified carboxylate carbon, the zinc ion, and a midway point of the C_2 carbons of the both inhibitors (Figure 2).

It is apparent that the covalent modification of the Glu-270 carboxylate is initiated by attacking of the carboxylate on the activated oxirane ring of the enzyme bound BEBA. In this S_N2 type ring cleavage reaction, the carboxylate is expected to attack the electrophilic center from behind (back side) with respect to the cleavable C−O bond of the zinc coordinated oxirane. Therefore, the geometry of the C_4−O bond with respect to the nucleophilic Glu-270 carboxylate and the distance between the C_4 and the carboxylate are critical for the covalent bond formation reaction to occur. The zinc bound water molecule has been thought to be responsible for the hydrolytic cleavage of the peptide bond in the enzymic reaction of CPA.[1]. Ac-

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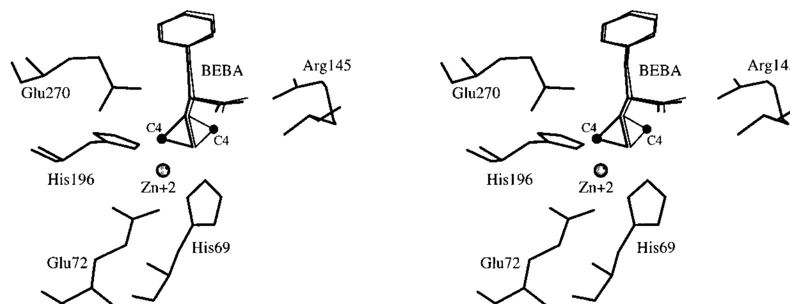

Figure 3. Model of noncovalent CPA·(2*R*,3*S*)-BEBA complex (thick line) built from the crystal structures of (2*R*,3*S*)-BEBA inactivated CPA is superposed with that of (2*R*,3*R*)-BEBA.

cordingly, the water molecule can also serve as a nucleophile, attacking the activated oxirane ring of the bound (2*R*,3*S*)- and (2*S*,3*R*)-BEBA to generate glycol derivatives. However, this possibility is precluded as seen from the inhibitory kinetic data[5d] and X-ray crystal structures of the (2*S*,3*R*)-[5b] and (2*R*,3*S*)-BEBA inactivated CPA. Failure of the water molecule to function as a nucleophile in attacking the oxirane may be to be due to its poor alignment with respect to the C_4−O bond. However, an alternative explanation that the water molecule may just be displaced by the oxirane oxygen of BEBA upon the inhibitor binding the enzyme cannot be excluded. It has been reported that the zinc bound water molecule becomes mobile and is easily displaceable when a ligand coordinates to the zinc ion.[9]

The lack of CPA inactivating activity observed previously with (2*R*,3*R*)- and (2*S*,3*S*)-BEBA may now be envisaged. It is apparent that if these BEBA stereomers bind the enzyme the way their diastereomers bind, that is, its benzyl group anchors in the S_1' pocket and the carboxylate hydrogen bonds to the guanidinium moiety of Arg-145, then the C_4 reaction center would not be rested at a position amenable for the S_N2 type reaction with the Glu-270 carboxylate. This is illustrated in Figure 3, in which one can see clearly that the C_4−O bond of (2*R*,3*R*)-BEBA is not only improperly aligned for the S_N2 type reaction but also the C_4 atom is too far distanced from the nucleophilic carboxylate. Similarly, the C_4−O bond of (2*S*,3*S*)-BEBA is misaligned for the S_N2 reaction. However, one should also consider the possibility that the failure of the CPA inactivation by these stereomers may have resulted as a consequence of their poor binding affinity to the enzyme by virtue of uncompatible configuration of the stereomers to the CPA active site. The formation of complexes of Michaelis type is prerequisite for the covalent modification. In fact, binding of these compounds to the enzyme was so poor that attempts at determining binding affinity were not successful.

A question that remains unanswered is why (2*S*,3*R*)-BEBA having the stereochemistry corresponding to the D series at the α-position inactivates the enzyme more effectively over its enantiomer. This question may perhaps be answered with the proposition that in the case of (2*S*,3*R*)-BEBA the unnatural mode of anchoring of the benzyl side chain in the S_1' subsite pocket would place the C_4 electrophile at a position where its interaction with the carboxylate is more conducive, possibly with an accompanying conformational change of the Glu-270 carboxylate caused by the anchoring. A very recent report of Nienaber *et al*. that a minor alteration of the structure of an inhibitor can lead to a small but significant reorganization of neighboring protein structure can be cited as a supportive argument for the proposition.[10]

In conclusion, it has been difficult to comprehend that both forms of enantiomeric BEBA, that is, (2*R*,3*S*)- and (2*S*,3*R*)-BEBA, inactivate the enzyme almost equally well, while their diastereomers are totally ineffective. The present study sheds light on this seemingly inexplicable stereochemistry associated with the inhibition of CPA by the nonpeptidic oxirane containing substrate analog inhibitors and thus provides valuable information for the design of inhibitors effective against other zinc containing enzymes. In addition, the present study demonstrates that *inhibitory stereochemistry of enzyme is far more complicated than that of substrates and cannot be simply inferred from* the *stereospecificity of substrates*. In this respect, it may be noteworthy that L-2-benzylsuccinic acid binds CPA much more tightly than its enantiomer,[11] while the D-form is the preferred inhibitor for thermolysin, a zinc containing endopeptidase of bacterial origin whose active site structure, mechanism of action, and stereospecificity resemble those of CPA.[12] Furthermore, D-*N*-chloroacetyl-*N*-hydroxyleucine methyl ester is reported to be a potent irreversible inhibitor for thermolysin, but its enantiomer is devoid of such activity.[13]

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