X-ray Crystallographic Study of Covalently Modified Carboxypeptidase A by 2-Benzyl-3,4-epoxybutanoic Acid, a Pseudomechanism-Based Inactivator

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In recent years, zinc-containing metalloenzymes have received special attention because of the roles that they play in the etiology of many serious diseases;[1] thus they serve as target enzymes in the design of therapeutically useful inhibitors of enzymes.[2] In this respect, carboxypeptidase A (CPA), a well-studied Zn^{2+}-containing metalloexopeptidase,[4] bears an unusual importance because it serves as a model for many metalloenzymes.[1,3,5]

Recently, we reported that 2-benzyl-3,4-epoxybutanoic acid (BEBA) is a novel type of CPA inhibitor which inactivates CPA irreversibly in high efficiency.[6] This inactivator was shown to be a pseudomechanism-based inactivator as designed on the basis of a proposed enzymic mechanism of CPA.[6] At the time, however, its nature as an inactivator and the proposed mode of inhibition were solely based on the kinetic studies and the chemical rationale used for the design. Now this communication reports the single-crystal X-ray characterization of the inactivated CPA by BEBA at 2-Å resolution,[7] showing clearly that the carboxylate of Glu-270 is indeed covalently modified by the inactivator.[6] Furthermore, the stereochemistry of the effective BEBA is established as the 2*S*,3*R* configuration on the basis of the electron density in $2F_o - F_c$ maps.

The electron density map (Figure 1) obtained from a single crystal[7] of inactivated CPA shows the presence of a continuous electron density between Glu-270 and the ring-cleaved BEBA to indicate clearly the formation of a covalent bond between the carboxylate of Glu-270 and BEBA at the 4-position with a concomitant ring opening. The length of the newly formed C–O bond was modeled to be 1.4 Å, a typical value for an ester linkage.[10] The hydroxyl at the C_3 of the chemically transformed inhibitor is coordinated to Zn^{2+} with the bond length 2.1 Å and the C–O···Zn^{2+} angle 103°, replacing the water molecule that is coordinated to the Zn^{2+} in the native CPA. The position of the carboxylate has changed little upon this covalent bond formation. As expected, the aromatic ring of Tyr-248 moved to the "down" position as seen in structures of CPA–ligand complexes.[11] The phenyl ring of the inactivator is seated in the S_1' subsite, having close contacts with Leu-203 and Ile-243 in a perpendicular fashion on each side of the ring with distances of 3.9 and 3.5 Å, respectively. This may be the result of CH/π interactions. The existence and importance of such interactions have recently been pointed out by Nishio and Hirata.[12]

The stereochemistry[7] of the inactivator deserves a special mention. We were surprised to learn that the stereochemistry of the C_2 stereogenic center is the *S* configuration because it corresponds to the D series, which is opposite to the known stereochemistry of CPA substrates.[13] CPA is shown to have L stereospecificity.[1,4] This reversal of stereochemistry became, however, apparent when molecular models of four possible stereoisomers of BEBA were examined in comparison with those of substrates. Thus, when the benzyl and the carboxylate moieties of each BEBA stereoisomer were superimposed onto the corresponding moieties of *N*-acetyl-L-phenylalanine, only the epoxy oxygen of the 2*S*,3*R* isomer, which is presumed to ligate to the Zn^{2+} at the active site, was positioned in close proximity to the amide oxygen of the substrate.

To our best knowledge, the present communication describes the first single-crystal X-ray structure of CPA covalently modified at the carboxylate of Glu-270, which has been implicated as the catalytic reaction site of CPA. Furthermore, it confirms the kinetic results reported in the previous communication[6] and strongly supports the letigimacy of the rationale used in the design of BEBA as a CPA inactivator.[6] Lastly, the present study bears a con-

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(2) This aspect has been amply demonstrated by the rational design of captopril, an antihypertensive agent which lowers high blood pressure by inhibiting the catalytic activity of angiotensin-converting enzymes.

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(7) The BEBA complex was cocrystallized with chromatographically separated BEBA in a racemic form by dialysis as described for the native crystals.[8] The BEBA·CPA crystals belong to space group $P2_1$ with a = 65.5, b = 60.6, c = 74.4 Å, β = 98.2°, and two molecules in the asymmetric unit. Data were measured using a FAST system to 2-Å resolution from two crystals (R_{merge} = 0.071). The structure was solved using the molecular replacement method with the native structure[8] as the starting model and was refined with X-PLOR.[9] The stereochemistry and conformation of bound BEBA were determined unambiguously from well-defined electron density in $2F_o - F_c$ maps. The present model, including two CPAs, two BEBAs, and 384 water molecules, is characterized by an *R* factor of 0.18 for 29 062 reflections between 15 and 2 Å, with the deviations from ideality of 0.015 Å and 3.08° for bond lengths and bond angles, respectively. Crystallographic details shall be included in a forthcoming full paper.

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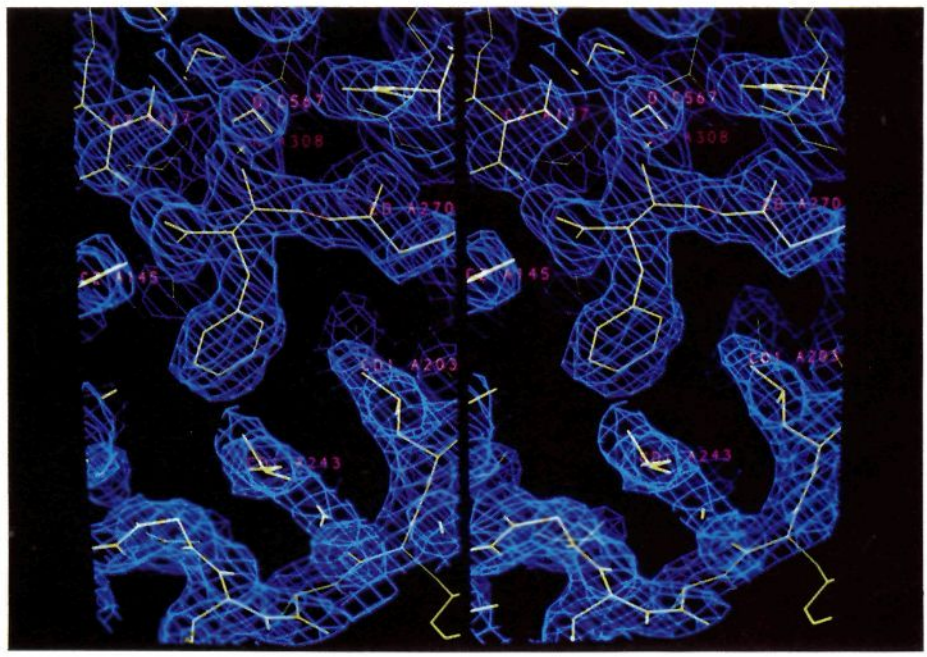

Figure 1. Stereoview of the electron density map and the final atomic model in the active site. The newly formed C–O bond between BEBA and Glu-270 of CPA is shown in red. The following residues are labeled: Arg-127, Arg-145, Leu-203, Ile-243, Glu-270, Zn^{2+} 308 (cross), and water 567.

siderable mechanistic significance,[1,4] tending to support the anhydride mechanism of CPA.[15] No covalent modification is expected to occur at Glu-270 by the alternative mechanism where the carboxylate of Glu-270 functions as a general base;[1,4] instead, a diol may be produced.

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