Crystallographic and Computational Insight on the Mechanism of Zinc-Ion-Dependent Inactivation of Carboxypeptidase A by 2-Benzyl-3-iodopropanoate

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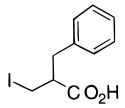
We reported recently a new strategy for inactivation of carboxypeptidase A (CPA), a prototypic metalloprotease, which exploited the active site zinc ion in its irreversible inactivation of the enzyme.[1] The inactivator, 2-benzyl-3-iodopropanoic acid (compound **1**), was designed to bind the active site of CPA, whereby the iodo group was expected to coordinate with the zinc ion. This binding interaction would activate the carbon−



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iodine bond by polarization toward nucleophilic substitution, hence promoting modification of the protein at a nucleophilic amino acid side chain within the active site (Scheme 1). We showed that compound **1** inactivated CPA in a time-dependent, active site directed, and irreversible manner and that the rate of covalent modification of the enzyme by **1** was enhanced by the protein active site by as much as 10^8- to 10^9-fold.[1] Inactivators of this type are of considerable interest in light of the fact that the key feature of the inactivation chemistry is the enhancement of the leaving ability of an *unactivated* halide by the active site transition metal for an S_N2-type displacement. *In principle, the chemistry should be generally applicable to any metalloenzyme, as long as tailor-made molecules could be designed to take advantage of the active site metal in the requisite rate enhancement process for covalent modification of various metalloenzymes*. We describe herein our structural analyses of the process of CPA modification by our inactivator **1**, which sheds light on a number of mechanistic features of the first inactivator of this type.

The iodo moiety of compound **1** was expected to coordinate to the active site zinc ion by design (Scheme 1).[2] As stated earlier, such a metal coordination would polarize the carbon−halogen bond, predisposing it to nucleophilic substitution. We have determined the X-ray structure of CPA modified by racemic compound **1** (Figure 1).[3] Crystals of the inhibited CPA were prepared by diffusing the inhibitor into the active site of native CPA crystals, as well as by completely inhibiting the enzyme in solution prior to crystallizing the inactive enzyme.

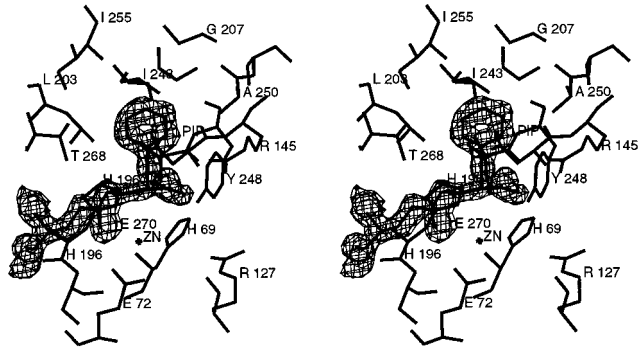
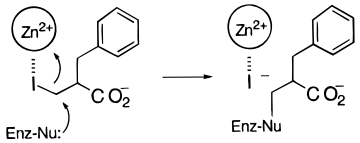


Figure 1. Stereoview of the crystal structure of CPA modified by 2-benzyl-3-iodopropanoate. The electron density within 2 Å of Glu-270 and its covalently attached inhibitor molecule is shown contoured at 1.5σ in a $2F_{obs} - F_{calc}$ map (7.0−1.6 Å). The electron density fits best for the enantiomer of compound **1** corresponding to D-Phe.

Scheme 1



Crystals from the latter method were used in this study because they gave much better data and were certain to have full occupancy of the inhibitor. The crystal structure of the inhibited CPA refined to an *R* value of 0.17 and an R_{free} value of 0.21 in a unit cell with four CPA molecules in the asymmetric unit. This unit cell, which retains the native *b*-axis, but has both the native *a*- and *c*-axes doubled, is reported here for the first time. The native unit cell was precluded by reflections at 0.5 intervals in *h* and *l*. A unit cell based on the diagonals of the native *a*−*c* plane with two molecules in the asymmetric unit[4] did not refine below an *R* value of 0.29. The four molecules are similar, as evidenced by root-mean-square overlaps of 0.3 Å or less in the C_α coordinates, but they are rotated sufficiently to break the symmetry of the native cell. The electron-density map unambiguously indicated that the side chain carboxylate of Glu-270 was modified by **1** to give an ester linkage (C−O bond length of 1.4 Å), with concomitant displacement of the iodide, which is not seen in the electron density. Whereas the racemic mixture of **1** was used to inactivate the enzyme, we only detected

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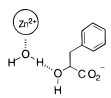
(1) Tanaka, Y.; Grapsas, I.; Dakoji, S.; Cho, Y. J.; Mobashery, S. *J. Am. Chem. Soc.* **1994**, *116*, 7475.

(2) The crystal structure for CPA inhibited by phenylacetate is now available (2CTC, Brookhaven Data Bank). The hydrolytic water was not displaced by this compound; however, as shown in the scheme below, the hydroxyl of the inhibitor makes a hydrogen bond to the hydrolytic water. This structure supports the coordination scheme for compound **1** that we have proposed.



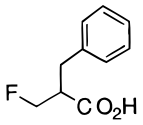
(3) Carboxypeptidase A, inactivated by a racemic mixture of **1**, gave crystals in space group $P2_1$ with $a = 105.7$ Å, $b = 60.5$ Å, $c = 91.8$ Å, $\beta = 97.2°$ and four protein molecules in the asymmetric unit. A total of 65 817 unique reflections with $F > 2\sigma$ were collected from two crystals ($R_{sym} = 0.039$) to 1.6 Å resolution on a Siemens X1000 multiwave detector. The structure was solved by molecular replacement with XPLOR using a CPA "tetramer" from the native crystals (Rees, D. C.; Lewis, M.; Lipscomb, W. N. *J. Mol. Biol.* **1983**, *168*, 367) as the search model. The current model, which includes four CPA molecules, four inactivator molecules, and 605 water molecules, gives an *R* factor of 0.17 ($R_{free} = 0.21$, $\Delta_{bond\ lengths} = 0.014$ Å, $\Delta_{bond\ angles} = 2.0°$) for data between 7.0 and 1.6 Å. Since the four molecules in the asymmetric unit are in crystallographically different environments, there is no restriction which forces a water molecule that is bound to one enzyme molecules to be bound also to any other, hence the number of water molecules need not be divisible by 4. Rigid-body refinement of the four independent molecules at 7−4 Å brought the *R* value to 0.244 and R_{free} to 0.246 (*R* is a measure of the "correctness" of the structure and is defined as $\Sigma||F_{obs}| - k|F_{calc}||/\Sigma|F_{obs}|$). The sum is taken over all of the reflections in the refinement, and *k* is a scaling constant. A small random sample of 10% of the data was set aside and was a cross validation of the *R* value in the refinement (Brünger, A. T. *Nature* **1992**, *355*, 472). Positional refinement at 7−2.8 Å was performed with 300 kcal mol^{-1} $Å^{-2}$ noncrystallographic symmetry (NCS) restraints in place, which brought *R* and R_{free} to 0.214 and 0.267, respectively. The data to 1.6 Å was added, the NCS restraints removed, water oxygens added at stereochemically reasonable positions where there was electron density in both $F_{obs} - F_{calc}$ and $2F_{obs} - F_{calc}$ maps, and the refinement continued to convergence.

(4) Yun, M.; Park, C.; Kim, S.; Nam, D.; Kim, S. C.; Kim, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 2281.

S0002-7863(96)03234-9 CCC: $12.00

the configuration for the C_2 of the inactivator bound to the enzyme which corresponds to D-Phe, a residue not found in typical substrates for CPA.[5] The side chain of Tyr-248 in the modified CPA was found in the "down" position, making a hydrogen bond to the inactivator carboxylate.[6] The Tyr-248 side chain experiences a motion of as much as 12 Å to make a hydrogen bond to substrates or substrate analogs, the contribution of which to turnover chemistry or the stability of the complex is estimated at approximately 2.5 kcal/mol.[7] The geometry of the zinc ion is tetrahedral, with a water molecule occupying the fourth coordination site.

Several attempts were made to obtain the X-ray structure for the noncovalent complex of **1** bound in the active site, in order to gain structural insight for the noncovalent enzyme−inhibitor complex prior to protein modification. In light of the rapid enzyme modification, all of these attempts were unsuccessful. We also used unsuccessfully the fluorinated analog **2** in these efforts. The fluoride moiety of **2** cannot be displaced in an S_N2



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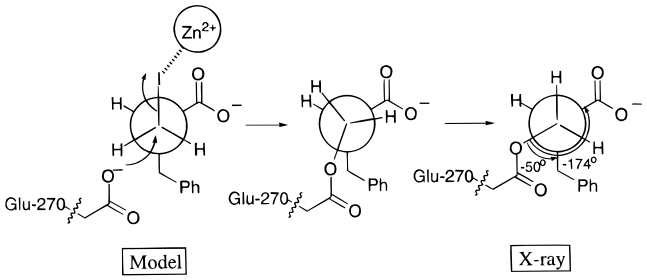
displacement, and compound **2** was shown to be merely a competitive inhibitor of CPA.[1] We were not able to obtain crystals of CPA with **2** in its active site.

We have generated energy-minimized models for the noncovalent enzyme−inactivator complex (data not shown). The bound inactivator was fitted within the active site in a manner similar to that for the crystal structures reported for known inhibitors for CPA. In the "down" orientation for Tyr-248, the phenolic hydroxyl of the side chain function forms a hydrogen bond with the inactivator carboxylate, as reported for several crystal structures for other inhibitors previously.[6] The carboxylate moiety of the inactivator makes additional hydrogen bonds with the side chain functions of residues Asn-144, Arg-127, and Arg-145.

We believe that it is likely that Tyr-248 would come down to make the hydrogen bond with the carboxylate of the inactivator in the noncovalent enzyme−inactivator complex, since there is ample precedent for such interaction.[6] However, in the course of the S_N2-type displacement of the iodo moiety, the attack of the nucleophile at the methylene carbon would necessitate an inversion of configuration. The inversion of configuration, its influence on the position of the Glu-270 side chain, and ultimately the generation of the modified amino acid contribute to the repositioning of the inhibitor in the active site.

Scheme 2



The stereoelectronic environment of the active site of CPA is well-suited for the proper positioning of the inactivator, the requisite coordination of the halogen moiety with the zinc ion, and the approach of the incoming nucleophile (the Glu-270 side chain). As shown in the Newman projections of Scheme 2, the approach of the Glu-270 carboxylate is from the distal orientation to the inactivator carboxylate and is flanked only by the hydrogen atoms of the methylene moiety. The distance between the Glu-270 O_ϵ and the inactivator methylene carbon is 2.7 Å in the model for the zinc-coordinated species. Furthermore, the improper torsion angle for Glu-270 O_ϵ−(I−C_3−C_2)$_{inhibitor}$ is 172°. The approach of Glu-270 O_ϵ to the methylene carbon would have to be initiated by the local motion of the protein. Hence, such a protein motion would not only bring the side chain of Glu-270 closer to the electrophilic carbon of the coordinated inactivator but also it would bring the requisite torsion angle closer yet to the ideal 180°. On the approach of the nucleophile, the attendant depature of the leaving group and inversion of configuration of the methylene, the complex would go through a high-energy eclipsed conformation, prior to the formation of the covalent species (Scheme 2). The dihedral angles that are shown for the product in Scheme 2 (−174° and −50°) deviate from the ideal situation somewhat (*i.e.*, −180° and −60°, respectively).

As stated earlier, carboxypeptidase A shows preference for L-amino acids in the C-terminal portion of its substrates. The fact that the enantiomer of compound **1** found in the active site of the modified CPA corresponds to D-Phe is unusual. Indeed, this type of preference for stereogenicity in inhibitors for this enzyme is documented only once previously.[4]

A sizable proportion of proteins of known structure possess transition metals in their active sites. Because of the generality of the principles disclosed in this manuscript, in principle, one should be able to prepare tailor-made inactivators of this type for any number of metalloenzyme; hence, the inactivation strategy should be of considerable interest with potential for many applications in the future.[8]

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(5) The stereogenecity corresponds to (*R*) for the noncovalently bound inactivator and to (*S*) for the covalently bound species.

(6) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62.

(7) Gardell, S. J.; Craik, C. S.; Hilvert, D.; Urdea, M. S.; Rutter, W. J. *Nature* **1985**, *317*, 551. Hilvert, D.; Gardell, S. J.; Rutter, W. J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1986**, *108*, 5298.

(8) The crystallographic coordinates have been deposited with the Brookhaven Protein Data Bank.