Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 13, pp. 1449-1452, 1996
Copyright © 1996 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00251-X

ELECTROSPRAY IONIZATION MASS SPECTROMETRIC ANALYSIS FOR INTERACTION OF α-CHYMOTRYPSIN WITH BEBAME, AN ACTIVE-SITE DIRECTED ENZYME INHIBITOR

Yeoun Jin Kim, Zhi-Hong Li, Dong H. Kim,* and Jong Hoon Hahn*

Center for Biofunctional Molecules and Department of Chemistry, Pohang University of Science and Technology, San 31 Hyoja Dong, Pohang 790-784, Korea

Abstract: Using the electrospray ionization mass spectrometric method, we have shown that (2S,3R)-2-benzyl-3,4-epoxybutanoic acid methyl ester (BEBAME) inactivates α-chymotrypsin most probably by modifying covalently the hydroxyl of Ser-195. Copyright © 1996 Elsevier Science Ltd

Recently, we have reported that (2S,3R)-2-benzyl-3,4-epoxybutanoic acid methyl ester (BEBAME) is a novel type of active-site directed inhibitor for α-chymotrypsin (α-CT) designed on the basis of the designing principle that makes use of the stereospecificity of the enzyme: the oxirane ring of the inhibitor is expected to be rested in the catalytic site and thus to experience a nucleophilic attack by the hydroxyl of Ser-195, forming an ether linkage to the inhibitor with opening of the oxirane ring (Figure 1).[1,2] The inhibitor showed a time dependent inactivation for α-CT to suggest strongly that the covalent modification was indeed occured at the active site of the enzyme. This communication describes the electrospray ionization (ESI) mass spectrometric study of α-CT/BEBAME complex to show that BEBAME inactivates the enzyme through the formation of a covalent bond and that Ser-195 is the probable site for the covalent modification.

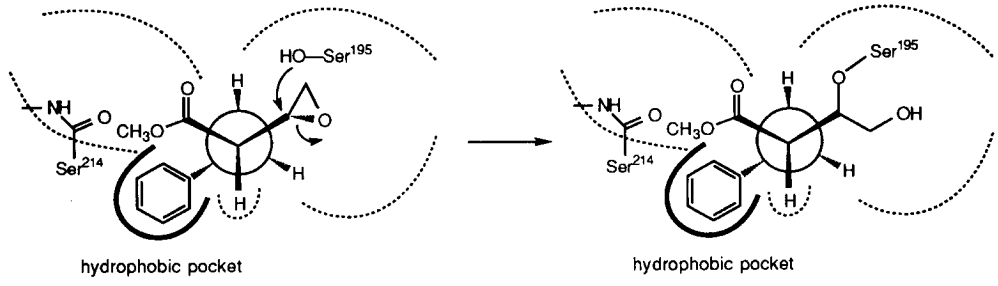

Figure 1. Schematic representation of the interaction of BEBAME with α-CT to covalently modify the enzyme at the hydroxyl of Ser-195.

Figure 2 shows transformed ESI mass spectra[3] of α-CT before and after incubation with BEBAME. It can be seen from the figure that as the incubation period is lengthened, the peak at 25440 Da which corresponds to the molecular weight of covalently modified α-CT by the inhibitor becomes predominant at the

expense of the peak of the free enzyme at 25234 Da. Although these data suggest strongly that the incubation causes BEBAME to interact with the enzyme, this does not necessarily prove that a covalent bond formation is resulted between them, because ESI can generate ions of noncovalent complexes as well.[4-9] However, the possibility that the peak of 25440 Da is due to the formation of a noncovalent complex of the enzyme with the inhibitor is highly unlikely, because the peak of 25440 Da was observed even after the incubation was quenched with a strong denaturant, trichloroacetic acid. If the peak at 25440 Da is due to a noncovalent complex, the complex is expected to dissociate upon the quenching, and the peak will be no longer observed.

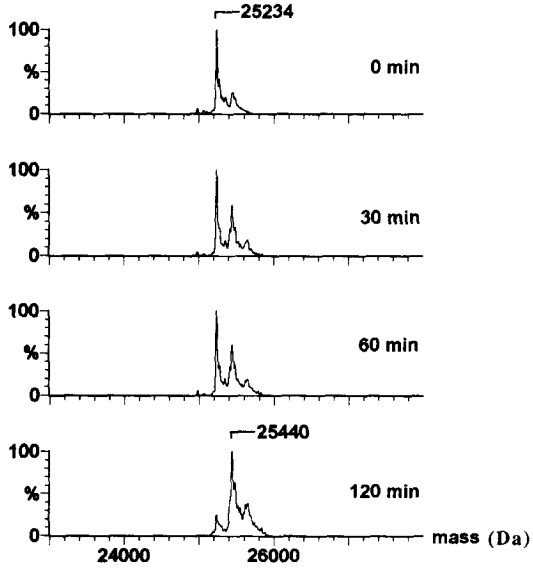

Figure 2. ESI mass spectrometric monitoring of the covalent modification of α-CT by BEBAME. A mixture of 9 x 10^{-5} M α-CT and 3 x 10^{-3} M BEBAME in 0.04 M Tris buffer (pH 7.8) was incubated at 25°C. After the time shown on each mass spectrum, the incubation was quenched by adding trichloroacetic acid. For a MS analysis, the product proteins were reconstituted with an acidified 50/50 water/acetonitrile solution (1% acetic acid). ESI mass specta of the proteins were obtained using a VG Biotech QUATTRO mass spectrometer.

The covalent complex of α-CT with BEBAME was digested with trypsin for 20 h at pH 8, the cleavage products were separated by reversed-phase HPLC, and each band thus obtained was examined by ESI mass spectrometry for the covalently modified amino acid residue. T17 peptide[10] obtained from the digestion has the following amino acid sequence: Asp^{178}-Ala-Met-Ile-Cys*-Ala-Gly-Ala-Ser^{186}-Gly-Val-Ser^{189}-Ser^{190}-Cys*-Met-Gly-Asp-Ser^{195}-Gly-Gly-Pro-Leu-Val-Cys*-Lys^{202} where Cys* represents a S-carbamoylmethyl cystein residue obtained by treating Cys with iodoacetamide to prevent thiols from forming disulfide linkages. Since T17 peptide contains Ser-195 residue, it should be the first target in checking for the presence of covalent modification. The ESI-generated ions of T17 peptide at pH 2 are expected to have two or more positive charges, because the peptide has two amino groups (one at the N-terminal and the other at the side chain of Lys-202) and three slightly basic amide groups (at Cys* residues). The calculated m/z values of $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions generated from T17 peptide modified covalently with BEBAME are 1348 and 899, respectively.[11] Each scan point in the total-ion-current (TIC) chromatogram (Figure 3a) was searched for those ion peaks. Plotting of the two ion currents against scan number produced single-ion-current (SIC) chromatograms corresponding to $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions (Figures 3b and 3c, respectively).

Comparing of the SIC chromatograms with the TIC chromatogram shows that the fraction indicated by an arrow in Figure 3a corresponds to BEBAME-linked T17 peptide. The ESI mass spectrum for the fraction (Figure 3d) was obtained by averaging the mass spectra of scan points under the band. Two of three major peaks in the spectrum corresponds to $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions of the peptide, and the peak at m/z 813.9 comes from a component that was not separated from the modified T17 peptide under our HPLC separation conditions. These analyses of mass spectrometric data suggest strongly that a nucleophile in T17 peptide, probably the serine hydroxyl, is responsible for the covalent modification.

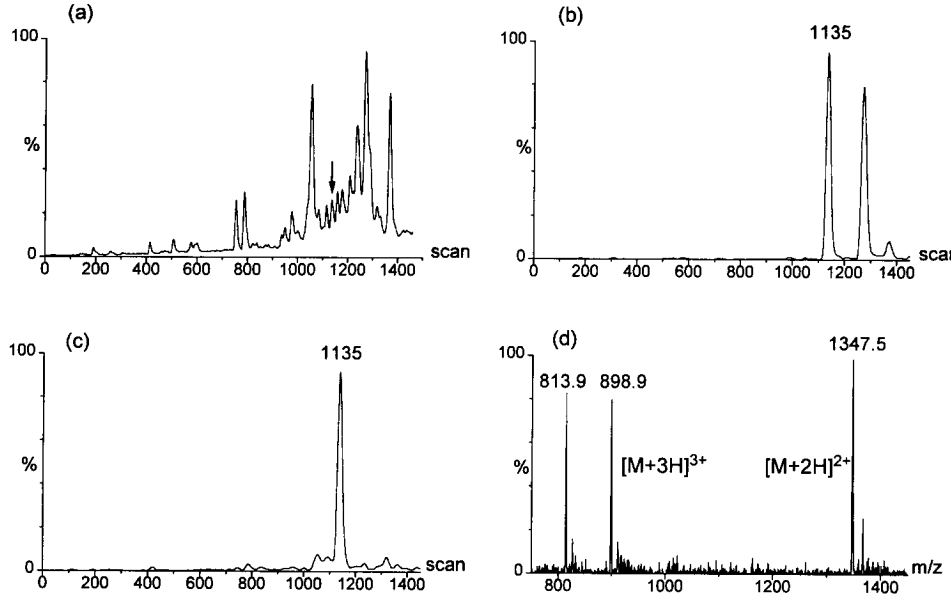

Figure 3. (a) An HPLC/MS TIC trace (m/z 650 – 1450) of a tryptic digest of the inactivated α-CT. A microbore (1 mm x 250 mm) C18 column was used. The entire effluent (50 μL/min) entered an ESI source. (b) and (c) SIC chromatograms of (b) m/z 1348 and (c) m/z 899 ions. (d) An ESI mass spectrum of the fraction indicated by an arrow in (a).

There are four serine residues (Ser-186, Ser-189, Ser-190, and Ser-195) in T17 peptide, but those serines except Ser-195 are highly unlikely to undergo a covalent bond formation with BEBAME since they are not suitably located for the chemical interaction with the oxirane ring of the active site bound inhibitor. The X-ray crystal structures of the free and inactivated α-CT revealed that Ser-186 is rested at the surface of the enzyme and the hydroxyl of Ser-190 is involved in the stabilization *via* hydrogen bonding of the tetrahedral transition state in the enzyme hydrolysis reaction.[12-18] Therefore, both serine residues are unlikely to interact with the oxirane ring of the inhibitor. Ser-189 is located also too remotely for its hydroxyl to attack the oxirane ring,[15] thus leaving the hydroxyl of Ser-195 as the only nucleophile which can undergo a reaction with the oxirane ring.

In conclusion, we have demonstrated using the ESI mass spectrometric method that the hydroxyl of Ser-195 is the most probable site for the covalent modification when BEBAME inactivates α-CT as expected from the designing rationale. Furthermore, together with the result from the kinetic analysis of the inactivation, the present study shows that the principle used in designing of BEBAME as a site-directed inactivator for α-CT is valid and is potentially applicable to the design of inhibitors of other serine proteases.

References and Notes

1. Kim, D. H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1313.
2. Kim, D. H.; Li, Z.-H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2297.
3. With appropriate computer algorithms, an ESI mass spectrum with differently charged molecular ion peaks of a component can be transformed to one which yields directly the molecular mass for the component.
4. Ganem, B.; Li, Y.-T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 7818.
5. Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1991**, *113*, 8534.
6. Light-Wahl, K. J.; Springer, D. L.; Winger, B. E.; Edmonds, C. G.; Camp, D. G., II; Thrall, B. D.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 803.
7. Ogorzalek Loo, R. R.; Goodlett, D. R.; Smith, R. D.; Loo, J. A. *J. Am. Chem. Soc.* **1993**, *115*, 4391.
8. Light-Wahl, K. J.; Winger, B. E.; Smith, R. D. *J. Am. Chem. Soc.* **1993**, *115*, 5869.
9. Cheng, X.; Chen, R.; Bruce, J. E.; Schwartz, B. L.; Anderson, G. A.; Hofstadler, S. A.; Gale, D. C.; Smith, R. D.; Gao, J.; Sigal, G. B.; Mammen, M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1995**, *117*, 8859.
10. Since trypsin cleaves amide bonds at the C-terminal side of arginine and lysine residues, it is expected from the known amino-acid sequece of α-CT that the tryptic digestion generates 20 different oligopeptides. T17 peptide thus obtained from the digestion is the 17th peptide counted from the N-terminal of α-CT.
11. Molecular weights of the doubly and triply protonated T17 are 2489 Da and 2490 Da, respectively, from amino acid sequence. The molecular weights of the protonated T17/BEBAME (206 Da) complexes are 2695 Da and 2696 Da, respectively. Since the number of charge of a T17/BEBAME complex is equal to the degree of protonation, the m/z values of the doubly and triply protonated complexes are calculated to be 1348 (2695/2) and 899 (2696/3), respectively.
12. Steitz, T. A.; Henderson, R.; Blow, D. M. *J. Mol. Biol.* **1969**, *46*, 337.
13. Freer, S. T.; Kraut, J.; Robertus, J. D.; Wright, H. T.; Xuong, N. H. *Biochemistry* **1970**, *9*, 1997.
14. Henderson, R. *J. Mol. Biol.* **1970**, *54*, 341.
15. Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145.
16. Blevins, R. A.; Tulinsky, A. *J. Biol. Chem.* **1985**, *260*, 4264.
17. Ringe, D.; Seaton, B. A.; Gelb, M. H.; Abeles, R. H. *Biochemistry* **1985**, *24*, 64.
18. Tsukada, H.; Blow, D. M. *J. Mol. Biol.* **1985**, *184*, 703.

(*Received in Japan* 1 *March* 1996; *accepted* 22 *May* 1996)