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THE FUNCTION OF S_1' SUBSITE POCKET OF CARBOXYPEPTIDASE A

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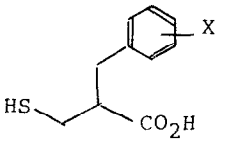
Abstract: Our study on the mechanism involved in the substrate recognition of Carboxypeptidase A using transition state analog inhibitors indicated that, on the contrary to the general believe, there is no hydrophobic interaction involved. The bulky hydrophobic moiety entered the narrow opening of the subsite pocket is held by Tyr-248 which moved from the enzyme surface until the catalytic action is over.

Carboxypeptidase A[1] (CPA, EC 3.4.17.1) is a digestive protease having a Zn^{2+} at the active site. It is one of most studied enzymes, and serves as a model for other metalloenzymes. The enzyme hydrolyzes preferentially the peptide bond of C-terminal amino acid having an aromatic moiety as the side chain. It is generally believed that such substrate specificity results from favorable interaction occuring between the hydrophobic side chain of the substrate and the S_1' subsite pocket present at the active site. The pocket has been known for a long time, but precise nature of the pocket and the mechanism involved in the recognition of potential substrate has not yet been clarified in spite of myriad of literature on CPA have been accumulated. In the preceding paper, we have reported that the principal recognition subsite, S_1' subsite is a pocket-shaped cavity having a narrow opening with approximate dimensions of 3.5 Å X 7.1 Å. In this report, we address on the question of the role that the S_1' subsite plays in the binding.

In biological system, hydrophobic interactions play an important role, their forces being 10 - 100 times stronger than van der Waals forces[2]. The hydrophobicity constant[3] that is defined as $\pi_x = \log P_{RX} - \log P_{RH}$ where P is experimentally determined partition coefficient between water and octanol represents a measure of hydrophobicity of the

substituent. It has been used successfully for an estimation of hydrophobic interactions involved when moderate size molecules bind an enzyme[4]. The inhibitor having a substituent of high π value binds more strongly than the one with low π value substituent, provided the hydrophobic interactions are the major binding force involved in the complex formation. Accordingly, one can evaluate the involvement of hydrophobic interactions in the complex formation by examining the relationships between the π values and the binding affinities of ligands, preferably of inhibitors.

Table I. Inhibitory constants (K_i) of 2-benzyl-3-mercaptopropanoic acid and its derivatives, and van der Waals volumes (V_w) and hydrophobic constants(π) of the substituents(X).



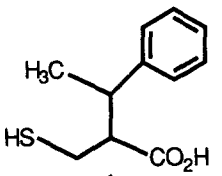
Compound no.	X	K_i[a]	V_w[b](A^3)	π[c]
1	H	11	5.6	0.00
2	4-F	37	11.5	0.14
3	4-CH_3	112	24.5	0.56
4	4-Cl	160	24.4	0.71
5	4-Br	150	28.7	0.86

[a] See the preceding paper. [b] From Moriguchi, I.; Kanada, Y. Chem. Pharm. Bull. 1977, 25, 926-935. [c] From Hansch, C.; Leo, A.; Unger, S.H.; Kim, K.H.; Nikaitani, D.; Lien, E.J. J. Med. Chem. 1973, 16, 1207-1216.

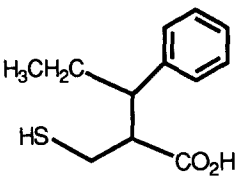
3-Mercapto-2-benzylpropanoic acid is a potent inhibitor of CPA reported by Ondetti et al[5]. In the binding of the inhibitor to CPA the aromatic ring is known to occupy the S_1' subsite of the enzyme. Table 1 shows the changes of the K_i values caused by various substituents on the phenyl ring of 3-mercapto-2-benzylpropanoic acid[6]. As expected, the K_i value increased as the van der Waals volume of the substituents increased irrespective of the nature of the substituent (Table I). Quite surprisingly, however, the K_i value also increased as the π values increased. The latter observation strongly suggests that hydrophobic interactions are not involved in the complexing of the aromatic moiety with the S_1'subsite pocket. Furthermore, when one compares K_i

values of 3 with that of 4, each having a different substituent of nearly equal van der Waals volume, it is found that 3 having substantially lower π value (0.56) than that (0.76) of 4 binds more favorably than 4 does. This observation suggests that not the hydrophobicity but the size of substituent governs the binding affinity. Apparently, the lack of hydrophobic interaction is due to the excessive inside volume of the pocket[7,8], where the interacting moieties are not within the effective distance for such interactions[9].

The question is then how the phenyl group is held inside the pocket during the cleavage reaction of peptide bond is occuring. Lipscomb and coworkers reported that CPA upon complexing with a inhibitor experiences conformational changes with the resultant movement of the aromatic ring of Tyr-248 by about 8 A from the surface of the enzyme to the vicinity of the S_1' subsite pocket[10]. Recently, Hilvert and coworkers[11] proposed based on their X-ray crystallographic data that the role of Tyr-248 is to provide a "hydrophobic lid[12]" in the binding of substrate. In order to elaborate this proposition we have examined the effect on the capping caused by introducing alkyl branches at the α-position to the phenyl ring of the side chain. Compound 6 that bears a methyl group at the position caused about 10-fold decrease in binding affinity compared with the parent compound. The decrease was drastically enhanced in the case of 7 where a bulkier ethyl group was introduced to about 140-fold. These decreases in the binding affinity may be ascribed to the inefficient capping of the subsite pocket by the Tyr-248 aromatic moiety because of the steric hinderance generated by the α-alkyl groups.



6
(K_i: 105 nM)



7
(K_i: 1,500 nM)

In conclusion, the present preliminary study together with x-ray crystallographic examinations[7,10] of CPA by others strongly suggest that the principal function of the S_1' subsite pocket of CPA in the catalytic action is to provide an anchoring space for the bulky side chain of the C-terminal amino acid of its ligand. Once the side chain enters the pocket it is then held there physically until the catalytic action is over by Tyr-248 which moved

down from the surface of the enzyme to the pocket opening rather than hydrophobic interactions between the hydrophobic group of the amino acid and the subsite. In this respect, the commonly used expression of hydrophobic pocket for the S_1' subsite appears to be inappropriate, and discontinuation of such usage is desirable.

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References and Notes

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5. Ondetti, M.A.; Conden, M.E., Reid, J.; Sabo, E.F.; Cheung, H.S.; Cushman, D.W. Biochemistry 1979, 18, 1427-1430.

6. The syntheses of these inhibitors and the determination of their K_i values, see preceding paper in this issue.

7. In the complex of CPA with substrate no hydrogen bonding appears to be in operation at the S_1' subsite. In the X-ray crystallographic study on the CPA complex with a transition state analog inhibitor (ZGP'), Christianson and Lipscomb failed to observe hydrogen bond contacts in the S_1' subsite pocket: Christianson, D.W.; Lipscomb, W.N. J. Am. Chem. Soc. 1986, 108, 545-546.

8. Kim, D.H.; Shin, Y.S.; Kim, K.B. Bioorg. Med. Chem. Lett. preceding paper in this issue.

9. Recently, Burley, S.K. and Petsko, G.A (Science, 1985, 229, 23-28) noted the importance of edge-to-face aromatic interaction in the biological system. From the X-ray crystallographic studies Christianson and coworkers claimed that such interactions are also found in the complex of CPA with an inhibitor, 5-amino-(N-t-butoxycarbonyl)-2-benzyl-4-oxo-6-phenylhexanoic acid: Shoham, G.; Christianson, D.W.; Oren, D.A. Proc. Natl. Acad. Sci. U.S.A. 1988, 85, 684-688. So far no such interaction has been, however, reported to operate in binding of S_1' subsite with substrare or inhibitor.

10. (a) Lipscomb, W.N.; Hartsuck, J.A.; Reeke, G.J., Jr.; Quicho, F.A.; Bethge, P.H.; Ludwig, M.L.; Steitz, T.A.; Muirhead, H.; Coppsia, J.C. Brookhaven Sym. Biol. 1968, 21, 24-90. (b) Reed, D.C.; Lipscomb, W.N. Proc. Natl. Acad. Sci. U.S.A. 1981, 78, 5455-5459. (c) Lipscomb, W.N. Tetrahedron, 1974, 30, 1725-1732.

11. Hilvert, D; Gardell, S.J.; Rutter, W.J.; Kaiser, E.T. J. Am. Chem. Soc. 1986, 108, 5298-5304.

12. It is not clear what they meant by the "hydrophobic lid".