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THE STRUCTURAL FEATURE OF S_1' SUBSITE OF CARBOXYPEPTIDASE A

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Abstract: The study on the structural feature of carboxypeptidase A using transition state analog inhibitors suggests that the principal substrate recognition subsite of the enzyme is a pocket-shaped cavity having a rectangular opening with effective dimensions of 3.5 Å X 7.1 Å.

Carboxypeptidase A (CPA, EC 3.4.17.1) is a prototypic zinc containing metalloenzyme which preferentially cleaves the C-terminal amino acid residue from a polypeptide substrate[1]. This hydrolytic cleavage is known to occur most rapidly when the C-terminal amino acid possesses a highly hydrophobic side chain such as a benzyl group[2]. This substrate specificity has long been thought to be due to hydrophobic interactions occuring between the hydrophobic side chain of the terminal amino acid and hydrophobic groups constituting the hydrophobic pocket(S_1' subsite[3]) present at the active site of CPA[4]. Even though the pocket has been known as the principal site for substrate recognition,the fundamental facet regarding its nature and function at the molecular level has not yet been addressed properly.

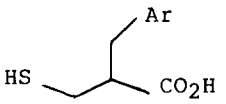
Detailed structural informations on the active site of an enzyme may be obtained through extensive computer analyses of its X-ray crystal structure, but this approach has its limitation, besides the high cost, since only limited number of enzymes have thus far been characterized the X-ray method. Such informations may also be available from the examination of molecular structures of its ligands. The structural features of the active site thus obtained are effective structural features rather than detailed actual absolute structures, and are of higher practical value for inhibitor design than those obtained from the X-ray crystal structures.

Pauling's proposition[5] that there should exist structural complementarity between the enzyme and the transition state of its substrate for effective enzymic action laid a foundation for the design of transition state analog inhibitors[6]. Thus, in order for a compound to be functioning as an effective transition state analog inhibitor, it must possess the molecular structure that is complementary to the active site structure of its target enzyme. Therefore, transition state analog inhibitors can be served as valuable tools in the study of the active site structure: The active site structure of an enzyme may be deduced from the molecular structure of its inhibitors. Besides the structural aspect, the transition state analog inhibitors have an additional advantage for such use over substrates which have been used occasionally in the past, for the K_i values of inhibitors represent pure binding affinity of the ligands unlike K_m values of substrates which frequently are complex quantities incorporating rate constants for multiple steps.

2-Benzyl-3-mercaptopropanoic acid is a tight-binding transition state analog inhibitor of CPA reported by Ondetti et al[7]. The mercapto group of this inhibitor is thought to be coordinating to the Zn^{2+} present at the active site[8], and the phenyl group anchores in the recognition pocket. A series of 2-arylmethyl-3-mercaptopropanoic acids having various substituents at the ring were synthesized by following the procedures described by Ondetti et al[7] with minor modifications, and their K_i values(Table 1) against CPA were determined from the kinetic measurements made at 25 °C in Tris buffer pH 7.5 containing 0.5 M NaCl using hippurylphenylalanine as substrate by the methods of the Lineweaver-Burk and the Dixon.

It can be seen from Table I that in general the K_i values are insensitive to the positions of the substitutions, but are markedly affected by the size and shape of the substituents. It is especially noteworthy that the replacement of the phenyl with a cyclohexyl group (17) causes a drastic increase of K_i value. Since the major difference between the two is found in their shapes, i.e., whereas the phenyl group has a planar shape, the cyclohexyl exists in a chair-like form[9], the decreased affinity shown by the cyclohexyl derivative may be ascribed to the difficulty of the subsite to accommodate the non-planar cyclohexyl

Table I. Inhibition Constants (K_i) for Carboxypeptidase A



Compound no.	Ar	K_i(nM)[a]	Compound no.	Ar	K_i(nM)[a]
1	C_6H_5	11	9	2-F-C_6H_4	45
2	2-CH_3-C_6H_4	120	10	3-F-C_6H_4	35
3	3-CH_3-C_6H_4	170	11	4-F-C_6H_4	37
4	4-CH_3-C_6H_4	112	12	3,4-F_2-C_6H_3	250
5	2,3-$(CH_3)_2$-C_6H_3	350	13	3,4,5-F_3-C_6H_2	590
6	3,4-$(CH_3)_2$-C_6H_3	492	14	2,3,4,5,6-F_5-C_6	2,300
7	4-$(CH_3)_2CH$-C_6H_4	4,500	15	1-naphthyl	480
8	4-$(CH_3)_3C$-C_6H_4	7,700	16	2-naphthyl	200
			17	cyclohexyl	2,350

[a]. K_i values were calculated from kinetic data plotted by the methods of Lineweaver-Burk and Dixon. Inhibitory measurements were made at 25 °C in Tris buffer hippurylphenylalanine as substrate. CPA (bovine) was purchased from Sigma.
[b]. From reference 7.

moiety. Extremely high K_i values observed with bulky isopropyl and tert-butyl derivatives (7 and 8, respectively), and the relatively low K_i values affinities shown by the naphthyl compounds (15 and 16) further support the view that the subsite preferentially accommodates only the planar shaped side chains. Recently, Suh et al[10] have made similar observations in the kinetic study of the CPA catalyzed hydrolysis of 2-(benzoylamino)cinnamoyl derivatives[11].

When the K_i value of 1-naphthyl derivatives (15) is compared with that of 2-naphthyl derivative (16), it is noted that the former binds less tightly than the latter, suggesting that 1-naphthyl moiety experiences substantially more difficulty in occupying the subsite than the 2-naphthyl moiety does. These observations may be explained in the following: The 1-naphthyl moiety is hard to enter the subsite because it is considerably larger in size compared with

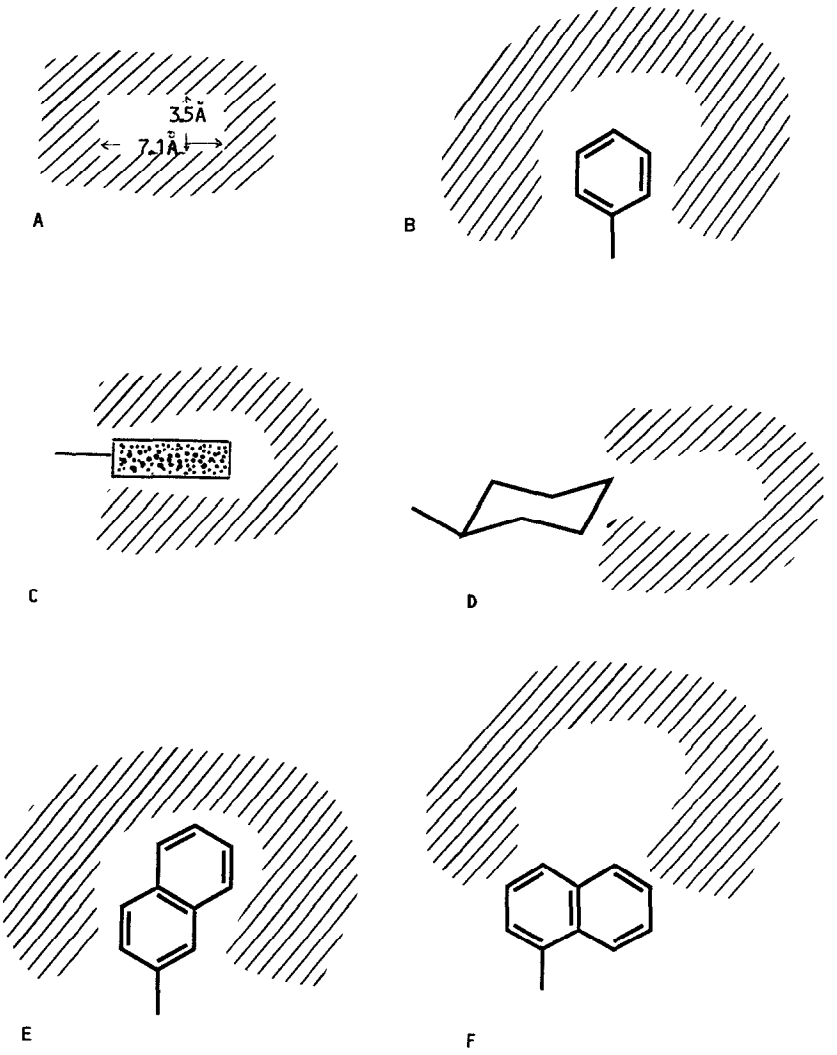


Figure 1. Schematic representation of the S_1' subsite pocket of Carboxypeptidase A. The pocket has a rectangular opening of effective dimensions of 3.5 A x 7.1 A (A). While planar phenyl ring enters the pocket snugly (B and C), cyclohexyl moiety that has a non-planar chair-like conformation can not enter the narrow opening (D). The 2-naphthyl moiety enters the pocket diagonally without much trouble (E), but the 1-naphthyl moiety experiences a difficulty in entering the pocket (F).

phenyl group(Figure 1). In case of 2-naphthyl, however, it may experience considerably less difficulty in entering the subsite pocket than the 1-naphthyl isomer does because the naphthyl ring in this case can enter the pocket in a diagonal fashion as shown in Figure 1F. Furthermore, the moderate binding affinity shown by the 2-naphthyl derivative seems to indicate that

the subsite is sufficiently deep, thus enough to accommodate the slantly entered ring(Figure 1C), suggesting the depth greater than 6.7 Å.

The above observations taken together suggest that the subsite has a rather narrow opening with the width just big enough for a planar phenyl ring to pass through snugly. Accordingly, based on the known thickness of benzene in terms of van der Waals interaction, i.e., 3.5 Å, the width of the pocket opening is estimated to be approximately 3.5 Å at the mimimum. As to the length of the opening of the pocket, since any moiety larger than benzene appears to be severly hampered in passing the opening as shown by the high K_i values of the 2-methylphenyl and naphthyl inhibitors, the length of the opening is figured to be roughly 7.1 Å[12].

In a study to locate hydrophobic portions of CPA, Kuntz[13] previously examined section maps which are 5.1 Å thicks perpendicular to the X-axis of Quicho and Lipscomb's X-ray crystal structure of the enzyme[14]. In this study he found seventeen channel-like hydrophobic regions in the interior of the enzyme molecule, of which several of larger regions were found to be extended to the surface of the enzyme molecule. When combining these findings with our results, it would appear that the S_1' subsite of CPA has the physical appearance of a channel-shaped pocket with rather voluminous interior but a narrow opening[15].

In conclusion, the preliminary results of present study directed towards the elucidation of the principal substrate recognition subsite (S_1') of CPA using its transition state analog inhibitors suggest that it is a pocket having at least 6.7 Å depth and a roughly rectangular shaped opening with effective dimensions of approximately 3.5 Å x 7.1 Å.

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3. The nomenclature used for subsites at the active site of CPA follows that of Schecter and Berger: Schecter, L.; Berger, A. Biochem. Biophys. Res. Commun. 1967, 27, 157-176.

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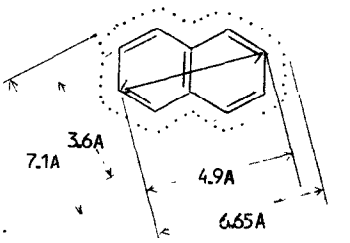
9. The chair-shaped cyclohexyl ring connected in equatorial fashion is believed to be oriented about 20° swayed from the nominal plane of the ring. However, the examination of the molecular model of compound 17 revealed that there is no noticeable misalign of the ring compared with its phenyl analog (compound 7). Furthermore, since there exists an fast chair-to-chair conformational equilibrium with energy barrier of 10.8 kcal/mole in the case of cyclohexane the difficulty of entering the ring to the pocket caused by the misalign would be rather insignificant, and the observed high K_i value appears to be mainly due to the nonplanarity of the ring.

10 Suh, J.; Cho, M. Bioorg. Chem. 1990, 18, 276-282.

11. They suggested that the hydrophobic pocket has a shape of cleft with a height comparable to the thickness of the benzene ring. This cleft model with a limited height can not explain satisfactorily the observed fact that the introduction of higher hydrophobic substituents with comparable size on the phenyl ring attenuates the binding affinity. Increase of hydrophobic interactions is expected between the hydrophobic group and the subsite cleft.

12. The van der Waals dimensions of naphthalene pertaining to the present discussion are calculated to be as the following:



13 Kuntz, I.D. J. Am. Chem. Soc. 1972, 94, 8568-8572.

14. Quicho, F.A; Lipscomb, W.N. Advan. Protein Chem. 1971, 25, 1-78.

15 The term "pocket" seems to reflect the subsite more closely to the actual entity than the term "cleft" because the latter gives the impression of being rigid, whereas the former has the implication of being flexible.