ON THE SIZE OF THE ACTIVE SITE IN PROTEASES
II. CARBOXYPEPTIDASE-A

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The size of the active site of carboxypeptidase-A was investigated by studying the kinetics of hydrolysis of peptides of L-alanine, D-alanine and L-phenylalanine, as well as of a number of their N-benzyloxycarbonyl, N-acetyl, N-phenylproprionyl and N-methyloxycarbonyl derivatives. From a comparison of the various kinetic parameters ($\overline{K}_m$, k_{cat}) it was concluded that the active site of this enzyme extends over about 18 Å. The binding area can be divided into 5 "subsites", each accommodating one amino acid residue (or blocking group) of the substrate. By comparing $\overline{K}_m$ values of pairs of substrates containing either a methyl or a benzyl side-chain in equivalent positions, it was shown that the binding area as a whole has a larger affinity towards the aromatic residues. Substitution of a D-residue for an L-residue reduced k_{cat} values rather than $\overline{K}_m$. In addition a remarkable affinity for the urethane-grouping located specifically at subsite S_3 was found. A methyloxycarbonyl or benzyloxy - carbonyl group occupying this subsite caused a 5-fold increase in $\overline{K}_m$ as compared with an acetyl or phenylproprionyl group, respectively. Methyloxycarbonyl or benzyloxycarbonyl groups in subsites S_2 or S_4 showed no such effect.

The method of measuring the size of an enzyme's active site by presenting it with substrates big enough to show up interactions with the further-most parts of the site has already been applied to papain (Schechter and Berger, 1967). The active site there was found to cover 25 Å, accommodating 7 amino acids of the substrate. It is shown here that in the case of carboxypeptidase-A too the reactivity of the susceptible bond depends not only on the two residues forming the bond but to a very large extent also on the nature of the residues further removed. In addition to comparing the reactivity of diastereoisomeric pairs, we also investigated the effect of replacing one side-chain by another. Special emphasis was given to the affinities of the substrates as expressed by $\overline{K}_m$ values, in the hope of bringing out specific interactions in the various subsites. Indeed, a certain degree of mapping of the active site was achieved.

METHODS. Carboxypeptidase-A (bovine pancreas), DFP treated, 3 x crystallized (Worthington) was used. Enzyme stock solutions in 10% LiCl were kept at 4°C. Enzyme activity was checked against $Ala_3(L_3)$ before every set of experiments, and was found to be constant for at least 3 weeks. The enzymic reactions were performed at 27°C in a medium containing 0.0375 M veronal buffer, pH 7.6, and 2.5% LiCl.

Initial rates were determined either polarimetrically (Bendix-Ericsson recording polarimeter, Type 143A) at 589 mμ, or by quantitative paper electrophoresis (Schechter and Berger, 1966), both methods giving identical results. The latter method was an important check in cases of consecutive reactions of comparable rates. Enzyme concentrations were chosen so as to give about 10% hydrolysis in 10 min. The kinetic constants were computed from initial rates over a suitable range of substrate concentrations, the Lineweaver-Burk plots being evaluated by least squares. Representative plots are given in Fig. 1.

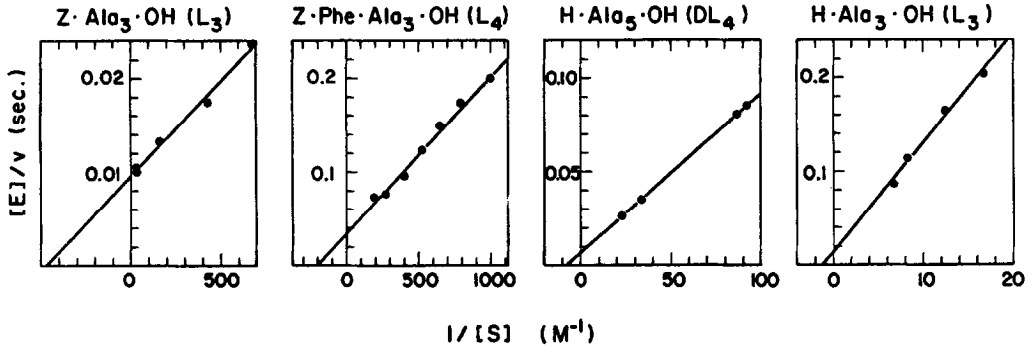

Fig. 1. Typical Lineweaver-Burk plots for the hydrolysis of a number of substrates by CP-A. For experimental conditions see text.

RESULTS. Kinetic parameters obtained with the various substrates are given in Fig. 2, which shows $\overline{K}_m$ values on a logarithmic scale; k_{cat} values are also shown. Low $\overline{K}_m$ values (1 to 10) are somewhat uncertain since substrate concentrations high enough for exact extrapolations were difficult to attain, but even in these cases the standard deviation for the intercept did not exceed 25%. Substrate inhibition was observed only with a few substrates, and in these cases points showing an upward trend at high [S] were omitted in the computations.

DISCUSSION. The observed data are interpreted on the basis of the following assumption: the substrate is lined up on the enzyme so that the C-terminal residue always occupies subsite S'_1 (on one side of the point of attack), and subsequent residues occupy subsites S_1, S_2 etc. (on the other side of the catalytic site, see Fig. 3). Variations in the kinetic parameters are taken as reflecting the interactions in the various subsites. For example, if the replacement of one residue (or blocking group) by another causes a significant change in either $\overline{K}_m$ or k_{cat}, this serves as evidence that the residue occupied part of the active site. Ratios of the kinetic parameters of pairs of substrates differing in one residue are given in Table I, which also shows the subsite occupied by the test residues. It is a well known fact that subsites S'_1 and S_1, flanking the catalytic site (see Fig. 3), are highly

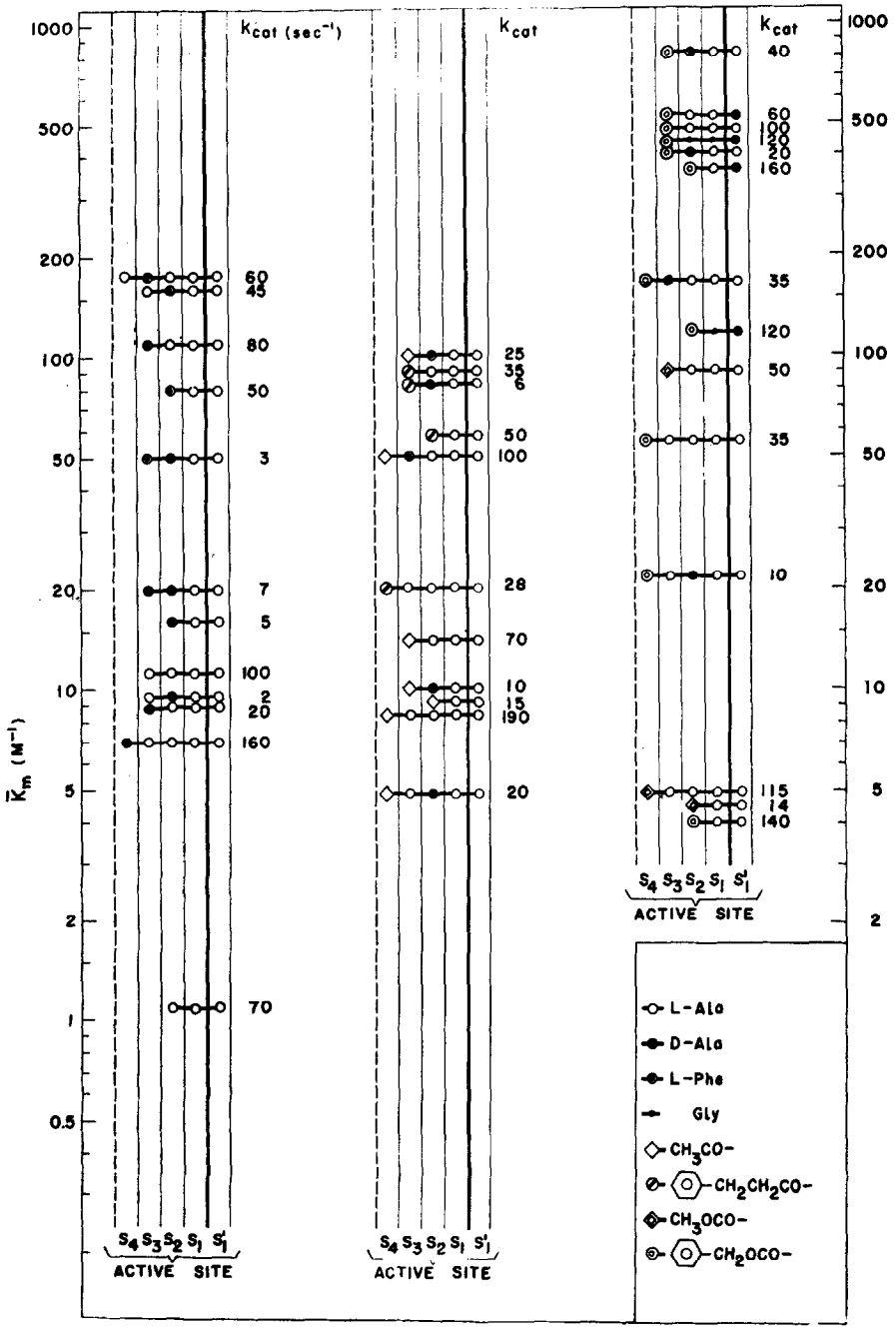

Fig. 2. The reciprocal Michaelis constants $\bar{K}_m$ (in M^{-1}) for the hydrolysis of the C-terminal peptide bond of a series of free and N-blocked peptides. The substrates are drawn schematically at the height corresponding to the $\bar{K}_m$ value, the heavy vertical line indicating the bond split. The subsites (S_1, S_2 etc) of the enzyme's active site occupied by the various residues during the splitting of the bond are indicated. The numbers at the right of each substrate symbol give the catalytic rate constant k_{cat} (in sec^{-1}) for the reaction (ES) $\longrightarrow$ E + products.

sensitive to substrate structure: peptides with D-residues in these positions are hydrolyzed extremely slowly; aromatic residues enhance rates considerably (Neurath, 1960; Schechter and Berger, 1966). The present data show that S_2 and S_3 also show strong discrimination with respect to their occupant. Even in S_4 one can see large differences between the acetyl and the benzyloxycarbonyl groups. Thus changes in substrate composition are felt over a range of five amino acid residues; this means that the size of the active site of carboxypeptidase-A corresponds to at least five amino acid residues

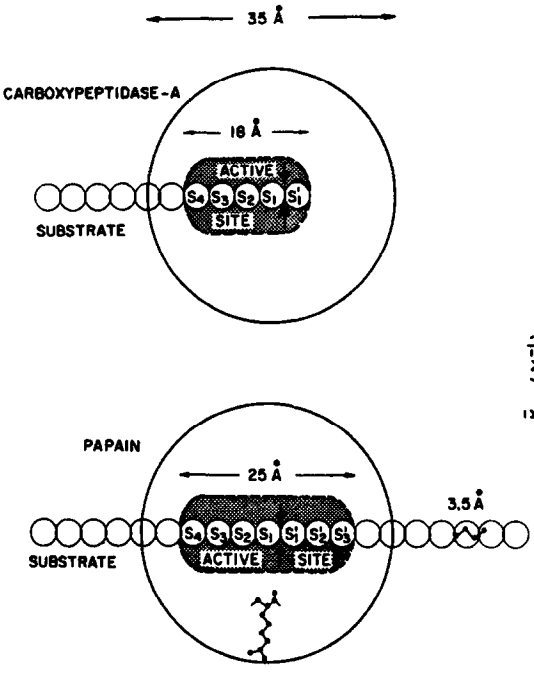

Fig. 3. Schematic representation of the enzyme substrate complexes of carboxypeptidase-A and of papain with a polypeptide chain. The shaded areas indicate the minimal extent of the active sites, the arrows the point of catalytic hydrolysis. Subsites are numbered from this point in either direction ($S_1, S_2 \ldots$ and S_1', S_2' etc.).

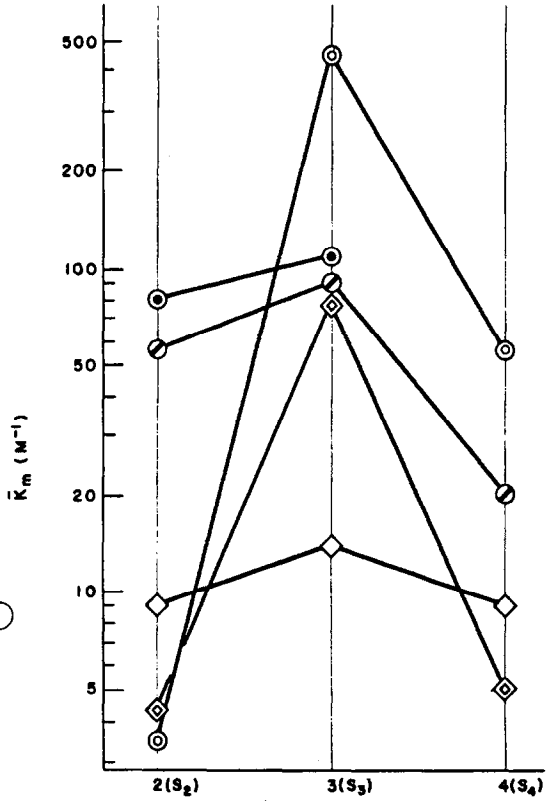

Fig. 4. Comparison of the $\bar{K}_m$ values for a series of substrates of the structure X·Ala$_n$ (L_n). X-groups are represented by: ◇ , acetyl; ◈ , methyloxycarbonyl; Ø , phenylpropionyl; ◎ , benzyloxy - carbonyl; ◉ , phenylalanyl. The abscissa gives the value of n and (in parentheses) the subsite occupied by the X - group.

(subsites S'_1 and S_1 to S_4, Fig. 3), or about 18 Å (taking 3.5 Å per residue). A schematic representation of the relationship between the length of the active site and the dimensions of the enzyme molecule is given in Fig. 3 for carboxypeptidase-A and for papain.

The catalytic rate constant k_{cat} is very sensitive to the steric configuration of the residues occupying subsites S_2 and S_3, whereas $\overline{K}_m$ is not. This is true for substrates of low affinity (the tetrapeptides Ala_4 LLLL, DLLL and LDLL) as well as for strongly

TABLE I. The Effect of Changes in Substrate Structure on Kinetic Parameters

The numbers are the ratios of $\overline{K}_m$ and k_{cat} for the pairs of substrates given. Abbreviations: L and D stand for L-alanine and D-alanine respectively; Phe, L-phenylalanine; Ac, acetyl; Moc, methyloxycarbonyl; Ø, phenylpropionyl; Z, benzyloxycarbonyl.

Replacement	Subsite occupied: S_4			S_3			S_2		
		$\overline{K}_m$	k_{cat}		$\overline{K}_m$	k_{cat}		$\overline{K}_m$	k_{cat}
L/D				L_4/DL_3	1.2	5	L_3/DL_2	0.1	14
				LDL_2/D_2L_2	0.5	0.3	L_4/LDL_2	1.1	50
							DL_3/D_2L_2	0.5	3
							$PhL_3/PhDL_2$	2	27
							$AcL_3/AcDL_2$	1.4	7
							$AcL_4/AcLDL_2$	2	10
							$ØL_3/ØDL_2$	1	6
							ZL_3/ZDL_2	1.1	5
							$ZL_4/ZLDL_2$	3	4
Ph/L				PhL_3/L_4	9	0.8	PhL_2/L_3	72	0.7
				$PhDL_2/LDL_2$	6	1.5	$LPhL_2/L_4$	14	0.5
				$AcPhL_3/AcL_4$	7	0.5	$AcPhL_2/AcL_3$	7	0.4
				$ZPhL_3/ZL_4$	3	1.0	$ZPhL_2/ZL_3$	1.7	0.4
Z/Ac	ZL_4/AcL_4	7	0.2	ZL_3/AcL_3	34	1.4	ZL_2/AcL_2	0.5	9
	$ZLDL_2/AcLDL_2$	5	0.5	$ZDL_2/AcDL_2$	42	2			
	$ZPhL_3/AcPhL_3$	3	0.3	$ZPhL_2/AcPhL_2$	8	1.6			
Moc/Ac	$MocL_4/AcL_4$	0.6	0.6	$MocL_3/AcL_3$	6	0.7	$MocL_2/AcL_2$	0.5	0.9
Ø/Ac	$ØL_4/AcL_4$	3	0.2	$ØL_3/AcL_3$	7	0.5	$ØL_2/AcL_2$	7	3
				$ØDL_2/AcDL_2$	9	0.6			
Z/Ph				ZL_3/PhL_3	4	1.2	ZL_2/PhL_2	0.05	3
				$ZDL_2/PhDL_2$	9	7			
Z/Ø	$ZL_4/ØL_4$	3	1.2	$ZL_3/ØL_3$	5	3	$ZL_2/ØL_2$	0.1	3
				$ZDL_2/ØDL_2$	5	3			
Ph/Ø				$PhL_3/ØL_3$	1.2	2	$PhL_2/ØL_2$	1.5	1.0
				$PhDL_2/ØDL_2$	0.7	0.5			

bound ones ($ZAla_3$ LLL vs. DLL, and $PheAla_3$ LLLL vs. LDLL), and indicates that the D residues do not interfere with binding but rather cause misalignment of the susceptible bond at the catalytic site.

Except for the influence of D-residues mentioned, the dependence of the k_{cat} values on substrate structure does not follow an easily recognizable pattern. It is intersting that a D-residue in S_2 followed by an L-residue with a free amino end-group in S_3 leads to exceptionally low k_{cat} values. The tetraalanine LDL_2 is 50 times slower than L_4, whereas D_2L_2 is only 3 times slower than DL_3. A similar effect is seen in the pair $PheAla_3$, where the LDL_2 compound is 27 times slower than the L_4 one.

From comparison of substrates with different side chains one can state something about the nature of the active site as a whole. Replacement of an acetyl group by a phenylpropionyl group or an alanine residue by a phenylalanine residue always leads to higher affinity (larger $\overline{K}_m$ values; see Table I and Fig. 4). In fact, all 21 substrates having $\overline{K}_m$ values larger than 20 M^{-1} (except $Moc{\cdot}Ala_3, L_3$) contain benzyl groups. The effect of the benzyl group is felt whichever of the five subsites it occupies, and is tentatively interpreted as indicating a hydrophobic environment.

An unexpected feature of the active site can be seen in Fig. 4. All the substrates compared here show a maximal $\overline{K}_m$ when the N-terminal end occupies subsite S_3, but this effect is several fold stronger with the urethane-grouping. The $\overline{K}_m$ of the methyloxycarbonyl compound $Moc\text{-}Ala_3$ is 6 times larger than that of $AcAla_3$, and it is the only aliphatic compound of the series with a $\overline{K}_m$ close to 100. Similarly, $ZAla_3$ has a $\overline{K}_m$ 5 times larger than phenylpropionyl-Ala_3. Indeed, the group of substrates with $\overline{K}_m$ of about 500 (see Fig. 2) all have Z-groups occupying S_3. This high value seems to result from the combined effect of the aromatic part and the urethane-grouping of the Z-group. It is significant that $Z{\cdot}Gly_2Phe$ has a $\overline{K}_m$ of 450, four times larger than that of the "classical" substrate Z·Gly·Phe, and that the "nonspecific" Ala-Ala bond in $ZAla_3(L_3)$ is likewise more susceptible than the Gly-Phe bond in Z·Gly·Phe.

References.

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