Size and Stereospecificity of the Active Site of Porcine Elastase†

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ABSTRACT: Two diastereomeric groups of compounds, peptides of the general formula Ala_4-Lys-Phe and Ala_5-Lys-Phe and *p*-nitrobenzyl esters of tri-, tetra-, and pentaalanine residues, were synthesized and subjected to enzymic hydrolysis by porcine elastase. The bond cleaved in the peptide substrates is between Ala and Lys, and in the *p*-nitrobenzyl esters the ester bond is cleaved. By comparing ring $\bar{K}_m$ and k_{cat} values of the appropriate pairs of substrates, we can show that the active site of elastase extends over about 25 Å (seven subsites). Elastase does not catalyze the hydrolysis of esters of the D form, and stereospecificity decreases as the distance from the cleaved bond increases. When a D residue is placed at the N terminal of the substrate, no effect on the mode of binding ($\bar{K}_m$) or on the catalytic power (k_{cat}) is observed.

Elastase (EC 3.4.4.7), a pancreatic porcine protease which belongs to the serine enzymes (Brown *et al.*, 1967; Hartley, 1970), possesses at least six subsites at the active site (Atlas *et al.*, 1970; Thompson and Blout, 1970). The active site is divided into two subsite categories according to their orientation *vis-à-vis* the cleaved bond; four subsites (S_1, S_2, S_3, S_4), or more, from the cleaved bond toward the N terminal of the substrate and at least two subsites (S_1', S_2') toward the C terminal of the substrate, according to the accepted nomenclature (Schechter and Berger, 1967). The above conclusions were established by studies of synthetic substrates where it was shown that the hydrolysis rates are strongly dependent on the chain length of the substrate (Gertler and Hofmann, 1969; Thompson and Blout, 1970; Atlas and Berger, 1972).

The object of the present study was to gain better insight into the length of the active site, from the scissile bond toward the amino terminal of the substrate, by comparing rates of hydrolysis of diastereomeric pairs of substrates containing D residues at the various positions (Schechter and Berger, 1967, 1968). In addition, this method established the stereospecificity of the subsites and sheds light on the nature of the local enzyme–substrate interactions. Having ester substrates enabled us to extend the study of the length and stereospecificity of the active site of porcine elastase. The alcohol residue of the ester chosen was *p*-nitrobenzyl alcohol, which was shown to possess considerable affinity toward subsite S_1' of the active site (Atlas and Berger, 1972). Our present work consists of comparison of $\bar{K}_m$ and k_{cat} values of diastereomeric hexa- and heptapeptides with the general structure Ala_4-Lys-Phe and Ala_5-Lys-Phe, as well as a series of *p*-nitrobenzyl esters of tri-, tetra-, and pentaalanine peptides.

Materials and Methods

Enzyme Kinetics. Porcine pancreatic elastase (EC 3.4.4.7) was a gift from Dr. D. M. Shotton, who described its preparation (Shotton, 1970). Rates of enzymic hydrolysis of peptides were determined by measuring the decrease of peptide-bond absorbance at 225 nm on a Gilford 2400-S spectrophotometer. The assays were done in Tris buffer, pH 8.6, 0.05 M at 25° (Atlas *et al.*, 1970). Ester hydrolysis was followed titrimetrically by alkali uptake in a pH-Stat (Radiometer, Copenhagen), type TTT 11, in conjunction with a pH meter, type 26, at pH 8.6 and 0.1 M KCl, in a thermostated reaction vessel at 25°, under an argon atmosphere. Titrant (NaOH) concentrations of 0.002–0.2 M and enzyme concentrations of 1×10^{-9}–2×10^{-7} M were chosen so that initial slopes of 0.5–10 divisions/min were obtained. Blank values (less than 0.5 division/min) were subtracted from the initial rates. Concentrations of substrate were determined on a dry weight basis, as well as by evaluation of alkali uptake at total enzymic hydrolysis. When measuring peptidase activity at the pH-Stat, corrections of the observed rates were made for ionization of the α-amino group at the cleaved peptide bond. All substrates were checked by high-voltage paper electrophoresis (at pH 1.9 and 6.5, 3000 V). It was affirmed that only esterolysis occurred during enzymic hydrolysis of the peptide–esters and products of peptide hydrolysis were identified by comparing them to synthetic markers.

Enzyme concentrations were based on absorption at 280 nm ($E^{1\%}_{1\,cm,\,280\,nm} = 19.0$). The specific activity of the enzyme was checked periodically using Ala_2LysAlaOMe as a substrate ($\bar{K}_m = 1.4 \times 10^4$ M^{-1}; $k_{cat} = 48$ sec^{-1}). Solutions of porcine elastase (0.5 mg/ml) in deionized water were kept at 0°. These solutions were found to be stable for periods longer than 2 weeks.

Substrates. (a) *p*-NITROBENZYL ESTERS OF ALANINE were prepared according to the procedure described elsewhere (Schechter and Berger, 1966; Atlas and Berger, 1972).

(b) PEPTIDES. Coupling of Blocked *N*-Hydroxysuccinimide Esters with Amino Acid *p*-Nitrobenzyl Ester Hydrochlorides. To a 0.2 M solution of amino acid (or peptide) *p*-nitrobenzyl ester hydrochloride in dimethylformamide, 1 equiv of triethylamine was added. An equimolar amount of the blocked *N*-hydroxysuccinimide ester was dissolved in an equal volume of dimethylformamide and the two solutions were combined. After 3–5 hr the reaction mixture was poured into 10 vol of water with stirring. The precipitate formed was collected, washed with water, and dried. The material was then dissolved in a small volume of methanol and precipitated by 20–30 vol of ether and dried *in vacuo* over H_2SO_4. Yields were 80–95%. An elementary analysis of the compounds is given in Table I.

Coupling with Dicyclohexylcarbodiimide. To a 0.2 M solution of the amino acid *p*-nitrobenzyl ester hydrochloride in ethyl acetate, 1 equiv of triethylamine was added. An equivalent amount of a *tert*-butyloxycarbonylamino acid, dissolved in an equal volume of ethyl acetate, was then added and the reaction mixture cooled to 4°. Dicyclohexylcarbodiimide (1

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TABLE I: Analytical Data of Peptide (Intermediate Compounds).

Compound[a]	Formula (Mol Wt)	Mp (°C)	Anal. (%) Calcd C	H	N	Found C	H	N
(L_2) Boc-ε-Z-Lys-Phe-ONb	$C_{34}H_{42}O_9N_4$ (660.7)	118	63.62	8.48	5.90	63.56	8.47	5.94
(L_2) HCl-ε-Z-Lys-Phe-ONb	$C_{30}H_{35}O_7N_4Cl$ (599.07)	146–149	60.14	5.98	9.35	59.90	5.89	9.40
(L_3) Boc-Ala-ε-Z-Lys-Phe-ONb	$C_{38}H_{47}O_{10}N_5$ (733.796)	130–133	62.19	6.46	9.54	62.02	6.51	9.24
(L_3) HCl-Ala-ε-Z-Lys-Phe-ONb	$C_{33}H_{40}O_8N_5Cl$ (670.147)	134–137	59.14	6.02	10.45	58.90	6.20	10.63
(L_4) Boc-Ala_2-ε-Z-Lys-Phe-ONb	$C_{41}H_{52}O_{11}N_6$ (804.374)	139–141	61.18	6.51	10.44	61.05	6.72	10.65
(L_4) HCl-Ala_2-ε-Z-Lys-Phe-ONb	$C_{36}H_{45}O_9N_6Cl$ (741.225)	158–160	58.33	6.12	11.34	58.10	6.22	11.10
(DL_3) Boc-Ala_2-ε-Z-Lys-Phe-ONb	$C_{41}H_{52}O_{11}N_6$ (804.374)	140–144	61.18	6.51	10.44	60.97	6.38	10.20
(DL_3) HCl-Ala_2-ε-Z-Lys-Phe-ONb (+ H_2O)	$C_{36}H_{47}O_{10}N_6Cl$ (759.241)	102–105	56.95	6.24	11.07	57.09	6.50	10.80
(L_5) Boc-Ala_3-ε-Z-Lys-Phe-ONb	$C_{44}H_{57}O_{12}N_7$ (875.374)	130–135	60.33	6.56	11.19	59.18	6.59	11.42
(L_5) HCl-Ala_3-ε-Z-Lys-Phe-ONb (+ H_2O)	$C_{39}H_{52}O_{11}N_7Cl$ (830.319)	180–183	56.41	6.31	11.81	56.54	6.50	12.08
(L_2DL_3) Z-Ala_4-ε-Z-Lys-Phe-ONb	$C_{50}H_{60}O_{13}N_8$ (981.044)	168–171	61.20	6.10	11.41			
(LDL_4) Z-Ala_4-ε-Z-Lys-Phe-ONb	$C_{50}H_{60}O_{13}N_8$ (981.044)	190–193	61.21	6.16	11.42	60.46	6.25	11.44
(DL_5) Z-Ala_4-ε-Z-Lys-Phe-ONb	$C_{50}H_{60}O_{13}N_8$ (981.044)	187–190	61.21	6.16	11.42	60.01	6.18	10.96
(L_7) Z-Ala_5-ε-Z-Lys-Phe-ONb	$C_{53}H_{65}O_{14}N_9$ (1052.122)	236–240	60.50	6.23	11.42	59.15	6.25	12.20
(LDL_5) Z-Ala_5-ε-Z-Lys-Phe-ONb	$C_{53}H_{65}O_{14}N_9$ (1052.122)	200–203	60.50	6.23	11.41	59.56	6.45	11.41

[a] Abbreviations used in the tables are: ONb, *p*-nitrobenzyl ester; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl.

TABLE II: Analytical Data of Peptide Substrates.

Substrate	Formula (Mol Wt)	Mp (°C)	Anal. (%) Calcd C	H	N	Found C	H	N
(L_2DL_3) Ala_4-Lys-Phe ($2H_2O$)	$C_{27}H_{47}O_9N_7$ (613.700)	200–203	52.84	7.72	15.98	52.55	7.93	15.66
(LDL_4) Ala_4-Lys-Phe ($2H_2O$)	$C_{27}H_{47}O_9N_7$ (613.700)	190–194	52.84	7.72	15.98	52.60	7.90	15.94
(DL_5) Ala_4-Lys-Phe ($3H_2O$)	$C_{27}H_{49}O_{10}N_7$ (631.718)	190–200	51.33	7.82	15.52	51.68	7.87	15.21
(L_6) $(HCl)_2Ala_4$-Lys-Phe ($2H_2O$)	$C_{27}H_{49}O_9N_7Cl_2$ (680.700)	203–205	47.30	7.14	14.20	42.84	7.45	13.02
(L_7) Ala_5-Lys-Phe ($3H_2O$)	$C_{30}H_{54}O_{11}N_8$ (702.798)	227–230	51.27	7.75	15.93	51.50	7.76	
(LDL_5) Ala_5-Lys-Phe ($3H_2O$)	$C_{30}H_{54}O_{11}N_8$ (702.798)	210–215	51.27	7.75	15.93	51.93	8.29	

equiv) was dissolved in the mixture which was then kept for 12 hr at 4°. The dicyclohexylurea formed was filtered off, and the filtrate was concentrated *in vacuo* until precipitation began, which was completed by adding petroleum ether. The material was collected and dried *in vacuo* over H_2SO_4. Yields were between 73 and 85%. Elementary analysis of the compounds is given in Table I.

N-Deblocking of *tert*-Butyloxycarbonyl Peptide *p*-Nitrobenzyl Esters. Blocked *tert*-butyloxycarbonyl peptides were dissolved in 0.5 N HCl in acetic acid (threefold excess) and kept for 20 min at room temperature. The reaction mixture was evaporated *in vacuo* at 40° and the residue dissolved in methanol and precipitated with ether. The ether precipitation from methanol was repeated and the material collected and dried *in vacuo* over H_2SO_4 and KOH. Yields were between 90 and 100%. An elementary analysis is given in Table I.

Catalytic Hydrogenation. The *N*-benzyloxycarbonyl peptide *p*-nitrobenzyl ester was dissolved in acetic acid (100–200 ml/g of peptide, according to solubility), with heating, if necessary. Water (10% of the total volume of the reaction mixture) and palladium (5% on activated charcoal) were added, and a stream of hydrogen was passed through the stirred solution. After 12 hr at room temperature the catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo* at 40°. The solid was dissolved in water and the solution evaporated to remove most of the acetic acid. On addition of ethanol crystallization began and was completed by the addition of ether. The compound was washed with ether and dried *in vacuo* over H_2SO_4 and KOH. Yields were between 60 and 90%. An elementary analysis is given in Table II.

Analytical data, etc., for new compounds are given in Table III (peptide *p*-nitrobenzyl esters), Table I (intermediates of the peptide synthesis), and Table II (peptide substrates).

In addition to the elementary analysis of new compounds, the purity of substrates, as well as of their intermediates, was checked using thin layer chromatography. Blocked peptides were checked using two solvent systems: chloroform–methanol (9:1) and the organic layer of butanol–acetic acid–water (4:1:4). Free peptides were checked for purity using high-voltage paper electrophoresis at pH 1.9 and 6.5, 3000 V.

Results

The steady-state kinetic parameters $\bar{K}_m$ ($1/K_m$) and k_{cat} were evaluated from double reciprocal plots of [E]/v *vs.* 1/[s] (the total enzyme concentration ([E]) in M; v in M × sec^{-1}; [s]

TABLE III: Analytical Data of Peptide Ester Substrates.

Substrate	Formula (Mol Wt)	Mp (°C)	Anal. (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
(Ala_3) HBr-DLL-ONb	$C_{16}H_{23}O_6N_4Br$ (447.500)	112–115	42.90	5.14	12.50			
(Ala_3) HBr-LDL-ONb	$C_{16}H_{23}O_6N_4Br$ (402.833)	146–149	47.66	5.70	13.90			13.30
(Ala_3) HBr-LLD-ONb (0.5H_2O)	$C_{16}H_{23}O_6N_4Br$ (456.290)	115–118	42.11	5.30	12.28	42.03	5.18	12.50
(Ala_4) HBr-DLLL-ONb (3H_2O)	$C_{19}H_{34}O_{10}N_5Br$ (572.418)	195–198	39.86	5.99	12.24	39.68	5.75	12.49
(Ala_4) HBr-LDLL-ONb	$C_{19}H_{28}O_7N_5Br$ (518.370)		44.02	5.44	13.51			
(Ala_4) HBr-LLDL-ONb	$C_{19}H_{28}O_7N_5Br$ (518.370)	174–180	44.02	5.44	13.51	44.06	5.70	13.12
(Ala_4) HCl-LLLD-ONb (2H_2O)	$C_{19}H_{32}O_9N_5Cl$ (509.903)	116–120	44.75	6.33	13.78	44.95	6.40	13.52
(Ala_5) HBr-LDLLL-ONb (2H_2O)	$C_{22}H_{37}O_{10}N_6Br$ (625.452)	115–119	42.20	5.96		42.19	6.16	
(Ala_5) HBr-LLDLL-ONb	$C_{22}H_{33}O_8N_6Br$ (589.450)	90–95	44.78	5.59	14.25			

in M) based on initial rates. Binding constants ($\bar{K}_m$), rate constants (k_{cat}), and the proteolytic coefficients ($C = \bar{K}_m k_{cat}$) are presented in Table IV (*p*-nitrobenzyl esters of alanine) and Table V (free peptides). The inhibition constants (K_i) of the *p*-nitrobenzyl esters were determined using Ala_2LysAlaOMe as a substrate and are listed in Table IV.

Discussion

In the investigation of the active site of pancreatic porcine elastase, one of the important aspects is the determination of its size. A very fruitfully used method is a comparison of the enzyme activity toward substrates in which one amino acid is replaced by its optical diastereoisomer (Schechter and Berger, 1967; Abramowitz *et al.*, 1967). Apart from the size of the active site, one can determine the local interactions at the subsites of the active center of the enzyme.

In an attempt to define more precisely both the size and the stereospecificity at the different subsites of the active site of porcine pancreatic elastase, we synthesized a series of alanine-*p*-nitrobenzyl esters and a series of peptides in which D- and L-alanine residues were exchanged systematically (see Tables IV and V).

TABLE IV: Kinetic Parameters for the Ester Hydrolysis of Alanine Peptide *p*-Nitrobenzyl Esters (the Arrow Indicates the Point of Cleavage).

Substrate	$\bar{K}_m$ (M^{-1})	k_{cat} (sec^{-1})	C (M^{-1} sec^{-1})	$\bar{K}_i$ (M^{-1})
L LL ↑ ONb	6,000	66.7	400,000	
D LL | ONb	6,000	64.5	387,000	
L DL | ONb	900	21.0	18,800	
L LD | ONb				450
LL LL ↑ ONb	24,000	77.0	1,800,000	
DL LL | ONb	18,000	70.0	1,260,000	
LD LL | ONb	1,400	125.0	175,000	
LL DL | ONb	3,000	25.0	75,000	
LL LD | ONb				560
LL L LL ↑ ONb	12,000	100.0	1,200,000	
LD L LL | ONb	2,000	77.0	154,000	
LL D LL | ONb	3,140	50.0	175,000	

Enzymic Hydrolysis of p-Nitrobenzyl Esters of Alanine Peptides. The derived steady-state parameters of the hydrolysis of *p*-nitrobenzyl esters of the D- and L-alanine peptides allow us to draw the following conclusions. Porcine elastase displays a considerable degree of stereospecificity at subsites S_1, S_2, S_3, and S_4. No hydrolysis could be detected (up to as high a concentration of enzyme as 1×10^{-6} M) when the D-alanine residue was at position P_1 of the substrate. Inhibition constants (K_i) of Ala_3ONb (L_2D) and Ala_4ONb (L_3D) (calculated from V_0/V *vs.* [I] plots) were found to have similar values (see Table IV).

Neither α-chymotrypsin (Ingles and Knowles, 1968) nor porcine elastase hydrolyzes ester substrates containing a D residue at the scissile bond. In both enzymes these esters act as inhibitors of the hydrolysis of the L isomer. Trypsin, on the other hand, displays a considerably lower degree of stereospecificity (Purdie *et al.*, 1971).

Subsite S_2 shows the highest stereospecificity ratio $(\bar{K}_m \cdot k_{cat})_L/(\bar{K}_m k_{cat})_D = C_L/C_D$, where C, the proteolytic coefficient, is defined as $\bar{K}_m k_{cat}$ (Ingles and Knowles, 1968). The 24-fold decrease in C value is manifested strongly in the binding ($\bar{K}_m$, eightfold) and to a considerably smaller extent in the catalytic activity (k_{cat}, threefold). The result was shown to be consistent in both the tri- and the tetrapeptide esters (Table IV). A high stereospecificity of subsite S_2 is also manifested by chymotrypsin A (Segal *et al.*, 1972).

As for the stereospecificity of subsites S_3 and S_4, we have to consider previous results (Atlas and Berger, 1972). It has been reported that the rate of catalysis (k_{cat}) of *p*-nitrobenzyl esters of dialanine hardly changes upon adding the third and the

TABLE V: Kinetic Parameters for the Hydrolysis of Peptide Substrates (the Arrow Indicates the Point of Cleavage).

Substrate[a]	$\bar{K}_m$ (M^{-1})	k_{cat} (sec^{-1})	C (M^{-1} sec^{-1})
LL LL ↑ Lys-Phe	4500	32.0	144,000
DL LL | Lys-Phe	1000	25.0	25,000
LD LL | Lys-Phe	300	2.0	600
LL DL | Lys-Phe	250	2.5	625
LL L LL ↑ Lys-Phe	6000	50.0	300,000
LD L LL | Lys-Phe	1000	2.0	2,000

[a] L and D denote the two stereoisomers of alanine.

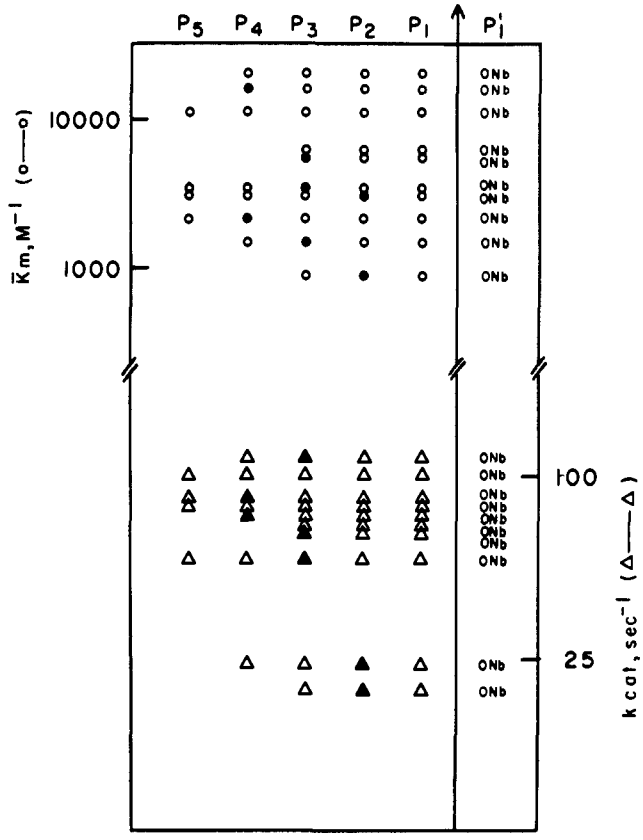

FIGURE 1: $\bar{K}_m$ and k_{cat} value for the hydrolysis of *p*-nitrobenzyl esters of alanine peptides by porcine elastase: L-amino acids, K_m (○), k_{cat} (△); D-amino acids, K_m (●), k_{cat} (▲).

fourth L-alanine residues. It was suggested that the strong mode of binding of the *p*-nitrobenzyl group does not change *vis-à-vis* the catalytic system, with additional alanine residues at P_3 and P_4; thus the contribution of the interactions at subsites S_3 and S_4 is minor. Therefore, it is unlikely to expect changes in the rate of catalysis due to diastereomeric changes at subsites S_3 and S_4. If, however, subsites S_3 and S_4 are important for catalysis, it will be expressed in peptide hydrolysis, where we deal with a chain containing amino acid residues only.

Stereospecificity of subsites S_3 and S_4 expressed in the ester hydrolysis stems from the binding only (see Tables IV and VIa and Figure 1).

Quite remarkable is the lack of almost any stereospecificity at subsites S_3 and S_4 when the D residue is the N-terminal one (Table IV). However, upon adding an L residue to the N terminal, the effect of the D residue at S_3 and S_4 is expressed (Tables IV and VIa). A possible explanation for the appearance of stereospecificity at S_3 and S_4 is that the added peptide bond considerably restricts the freedom of rotation of the bond between C^α and the carbonyl carbon (dihedral angle ψ) due to the presence of the methyl group of the D residue and thus induces an unfavorable geometry at the catalytic site.

As for subsite S_5, it is interesting to note that although position P_5 is quite remote from the bond split, it contributes to enzymic catalysis, either by full occupancy at subsite S_5, or by abolishing the charge on the α-amine at the P_4 alanine residue *via* the formation of the P_4–P_5 peptide bond.

Enzymic Hydrolysis of Peptides. As mentioned previously (Atlas and Berger, 1972), enzyme specificity does not always overlap when comparing data of esterase and peptidase activity.

TABLE VI: Ratio of the Kinetic Parameters of Substrates of All L Configuration and Substrates with D Configuration at the Various Positions.

Subsite Occupied by a D Residue	Substrate Ratio	$\bar{K}_m/\bar{K}_m$	k_{cat}/k_{cat}	C/C
	(a) Esters			
S_2	LLL ONb / LDL ONb	6.70	3.2	21.50
S_2	LLLL ONb / LLDL ONb	8.00	3.00	24.00
S_3	LLL ONb / DLL ONb	1.00	1.00	1.00
S_3	LLLL ONb / LDLL ONb	17.50	0.616	10.30
S_3	LLLLL ONb / LLDLL ONb	3.82	2.00	7.65
S_4	LLLL ONb / DLLL ONb	1.33	1.00	1.46
S_4	LLLLL ONb / LDLLL ONb	6.00	1.30	7.8
	(b) Peptides			
S_2	LLLL Lys-Phe / LLDL Lys-Phe	18.0	12.0	230.00
S_3	LLLL Lys-Phe / LDLL Lys-Phe	15.0	16.0	240.0
S_4	LLLL Lys-Phe / DLLL Lys-Phe	4.5	1.3	5.0
S_4	LLLLL Lys-Phe / LDLLL Lys-Phe	6.0	25.0	150

In the case of porcine elastase, the strong orienting power of the *p*-nitrobenzyl group toward subsites S_1' may mask some of the relatively smaller interactions at remote subsites. Therefore, it seemed necessary to use peptidase activity as a more sensitive parameter to interpret small changes in the mode of catalysis.

The choice of peptide substrates was guided by previous findings. The hexapeptide $Ala_4LysPhe$ (L_6) was shown to be a good substrate for elastase ($\bar{K}_m = 4500\ M^{-1}$; $k_{cat} = 32\ sec^{-1}$) (Atlas *et al.*, 1970). Thus, the substrates used in the present study were diastereoisomers of $Ala_4LysPhe$.

From the results presented in Tables V and VIb and Figure 2, subsites S_2 and S_3 exhibit a strikingly high stereospecificity ratio. In both subsites the replacement of the L- with the D-alanine residue brings about a 15-fold decrease of the binding constant and about a 16-fold decrease in the rate of catalysis. The stereospecificity ratios in the peptide–ester substrates are 10 and 24 times smaller than in peptide substrates at subsites S_2 and S_3, respectively. Unlike esterase activity, the rate observed responds considerably to the changes in the optical configuration of the substrates. We may attribute this difference to the fact that the rate measured in peptide hydrolysis is not influenced by a group like the *p*-nitrobenzyl ester that displays a considerable affinity to the active site, at subsite S_1' (Atlas and Berger, 1972). This explains our earlier suggestion that hydrolysis of the appropriate peptides provides us with a

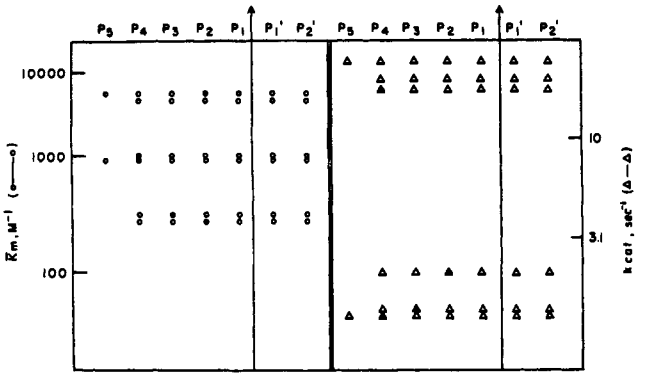

FIGURE 2: $\bar{K}_m$ and k_{cat} values for the hydrolysis of peptide substrates by porcine elastase: L-amino acids, K_m (○), k_{cat} (△); D-amino acids, K_m (●), k_{cat} (▲).

more sensitive as well as a more objective method for obtaining stereospecificity values at remote subsites of the active site.

Subsite S_4 displays a more complex role. When the D-alanine residue occupying S_4 is the N terminus of the chain (DL_5) (Table V), it causes a 4.5-fold decrease of the binding constant and a 1.3-fold decrease in the k_{cat}. However, by introducing another alanine residue to form LDL_5, a remarkable decrease in k_{cat} (25-fold) and in the $\bar{K}_m$ (6-fold) is obtained. Rates of hydrolysis and binding ($\bar{K}_m$) of the substrate were compared with the corresponding hepta-L-peptide (L_7) (Tables V and VI). A similar phenomenon was observed in the peptide–ester system. The additional L residue at P_5 probably determines the mode of binding of the D residue at S_4 and only then is stereospecificity expressed to its full extent.

Our conclusions as to the existence of subsite S_5 are strongly supported by our findings on the enzymic hydrolysis of the peptide series. The alanine residue at P_5 influences the mode of catalysis ($\bar{K}_m$ increases from 4500 to 6000 M^{-1} and k_{cat} from 32 to 50 sec^{-1}) and affects the enzyme kinetics *vis-à-vis* a D-alanine residue at P_4. The conclusion drawn above concerning the existence of subsite S_5 is confirmed by the work of Thompson and Blout (1970) where it was shown that the charge of the amino residue at P_4 interferes in the binding ($\bar{K}_m$) (fourfold decrease) and in the catalysis (2.4-fold decrease). The active site of porcine elastase extends at least over 25 Å, accommodating seven amino acid residues (or more), five from the bond cleaved toward the N terminal of the substrate and two indispensable subsites toward the C terminal of the substrate. Experiments are now being carried out in order to determine the size of the active site from the bond cleaved toward the C terminal.

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