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Effect of Secondary Substrate Binding in Penicillopepsin: Contributions of Subsites S_3 and S_2' to k_{cat}†

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ABSTRACT: The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were determined at 25 °C and pH 4.5, 5.5, and 6.0 for the series of penicillopepsin substrates Ac-Ala$_m$-Lys-(NO$_2$)Phe-Ala$_n$-amide, where (NO$_2$)Phe is *p*-nitrophenylalanine and *m* and *n* equal 0–3. K_M values at pH 6.0 were the same for all 12 peptides and averaged 0.088 ± 0.02 mM but increased to different degrees at lower pH. In contrast, k_{cat} values increased with increasing chain length. At pH 6 and at the pH optimum of k_{cat}, the largest increases (about 37-fold on average) were obtained when alanine residues were added in positions P_2' and P_3. Only 1–2-fold increases were observed for positions P_2, P_3', P_4, and P_4'. These results show that occupation of subsites S_2' and S_3 is largely responsible for the rate enhancements caused by secondary substrate interactions with this series of peptides. Additional support for an important role of subsite S_3 comes from the observation that the two peptides where $m = 1$ and $n = 1$ or 2, respectively, are cleaved not only between lysine and *p*-nitrophenylalanine but also between the latter and alanine, suggesting that occupation of subsite S_3 by the N-terminal alanine overcomes the unfavorable interaction of alanine in subsite P_1'. Subsite S_3 is also important in the binding of pepstatin analogues and in transpeptidation reactions. It is proposed that the roles of subsites S_3 and S_2' are to facilitate the conversion of the first enzyme–substrate complex into a productive complex and to assist in the distortion of the scissile bond. Sequence comparisons suggest that the effects observed for penicillopepsin may be common to most aspartic proteinases.

Penicillopepsin is a well-characterized member of the family of aspartic proteinases. Its three-dimensional structure, which has been determined at high resolution (James & Sielecki, 1983), is very similar to that of pig pepsin (Andreeva et al., 1984) and to those of the aspartic proteinases from *Rhizopus chinensis* (Suguna et al., 1987a) and *Endothia parasitica* (Pearl & Blundell, 1984). Other representatives of this family of enzymes, the mammalian renins, gastricsins, and cathepsins D (Tang, 1987) and the fungal proteinases from *Mucor miehei* (Bech & Foltmann, 1981; Gray et al., 1986), *Mucor pusillus* (Tonouchi et al., 1986), *Saccharomyces cerevisiae* (Dreyer

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et al., 1986; Ammerer et al., 1986; Woolford et al., 1986), and *Aspergillus awamori* (Ostoslavskaya et al., 1986), have amino acid sequences that show a high degree of homology and have nearly identical sequences around the two aspartic residues that are primarily responsible for the catalytic activity. It is therefore highly probable that their three-dimensional structures are also similar. These close structural similarities imply that the mechanism of action of these enzymes is probably the same and that other functional properties may be similar. One of the apparently common features is the presence of an extended active site cleft (Tang et al., 1978) which can bind at least seven amino acids. Secondary binding in this extended site has a large effect on the catalytic efficiency of pig pepsin, cow cathepsin D, the aspartic proteinase from *Rhizopus chinensis* (Fruton, 1970; Sampath-Kumar & Fruton, 1974), and penicillopepsin (Hofmann & Hodges, 1982; Blum et al., 1985). The primary effect of increasing the chain length of a substrate molecule is on k_{cat} values, which can increase by several orders of magnitude, whereas K_M values are insensitive. The studies with pig pepsin (Fruton, 1970) suggested that the effects were additive; i.e. k_{cat} values increased for each amino acid added to the peptide chain of the substrate. However, systematic studies of these effects with pig pepsin are hampered because of the poor solubility of most good substrates which require large hydrophobic side chains in positions P_1 and P_1'.[1] In order to make soluble substrates, charged amino acids, such as histidine, or other groups, such as the 3-(4-pyridyl)-propyl-1-oxy group (Fruton, 1976), must be incorporated into the peptide.

In the present work we have taken advantage of the fact that penicillopepsin has a specific binding site for lysine in S_1 (Hofmann & Hodges, 1982), although like most, if not all, other aspartic proteinases it also has a high specificity for large hydrophobic side chains (Mains et al., 1971; Powers et al., 1977). The use of lysine in P_1 allows one to synthesize long substrate peptides with solubilities in the range of 20–100 mM. In this paper we describe the effect of increasing the chain length on either side of the scissile peptide bond -Lys–(NO_2)Phe- by adding alanyl residues and of replacing the alanyl residue in P_3 with other residues. A preliminary report of some of the experiments reported here has been published (Blum et al., 1985).

Experimental Procedures

Materials

Penicillopepsin was prepared as described by Hofmann (1976) and was of the highest specific activity obtained so far. Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala-amide and Ac-Lys-(NO_2)Phe-amide were prepared as described previously (Hofmann & Hodges, 1982). The synthesis of other peptides containing the -Lys–(NO_2)Phe- bond is described below. Leu-Ser-(NO_2)Phe-Nle-Ala-Leu-OMe was from Bachem (Bubendorf, Switzerland).

Peptide Synthesis

Materials. FMOC-alanine, N^{α}-FMOC-N^{ϵ}-*t*-BOC-L-lysine, HOBt, and DCC were obtained from the Institut Armand Frappier, 531 Blvd des Prairies, Laval, Montreal. FMOC-L-*p*-nitrophenylalanine was prepared by mixing 9.1 mmol of *N*-FMOC-*N*-hydroxysuccinimide ester in a minimum volume of dry dioxane with 9.1 mmol of L-*p*-nitrophenylalanine in 11 mL of 10% (w/v) sodium carbonate and by stirring for 22 h. The mixture was extracted twice with ethyl ether. The aqueous phase was acidified to pH 2.0 and extracted with ethyl acetate. The ethyl acetate layer, after being washed with dilute HCl and water and dried over $MgSO_4$, was concentrated by rotary evaporation. FMOC-L-*p*-nitrophenylalanine was crystallized from ethyl acetate–hexane and obtained in about 80% yield. DMF was dried over CaO and distilled over ninhydrin at reduced pressure.

Synthesis. The peptides of the series Ac-Ala_m-Lys-(NO_2)Phe-Ala_n-amide, where m and n equal 1–3 (with the exception of the peptide in which $m = n = 3$), and the peptides of the series Ac-Xaa-Ala-Lys-(NO_2)Phe-Ala-Ala-amide were synthesized by the general procedures for solid-phase synthesis (Erickson & Merrifield, 1976) on a Beckman peptide synthesizer, Model 990, as described for Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala-amide (Hofmann & Hodges, 1982). Ac-Ala_3-Lys-(NO_2)Phe-Ala_3-amide was synthesized on a semiautomatic peptide synthesizer, Labortec SP640 (Labortec AG, Bubendorf, Switzerland), by the solid-phase synthesis method of Atherton et al. (1978) in which FMOC derivatives of the amino acids are used. Copoly(styrene-2%-divinylbenzene)–benzhydrylamine–resin (0.65 mmol of NH_2/g of resin; Institut Armand Frappier, Montreal) was shaken for 2 min with FMOC-alanine (1.95 mmol/g of resin) and HOBt (1.96 mmol/g of resin) in DMF (15 mL/g of resin). DCC (1 M in dichloromethane, 1.95 mmol/g of resin) was added, and shaking was continued overnight. The resin was washed 4 times alternately with 2-propanol and DMF (15 mL/g of resin). Unblocked amino groups were acetylated with 20% acetic anhydride in dichloromethane. The FMOC group was cleaved with 20% piperidine in DMF (15 mL/g of resin) once for 2 min and a second time for 10 more minutes. The resin was washed 3 times with DMF, 4 times with 2-propanol, and finally with DMF. Subsequent FMOC-amino acids were coupled to the resin analogously. Coupling was monitored at each step with ninhydrin as described by Kaiser et al. (1970). At each step >99% coupling was obtained. Before the completed peptide was cleaved from the resin it was acetylated with 20% acetic anhydride in dichloromethane. The peptide was cleaved with HF at 0 °C as described (Hofmann & Hodges, 1982).

Purification. All peptides were freed from non-peptide contaminants by gel filtration. Each peptide (100 mg to 1 g) was dissolved in a minimum volume of 2 mM ammonium acetate, pH 5.5, and applied to a Sephadex G-15 column (85 cm × 1.5 cm) equilibrated in the same buffer. The column was developed at a flow rate of about 1.1 mL/min. The eluate was monitored for absorbance at 277 nm, the absorption maximum of the (NO_2)Phe residue. The fractions containing the peptide were pooled and freeze-dried. Whereas the di- to tetrapeptides were homogeneous after this step, as shown by HPLC analysis (see below), the longer peptides had to be purified further by ion-exchange chromatography as described by Hofmann and Hodges (1982).

HPLC. Reverse-phase HPLC on a Waters C-18 column

[1] Abbreviations: Ac, acetyl; Iva, isovaleryl; Nle, norleucyl; (NO_2)Phe, *p*-nitrophenylalanyl; Sta, statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]; Lysta, (3*S*,4*S*)-4,8-diamino-3-hydroxyoctanoic acid; -PheΨ[CH_2-NH]Phe-, a dipeptidyl residue in which the carbonyl group is reduced to methylene; Xaa, any one of the amino acids occurring in proteins; FMOC, fluorenylmethoxycarbonyl; *t*-BOC, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; OEt, ethyl ester; OMe, methyl ester. The definition of Schechter and Berger (1967) for denoting amino acid residues in peptide substrates as P_1 to P_n and P_1' to P_n' and subsites in the enzyme to which the side chains of these residues bind as S_1 to S_n and S_1' to S_n' is used throughout. Amino acids in the sequence of penicillopepsin are numbered sequentially; the analogous pepsin numbers are in parentheses.

(25 mm × 4 mm) was used to assess the purity of each peptide. The peptide (50–100 μg) was applied to the column in 0.1% aqueous TFA and eluted with a linear gradient formed with acetonitrile–0.06% TFA from 0 to 25% acetonitrile over 50 min at a flow rate of 1 mL/min. The eluate was monitored at 220 and 277 nm. This system gave base-line separations of a penta- and a hexapeptide of the series under study. The peptides were judged sufficiently pure for use when the sum of their homologous contaminants was ≤3%.

Amino Acid Analysis. The identity of the peptides were confirmed by amino acid analysis after hydrolysis of the peptides in 5.7 N HCl (0.2 mL) at 107 °C for 20 h. The amino acid compositions were determined in a Beckman-Spinco Model 121C analyzer fitted with high-sensitivity colorimeter cells (18-mm light path; Evans Electroselenium, Halstead, Essex, U.K.). Amino acid analyses of the 17 peptides that were synthesized (Tables I and II) gave molar ratios of the expected amino acids that had a standard deviation of ±5%. All values were within 10% of the theoretical integral values.

Enzyme Assays and Kinetics. Before the kinetic analyses were carried out it was necessary to ascertain that all peptides studied were cleaved uniquely at the -Lys–(NO_2)Phe- bond. The substrates (1 mM in 10 mM sodium acetate, pH 5.5) were incubated for periods up to 24 h with 0.1–100 μg of penicillopepsin, the amount depending on the rate of cleavage. Samples of 20 μL were collected at intervals, immersed briefly in a water bath at 100 °C, and analyzed by high-voltage electrophoresis at pH 3.6 [acetic acid–pyridine–water, 200:20:1780 per volume, as described by Kurosky and Hofmann (1976)]. All peptides listed in Tables I and II, except V and VIII, were cleaved uniquely at the -Lys–(NO_2)Phe- bond (primary cleavage). With peptides V and VIII, however, four products were observed from the beginning of the reaction. Their analysis showed that these peptides had also been cleaved at the -(NO_2)Phe–Ala- bond. In the digests of peptide V this alternative cleavage (secondary cleavage) was only minor, and a kinetic analysis showed that it could be ignored. However, in the digest of peptide VIII the products from the secondary cleavage were present in amounts comparable to those of the primary cleavage. This was taken into account in the kinetic analysis as described below.

All enzyme assays were carried out at 25 ± 0.05 °C in 20 mM sodium acetate at pH 3.5, 4.0, 4.5, and 5.5 or in 10 mM sodium phosphate at pH 6.0. All buffers were adjusted to an ionic strength of 20 mM with NaCl. A Uvikon 820 spectrophotometer (Kontron AG, Zurich, Switzerland) was used with cells of 2- or 10-mm light paths and at wavelengths of 296, 306, or 320 nm. The cells and wavelengths were chosen so that the initial absorbance of the substrate solution did not exceed 2.0 (Hofmann & Hodges, 1982). The enzyme concentrations ranged between 0.5 and 300 μg/mL, depending on the rate of the reaction, and were chosen to give initial rates in the first 2–10 min.

Peptide VIII was cleaved at two alternative sites as mentioned. Whereas the cleavage on the N-terminal side of (NO_2)Phe leads to a decrease in absorbance at 296 nm (Hofmann & Hodges, 1982), the cleavage on the C-terminal side leads to an increase (Medzihradszky et al., 1970). However, the two cleavages could be measured separately by following the reactions at the isosbestic point of the respective difference spectra between substrate and products. The difference spectra were determined with the use of peptide IX for the primary cleavage and with Leu-Ser-(NO_2)Phe-Nle-Ala-Leu-OMe (peptide C) for the secondary cleavage, a peptide that is uniquely cleaved at the -(NO_2)Phe–Nle- bond by penicillopepsin (Hofmann et al., 1984). The isosbestic points were at 273 nm for peptide IX and at 284.5 nm for peptide C at all pHs measured.

Kinetically, a system in which an enzyme acts alternatively on two sites of a substrate can be treated for each of the cleavage sites as competitive inhibition, where K_M for the reaction that is not being measured replaces K_I and the substrate concentration replaces the inhibitor concentration. This gives the following equations for the reciprocal of the initial rates:

$$1/v_1 = (1/V_1)(K_{M1}/S + K_{M1}/K_{M2} + 1) \quad (1)$$

$$1/v_2 = (1/V_2)(K_{M2}/S + K_{M2}/K_{M1} + 1) \quad (2)$$

The Lineweaver–Burk plot of the two reactions gives two straight lines which intersect at the same point (a) on the $1/S$ axis, a point that corresponds to

$$-a = (K_{M1} + K_{M2})/(K_{M1}K_{M2}) \quad (3)$$

The intercepts on the $1/v$ axis are at points b and c, which correspond to

$$b = (K_{M1} + K_{M2})/(V_{M1}K_{M2}) \quad (4)$$

$$c = (K_{M1} + K_{M2})/(V_{M2}K_{M1}) \quad (5)$$

It is therefore not possible to estimate separately the four parameters k_{cat1}, k_{cat2}, K_{M1}, and K_{M2}; but by dividing eq 4 into eq 3 and eq 5 into eq 3, one obtains V_{M1}/K_{M1} and V_{M2}/K_{M2}, respectively. Fortunately at pH 6.0 the K_M values of peptides I–VII and IX–XII of the series are very similar (Table I), and it is therefore reasonable to assume that the K_M value for the primary cleavage would be close to the average (0.088 mM) of the K_M's of the other peptides. This value was therefore chosen as representing the real K_M of the primary cleavage of peptide VIII. This allowed us to calculate k_{cat} for this pH. However, for the other pH's only k_{cat}/K_M values for the primary cleavage are reported (Table I) because of the significant differences between the K_M's of the series.

Results and Discussion

Role of Subsites S_3 and S_2' in Penicillopepsin. The results of the determination of the kinetic parameters for the hydrolysis of 12 peptides of the series Ac-Ala$_m$-Lys-(NO_2)Phe-Ala$_n$-amide, where m and n are equal to 0–3, are given in Table I for pH 5.5 and 6.0 and for the pH or pH range in which k_{cat} is optimal. They show clearly the effect of secondary binding on the rate constant and the small or negligible effect on K_M. In a previous paper (Blum et al., 1985) we reported a comparison of k_{cat} values at pH 5.5 at which pH the K_M values range between 0.063 and 0.34 mM with an average of 0.18 ± 0.08 mM. We subsequently found that they converge to very similar values at pH 6.0 where they range only between 0.053 and 0.14 with an average of 0.088 ± 0.02 mM. This extends our earlier comparison of the action of penicillopepsin on peptides I and IX (Hofmann et al., 1984) and the work of Fruton (reviewed in 1976) on pig pepsin. The results in Table I also indicate a large increase in K_M with decreasing pH for all peptides, similar to that which we observed for peptide IX (Hofmann et al., 1984).

The primary purpose of the present experiments was to determine whether the relatively simple series of readily water-soluble peptides (Table I) showed similar cumulative rate increases as the more complex peptides that Fruton (1976) used with pig pepsin or whether the increases were confined to certain positions. The rate changes caused by the systematic

Table I: Kinetic Parameters for the Hydrolysis of Peptides of the Series Ac-Ala$_m$-Lys-(NO$_2$)Phe-Ala$_n$-amide[a]

		at pH optimum for k_{cat}				pH 5.5			pH 6.0		
peptide	no.	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	pH	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
Ac-Lys-(NO$_2$)Phe-amide	I	0.026 ± 0.006	0.6 ± 0.11	0.042	3.8–4.2	0.01 ± 0.003	0.22 ± 0.01	0.045	0.004 ± 0.001	0.08 ± 0.03	0.05
Ac-Ala-Lys-(NO$_2$)Phe-amide	II	0.021 ± 0.004	0.41 ± 0.04	0.049	4.3	0.009 ± 0.003	0.13 ± 0.015	0.071	0.006 ± 0.001	0.07 ± 0.01	0.086
Ac-Ala-Ala-Lys-(NO$_2$)Phe-amide	III	0.9 ± 0.1	0.25 ± 0.04	3.6	4.5	0.62 ± 0.02	0.13 ± 0.02	4.6	0.3 ± 0.05	0.1 ± 0.05	3
Ac-Lys-(NO$_2$)Phe-Ala-amide	IV	0.65 ± 0.07	0.55 ± 0.1	1.09	3.9–4.1	0.37 ± 0.09	0.34 ± 0.16	1.09	0.14 ± 0.06	0.1 ± 0.05	1.4
Ac-Ala-Lys-(NO$_2$)Phe-Ala-amide	V	0.8 ± 0.1	0.4 ± 0.06	2	4.5	0.57 ± 0.11	0.21 ± 0.03	2.7	0.29 ± 0.04	0.1 ± 0.02	2.9
Ac-Ala-Ala-Lys-(NO$_2$)Phe-Ala-amide	VI	29 ± 2.1	0.8 ± 0.1	36.2	4.5	21 ± 8.0	0.3 ± 0.11	70	9.3 ± 0.66	0.14 ± 0.024	66
Ac-Lys-(NO$_2$)Phe-Ala-Ala-amide	VII	1.6 ± 0.2	0.65 ± 0.08	2.46	4.5	1.4 ± 0.8	0.25 ± 0.1	5.6	0.29 ± 0.07	0.1 ± 0.05	2.9
Ac-Ala-Lys-(NO$_2$)Phe-Ala-Ala-amide	VIII	nd	nd	15.2	nd	nd	nd	15.6	0.7	0.088 ± 0.02[b]	8.1
Ac-Ala-Ala-Lys-(NO$_2$)Phe-Ala-Ala-amide	IX[c]	47 ± 3.8	0.2 ± 0.09	235	4.5	36 ± 2.8	0.078 ± 0.012	462	22 ± 5.1	0.072 ± 0.006	305
Ac-Ala-Ala-Ala-Lys-(NO$_2$)-Phe-Ala-Ala-amide	X	56 ± 15	0.075 ± 0.024	747	5.5	see preceding columns			40 ± 10	0.08 ± 0.03	500
Ac-Ala-Ala-Lys-(NO$_2$)Phe-Ala-Ala-Ala-amide	XI	45 ± 8.1	0.1 ± 0.04	450	5.5	see preceding columns			30 ± 1.9	0.07 ± 0.017	428
Ac-Ala-Ala-Ala-Lys-(NO$_2$)-Phe-Ala-Ala-Ala-amide	XII	–	–	–	–	28 ± 2.7	0.06 ± 0.02	470	17 ± 1.2	0.053 ± 0.004	320

[a] Assay conditions: 25 ± 0.05 °C; 20 mM sodium acetate for all pHs except pH 6.0, where 10 mM sodium phosphate was used; all buffers adjusted to an ionic strength of 20 mM with NaCl. Substrate concentrations ranged between 0.005 and 0.7 mM. Decreases in absorbance were followed at 296, 306, or 320 nm depending on the substrate concentration. The number of experiments for each parameter varied from 3 to 18. nd, not determinable, see text. (–) not determined. [b] This is the average and its standard deviation for the K_M values at pH 6.0 for the peptides I–VII and IX–XII, see text. [c] The values for this peptide were taken from Hofmann and Hodges (1982) and Hofmann et al. (1984).

Table II: Kinetic Parameters for the Hydrolysis of Peptides of the Series Ac-Xaa-Lys-(NO$_2$)Phe-Ala-Ala-amide[a]

		pH 4.5			pH 5.5			pH 6.0		
Xaa	no.	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
Gly	G3	7.6 ± 0.9	0.07 ± 0.01	105	1.9 ± 0.2	0.027 ± 0.003	70.4	0.8 ± 0.09	0.029 ± 0.003	28
Thr	T3	15.3	0.2	76.5	16.6 ± 1.1	0.16 ± 0.09	104	6.7 ± 0.9	0.083 ± 0.01	61
Val	V3	33.4	0.017	1960	28 ± 1.5	0.013 ± 0.007	2100	20.2	0.012	1680
Arg	R3							11 ± 1	<0.01	>1100
Asp	D3							~2.5	~2	~1.25

[a] Assay conditions: 25 ± 0.05 °C; 20 mM sodium acetate for all pHs except pH 6.0, where 10 mM sodium phosphate was used; all buffers adjusted to an ionic strength of 20 mM with NaCl. Substrate concentrations ranged between 0.005 and 0.7 mM except for peptide D3, where concentrations up to 5 mM were used. Decreases in absorbance were followed at 296, 306, or 320 nm depending on the substrate concentration, as explained in the text. The number of experiments for each parameter varied from two to five.

Table III: Changes in k_{cat} at pH 6.0 and at Its pH Optimum Observed as a Function of the Stepwise Addition of Alanine Residues to the Substrate[a]

position	peptides compared	ratio at pH 6	average for position	ratio at pH optimum	average for position
P_2	II and I	1.5		0.8	
	V and IV	2.07		1.2	
	VIII and VII	2.4	1.99		1.0
P_3	III and II	50		43	
	VI and V	32.1		36	
	IX and VIII	31.6	37.9		39.5
P_4	X and IX	1.9		1.2	
	XII and XI	0.56	1.23		(1.2)
P_2'	IV and I	35		25	
	V and II	48		38	
	VI and III	31	38	32	31.7
P_3'	VII and IV	2.07		2.5	
	VIII and V	2.4			
	IX and VI	2.4	2.3	1.6	1.95
P_4'	XI and IX	0.76		0.96	
	XII and X	0.41	0.59		(0.96)

[a] The ratios given were obtained by dividing the k_{cat} values for the peptide that has an alanine in the position given in column 1 by those for the next lower homologue. k_{cat} values were taken from Table I.

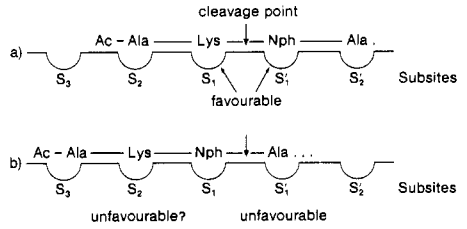

FIGURE 1: Schematic of alternative binding of peptides V and VIII.

addition of alanine residues to the parent peptide I are shown in Table III for k_{cat} at pH 6.0 and at its pH optimum. There are two positions, namely S_3 and S_2', whose interactions in the substrate binding site are responsible for the largest increases: the k_{cat} values increase by factors of 37.9 and 38, respectively, at pH 6 and 31.7 and 39.5 at the optimum of k_{cat}. This compares to at most about 2-fold rate increases for addition of alanine in positions P_2 and P_3'. Only small changes are observed in positions P_4 and P_4'.

Convincing though these results are for the series of peptides studied, they cannot by themselves be interpreted to indicate a special general role for the subsites S_3 and S_2', since other peptides could well provide different degrees of rate enhancements in a systematic analysis of chain elongation. Also, there are to the best of our knowledge no studies on related aspartic proteinases that give clear evidence for a specific role of any particular subsite related to the length of the substrate. However, there are other independent experiments, involving especially subsite S_3, that indicate strongly that these subsites play a very important and more general role.

As mentioned above (see Experimental Procedures), peptides V and VIII are not cleaved uniquely between lysine and *p*-nitrophenylalanine but also between *p*-nitrophenylalanine and alanine (Figure 1). This cleavage provides at least one unfavorable interaction, namely, that of alanine in S_1', a site that is specific for large hydrophobic side chains (Mains et al., 1971). The kinetic parameters of this secondary cleavage are comparable in peptide VIII to those of the primary cleavage (k_{cat}/K_M = 5.9–7.7 at all three pHs), indicating that the alternative cleavage in which an alanine residue occupies S_3 provides binding that gives a similar free energy change of the overall reaction as that for the primary cleavage. In peptide V, on the other hand, the secondary cleavage is only minor and negligible for kinetic experiments, probably because the alternative binding mode (Figure 1) leaves subsite S_2' unoccuppied and thus loses the rate-enhancing effect of this site. Other peptides in which P_3 was not occupied presumably did not undergo an alternative cleavage because the enzyme would have either to act as an amidase, as in peptides II and IV where neither S_1' nor S_2' would be occupied by a side chain, or to cleave between two alanine residues as in peptide VII.

A further indication for a special role of subsite S_3 comes from inhibition studies with pepstatin analogues. Thus, Iva-Val-Sta-OEt has a K_I for penicillopepsin that is at least 10000 times higher than that of Iva-Val-Val-Sta-OEt (Blum et al., 1985). Rich and Sun (1980) and Rich and Bernatowicz (1982) showed that the nature of the side chain in P_3 of pepstatin analogues determined not only the magnitude of the inhibition constant but also the pathway of binding and [lactoyl]pepstatin in which the N-terminal Iva-Val group was replaced by a lactoyl residue was a much poorer inhibitor for chymosin, gastricsin, and four fungal aspartic proteinases (Valler et al., 1985).

Additional evidence comes from transpeptidation studies, in which it was found that penicillopepsin catalyzes transpeptidation of the substrate (NO_2)Phe-Ala-Ala-amide with an oligomer of *p*-nitrophenylalanine and Ala-Ala-amide as the first products released (Blum et al., 1985). The oligomer has now been identified as tris(*p*-nitrophenylalanine) (M. Blum and T. Hofmann, unpublished observations). Thus, during the several reactions that lead to the formation of this trimer all the intermediate products, other than Ala-Ala-amide, are trapped in the active site until the N-terminal *p*-nitrophenylalanine residue reaches subsite S_3 when the transpeptidation product is released.

The question now arises as to the physical basis of these different effects. Four possibilities come to mind: (1) occupation of subsites S_3 and S_2' induces conformational changes that are responsible; (2) binding induces changes in the electronic state of the catalytically active residues without inducing conformational changes that can be detected by X-ray analysis; (3) binding in these "activating" subsites facilitiates the distortion of the scissile bond that has been suggested by Pearl (1985, 1987) and by Suguna (1987b); (4) specific interactions, such as hydrogen bonds in subsites S_2' and S_3, assist in the formation of the productive enzyme–substrate complex.

Conformational Changes. The major change that is observed when peptide inhibitors bind to penicillopepsin, rhizopuspepsin, and endothiapepsin is the movement of the large β-loop of the residues from Trp-71 (71) to Gly-83 (83), the so-called tyrosine-75 (75) "flap" (James et al., 1982), from a relatively flexible position to a more rigid interaction with the inhibitor (James et al., 1982, 1983, 1985; Bott et al., 1982; Foundling et al., 1987; Suguna et al., 1987b). This change, however, is unlikely to be responsible for the effects of binding to subsites S_2' and S_3 because the major interactions of residues of the flap with residues of the inhibitor, and by implication also a substrate, are formed with P_2, P_1, and P_1'. These include three hydrogen bonds and, in the case of an inhibitor with lysine in P_1 of a pepstatin analogue (James et al., 1985), also an ionic interaction. There is no evidence for any significant changes elsewhere in the molecule upon binding of a variety of inhibitors, as deduced from high-resolution structures, such as that of the penicillopepsin–Iva-Val-Val-Sta-OEt complex at 1.8 Å (James et al., 1983), complexes of renin inhibitors with endothiapepsin (Foundling et al., 1987), or the complex of D-His-Pro-Phe-His-PheΨ[CH_2-NH]Phe-Val-Tyr with rhizopuspepsin (Suguna et al., 1987b). In penicillopepsin any

movement of residues other than those of the flap is less than 0.3 Å (James et al., 1983).

Electronic Effects. A second possibility for explaining the effects of occupation of S_3 and S_2' is changes in the electronic environment of the catalytic site. Such changes could affect, for instance, the electronegativity of the active site carboxyl acting as the general base catalyst. However, such changes are unlikely to affect the binding of the transition-state analogues or to explain the transpeptidation reactions. In these reactions, exemplified by action of penicillopepsin on (NO_2)Phe-Ala-Ala-amide (Blum et al., 1985), it would appear phenomenologically that occupation of subsite S_3 triggers the release of tris(*p*-nitrophenylalanine).

Scissile Bond Distortion. A distortion of the scissile bond in the substrate as discussed by Pearl (1985, 1987) and Suguna et al. (1987b) might be facilitated by binding of substrate residues in S_3 and S_2'. This could readily explain the rate enhancements observed; it could also explain the alternative cleavage site in peptides V and VIII. However, by itself it would not explain the reduction in inhibitory power of pepstatin analogues that is seen when the P_3 residue is absent. Nor would it explain the transpeptidation reactions. However, as we propose below, facilitated scissile bond distortion could accompany the orientation effect discussed in the following section.

Facilitation of Productive ES Complex Formation by Specific Interactions in Subsites S_3 and S_2'. The specific interactions, other than van der Waals contacts, between amino acid residues in positions P_3 and P_2' of a substrate and the enzyme are one hydrogen bond each. In subsite S_3 a hydrogen bond is formed between the NH of P_3 and the O^δ of Thr-217. We suggested earlier (Blum et al., 1985) that this hydrogen bond plays a major role in determining the subsite effects. Analogous hydrogen bonds are found in three inhibitor complexes with endothiapepsin (Foundling et al., 1987) and in an inhibitor complex of rhizopuspepsin (Suguna et al., 1987b), a finding that suggests that this hydrogen bond may be a general feature of aspartyl proteinases. In fact, in 19 out 21 aspartic proteinases whose complete sequences are known the position that is analogous to that of Thr-217 (219) in penicillopepsin is occupied by either a threonine or a serine residue. Only in the sequences of *Mucor miehei* (Bech & Foltmann, 1981) and *Mucor pusillus* (Tonouchi et al., 1986) does another amino acid, aspargine, occupy that position. However, the side chain C=O of this amino acid could act as readily as a threonine or serine as a H acceptor from an NH group of a residue in P_3 with only minor displacement of the main peptide chain. The recent determination of the structure of the D-His-Pro-Phe-His-PheΨ[CH_2-NH]Phe-Val-Tyr–rhizopuspepsin complex (Suguna et al., 1987b) shows that the carbonyl group of P_2' forms a hydrogen bond with the N^ϵ of the ring of Trp-194. We postulate that an analogous hydrogen bond forms in penicillopepsin with Trp-191 (190) because (a) the hydrogen bonds that have been identified between the backbone of inhibitors in complexes of pepstatin analogues and penicillopepsin (James et al., 1982, 1985) and in complexes of inhibitors with endothiapepsin (Foundling et al., 1987) are very similar to those in rhizopuspepsin and (b) Trp-191 (190) is conserved in analogous positions in all 21 presently known complete sequences of aspartyl proteinases, as determined from all possible pairwise alignments of the sequences (unpublished observation).

As a working hypothesis we therefore suggest that the kinetic and binding effects associated with the occupation of subsites S_3 and S_2' are due to the formation of these hydrogen bonds in penicillopepsin and furthermore that analogous hydrogen bonds form in other members of the family of aspartic proteinases and induce comparable effects. The role of these hydrogen-bond interactions with the substrate would be to facilitate its orientation into its productive mode and the distortion of the scissile bond from its planar conformation after the formation of the initial enzyme–substrate complex. This latter complex would be the "first detectable enzyme: substrate complex" proposed by Fruton (1980) and is represented by ES in Scheme I, where ES* is the productive complex, i.e., the complex in which the substrate is in its final orientation, with its scissile bond distorted to give optimum fit in the active site, just before it is attacked by the nucleophile. [ET*] is the tetrahedral intermediate, and EP* and EP are product complexes before and after conformational changes, respectively, that lead to release of the products. We suggest that K_M represents K_S, the dissociation constant of ES in accordance with Fruton's evidence that showed that the kinetically determined K_M for three substrates of pig pepsin and rhizopuspepsin was nearly identical with K_S. At present it is not clear why K_M is independent of the length of the substrate, not only with penicillopepsin but also with other enzymes such as pepsin (Fruton, 1976). The reaction ES $\rightarrow$ ES* would represent the conversion of the first complex into the final, productive complex and include the interaction of the flap with the substrate as well as the distortion of the scissile bond. In the absence of amino acyl residues in P_3 and P_2', this step would be slow and may well be the rate-limiting step.

Scheme I

$$E + S \leftrightarrow ES \leftrightarrow ES^* \leftrightarrow [ET^*] \leftrightarrow EP^* \leftrightarrow EP \rightarrow E + P$$

Amino Acid Side-Chain Effects in S_3. In addition to the probable role that the hydrogen bond between the NH of P_3 and the O^δ of Thr-217 (219) plays, there are side-chain interactions that are also important as is shown when alanine in P_3 is replaced by other amino acids. Both k_{cat} and K_M are affected (Table II). The replacement of alanine by glycine (peptides IX and G3, respectively) leads to considerably lower K_M and k_{cat} values at corresponding pH. K_M's for T3 are similar to those for peptide IX, but k_{cat}'s are only between one-half and one-third as large; a valine side chain (V3) causes a large decrease in K_M with k_{cat} being the same as that for peptide IX. The difference in kinetic parameters between these two peptides is unexpected since the threonine and valine side chains are sterically very similar. The lower K_M of V3 suggests a strongly hydrophobic binding site for the side chain. The fact that arginine in P_3 has an even lower K_M at first appears to contradict this, but the three-dimensional model shows that the arginine side chain is sufficiently long to extend through the hydrophobic area and to enable the positive charge to interact with two carboxylate groups [Glu-15 (12) and Glu-16 (13)]. In pig pepsin arginine in P_3 is highly unfavorable (Powers, 1977). The negative charge on the aspartic acid of peptide D3 would probably be placed directly into the hydrophobic site. Such an unfavorable interaction explains the high K_M of this peptide.

Extensive studies on the effects of side chains in S_3 of a variety of different aspartic proteinases have been carried out by Dunn et al. (1986) with different substrates of the series Lys-Pro-Xaa-Glu-Phe-(NO_2)Phe-Arg-Leu. In general agreement with our results they found that the fungal proteinases gave favorable kinetics with aliphatic side chains and with histidine and lysine, whereas the mammalian enzymes showed a great deal of specificity and showed mostly unfavorable interactions with basic side chains. Actual kinetic

parameters cannot be compared because the experiments were carried out at 37 °C and pH 3.1. In a more recent study Dunn et al. (1987) investigated the effect of pH on the hydrolysis by pig pepsin, calf chymosin, and endothiapepsin of several substrates of the same series with changes also in P_2. They observed pH optima or pH plateaus for k_{cat} that are similar to those of the peptides described in this paper. Both studies highlight the importance of subsite S_3 in determining the specificity differences between different aspartic proteinases.

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