CLEAVAGE POINTS AND REACTION KINETICS FOR THE INTERACTION OF MODEL PEPTIDES WITH SUBTILISIN-BPN'

W. BLACKBURN*, R.F. BILTON*, M.J. CRUMPTON† and H.D. LAW‡

*Department of Chemistry, Liverpool Polytechnic, Liverpool L3 3AF,

† National Institute for Medical Research, Mill Hill, London NW7 1AA

and

‡ Glasgow College of Technology, Glasgow G4 0BA, U.K.

Received 21 May 1973

1. Introduction

A hypothesis was recently proposed to explain the molecular nature of the interaction between subtilisin-BPN' and its substrates [1]. This proposal was based on X-ray crystallographic studies of the enzyme [2, 3], inactive peptidylalkyl complexes formed by reacting the enzyme with chloromethylketone derivatives of peptide substrates [4, 5] and complexes formed between 'virtual substrates' and the enzyme [1]. Alkylation of the enzyme by the chloromethylketone derivatives was postulated to involve a methylene link to the Nε2 atom of histidine-64. Model building studies showed that this link can be replaced by a carbonyl (ester) link to the O atom of serine-221 without disturbing the conformation of the molecule and independent evidence has been presented for the existence of this link in the normal acyl-enzyme intermediate [6]. The binding of the peptide portion of the inhibitor involved i) inclusion of a relatively large hydrophobic side chain at P_1 (for nomenclature see [7]) into a crevice on the enzyme surface (the S_1 subsite); ii) a H-bond between the NH of P_1 and the CO of serine-125; iii) a H-bond between the CO of P_3 and the NH of glycine-127; iv) a H-bond between the NH of P_3 and the CO of glycine-127 and v) incorporation of a hydrophobic group at P_4 into a large depression on the enzyme surface; the size and orientation of the side chain at P_2 was also important. In the case of virtual substrates binding to the enzyme was similar to that described above except that H-bond (ii) was not detected. It was also argued that the binding of true substrates probably involves the same types of interactions and that H-bond (ii) is of especial importance in the formation of the Michaelis complex. The above postulated modes of binding are supported by kinetic evidence obtained using a homologous series of small synthetic peptides [8]. The present paper describes a study of the interaction of the enzyme with more complex substrates, namely bradykinin and peptides derived from the *C*-terminal sequence of sperm-whale myoglobin. The results revealed that subtilisin BPN' can function as an exopeptidase and that the cleavage position depends on the nature of the amino acid side-chains remote from the point of cleavage. These observations are discussed relative to the structure of the binding site.

2. Experimental and results

Subtilisin BPN' was obtained from Nagarse Co., Japan and from Sigma (London) Chemical Co. Ltd. Di- and tri-glycine were purchased from BDH Chemicals Ltd., and tetra- and penta-glycine from Ralph N. Emmanuel Ltd. Synthetic analogues of bradykinin and of the *C*-terminal amino acid sequence of myoglobin were prepared as previously described [9, 10]. Glycyl-(*O*-methyl-L-tyrosyl)-glycylglycine was synthesised using the solid phase technique [11, 12] and was purified by elution from Sephadex G-25 in 20 mM acetic acid and by paper chromatography in butan-1-ol, acetic acid, water (12:3:5 by vol). Analysis of the product gave $Gly_{3.06}$, *O*-methyl tyro-

sine$_{1.00}$, and $R_f{}^a$, 0.10; $R_f{}^b$, 0.04; $R_f{}^c$, 0.38 and $R_f{}^d$, 0.52 where a, b and c refer to TLC (Kieselgel-G) and d to paper chromatography in the following solvent systems: a) butan-2-ol, 3% aqueous ammonia (30:11, v/v); b) butan-1-ol, pyridine, acetic acid, water (15:10:3:12, by vol); (c and d) butan-1-ol, acetic acid, water (12:3:5, by vol).

Concentrations of tyrosine-containing peptides were estimated spectrophotometrically at 274.5 nm relative to tyrosine (ϵ, 1340), whereas peptides containing phenylalanine were measured at 257.5 nm relative to phenylalanine (ϵ, 195).

For cleavage point determinations, the peptides (3–29 mM in 50 mM Tris-HCl buffer, pH 7.5) were incubated with 6–18 μM-subtilisin BPN' for 2–4 hr at 37°C. The components were separated by paper chromatography and eluted with 10% (v/v) acetic acid prior to amino acid analysis.

Samples for amino acid analysis were hydrolysed as previously described [10]. The analyses were carried out using an automatic analyser (Lobell–Locarte) which was also adopted for the kinetic analysis of the myoglobin-like peptides. Reaction kinetics were determined using 0.3–8.5 mM peptide and 14–50 μM enzyme in a total vol of 500 μl of 50 mM Tris-HCl buffer, pH 7.5, at 37°C. Samples (50 μl) were removed at intervals, added to 50 μl of 0.2 M HCl and 30 μl of the mixture was injected into the buffer line of the analyser; the extent of hydrolysis was calculated from the area of the peak on the chart. Alternatively, the hydrolysis of bradykinin was followed by using a slight modification of the method described by Yemm and Cocking [13].

The cleavage points and reaction kinetics for the bradykinin peptides are summarised in table 1 and for the myoglobin peptides in table 2.

3. Discussion

The results shown in tables 1 and 2 contain two notable observations. Firstly it is evident from the cleavage of peptides no. 1, 2, 3 and 11 that subtilisin BPN' can function as an exopeptidase and secondly, that the cleavage point can vary markedly between closely-related peptides (no. 4 and 5). Although the former observation is entirely explicable in terms of the proposed enzyme–substrate interactions, the latter implies that other interactions are involved or, as recently suggested [14], that an alternative mode of productive binding is possible.

Table 1

Cleavage points for the hydrolysis of bradykinin and of related peptides by subtilisin BPN'.

Peptide no.	Sequence and cleavage points*	K_m (mM)
1	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe↓Arg	1.26
2	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe↓Agm	n.d.
3	Guv–Pro–Pro–Gly–Phe–Ser–Pro–Phe↓Agm	n.d.

Abbreviations:

Agm, agmatine (des-carboxyarginine); Guv, γ-guanyl-valeric acid (des-aminoarginine); n.d., not determined.

* The cleavage point is indicated by the arrow.

Subtilisin BPN' might have been expected to act as an endopeptidase with the bradykinin series of peptides (table 1) especially since the central phenylalanine residue seems ideally suited to occupy the P_1 position. No cleavage was, however, detected in this position. Although the reason for this lack of cleavage is not known, it seems possible it is related to the presence of proline in the P_3, P_4 or $P_2{}^1$ positions which would be expected to introduce stereochemical restraints and to restrict H-bonding. On the other hand, compelling reasons can be adduced, in retrospect, to account for the exopeptidase action. In this case, the phenylalanine residues at the P_1 and P_4 positions can be accommodated in the hydrophobic crevices at the S_1 and S_4 sub-sites on the enzyme surface, the side chain of the serine residue at P_3 is free to interact with the aqueous environment and the proline residue at P_2 should favour the formation of a β-conformation which would promote interaction of the peptide with the enzyme. The importance of the above interactions in orienting the bradykinin peptides on the enzyme surface is emphasised by the insensitivity of the cleavage point to the presence or absence of a free α-carboxyl group, particularly since inhibition of hydrolysis by such a group has been reported [8, 15]. Subtilisin BPN' also acts as an exopeptidase on peptide no. 11 (table 2) which resembles bradykinin in possessing two nonpolar residues with bulky side-chains separated from each other by two

Table 2

Cleavage points and reaction kinetics for the hydrolysis of peptides related to the C-terminus of sperm-whale myoglobin by subtilisin BPN'.

Peptide no	Sequence and cleavage points*	K_M (mM)	$\frac{V_{max}}{[E]}$ §	Rate‡
4†	Lys–Glu–Leu↓–Gly–Tyr ⋮ Gln–Gly	0.70	1.74	1.02
5	Glu–Leu–Gly–Tyr ↓ Gln–Gly	1.35	1.33	0.52
6	Glu–Leu–Gly–OMT↓Gln–Gly	n.d.	n.d.	n.d.
7	Leu–Gly–Phe ⋮ Gln–Gly	n.d.	n.d.	n.d.
8	Leu–Gly–Tyr ↓ Gln–Gly	2.97	1.62	0.41
9	Leu–Gly–OMT↓Gln–Gly	2.46	3.29	0.95
10	Leu–Gly–Tyr ↓ Gly–Gly	3.09	7.41	1.79
11	Leu–Gly–Gly ↓ Gln–Gly	0.73	0.57	0.33
12‡‡	Gly–Gly–Gly – Gly–Gly	–	–	–
13	Gly–Tyr ↓ Gln–Gly	1.63	0.87	0.33
14	Gly–OMT↓Gly–Gly	3.50	1.49	0.33
15‡‡	Gly–Gly –Gly–Gly	–	–	–
16‡‡	Gly –Gly–Gly	–	–	–
17‡‡	Gly–Gly	–	–	–

Abbreviation:

OMT, *O*-methyl-L-tyrosine; n.d., not determined.

* The cleavage point is indicated by the arrow.

† The major cleavage point occurred at the leucyl–glycyl bond, but about 15% of the heptapeptide was cleaved at the tyrosyl–glutamyl bond. In addition, the C-terminal tetrapeptide formed by cleavage of the heptapeptide was subsequently hydrolysed at the tyrosyl–glutamyl position (cf. peptide no. 13).

‡‡ No digestion was detected.

§ Expressed as peak area units min^{-1} mM (enzyme)$^{-1}$ (calculated from a modified Lineweaver–Burk plot of $[E]/v$ *versus* $1/[S]$).

‡ Rate of hydrolysis of 1 mM substrate by 1 mM enzyme, calculated from modified Lineweaver–Burk plot and expressed as peak area units min^{-1}.

residues. As noted previously, exopeptidase activity would be favoured by the location of these nonpolar residues in the S_1 and S_4 crevices. Furthermore, although the leucine and glutamine residues may appear capable of fulfilling the requirements of the P_1 position, the *N*-terminal leucine is presumably excluded from the S_1 sub-site due to the solvation of the α-amino group which is hence, unable to H-bond to the carbonyl group of serine-125; a similar explanation has been proposed for the resistance of leucinamide to hydrolysis [1, 8].

The difference between the cleavage points of the heptapeptide and the hexa- and penta-peptides in the myoglobin series (table 2) is not so easily rationalised. Thus, for the major cleavage point of the heptapeptide the absence of a residue in the P_4 position is particularly surprising [1]. Furthermore, since the size of an alanine side-chain is optimal for the P_2 position and larger side-chains inhibit binding [5, 8], a glutamic acid residue in this position is also unexpected. Examination of molecular models suggests, however, that the S_2 sub-site may be able to accommodate a

glutamic acid residue with, possibly, the formation of an electrostatic bond to histidine-64. It is also conceivable that a lysine side-chain at P_3 could form a H-bond to the phenolic group of tyrosine-104 which blocks the entrance of the S_4 crevice in the native enzyme. The above hypothetical interactions together with the location of the leucine residue in the S_1 cleft may be responsible for the unusual cleavage of the heptapeptide. However, an alternative explanation is possible. *N*-Benzoyl-L-arginine which competitively inhibits the hydrolysis of acetyltyrosine ethyl ester by subtilisin BPN' [16], occupies a different position on the enzyme surface from the peptidylalkyl chains and virtual substrates [17]. This may represent an alternative mode of productive binding. The apparent anomalies in the interaction of the heptapeptide with the enzyme would be readily explicable if a different binding mode was involved, although the results of studies with other substrates [14] suggest that this explanation is unlikely to be correct.

The kinetic results (table 2) revealed that an increase in molecular size from the penta- to the heptapeptides is associated with a progressive rise in the strength of binding of the peptide by the enzyme; a similar relationship has been established previously for the hydrolysis of a series of peptides of increasing size by elastase [18]. If it is assumed that the different peptides occupy the same binding mode of subtilisin BPN', then the above relationship indicates that there are greater binding-opportunities for the larger peptides.

Acknowledgements

We are indebted to Drs. D.M. Blow and J.D. Robertus for helpful discussions and for allowing us to examine a molecular model of subtilisin BPN'. One of us (H.D.L.) wishes to acknowledge a grant from the Science Research Council for the provision of an amino acid analyzer.

References

[1] Robertus, J.D., Kraut, J., Alden, R.A. and Birktoft, J.J. (1972) Biochemistry 11, 4293.
[2] Wright, C.S., Alden, R.A. and Kraut, J. (1969) Nature 221, 235.
[3] Alden, R.A., Wright, C.S. and Kraut, J. (1970) Phil. Trans. Roy. Soc. Lond. B 257, 119.
[4] Kraut, J., Robertus, J.D., Birktoft, J.J., Alden, R.A., Wilcox, P.E. and Powers, J.C. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 117.
[5] Robertus, J.D., Alden, R.A., Birktoft, J.J., Kraut, J., Powers, J.C. and Wilcox, P.E. (1972) Biochemistry 11, 2439.
[6] Hess, G.P. (1971) in: The Enzymes (Boyer, P.D., ed.), Vol. 3, p. 213, Academic Press, New York.
[7] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157.
[8] Morihara, K., Tsuzuki, H. and Oka, T. (1971) Biochem. Biophys. Res. Commun. 42, 1000.
[9] Johnson, W.H., Law, H.D. and Studer, R.O. (1971) J. Chem. Soc. C., 748.
[10] Crumpton, M.J., Law, H.D. and Strong, R.C. (1970) Biochem. J. 116, 923.
[11] Merrifield, R.B. (1963) J. Am. Soc. 85, 2149.
[12] Stewart, J.M. and Young, J.D. (1969) in: Solid Phase Peptide Synthesis, W.H. Freeman, San Francisco.
[13] Yemm, E.W. and Cocking, E.C. (1955) The Analyst 80, 209.
[14] Bosshard, H.R. (1973) FEBS Letters 30, 105.
[15] Morihara, K., Oka, T. and Tsuzuki, H. (1970) Arch. Biochem. Biophys. 138, 515.
[16] Glazer, A.N. (1967) J. Biol. Chem. 242, 433.
[17] Wright, C.S., Alden, R.A. and Kraut, J. (1972) J. Mol. Biol. 66, 283.
[18] Atlas, D. and Berger, A. (1972) Biochemistry 11, 4719.