On the active site of elastase: Partial mapping by means of specific peptide substrates

ARTICLE *in* FEBS LETTERS · JANUARY 1971

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(70)80548-8 · Source: PubMed

4 AUTHORS, INCLUDING:

Daphne Atlas
Hebrew University of Jerusalem
158 PUBLICATIONS **4,627** CITATIONS

SEE PROFILE

ON THE ACTIVE SITE OF ELASTASE: PARTIAL MAPPING BY MEANS OF SPECIFIC PEPTIDE SUBSTRATES

Daphna ATLAS, S.LEVIT, I.SCHECHTER and A.BERGER
The Weizmann Institute of Science, Rehovot, Israel

Received 15 October 1970

RNase-S peptide as well as some related octa- and hexapeptides were found to be highly reactive substrates of porcine elastase (e.g. Ala_4–Lys–Phe: $\bar{K}_m = 4500\ M^{-1}$, $k_{cat} = 32\ sec^{-1}$, $C = 1.4 \times 10^5\ M^{-1}\ sec^{-1}$). Comparison of the various peptides led to the conclusion that the active site of porcine elastase is composed of 6–7 subsites (c.f. [1]). Preliminary mapping shows that subsites S_2, S_1' and S_2' have hydrophobic character. Occupation of subsite S_4 by the substrate is important for efficient hydrolysis. Binding at this subsite was found to be stereospecific.

1. Introduction

The specificity of elastase towards insulin A and B chains was investigated by Narayanan and Anwar [2] who used a purified preparation of porcine pancreatic origin. Splits at alanine, serine, glycine and valine were reported, but the system did not lend itself to the determination of the kinetic constants. In their investigations on the reaction mechanism, Geneste and Bender [3] as well as Kaplan et al. [4] used as substrates mainly esters of *N*-substituted amino acids. These are rather poor substrates ($C = \bar{K}_m k_{cat} < 10^3\ M^{-1}\ sec^{-1}$). Gertler and Hofmann [5] showed that acetyl–Ala_3–OMe ($\bar{K}_m = 2300\ M^{-1}$, $k_{cat} = 73\ sec^{-1}$, $C = 1.7 \times 10^5\ M^{-1}\ sec^{-1}$ at pH 8). Esters of (unblocked) tetrapeptides may have still higher reactivity (Ala_4OMe $C \sim 3 \times 10^5$, Ala_2–Lys–Ala–OMe $C \sim 3 \times 10^5$, [6]. However, no kinetic data for the splitting of peptide bonds by elastase have been published. The present report shows that peptide substrates containing at least 6 residues can be highly reactive ($C > 10^5\ M^{-1}\ sec^{-1}$).

2. Materials and methods

Porcine pancreatic elastase was kindly provided by Dr. D.M.Shotton, who described its preparation [7].

Synthetic peptides were prepared by successive addition of BOC-amino acids (either by DCC or as their *N*-hydroxysuccinimide esters) or of BOC-peptide *N*-hydroxysuccinimide esters to the *p*-nitrobenzyl esters of the *C*-terminal amino acid or of the intermediate *C*-terminal peptides. The ε-amino group of lysine as well as the α-amino group of the final *N*-terminal residue were protected by the benzyloxycarbonyl group. The free peptides (table 1, no. III to IX) were obtained by catalytic hydrogenation. Purity of the products was better than 95% as judged by electrophoresis at pH 1.9, 3.5 and 6.5, and by paper chromatography.

RNase-S peptide [1] was prepared according to Richards an Vithayathil [8]. Trifluoroacetyl S-peptide was prepared according to Goldberger and Anfinsen [9]. The octapeptide (RNase 1–8) was prepared from S-peptide by chymotryptic digestion and purified on a phosphorylated cellulose column [10].

Products of cleavage were identified by paper electrophoresis at pH 1.9 and comparison with synthetic markers. When markers were not available (as in the cleavage of the S-peptide) spots were eluted and their amino acid composition determined on an automatic amino acid analyzer.

Kinetics of enzymatic cleavage were determined at 25° by measuring the rate of decrease of the peptide bond absorption at 225 nm in a Gilford recording 4-cell spectrophotometer. Substrate concentrations down to 10^{-4} M could be handled. Lineweaver–Burk plots (based on initial rates) were constructed over a 6 to 10-fold concentration range. They were linear in

all cases. Enzyme concentrations were adjusted to give half-lifes of 5–20 min.

Reaction mixtures contained substrate, enzyme, and tris-buffer pH 8.6, 0.05 M. The enzyme concentration used to calculate k_{cat} values is based on dry weight. Enzyme activity was periodically checked against Bz–Ala OMe (in 0.1 M KCl) in a pH-stat. (Found: $\bar{K}_m$ = 30 M^{-1}, k_{cat} = 55 sec^{-1} at pH 8.6).

A detailed account of the experimental procedures will be published elsewhere.

3. Results and discussion

The marked selectivity [2] of purified elastase in the type of peptide bond split in the A and B chains of insulin, led us to look for other substrates which might give a clue to the specificity of this enzyme as a protease. Since the results with insulin seemed to indicate that splitting occurs preferably at the carbonyl group of amino acid residues with small side-chains (glycine, alanine, serine), RNase S-peptide (RNase 1–20) seemed a good choice. It contains two distinct regions where splits appeared likely to occur. Indeed, total digestion of the S-peptide yielded the expected three products: Lys–Glu–Thr–Ala_3; Lys–Phe–Glu–Arg–Gln–His–Met–Asp–Ser_2–Thr–Ser; Ala_2. Semiquantitative paper electrophoresis showed that the bond 6–7 is split many times faster than the bond 18–19, and it was found possible to isolate the pure peptide RNase 7–20, in good yield, after digestion under controlled conditions.

The kinetics of hydrolysis of bond 6–7 could be followed by measuring the rate of inactivation of the S-peptide, since it was found that an equimolar mixture of S-protein and peptide 7–20 has practically no RNase activity. The parameters determined from a double reciprocal plot were $\bar{K}_m$ = 1450 M^{-1} and k_{cat} = 45 sec^{-1}.

In order to determine some of the structural factors responsible for this remarkable reactivity we prepared a number of related peptides and measured the kinetics of their hydrolysis by elastase. The kinetic parameters determined are summarized in table 1. The amino acid residues of the substrates are numbered P_1, P_2 etc. to the "left" of the susceptible bond and P'_1, P'_2 etc. to its "right" – corresponding to the possible subsites (S_1, S_2 etc. S'_1, S'_2 etc.) on the enzyme surface, which they occupy upon forming the enzyme substrate complex (cf. [1, 11]).

Our smallest model containing the reactive sequence, Ala_3–Lys–Phe (XI), is a relatively poor substrate (C = 100 M^{-1} sec^{-1}). Addition of one alanine residue in P_4, leading to Ala_4–Lys–Phe (III), causes an increase by a factor of 1400 in overall hydrolysis (a factor of 13 in k_{cat} and 110 in $\bar{K}_m$). Thus there is an extremely large effect due to binding of a residue in subsite S_4. The fact that $Ala_6(L_6)$ (V) is about 5 times more reactive than $Ala_6(DL_5)$ (VI) shows that the interaction in S_4 is considerably stereospecific. This is quite unexpected for a subsite so far removed from the catalytic site. Similar stereospecificity was found in the pair Ala_2–Phe–Ala_3 (L_6 and DL_5) (VII and VIII). The increase in $\bar{K}_m$ by a factor of 5 (from 250 to 1300) on exchanging an Ala by a Phe residue in P_2 shows strong hydrophobic binding in S_2 (compare

Table 1

No.	Substrate										$\bar{K}_m$ (M^{-1})	k_{cat} (sec^{-1})	C (M^{-1} sec^{-1})
	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4 .... P'_{14}			
I	Lys	Glu	Thr	Ala	Ala	Ala	Lys	Phe	Glu	Arg ... Ala	1450	45	65 000
II	Lys	Glu	Thr	Ala	Ala	Ala	Lys	Phe			4500	32	144 000
III			Ala	Ala	Ala	Ala	Lys	Phe			4500	32	144 000
IV			Ala	Ala	Ala	Ala	Lys	Ala			1700	22	37 000
V			Ala	Ala	Ala	Ala	Ala	Ala			250	60	15 000
VI			D-Ala	Ala	Ala	Ala	Ala	Ala			250	13	3 250
VII			Ala	Ala	Phe	Ala	Ala	Ala			1300	28	36 400
VIII			D-Ala	Ala	Phe	Ala	Ala	Ala			1370	8	11 000
IX				Ala	Ala	Ala	Lys	Phe			40	2.5	100

peptide V with VII, and VI with VIII). Further elongation of the peptide in the direction of the amino terminal (see octapeptide II) causes no change in either $\bar{K}_m$ or K_{cat}, indicating a limit of four subsites on this side of the active site of elastase.

"Elongation" of the octapeptide (II) in the direction of the carboxyl terminal by residues P'_3 to P'_{14} causes a 3-fold decrease in $\bar{K}_m$ and a 40% increase in k_{cat}. This effect indicates that the "right-hand" side of the active site of elastase may contain three subsites, making a total of six to seven subsites altogether. (It is interesting that seven subsites were also found in papain [1]).

We turn now to the significance of the Lys and Phe residues in P'_1 and P'_2 respectively. To investigate this point we studied the two hexapeptides Ala_4–Lys–Ala and Ala_6. The "non-specific" Ala_6 is indeed a poorer substrate ($\bar{K}_m = 250\ M^{-1}$; $k_{cat} = 60\ sec^{-1}$). Comparing it with the "specific" Ala_4–Lys–Phe it is seen that the Lys and Phe side-chains in P'_1 and P'_2 respectively improve the *C*-value 10-fold (18-fold increase in $\bar{K}_m$; 47% decrease in k_{cat}).

In order to decide whether the specificity of Ala_4–Lys–Phe resides in the Lys residue (P'_1), in the Phe residue (P'_2) or in both, we compare Ala_6 with Ala_4–Lys–Ala ($\bar{K}_m = 1700\ M^{-1}$; $k_{cat} = 22\ sec^{-1}$). It is seen that exchanging Ala for a Lys residue in P'_1 increases $\bar{K}_m$ 6-fold while k_{cat} decreases 3-fold. Making the further change Ala → Phe in P'_2 results in a 3-fold increase in $\bar{K}_m$ and a 50% increase in k_{cat}. Thus most of the side-chain effects stem from the Lys in P'_1, although Phe in P'_2 is also quite effective.

Qualitative experiments showed that trifluoroacetylated S-peptide was also split rapidly at bond 6–7, yielding the trifluoroacetylated hexapeptide 1–6 and the Lys(7)-ε-trifluoroacetyl-tetradekapeptide 7–20, which was isolated in the pure state. This shows that the specificity observed is not due to the free amino group of Lys(7), and we conclude that it stems from hydrophobic binding of the lysine methylenes and of the phenyl group in subsites S'_1 and S'_2 respectively.

The system described here readily lends itself to expansion aimed at the further study of the active site of elastase. The fact that the sequence –Ala–Lys–X–, or a similar one, in a hexa- or larger peptide efficiently directs hydrolysis by elastase to a defined bond makes it possible to explore systematically the binding properties of the various subsites and their influence on the catalytic rate. Also, exchanging the Ala residue for others will help to define the spatial restrictions obviously present at the active site (it is known from the X-ray structure of elastase that the "hole", which in the analogous chymotrypsin molecule accepts a phenyl group, is blocked off by a Val residue [12]). Finally, in preliminary experiments we have found that Lys–Phe is a quite effective inhibitor of elastase. This peptide as well as related ones should be of value in the direct observation of elastase-peptide complexes by X-ray diffraction.

In conclusion, it may be stated that in the pattern of hydrolysis by elastase, such as that observed in the RNase S-peptide, the preference for splitting at the CO of alanine is probably due to space restrictions in subsite S_1, and the preference for the splitting of the specific 6–7 bond is due to favorably hydrophobic interactions of the Lys and Phe residues in subsites S'_1 and S'_2. It also seems that elastase will not easily split the first three bonds at the amino terminal of a peptide chain, since interaction of a residue with subsite S_4 is necessary for effective hydrolysis by this enzyme.

References

[1] I.Schechter and A.Berger, Biochem. Biophys. Res. Commun. 27 (1967) 157.
[2] A.S.Narayanan and R.A.Anwar, Biochem. J. 114 (1967) 11.
[3] P.Geneste and M.L.Bender, Proc. Natl. Acad. Sci. U.S. 64 (1969) 683.
[4] H.Kaplan, V.D.Symonds, H.Dougas and D.R.Whitaker, Can. J. Biochem. 48 (1970) 649.
[5] A.Gertler and T.Hofmann, Can. J. Biochem. 48 (1970) 384.
[6] D.Atlas and A.Berger, unpublished results.
[7] D.M.Shotton, Methods Enzymology, in press.
[8] F.M.Richards and P.J.Vithayathil, J. Biol. Chem. 234 (1959) 1459.
[9] R.F.Goldberger and C.B.Anfinsen, Biochemistry 1 (1962) 401.
[10] S.Levit, (1971) Ph.D.Thesis, Feinberg Graduate School, The Weizmann Institute of Science, Rehovot, Israel.
[11] A.Berger and I.Schechter, Phil. Trans. Roy. Soc. Lond. B 257 (1970) 249.
[12] D.M.Shotton and H.C.Watson, Phil. Trans. Roy. Soc. Lond. B 257 (1970) 111.