Proceedings of the National Academy of Sciences
Vol. 67, No. 4, pp. 1734–1740, December 1970

Evidence for an Extended Active Center in Elastase*

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Communicated September 28, 1970

Abstract. Large increases in the esterase, amidase, and peptidase activities of elastase are observed on increasing the length of peptide substrates. The results suggest that at least five contiguous peptide units of the substrate (four N-terminal and one C-terminal to the scissile bond) can interact concurrently with the enzyme. When analyzed in terms of an acyl-enzyme mechanism, the increased effectiveness of the enzyme with larger substrates appears mainly in the acylation reaction and, to a lesser extent, in the binding and deacylation steps.

Elastase is a member of the family of serine proteinases isolated from the pancreas.[1] It shows considerable sequence homology with the chymotrypsins and trypsin,[2] and its three-dimensional structure, as deduced by x-ray crystallography, is closely related to that of α-chymotrypsin.[3] Both elastase and α-chymotrypsin hydrolyze small esters by similar mechanisms.[4] α-Chymotrypsin has been shown to cleave certain acetyl amino acid amides,[5] but elastase does not appear to cleave Ac-Ala-NH_2, although its specificity is commonly considered to include alanyl bonds.[6] In order to study the effect of the length of the peptide on the ability of elastase to hydrolyze peptide esters and amides, a series of substrates has been prepared in which the only bonds cleaved involve the carbonyl groups of alanine residues. We report here that the esterase, amidase, and peptidase activities of elastase depend markedly on the number of amino acid residues in the substrate. Large increases in proteolytic activity also have been observed when long chain substrates are hydrolyzed by papain,[7] carboxypeptidase A,[8] and pepsin.[9]

Experimental. All peptides were synthesized by methods known to lead to nonracemized products. Products were pure by thin-layer chromatography (TLC) and elemental analysis. Porcine elastase (>99.8% pure) was purchased from Whatman Biochemicals, England.

Rates of substrate hydrolysis at 37°C and pH 9.00 in 10^{-2} M aqueous $CaCl_2$ were determined in a pH-stat. Product ionization was corrected according to Kurtz and Niemann.[10] Data were analyzed in terms of the Michaelis-Menten equation. Inhibitor constants were determined from Dixon plots.[11]

Ester and amide cleavage was proved by the absence on thin-layer chromatograms of ninhydrin positive products in the reaction mixture, except where the starting material was ninhydrin positive. Peptide bond cleavage was established by identification of the ninhydrin positive product by thin-layer and ion-exchange chromatography. The kinetic parameters of concurrent peptidase and amidase activities were calculated from an analysis of the products (amino acid analyzer) and a knowledge of their pK_a values.

TABLE 1. *Elastase-catalyzed hydrolysis of esters.*

Substrate	k_{cat}/K_m(app) ($M^{-1}sec^{-1}$)	k_{cat} (sec^{-1})	K_m(app) (mM)	[S]* (mM)
Ac-Ala-OMe†	49	8.2	170	10.0–100
Ac-Ala-Ala-OMe	2,200	49	22	1.0– 30
Ala-Ala-Ala-OMe‡	980	N.D.	N.D.	2.0– 20
Ala-Ala-Ala-OMe	2,700	40	15	0.2– 30
Ac-Ala-Ala-Ala-OMe	300,000	120	0.4	0.2– 10
Ac-Ala-Ala-Ala-Ala-OMe§	1,800,000	120	0.067	0.1– 1

* Range of substrate concentrations.
† pH 8.00.
‡ pH 7.50. Estimation of k_{cat} and K_m(app) was not possible due to the high value of K_m(app).
§ In this one case it was necessary to solubilize the substrate with trifluoroethanol. Final reaction mixture is 1% trifluoroethanol (v/v).

Results. Esterase activity: Product analysis showed that the sole reaction catalyzed by elastase for the substrates listed in Table 1 is cleavage of the ester bond. The enzyme exhibits poor esterase activity toward ω-amino tripeptide esters and smaller substrates. However, the kinetic parameters for ester hydrolysis of Ac-Ala-Ala-Ala-OMe (k_{cat}/K_m(app) = 3.0×10^5 M^{-1} sec^{-1}) are similar to those observed for other serine proteinases acting on their specific substrates.

Amidase activity: Product analyses of enzymic digests of the compounds lised in Table 2 were generally consistent with the hypothesis that no cleavage of peptide bonds occurred. The only substrate we describe with which competing amidase and peptidase activities were observed was Ac-Ala-Ala-Ala-Ala-NH_2. Elastase cleaves the amide function from *N*-acetyl tripeptide amides at rates comparable to those found with the α-chymotrypsin-catalyzed hydrolysis of Ac-Trp-NH_2 (k_{cat}/K_m(app) = 3.56 M^{-1} sec^{-1})[5] and trypsin-catalyzed hydrolysis of Bz-Arg-NH_2 (k_{cat}/K_m(app) = 14 M^{-1} sec^{-1}).[12] However, as can be seen from Table 2, longer peptide amides are cleaved much more rapidly, and the increased rates are reflected mainly in the k_{cat} value.

In an attempt to clarify the reasons for elastase's inability to hydrolyze amide substrates smaller than acetyl tripeptide amides, inhibition constants were measured for several of these compounds and are presented in Table 3. In all cases the inhibition appears to be competitive. Compounds smaller than acetyl tripeptide amides show very poor binding to the enzyme, but it is unlikely that this factor alone causes the decreased reactivity.

TLC of reaction mixtures showed that cleavage of Pro-Ala-Pro-Ala-NH_2, but not Ac-Pro-Ala-Pro-Ala-NH_2, was strongly pH dependent between pH

TABLE 2. *Elastase-catalyzed hydrolysis of amides.*

Substrate	k_{cat}/K_m(app) ($M^{-1}sec^{-1}$)	k_{cat} (sec^{-1})	K_m(app) (mM)	[S]* (mM)
Ac-Ala-Ala-Ala-NH_2	15	0.038	2.5	0.5–5
Ac-Ala-Pro-Ala-NH_2	25	0.105	4.2	0.5–8
Ac-Ala-Ala-Ala-Ala-NH_2	2,070	6.0	2.9	0.5–5
Ac-Ala-Ala-Pro-Ala-NH_2	3,500	7.3	2.1	0.5–9
Ac-Pro-Ala-Pro-Ala-NH_2	3,700	9.3	2.5	0.5–5
Pro-Ala-Pro-Ala-NH_2	880	3.5	4.0	1.0–5

* Range of substrate concentrations.

TABLE 3. *Inhibition of elastase-catalyzed hydrolysis of amides.*

Inhibitor	K_i (mM)	$[I]$* (mM)
Ac-Ala-NH_2	150	10–100
Ac-Ala-Ala-NH_2	50	10– 50
Ac-Ala-Ala-Ala-NH_2	3	1– 5
Ac-Ala-Pro-Ala-NH_2	6	2– 10

* Range of inhibitor concentrations.

9.0 and 7.5. Since digestion of the free peptide was slower at the lower pH, where protonation of the amino terminus is expected, these results point to the unprotonated compound as the preferred amidase substrate.

Peptidase activity: Elastase digests of the *N*-acetyl tripeptide amides, Ac-Ala-Ala-Ala-NH_2 and Ac-Ala-Pro-Ala-NH_2, show the absence of significant amounts of ninhydrin positive products. Peptidase activity with these substrates is therefore much slower than the corresponding amide hydrolysis, and the k_{cat}/K_m(app) ratio is probably less than 0.5 M^{-1} sec^{-1}. However, cleavage of the C-terminal peptide bond of certain *N*-acetyl tetra- and pentapeptide amides is rapid (Table 4).

TABLE 4. *Elastase-catalyzed hydrolysis of peptides.**

Substrate	k_{cat}/K_m(app) ($M^{-1}sec^{-1}$)	k_{cat} (sec^{-1})	K_m(app) (mM)	$[S]$† (mM)
Ac-Ala-Ala-Ala↓Ala-NH_2	90	0.26	2.9	0.5–5
Ac-Ala-Pro-Ala↓Ala-NH_2	520	2.4	4.6	0.5–9
Ac-Pro-Ala-Pro-Ala↓Ala-NH_2	25,500	37	1.45	0.5–8
Ac-Pro-Ala-Pro-Ala↓Ala-OH	165	0.33	2.0	0.5–5

* The point of cleavage is shown by the arrow ↓.
† Range of substrate concentrations.

Discussion. Marked accelerations of the esterase, amidase, and peptidase activities of elastase appear with increasing substrate chain length (Tables 1, 2, and 4). The largest increment occurs between substrates having tripeptide and acetyl tetrapeptide moieties N-terminal to their scissile bond. In the notation of Schechter and Berger,[13] this acceleration corresponds to the substrate filling the S_4 and S_5 subsites of the enzyme's active center. It is possible to further localize the important features of the substrate. The amide groups of Pro-Ala-Pro-Ala-NH_2, Ac-Pro-Ala-Pro-Ala-NH_2, and Ac-Ala-Ala-Pro-Ala-NH_2 are all cleaved at similar rates, which are much faster than the rate of the same reaction in Ac-Ala-Pro-Ala-NH_2 (Table 2). The increased rate seems, therefore, to be due to the nitrogen atom and side-chain of the P_4 amino acid residue. On similar grounds, the important element of the substrate's peptide bond fitting between S_4 and S_3 may be thought to be the carbonyl group, since Ac-Ala-Ala-Ala-OMe reacts much faster than Ala-Ala-Ala-OMe, even at pH 9 where the latter compound is largely unprotonated (Table 1).

A peptide bond between two alanine residues seems to be cleaved more easily than a terminal alanine amide bond (Tables 2 and 4). This may be due simply to changes in the basicity of the nitrogen atom, but could also be due to a specific interaction of the P_1'–P_2' amide bond with the S_1'–S_2' subsites of the enzyme. An enzyme–substrate interaction in the S_1'–S_2' subsite of α-chymotrypsin has recently been reported by Cohen *et al.*[14]

In an attempt to define more exactly the factors leading to the increased efficiency of elastase with long chain substrates, we consider now the steps of the reaction sequence leading to hydrolysis. It appears that elastase-catalyzed hydrolyses proceed by an acyl-enzyme mechanism.[4] Initial formation of the Michaelis complex (dissociation constant, K_s) leads to acylation of the enzyme with the release of one product (rate constant, k_2) and subsequent decomposition of the acyl-enzyme to enzyme and a second product (rate constant, k_3).

We use Eq. (1)[15] to relate the above constants to those of the Michaelis–Menten equation:

$$k_{cat}/K_m(\text{app}) = k_2/K_s \qquad (1)$$

Also, we have assumed that k_{cat} (ester) is approximately equal to the rate of deacylation (k_3) in the hydrolysis of analogous esters, amides, and peptides[15] with the knowledge that this may underestimate k_3 for the smaller substrates. [Such an underestimate would only reinforce our conclusions with regard to the insensitivity of k_3 to substrate chain length (see below)].

From the data in Tables 1, 2, and 4 and a knowledge of the immeasurably slow rate of amidase and peptidase action on acetyl dipeptide amides, it can be seen that k_{cat}/K_m(app) for hydrolysis of long substrates is over 10,000 times greater than that for the same reaction with small substrates. For the same increase in chain length, the increase in the rate of the deacylation reaction ($k_3 = k_{cat}$, Table 1) is only 15-fold. Acceleration of deacylation with long substrates is therefore unlikely to be of prime importance in determining the high reactivity of these compounds.

The dependence of k_{cat}/K_m(app) on substrate chain length (see above) makes it obvious to examine the constants, k_2 and K_s [Eq. (1)], for the factor leading to high reactivity in large substrates. However, to obtain the fundamental constants k_2 and K_s from the ratio k_2/K_s, it is necessary to make the further assumption that the measured values of K_i and K_m(app) for amides and peptides represent mainly binding in a single mode.[16,17] This is not consistent with our evidence that the enzyme can act as both an amidase and peptidase on the same substrate. Moreover, it appears that a substantial part of the substrate binding energy of elastase comes from its interaction with peptide bonds of the substrate rather than with a specific side-chain as in α-chymotrypsin and trypsin. Since the peptide bond is a repeating unit, the assumption of a unique binding mode is impossible to justify. For this reason we consider first the extreme position that the poor rates of reaction of small substrates are due to their being bound on the enzyme without the scissile bond being close to the active site. Possible binding modes for acetyl dipeptides, for example, are the nonproductive modes,

S_4–S_3 and S_3–S_2, as well as the productive mode, S_2–S_1. It is important to note that to account completely for the variation in proteolytic coefficients in these terms requires that more than 99.9% of Ac-Ala-OMe and 99% of Ac-Ala-Ala-OMe are bound in nonproductive modes. An example of an enzyme in which nonproductive binding plays such an important role is lysozyme, where the subsite immediately adjacent to the active site has a positive free energy of binding, and excellent binding subsites exist elsewhere in the enzyme's active center.[18] It appears, however, that a characteristic feature of this situation, in which high reactivity necessitates occupation of an unfavorable binding site, would be an increasing K_m(app) associated with an increasing k_{cat}. This is clearly not the case with elastase where the striking increases in proteolytic coefficient associated with extension of the polypeptide chain are accompanied by decreasing or unchanged K_m(app)s (Tables 2, 3, and 4). For this reason we feel justified in assuming that binding of small substrates in nonproductive modes does not occur to the extent necessary to completely account for their poor reactivity.

If the equally extreme viewpoint is taken that there is no binding of substrates or inhibitors other than in a single "productive" mode; i.e., with the scissile bond between subsites S_1 and S_1', the values of K_m(app) and K_i measured for amides and peptides must correspond directly to the K_s for the productive mode. In this case the values of k_{cat} determined for the substrates listed in Tables 2 and 4 are equal to k_2, the rate at which they acylate the enzyme. It appears in this analysis that the most important factor in the increased reactivity of long chain substrates is the rate of the acylation reaction, although the exact increase in this rate is impossible to calculate due to the poor reactivity of the smaller substrates.

If one further assumption is made, that the K_s of an ester substrate is not greatly different from the K_s of the corresponding amide, we can calculate k_2 for the ester substrates listed in Table 1 with the aid of Eq. (1). As with the amidase and peptidase activities, the k_2 factor responds most strongly to increasing substrate chain length. Thus, k_2 increases about 1000 times on going from Ac-Ala-OMe to Ac-Ala-Ala-Ala-Ala-OMe.

With this large increase in k_2, it appears that it would not greatly alter our conclusions if as much as 70% of the binding of small substrates and inhibitors occurred in nonproductive modes. Therefore, we consider that the specificity of this enzyme for long chain peptides appears mainly in the acylation reaction and, to a lesser extent, in the binding and deacylation steps.

Studies of the hydrolysis of *N*-acetyl-L-amino acid methyl esters by α-chymotrypsin have shown that an increase in the hydrophobic nature of the amino acid side-chain not only increases the enzyme-substrate binding, but also the rate constants for acylation and deacylation.[19,20] Indeed, if these parameters are compared with those for Ac-Gly-OMe,[20] it can be inferred that the dominant factor in the increasing reactivity is the increased rate of the acylation reaction. The increase in enzyme-substrate binding is easily rationalized in α-chymotrypsin by a simple "lock-and-key" mechanism, and part of this "lock" has been identified as an hydrophobic pocket in the S_1 subsite of the enzyme.[21] However,

the increased rates of acylation which accompany the increase in enzyme-substrate binding are more difficult to explain. It has been postulated that binding of the side-chain in the hydrophobic pocket, in conjunction with binding of the adjacent nonscissile amide group and the α-hydrogen atom at other specific sites, has the effect of orienting the scissile bond in exactly the right position for attack by the active serine–histidine residues.[20,21] In the absence of an hydrophobic side-chain to fit into the pocket, the scissile bond has greater freedom of movement, and orientation with respect to the catalytic residues at any given time is likely to be less than optimal. This loss of orientation is proposed to explain the fall in k_2 and k_3 that accompanies the high K_m(app) associated with α-chymotrypsin-catalyzed hydrolysis of esters of amino acids with small side-chains.[20,21]

The active site of elastase is very similar to that of α-chymotrypsin, except in the area of the side-chain binding site at S_1.[3] In elastase, the mouth of the hydrophobic pocket is partially occluded by the side-chain of valine-216; the analagous residue is glycine in α-chymotrypsin.[2,3] The presence of valine in this position restricts the possibility of side-chain interaction at the S_1 subsite of elastase leading to poor enzyme-substrate binding and concomitant difficulty in orienting the scissile bond in the active site. Thus, the large K_m(app) and low acylation rates observed for elastase-catalyzed hydrolysis of small substrates find a ready explanation in the geometry of the active site. The point of interest is that elastase manages to overcome these deficiencies if it is presented with larger substrates, although the mechanism by which this is accomplished is not obvious. Binding the substrate at points remote from the cleavage site, while increasing the total enzyme-substrate binding energy, would not be expected to orient the scissile bond with any precision.[22]

With elastase apparently responding to a part of the substrate some distance from the active site, the question of whether we are dealing with an absolutely rigid enzyme arises. The kinetic data reported here are best explained by a flexible, or "induced fit,"[23] enzyme. In such an enzyme, the fit and orientation of substrate at the cleavage site could be improved by a conformational change resulting from substrate binding to other parts of the active center. Although the evidence that elastase is an induced-fit enzyme is not conclusive, it is now a reasonable hypothesis that the enzyme assumes its most favorable hydrolytic conformation only in the presence of large peptide substrates.

We wish to thank Dr. Richard M. Schultz for his constructive criticism and helpful advice.

* Supported in part by United States Public Health Service grant AM07300.

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