Substrate Specificity of Human Pancreatic Elastase 2†

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ABSTRACT: The substrate specificity of human pancreatic elastase 2 was investigated by using a series of peptide *p*-nitroanilides. The kinetic constants, k_{cat} and K_m, for the hydrolysis of these peptides revealed that this serine protease preferentially hydrolyzes peptides containing P_1 amino acids which have medium to large hydrophobic side chains, except for those which are disubstituted on the first carbon of the side chain. Thus, human pancreatic elastase 2 appears to be similar in peptide bond specificity to the recently described porcine pancreatic elastase 2 [Gertler, A., Weiss, Y., & Burstein, Y. (1977) *Biochemistry 16*, 2709] but differs significantly in specificity from porcine elastase 1. The best substrates for human pancreatic elastase 2 were glutaryl-Ala-Ala-Pro-Leu-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Met-*p*-nitroanilide. However, there was little difference among substrates with leucine, methionine, phenylalanine, tyrosine, norvaline, or norleucine in the P_1 position. Changes in the hydrolysis rate of peptides with differing P_5 residues indicate that this enzyme has an extended binding site which interacts with at least five residues of peptide substrates. The overall catalytic efficiency of human pancreatic elastase 2 is significantly lower than that of porcine elastase 1 or bovine chymotrypsin with the compounds studied.

Elastases, because of their ability to hydrolyze elastin, have been implicated in the etiology of emphysema (Kaplan et al., 1973; Loeven, 1972), atherosclerosis (Balo & Banga, 1953), and aging (Hall, 1964). Porcine pancreatic elastase 1 has been characterized, and its substrate specificity has been determined with peptide *p*-nitroanilides (Kasafirek et al., 1976), chloromethyl ketone inhibitors (Powers et al., 1977), and a natural peptide (Narayanan & Anwar, 1969). These studies indicate that porcine elastase 1 preferentially hydrolyzes substrates with a P_1 alanine residue.[1] Based on this specificity, it has been proposed that elastolytic activity results from the ability of a protease to hydrolyze peptides at amino acids with short aliphatic side chains and to be absorbed onto elastin in the pH range 7–10 (Gertler, 1971).

Human pancreatic elastase 2 was first isolated in this laboratory (Largman et al., 1976) by assaying pancreatic tissue extracts for activity against RBB elastin.[2] Initial investigation of this enzyme revealed that it was a serine endopeptidase which had approximately 40% of the activity of porcine elastase 1 against undyed elastin. However, the human enzyme did not fit the pattern of a "traditional" elastase in that it did not rapidly hydrolyze substrates designed for the porcine enzyme such as Suc-$(Ala)_3$-pNA (Bieth et al., 1974) or Ac-$(Ala)_3$-OMe (Gertler & Hofmann, 1970). In order to more clearly define the nature of elastolysis, and to further characterize human pancreatic elastase 2, we undertook the present study to determine the substrate specificity of human pancreatic elastase 2. During the course of this work, two other enzymes with elastolytic activity have been characterized: human leukocyte elastase (Zimmerman & Ashe, 1977) and porcine pancreatic elastase 2 (Gertler et al., 1977). These enzymes also showed a peptide bond specificity which differs from that of porcine elastase 1. In the present report, the peptide bond specificity of human pancreatic elastase 2 is compared with those of the other characterized elastases and with the specificity of bovine chymotrypsin.

Materials and Methods

Human pancreatic elastase 2 was purified as previously described (Largman et al., 1976). Porcine pancreatic elastase (type III) was purchased from Sigma Chemical Co., St Louis, MO. Cbz-amino acid *p*-nitroanilides were synthesized from Cbz-amino acids and *p*-nitroaniline by using the phospho–azo method of Kasafirek et al. (1976), except that the reactions were carried out at room temperature for 24 h instead of at 115 °C for 3 h. The majority of the substrates were synthesized by coupling Cbz-Ala-Ala-Pro with the appropriate amino acid *p*-nitroanilide by using EDAC·HCl, followed by HBr–HOAc removal of the Cbz group and acylation with succinic anhydride as previously described (Del Mar et al., 1979b). Some of the compounds were synthesized by the active ester method as summarized for Suc-Ala-Ala-Pro-Leu-pNA as follows: Cbz-Leu-pNA was reacted with 16% HBr in HOAc for 1 h; the resulting Leu-pNA was reacted with Cbz-Pro-*p*-nitrophenyl ester and triethylamine in methylene chloride for 48 h; the resulting product was treated with alternating steps of HBr in HOAc and reaction with Cbz-Ala-*p*-nitrophenyl ester to yield Ala-Ala-Pro-Leu-pNA; this compound was reacted with succinic anhydride as described above; the overall yield was 28%. All of the compounds synthesized had an analytical analysis consistent with the assigned structure.[3]

Assays. Enzyme assays were conducted at 25 °C in 0.2 M Tris-HCl (pH 8.0). The absorbance of the *p*-nitroaniline

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[1] The nomenclature introduced by Schechter & Berger (1967) is used to describe the positions of amino acids in a substrate. Amino acid residues and terminal acyl substituents (succinyl, acetyl, etc.) are numbered P_1, P_2, P_3, etc., in the N-terminal direction from the scissle bond. The corresponding subsites of the enzyme's active site are numbered S_1, S_2, S_3, etc., in an analogous fashion.

[2] Abbreviations used: RBB elastin, Remazol brilliant blue stained elastin; Suc, succinyl (3-carboxypropionyl); MeO-Suc, methylsuccinyl (3-carbomethoxypropionyl); Glt, glutaryl (4-carboxybutanoyl); pNA, *p*-nitroanilide; Cbz, carbobenzyloxy; Ac, acetyl; EDAC·HCl, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; Nva, norvaline [(*S*)-2-aminopentanoic acid]; Nle, norleucine [(*S*)-2-aminohexanoic acid]; Abu, α-aminobutyric acid; Met(O), methionine sulfoxide; OEt, ethyl ester.

[3] Data on analytical analysis, melting points, and details of substrate synthesis were submitted for review and can be obtained by writing to Dr. Michael C. Geokas, Martinez VA Medical Center.

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Table I: Kinetic Parameters for the Hydrolysis of Succinyl Tetrapeptide *p*-Nitroanilides by Human Pancreatic Elastase 2

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Suc-Ala-Ala-Pro-Gly-pNA	7.3	0.01	1
Suc-Ala-Ala-Pro-Ala-pNA	2.4	0.34	140
Suc-Ala-Ala-Pro-Abu-pNA	2.4	1.4	600
Suc-Ala-Ala-Pro-Nva-pNA	1.6	4.3	2800
Suc-Ala-Ala-Pro-Nle-pNA	1.5	3.3	2200
Suc-Ala-Ala-Pro-Met-pNA	1.3	6.4	4900
Suc-Ala-Ala-Pro-Met(O)-pNA	3.5	0.56	160
Suc-Ala-Ala-Pro-Leu-pNA	1.4	5.1	3600
Suc-Ala-Ala-Pro-Phe-pNA	2.5	5.1	2000
Suc-Ala-Ala-Pro-Tyr-pNA	1.0	2.8	2700
Suc-Ala-Ala-Pro-Val-pNA	3.5	0.01	3
Suc-Ala-Ala-Pro-Ile-pNA	6.0	0.01	2

produced was measured continuously on a Gilford Model 252 spectrophotometer at 410 nm. In the kinetic determinations for human pancreatic elastase 2 and porcine elastase 1, duplicate measurements were made at eight substrate levels between approximately 0.2 and 5 times K_m (where solubility allowed). The data obtained were first plotted according to the method of Lineweaver & Burk (1934) to check for linearity of the reciprocal plot and to allow for rejection of obviously erroneous data points. The parameters K_m and V_{max} were then estimated by an iterative least-squares fit to the Michaelis–Menten equation by use of the computer program described by Cleland (1967). For individual determinations, the standard deviation in the K_m and V_{max} terms was less than 10% and often less than 5% as determined by the computer program. The data presented in Tables I–III are generally the average of two or three separate determinations. For each substrate, it was also determined that for a fixed concentration (1 mg/mL) of that substrate, the rate of hydrolysis was a linear function of the amount of enzyme added.

Results

The kinetic parameters for the hydrolysis of a series of compounds, Suc-Ala-Ala-Pro-AA-pNA (AA = an α-amino acid), by human pancreatic elastase 2 are presented in Table I. The data for the first five compounds show that among the substrates with unbranched amino acids in the P_1 position k_{cat}/K_m increases substantially with increasing side-chain length up to norvaline. The increase in catalytic efficiency can be seen to be mainly due to increases in k_{cat}; however, the parameters for AA = Nle or Nva are essentially the same. Incorporation of methionine, which is the same length as norleucine, into the P_1 position yields the best substrate of this series with respect to the parameter k_{cat}/K_m. However, oxidation of the methionine to methionine sulfoxide results in a large decrease in the k_{cat}/K_m for the oxidized peptide. The next three substrates listed in the table, containing P_1 leucine, phenylalanine, and tyrosine, which are branched at the second carbon of the side chain, are also good substrates, but there is little difference among them. In contrast, two compounds containing P_1 amino acids with a branch at the first carbon of the side chain, valine and isoleucine, are very poor substrates due to the very low values of k_{cat}.

Table II illustrates the effect of increasing the peptide chain length, and the effect of replacing the P_2 alanine with a proline, on the kinetic parameters for the hydrolysis of a series of succinyl peptide *p*-nitroanilides containing leucine or alanine at the P_1 position by human and porcine pancreatic elastases. For the human enzyme, with substrates containing leucine at the P_1 position, substitution of proline at P_2 increases k_{cat} twofold while raising K_m to a lesser extent. In contrast, the same substitution with alanine in P_1 affects K_m only; however, k_{cat} for human elastase 2 with these compounds is much less than for the leucine substrates. Increasing the peptide chain length from three to four residues yields better values for both K_m and k_{cat}, for substrates with leucine at P_1, but the corresponding pentapeptide is a less efficient substrate for human elastase because of a significant decrease in k_{cat}.

Similar results are shown in Table II for porcine elastase with respect to both substitution of proline for alanine and increasing peptide chain length. It is noteworthy, however, that these results indicate that Suc-Ala-Ala-Pro-Leu-pNA is a good substrate for the porcine enzyme. Furthermore, it can be seen that an increase in peptide chain length from three to four residues in substrates with alanine at P_1 yields an eightfold decrease in K_m without a significant effect on k_{cat}.

The effect of changing the substituent corresponding to the P_5 position on the kinetic parameters for the hydrolysis of N-substituted Ala-Ala-Pro-Leu-*p*-nitroanilides by human elastase 2 is illustrated in Table III. Negatively charged P_5 groups (glutaryl or succinyl) yield better substrates than a neutral (methylsuccinyl) or positively charged group (alanyl), the effect being mainly due to a decrease in K_m.

Discussion

A series of succinyl peptide *p*-nitroanilides was synthesized in order to determine the primary specificity of human pancreatic elastase 2. The succinyl group was employed to increase the water solubility of the peptides, thus eliminating the need for organic solvents, which have been shown to affect the rate of hydrolysis of peptides by porcine elastase 1 (Bieth & Wermuth, 1973). The succinyl moiety was successfully employed by Bieth et al. (1974) in the design of Suc-Ala-Ala-Ala-pNA, a specific peptide substrate for porcine elastase 1. Kasafirek et al. (1976) subsequently reported the synthesis of a series of succinyl peptide *p*-nitroanilides and the kinetic parameters for their hydrolysis by porcine elastase 1 and bovine chymotrypsin. In the current investigation *p*-nitroanilides were employed as well, because as amides they more closely resemble polypeptide substrates than do esters. In addition, these compounds are more stable to alkaline hydrolysis than esters,

Table II: Effect of Chain Length and P_2 Proline Substitution on the Hydrolysis of Peptide *p*-Nitroanilides by Human Elastase 2 and Porcine Elastase 1

	human pancreatic elastase 2			porcine elastase 1		
substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Suc-Ala-Ala-Leu-pNA	2.7	2.3	860	2.0	3.2	1 500
Suc-Ala-Pro-Leu-pNA	3.6	4.8	1300	4.9	18	3 700
Suc-Ala-Ala-Pro-Leu-pNA	1.4	5.1	3600	1.2	18	14 000
Suc-Ala-Ala-Ala-Pro-Leu-pNA	1.0	2.0	2000	0.49	4.9	10 000
Suc-Ala-Ala-Ala-pNA	8.1	0.15	19	1.4	17	12 000
Suc-Ala-Pro-Ala-pNA	3.6	0.16	44	1.5	59	40 000
Suc-Ala-Ala-Pro-Ala-pNA	2.4	0.34	140	0.19	54	290 000

Table III: Effect of Variation of the P_5 Substituent on the Kinetic Parameters for the Hydrolysis of N-Substituted Ala-Ala-Pro-Leu-*p*-nitroanilides

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Glt-Ala-Ala-Pro-Leu-pNA	1.1	7.4	6500
Suc-Ala-Ala-Pro-Leu-pNA	1.4	5.1	3600
MeO-Suc-Ala-Ala-Pro-Leu-pNA	8.2	8.5	1000
Ala-Ala-Ala-Pro-Leu-pNA	11.0	5.8	530

and the *p*-nitroaniline released during hydrolysis can be accurately and conveniently determined spectrophotometrically.

The results presented in Table I demonstrate the relatively low selectivity of human pancreatic elastase 2 with respect to the P_1 amino acid residue. Comparison of the kinetic constants for hydrolysis of substrates containing unsubstituted side chains longer than alanine (Abu, Nva, Nle, and Met) in the P_1 position and amino acids branched at the side-chain β-carbon (Leu, Phe, and Tyr) demonstrates that there is only a twofold difference in K_m and an eightfold difference in k_{cat}/K_m. Furthermore, there is only a 3.5-fold difference in the K_m values for all the compounds in Table I except for those containing isoleucine and glycine in the P_1 position. The k_{cat} values for the same group of peptides vary by only a factor of 5 if the alanine compound is excluded. These results contrast markedly with the high degree of specificity for the P_1 residue shown by porcine elastase and bovine chymotrypsin A. Porcine elastase 1 showed a 123-fold decrease in the parameter k_{cat}/K_m for the hydrolysis of the compounds Suc-Ala-Ala-Ala-pNA vs. Suc-Ala-Ala-Val-pNA (Kasafirek et al., 1976) and did not hydrolyze similar tripeptides containing tyrosine or phenylalanine in the P_1 position to a measurable extent. These authors also demonstrated a 55-fold decrease in the parameter k_{cat}/K_m for hydrolysis by bovine chymotrypsin of the corresponding tripeptides containing tryosine or methionine in the P_1 position. Related compounds containing glycine, alanine, valine, or isoleucine were not hydrolyzed to a measurable extent by chymotrypsin. Very similar results were obtained by Bauer et al. (1976) with acetyl tetra- and pentapeptide amides.

The great difference in substrate specificity observed between human elastase 2 and porcine elastase 1 indicates that the human enzyme has a significantly larger binding "pocket" for the P_1 amino acid side chain. Furthermore, the observation that elastase 2 does not discriminate among the leucine, tyrosine, and phenylalanine derivatives, as does chymotrypsin, suggests that the S_1 site in elastase 2 does not contain the precise geometry to allow the extensive contacts which have been shown by X-ray analysis to characterize the interaction of the phenylalanine or tyrosine side chains with the S_1 site in chymotrypsin (Steitz et al., 1969).

A comparison of the kinetic parameters for hydrolysis of peptide *p*-nitroanilide substrates by elastase 2 and several other neutral proteases is presented in Table IV. These data show that elastase 2 has a high K_m for its best substrate compared to the values for the best substrates reported for the other enzymes. The relatively high K_m values obtained for human pancreatic elastase 2 with the substrates studied to date may also reflect a less efficient binding of the P_1 amino acid. As seen in Table IV, the highest values of the parameter k_{cat}/K_m for elastase 2 are significantly lower than those obtained for porcine elastase 1, leukocyte elastase, and bovine chymotrypsin. These observations suggest that the inherent catalytic efficiency of human pancreatic elastase 2 is much less than that of these enzymes. This finding is similar to that recently reported for human cathepsin G, a serine endopeptidase which appears to possess a peptide bond specificity similar to that of human pancreatic elastase 2 but does not hydrolyze any synthetic substrates at a very high rate (Nakajima et al., 1979).

Three substrates, Suc-Ala-Ala-Pro-Gly, Val, and Ile *p*-nitroanilides, are not substantially cleaved by human pancreatic elastase 2, primarily due to their very low k_{cat} values. The low k_{cat} for Suc-Ala-Ala-Pro-Gly-pNA indicates that the anchoring of the P_1 amino acid side chain is important for the catalytic efficiency of human pancreatic elastase 2, even though the binding of P_1 residues to the S_1 site seems to be somewhat nonspecific. Porcine pancreatic elastase 1 and bovine chymotrypsin, as well as human leukocyte elastase, also fail to cleave glycine substrates to any significant degree (Kasafirek et al., 1976; Zimmerman & Ashe, 1977). Since the valine and isoleucine substrates for human elastase 2 have K_m values comparable to those obtained for much better substrates, the very low k_{cat} values for these compounds probably reflect steric interference between the α-methyl group of these substrates and the enzyme which results in a misalignment of the acyl–aniline bond with respect to the active site in the enzyme–substrate complex. This is in contrast to human leukocyte elastase, which prefers valine or isoleucine substrates (Zimmerman & Ashe, 1977), and to porcine elastase 1, which hydrolyzes valine substrates quite well, although more slowly than those with alanine at P_1 (Zimmerman & Ashe, 1977).

It has been reported that the presence of a proline in the P_2 position precludes the binding of peptides in nonproductive modes and therefore affects the apparent rate of hydrolysis of peptide substrates by porcine elastase 1 (Thompson & Blout, 1973a) and chymotrypsin (Segal, 1972). As can be seen from Table II, the replacement of the P_2 alanine with proline increases both the k_{cat} and the K_m for the hydrolysis of the P_1 leucine tripeptides by human elastase 2 and porcine elastase 1. This result is consistent with the observation of Thompson & Blout (1973b) that the main effect of proline in the P_2 position is to restrict nonproductive binding. However, for the P_1 alanine tripeptides, the effect of replacing a P_2 alanine with proline cannot be explained by a decrease in nonproductive binding. The data would suggest that both the human and porcine enzymes inherently prefer proline to alanine in the P_2 position (at least for this particular set of substrates). With human elastase 2 the increased binding energy is reflected in

Table IV: Comparison of Kinetic Constants for Hydrolysis of Peptide *p*-Nitroanilides by Elastase 2 and Other Neutral Proteases

enzyme	substrate	K_m (mM)	k_{cat} (s^{-1})	K_m/k_{cat} (M^{-1} s^{-1})
human elastase 2	Suc-Ala-Ala-Pro-Met-pNA	1.3	6.4	4 900
human leukocyte cathepsin G[a]	MeO-Suc-Ala-Ala-Pro-Met-pNA	0.31	0.52	1 700
human leukocyte elastase[a]	MeO-Suc-Ala-Ala-Pro-Val-pNA	0.14	17	120 000
porcine elastase 2	Suc-Ala-Ala-Pro-Ala-pNA	0.19	54	290 000
bovine chymotrypsin[b]	Suc-Ala-Ala-Pro-Phe-pNA	0.043	45	1 000 000

[a] Data from Nakajima et al. (1979). [b] Data from Del Mar et al. (1979b).

the lower value of K_m, but for porcine elastase 1 this increased binding energy might only be realized in the transition state and therefore appears as a higher k_{cat}.

The k_{cat}/K_m ratio is also increased by lengthening the peptide from three to four amino acids. The increase is approximately threefold for the substrates with P_1 leucine and for human elastase 2 with the P_1 alanine substrates. There is a larger increase, sevenfold, for porcine elastase 1 with the P_1 alanine substrates; however, Kasafirek et al. (1976) reported only a 29% increase for porcine elastase 1 with the compounds Suc-$(Ala)_3$-pNA vs. Suc-$(Ala)_4$-pNA. Therefore, no simple conclusion can be drawn concerning the differential effect of peptide chain length on the hydrolysis of succinyl peptides by human pancreatic elastase 2 or porcine elastase 1, since the magnitude of that effect is dependent on other variables, such as the nature of the P_1 and P_2 substituents.

The decrease in the k_{cat}/K_m value for the pentapeptide Suc-$(Ala)_3$-Pro-Leu-pNA as compared to the corresponding tetrapeptide observed for both the human and porcine elastases is the result of a decrease in k_{cat} along with a smaller decrease in K_m. This result suggests the existence of contributions to the k_{cat} and K_m terms by nonproductive binding of the pentapeptide. In this regard, Thompson & Blout (1973a) have reported that there is probably not a unique binding mode between porcine elastase 1 and substrates longer than acetyl tetrapeptides.

As shown in Table III, substrates containing a negatively charged group in the P_5 position (Suc-Ala-Ala-Pro-Leu-pNA and Glt-Ala-Ala-Pro-Leu-pNA) have lower K_m values than compounds with a neutral substituent in this position (MeO-Suc-Ala-Ala-Pro-Leu-pNA) or a substrate with a positively charged group in the P_5 position (Ala-Ala-Ala-Pro-Leu-pNA). These results probably reflect the presence of a positive charge in the S_5 subsite in human pancreatic elastase 2. Such a site has previously been postulated for porcine elastase 1 (Kasafirek et al., 1976). However, conclusive data demonstrating the presence or absence of a S_5 binding site for a negative charge in leukocyte elastase or bovine chymotrypsin have not been presented.

The observation that a substrate with methionine in the P_1 position was among the best substrates for elastase 2 was intriguing since Johnson & Travis (1979) have recently shown that a P_1 methionine bond is cleaved during reaction of enzymes with α_1-protease inhibitor. These findings suggested the examination of the effect of oxidation of methionine-containing substrates on the kinetic parameters for hydrolysis by elastase 2 (Del Mar et al., 1979a). As shown in Table I, when Suc-Ala-Ala-Pro-Met-pNA is compared to the corresponding sulfoxide derivative, there is a large decrease in k_{cat}. This effect probably results from the inability of the hydrophobic binding pocket of human elastase 2 to make efficient contacts with the polar sulfoxide moiety. Both the k_{cat} and the K_m terms are more unfavorable for the oxidized peptide, but not to such a large extent as has been observed with a similar peptide for human leukocyte elastase or bovine chymotrypsin (Nakajima et al., 1979).

The accepted definition of elastase as a serine endopeptidase that preferentially hydrolyzes at alanine residues was based on earlier work on synthetic substrates for porcine elastase 1 (Geneste & Bender, 1969), which was confirmed by X-ray crystallographic data (Shotton & Watson, 1970), suggesting that the hydrophobic binding pocket, characteristic for chymotrypsin, was partially blocked in porcine elastase 1. The definition of the substrate specificity of human pancreatic elastase 2, together with the previously reported data on leukocyte elastase (Zimmerman & Ashe, 1977), as well as data on porcine elastase 1 (Kasafirek et al., 1976), means that three serine endopeptidases with elastolytic activity have now been well characterized in terms of peptide bond specificity. In addition, data on the peptide bonds cleaved by porcine pancreatic elastase 2 during elastolysis have been reported (Gertler et al., 1977). When the peptide bond specificity for these four enzymes is compared, it is apparent that the definition of an elastase as an endopeptidase with specificity for alanine is too restrictive. In fact, only porcine elastase 1 demonstrates a specificity for alanine (Kasafirek et al., 1976). Human leukocyte elastase preferentially cleaves at valine residues (Zimmerman & Ashe, 1977), while porcine elastase 2 hydrolyzes elastin at leucine, tyrosine, and phenylalanine bonds. The results of the present study suggest that human pancreatic elastase 2 is most similar to porcine elastase 2 in terms of peptide bond specificity. Both enzymes completely solubilize elastin,[4] although at a slower rate than that observed for porcine elastase 1 (Gertler et al., 1977; Largman et al., 1976). This difference in rates may be due to the higher content of alanine vs. leucine, phenylalanine, and tyrosine in elastin (Sandburg et al., 1977).

The finding that both human elastase 2 and porcine elastase 2 have "chymotrypsin-like" specificities poses the question of what property of an enzyme confers elastolytic capability. Gertler (1971) has suggested that the cationic nature of elastases leads to a strong ionic interaction with elastin, facilitating peptide bond cleavage. In this regard, an anionic neutral protease with alanine specificity has been isolated from human pancreatic tissue which has little (Largman et al., 1976) or no (Mallory & Travis, 1975) elastolytic activity. However, on the basis of elution from cation-exchange resins, the chymotrypsins are at least as cationic as the elastases[5] (Feinstein et al., 1974), yet human and bovine chymotrypsins have little or no demonstrable elastolytic activity. It is possible that the ability of a serine protease to solubilize elastin may depend on the specific distribution of charged groups on the surface of the enzyme molecule which might facilitate the productive binding of the protease to elastin, as well as on the primary peptide bond specificity.

The present results lend support to the suggestion of Gertler et al. (1977) that the classification of elastases as a separate family of serine endopeptidases, which is based on a defined peptide bond specificity (preferentially hydrolyzing at Ala residues) and the ability to hydrolyze elastin, is no longer adequate. However, we feel that a continued grouping of elastases based on elastolytic activity is useful because of physiological implications.

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[4] Complete solubilization of both bovine ligamentum nuchae elastin and human lung elastin has been demonstrated in this laboratory (C. Largman and W. M. Sischo, unpublished results).

[5] Canine pancreatic chymotrypsinogen and rat pancreatic chymotrypsinogen.

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Direct Observation of Substrate Distortion by Triosephosphate Isomerase Using Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The infrared spectrum of dihydroxyacetone phosphate bound to triosephosphate isomerase has been measured. There are two carbonyl bands corresponding to the bound substrate, with an intensity ratio of about 3:1. Relative to the carbonyl absorption of dihydroxyacetone phosphate in free solution, the major band is shifted by 19 cm^{-1} to 1713 cm^{-1}, providing direct evidence of enzyme-induced distortion of the substrate. This strain is probably attributable to an enzymic electrophile that polarizes the carbonyl group of the substrate and thereby promotes catalysis.

One of the devices that have been proposed to account for the catalytic effectiveness of enzymes is ground-state destabilization. The theoretical basis for the catalytic advantage thus achieved is well established. As formulated by Jencks (1975), the geometric and electronic distortion of the enzyme and its substrate in the enzyme–substrate complex toward the transition-state structure reduces the activation free energy required for reaction and thereby results in a rate acceleration.

Although the logic of this concept has held a good deal of appeal for enzymologists, it has been a considerable challenge to establish conclusively the involvement of ground-state destabilization in real enzyme systems. Various methods have been utilized toward this end, including X-ray crystallography (Phillips, 1967; Smith et al., 1978), ultraviolet and electron spin resonance spectroscopy (Vallee & Williams, 1968), binding isotope effects (Thomson et al., 1964; Bush et al., 1971; de Juan & Taylor, 1976), NMR spectroscopy (Sykes et al., 1971), extended X-ray absorption fine structure (Eisenberger et al., 1978; Tullius et al., 1978), and comparative studies of substrate and inhibitor dissociation constants (Thompson, 1974).

Vibrational spectroscopy has seen rather limited use in establishing ground-state strain. Although this method can, in principle, provide a very direct measure of bond strength on a time scale (10^{-14} s) that essentially freezes molecular motion, the lack of sensitivity has until recently limited its usefulness in biochemistry, with the notable exception of resonance Raman studies of chromophoric protein ligands (Carey, 1978). The only example of the use of vibrational spectroscopy in an attempt to detect strain in a nonchromophoric enzyme–substrate complex was by Riepe & Wang (1968), who used infrared spectroscopy to conclude that carbon dioxide bound at the active site of carbonic anhydrase is *not* under appreciable strain [see also the study of carboxyhemoglobin (Alben & Caughey, 1968)]. However, doubts have since been raised as to whether the carbon dioxide whose

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