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Reactivity of Human Leukocyte Elastase and Porcine Pancreatic Elastase toward Peptide 4-Nitroanilides Containing Model Desmosine Residues. Evidence That Human Leukocyte Elastase Is Selective for Cross-Linked Regions of Elastin†

Akira Yasutake and James C. Powers*

ABSTRACT: Elastin contains a number of cross-linking amino acid residues such as desmosine and isodesmosine which are primarily hydrophobic in character, but have a positively charged pyridinium ring. These cross-linking residues are formed by the action of lysyl oxidase upon Lys residues in tropoelastin, a precursor of elastin. A series of tetrapeptide 4-nitroanilides which contain Lys and a series of modified lysine residues were synthesized. The modified lysine residues [ϵ-carbobenzyloxy (Z), ϵ-benzoyl (Bz), ϵ-benzimidoyl (Bim), and ϵ-2-picolinoyl (Pic)] have various characteristics of desmosine and isodesmosine residues, such as a positive charge, a hydrophobic aromatic ring, or a pyridine ring. The reactivity of the tetrapeptide 4-nitroanilides containing the model desmosine residues at P_4, P_3, or P_2 with human leukocyte (HL) and porcine pancreatic (PP) elastase was measured at pH 7.5 and 25 °C. HL elastase exhibited high reactivity toward the substrates with P_4 or P_3 hydrophobic groups (Z, Bz, or Pic), and MeO-Suc-Lys(Pic)-Ala-Pro-Val-NA is 7 times more reactive than the previous best HL elastase substrate, MeO-Suc-Ala-Ala-Pro-Val-NA. The major change occurred in K_M values. The substrates containing Lys residues were either nonreactive or poor. Except for two substrates with P_2 hydrophobic residues (Bz and Pic), PP elastase was less reactive toward the substrates containing model desmosine residues than toward MeO-Suc-Ala-Ala-Pro-Val-NA. The data support the hypothesis that HL elastase cleaves elastin selectively near cross-linking residues. The results also indicate that HL elastase binds tightly to these regions and would be poorly effective toward regions of elastin or tropoelastin which contain Lys residues.

Elastin is a flexible, highly cross-linked protein found in high quantities in mammalian lung and arteries. The chemical structure of elastin is not yet known, but the fragmentary data available indicate that elastin has long sequences of small aliphatic amino acid residues such as Gly, Ala, Val, and Pro (Gray et al., 1973; Sandberg et al., 1977). Mature cross-linked elastin is formed by the action of lysyl oxidase on the soluble precursor protein tropoelastin. Lysyl residues of tropoelastin are oxidized to α-aminoadipic δ-semialdehyde residues which then condense with each other and with Lys residues to form

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received December 23, 1980.* This research was supported by a grant from the Council for Tobacco Research.

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aldol condensation products and cross-linking residues such as desmosine, isodesmosine, merodesmosine, and dehydrolysinonorleucine (Thomas et al., 1963; Lent et al., 1969; Franzblau et al., 1965; Starcher et al., 1967). Mature elastin is thus rich in these cross-linking residues, but poor in lysine (Anwar, 1966; Francis et al., 1973).

Destruction of elastin structure occurs in the pathogenesis of pulmonary emphysema. The elastase which is found in granules of human polymorphonuclear leukocytes is commonly believed to be the primary agent responsible for this destruction. Studies with both natural and synthetic substrates have shown that this elastase cleaves preferentially peptide bonds following small amino acid residues such as Val and Ala (Blow, 1977; Zimmerman & Ashe, 1977; Reilly & Travis, 1980). In addition, the enzyme has an extended substrate binding site and recognizes a number of amino acid residues on both sides of the scissile bond. This recognition extends at least to the P_5 residue in the N-terminal direction and to P_3' in the C-terminal direction (McRae et al., 1980).[1] At present, almost nothing is known about the sites of cleavages of native elastin either in vitro or in vivo by human leukocyte elastase. However, there does seem to be a slight preference for cleaving after Val rather than Ala residues (Reilly & Travis, 1980).

In this paper, we report kinetic studies with human leukocyte (HL)[2] elastase and porcine pancreatic (PP) elastase and peptide 4-nitroanilide substrates with lysine residues and residues which contain features of elastin's cross-linking residues. The results support the hypothesis that HL elastase cleaves elastin selectively near cross-linking residues.

Materials and Methods

Human leukocyte elastase was supplied to us by Dr. James Travis of the University of Georgia. Porcine pancreatic elastase was purchased from Worthington. *N,N'*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxybenzotriazole (HOBt), methyl succinate, benzoyl chloride, picolinic acid, and 1,1'-carbonyldiimidazole were from Aldrich Chemical Co. Sephadex LH-20 was from Pharmacia Fine Chemicals. Methyl benzimidate hydrochloride was prepared according to Hunter & Ludwig (1962). The syntheses of all new compounds are described (see paragraph at end of paper regarding supplementary material).

4-Nitroanilide Kinetics. The rates of hydrolysis of the 4-nitroanilides were measured by adding 10 μL of the appropriate enzyme solution to 0.4 mL of a substrate solution in 0.1 M Hepes buffer at pH 7.50 containing 0.5 M NaCl and 10% dimethyl sulfoxide at 25 °C. The increase in the absorbance at 410 nm was followed with a Beckman Model 25 spectrophotometer. An ϵ of 8800 at 410 nm was used (Erlanger et al., 1961). The kinetic constants were determined from the initial rates of hydrolysis by the Lineweaver–Burk method and are based on duplicate rate determinations at five separate substrate concentrations.

The concentrations of human leukocyte elastase and procine pancreatic elastase were determined with MeO-Suc-Ala-Ala-Pro-Val-NA by using the kinetic constants which were based on titrated enzyme (Nakajima et al., 1979).

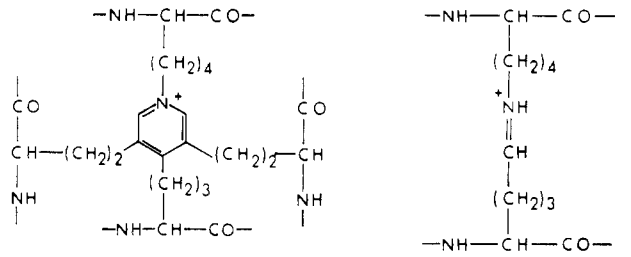

FIGURE 1: Structure of two common elastin cross-linking residues, desmosine and dehydrolysinonorleucine. Isodesmosine is an isomer of desmosine where the substituents are attached to the 1, 2, 3, and 5 positions of the pyridinium ring.

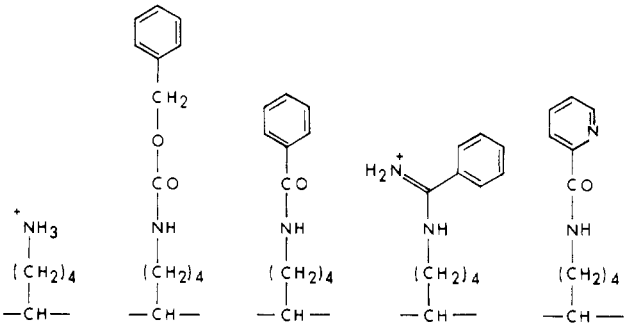

FIGURE 2: Structure of the models for elastin cross-linking residues. From left to right are shown a lysine, carbobenzyloxylysine [-Lys(Z)-], benzoyllysine [-Lys(Bz)-], benzimidate of lysine [-Lys(Bim)-], and a 2-picolinoyl derivative of lysine residue [-Lys(Pic)-].

Results and Discussion

When we began this study, we initially considered methods of incorporating desmosine residues into simple peptide structures. Upon reflection, it became apparent that the preparation of desmosine-containing peptides would present a considerable synthetic challenge and would be quite expensive. Previous studies with cross-linked elastin model peptides have used alternate methods of cross-linking, namely a peptide bond between the γ-carboxyl group of a glutamic acid of one chain and the ϵ-amino group of a lysine of the second chain (Urry et al., 1976). This model cross-link would have considerable resemblance to the dehydrolysinonorleucine residues of elastin, but less to the aromatic desmosine and isodesmosine residues.

Both desmosine and isodesmosine are bulky hydrophobic amino acid residues with an aromatic pyridinium ring and multiple methylene groups (Figure 1). Thus, we felt it likely that any binding site on elastase for such a residue would be hydrophobic in character. In addition, there might be recognition of the positive charge in the pyridinium ring. We therefore decided to investigate substrates containing the modified lysine side chains illustrated in Figure 2.

These particular model desmosine residues were chosen since their preparation would present no major practical synthetic problems. The carbobenzyloxy (Z) and benzoyl (Bz) derivatives of lysine are both large hydrophobic groups. The benzimidate (Bim) has both a hydrophobic aromatic ring and a positive charge. The 2-picolinyl derivative (Pic) has a pyridine ring which at pH 7.5 is uncharged since the p*K*s of simple pyridinecarboxamides occur in the range 3–4. Lysine itself was investigated to learn the effect of this residue on the susceptibility of molecules such as tropoelastin or regions of elastin containing un-cross-linked lysine residues.

In previous studies, we have studied the substrate specificity of HL elastase and have shown that substrates and inhibitors with the MeO-Suc-Ala-Ala-Pro-Val- sequence are highly reactive toward the enzyme (Nakajima et al., 1979; Powers

[1] The nomenclature used for the individual amino acid residues (P_1, P_2, etc.) of a substrate and the subsites (S_1, S_2, etc.) of the enzyme is that of Schechter & Berger (1967).

[2] Abbreviations used: NA, 4-nitroanilide; HL, human leukocyte; PP, porcine pancreatic; Z, carbobenzyloxy; Bz, benzoyl; Bim, benzimidoyl; Pic, 2-picolinoyl; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid.

Table I: Kinetic Constants for the Hydrolysis of Synthetic 4-Nitroanilide Substrates by Human Leukocyte Elastase[a]

substrate (P_4 P_3 P_2 P_1)	substrate concn range (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
MeO-Suc-Ala-Ala-Pro-Val-NA[b]	0.05–4.9	0.14	17	120 000
MeO-Suc-Lys-Ala-Pro-Val-NA	0.55–11	1.4	24	17 000
MeO-Suc-Ala-Lys-Pro-Val-NA	0.6–12	0.54	17	32 000
MeO-Suc-Ala-Ala-Lys-Val-NA	13			NR[d]
Z | MeO-Suc-Lys-Ala-Pro-Val-NA	0.007–0.14	0.026	18	710 000
Z | MeO-Suc-Ala-Lys-Pro-Val-NA	0.008–0.16	0.045	12	260 000
Z | MeO-Suc-Ala-Ala-Lys-Val-NA[c]	0.16			NR
Bz | MeO-Suc-Lys-Ala-Pro-Val-NA	0.017–0.17	0.045	20	440 000
Bz | MeO-Suc-Ala-Lys-Pro-Val-NA	0.03–0.3	0.090	16	180 000
Bz | MeO-Suc-Ala-Ala-Lys-Val-NA	0.0095–0.048	0.015	3.0	200 000
Bim | MeO-Suc-Lys-Ala-Pro-Val-NA	0.05–5.0	0.29	18	63 000
Bim | MeO-Suc-Ala-Lys-Pro-Val-NA	0.05–5.0	0.11	14	130 000
Bim | MeO-Suc-Ala-Ala-Lys-Val-NA	0.05–5.0	0.63	11	17 000
Pic | MeO-Suc-Lys-Ala-Pro-Val-NA	0.05–4.9	0.024	19	790 000
Pic | MeO-Suc-Ala-Lys-Pro-Val-NA	0.25–7.8	0.077	12	160 000
Pic | MeO-Suc-Ala-Ala-Lys-Val-NA	0.025–0.25	0.072	9.9	140 000

[a] Conditions were pH 7.50, 0.1 M Hepes buffer, 0.5 M NaCl, and 9.8% dimethyl sulfoxide at 25 °C. [b] Data of Nakajima et al. (1979). [c] The reaction was carried out in a 0.1 M Hepes buffer, pH 7.50, containing 0.5 M NaCl and 19.5% dimethyl sulfoxide at 25 °C due to the low solubility of the substrate. [d] NR, no reaction.

et al., 1977). In fact, MeO-Suc-Ala-Ala-Pro-Val-NA is the most reactive HL elastase 4-nitroanilide substrate yet reported. Therefore, we decided to systematically replace the P_2, P_3, and P_4 residues of MeO-Suc-Ala-Ala-Pro-Val-NA with the elastin cross-link model residues illustrated in Figure 2.

Human Leukocyte Elastase. The kinetic constants for the hydrolysis of 4-nitroanilide substrates by HL elastase are summarized in Table I. Our previous best substrate MeO-Suc-Ala-Ala-Pro-Val-NA is listed in the table first. There is a considerable range in the reactivity of the various peptide 4-nitroanilide substrates. Two did not react at all with HL elastase, while the most effective substrate, MeO-Suc-Lys-(Pic)-Ala-Pro-Val-NA, had a 7-fold higher k_{cat}/K_M value when compared with MeO-Suc-Ala-Ala-Pro-Val-NA.

The major changes in the kinetic constants occurred in the K_M values; the k_{cat} values for all of the substrates except one were 17 ± 7 s^{-1}. The K_M values varied over 2 orders of magnitude. In general, the substrates with a positive charge (Lys) or with a positive charge and a hydrophobic ring (Bim) bound very poorly to HL elastase, while those with hydrophobic groups (Z, Bz, and Pic) bound much more tightly.

The S_4 and S_3 subsites of HL elastase preferred bulky hydrophobic residues (Z, Bz, or Pic) over an Ala residue. All of the derivatives were more reactive than MeO-Suc-Ala-Ala-Pro-Val-NA, and some had 4–7 times higher k_{cat}/K_M values. Even the P_4 and P_3 lysyl benzimidate derivatives (Bim) were as reactive or almost as reactive as the Ala derivative. However, the S_4 and S_3 subsites of HL elastase do not like a simple lysine residue since those substrates with a P_4 or P_3 lysine were respectively 7 and 4 times less reactive.

The S_2 subsite of HL elastase was the most restrictive and would not accept either a P_2 Lys or a Lys(Z) residue. The P_2 Bim derivative was poorly reactive, while the benzoyl and picolinoyl derivatives were slightly more reactive than MeO-Suc-Ala-Ala-Pro-Val-NA. Thus, the S_2 subsite does not like a positive charge but can accommodate the less bulky aromatic derivatives (Bz, Bim, and Pic).

The three benzimidate derivatives were also tested with human cathepsin G, the other major leukocyte serine protease. This enzyme which prefers substrates with P_1 aromatic residues (Nakajima et al., 1979) did not cleave MeO-Suc-Ala-Ala-Lys(Bim)-Val-NA or MeO-Suc-Lys(Bim)-Ala-Pro-Val-NA. The P_3 isomer MeO-Suc-Ala-Lys(Bim)-Pro-Val-NA was cleaved extremely slowly (k_{cat} = 0.054 s^{-1}, K_M = 13 mM, k_{cat}/K_M = 4.2 M^{-1} s^{-1}). This indicates that these three substrates and probably most of the other good HL elastase substrates are highly specific. The high specificity and reactivity of the HL elastase substrates should make them useful tools for future studies of this enzyme.

Porcine Pancreatic Elastase. The kinetic constants for the hydrolysis of 4-nitroanilide substrates by porcine pancreatic elastase are reported in Table II. The MeO-Suc-Ala-Ala-Pro-Val- sequence is not an ideal one for PP elastase since this enzyme prefers P_1 Ala residues over Val residues (McRae et al., 1980; Zimmerman & Ashe, 1977), and the rates observed with this enzyme are thus much lower than those found with HL elastase. With PP elastase, K_M values varied by a factor of over 400, k_{cat} values by over 50, and k_{cat}/K_M by 60.

Table II: Kinetic Constants for the Hydrolysis of Synthetic 4-Nitroanilide Substrates by Porcine Pancreatic Elastase[a]

substrate (P_4 P_3 P_2 P_1)	substrate concn range	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
MeO-Suc-Ala-Ala-Pro-Val-NA[b]	0.25–7.8	6.2	17	2 700
MeO-Suc-Lys-Ala-Pro-Val-NA	11			NR[d]
MeO-Suc-Ala-Lys-Pro-Val-NA	0.6–12	3.0	5.4	1 800
MeO-Suc-Ala-Ala-Lys-Val-NA	13			NR
Z MeO-Suc-Lys-Ala-Pro-Val-NA	0.14			NR
Z MeO-Suc-Ala-Lys-Pro-Val-NA	0.008–0.16	920	18	19
Z MeO-Suc-Ala-Ala-Lys-Val-NA[c]	0.02–0.16	0.21	0.21	1 000
Bz MeO-Suc-Lys-Ala-Pro-Val-NA	0.34			NR
Bz MeO-Suc-Ala-Lys-Pro-Val-NA	0.074–0.59	0.76	9.0	12 000
Bz MeO-Suc-Ala-Ala-Lys-Val-NA	0.048–0.19	0.31	0.68	2 200
Bim MeO-Suc-Lys-Ala-Pro-Val-NA	2.5–10	1.6	0.33	210
Bim MeO-Suc-Ala-Lys-Pro-Val-NA	0.5–10	6.6	14	2 100
Bim MeO-Suc-Ala-Ala-Lys-Val-NA	0.5–50	3.3	6.7	2 000
Pic MeO-Suc-Lys-Ala-Pro-Val-NA	0.49			NR
Pic MeO-Suc-Ala-Lys-Pro-Val-NA	0.21–4.2	1.1	7.7	7 000
Pic MeO-Suc-Ala-Ala-Lys-Val-NA	0.093–0.74	1.7	3.6	2 100

[a] Conditions were pH 7.50, 0.1 M Hepes buffer, 0.5 M NaCl, and 9.8% dimethyl sulfoxide at 25 °C. [b] Data of Nakajima et al. (1979). [c] The reaction was carried out in a 0.1 M Hepes buffer, pH 7.50, containing 0.5 M NaCl and 19.5% dimethyl sulfoxide at 25 °C due to the low solubility of the substrate. [d] NR, no reaction.

The S_4 subsite of PP elastase is highly restricted and will not accept the Lys, Z, Bz, or Pic derivative, while the Bim derivative was very poorly hydrolyzed. In contrast, the S_3 subsite preferred the hydrophobic Pic and Bz derivatives. The P_3 charged Bim and Lys derivatives were almost as reactive as MeO-Suc-Ala-Ala-Pro-Val-NA, while the carbobenzyloxy derivative was very poor. The S_2 subsite of PP elastase would not accept a Lys residue. The other P_2 derivatives (Z, Bz, Bim, and Pic) were just slightly less reactive than MeO-Suc-Ala-Ala-Pro-Val-NA.

Elastin Degradation. We believe the data presented in this paper support the hypothesis that HL elastase is a cross-link selective enzyme. In particular, HL elastase probably prefers to cleave sequences such as cross-linking amino acid residue-$(AA)_n$-P_1*P_1' (asterisk indicates bond cleaved) where P_1 is Val or Ala and n is 1 or 2 but not 0. In addition, the enzyme would not like lysine-rich regions of tropoelastin or regions of elastin containing un-cross-linked lysine residues. HL elastase must also bind very tightly to cross-link regions of elastin since the introduction of a hydrophobic lysine derivative into a substrate resulted in a significant lowering of the K_M values. It has recently been shown that HL elastase preadsorbed onto elastin is incompletely inhibited by α_1-protease inhibitor (Reilly & Travis, 1980; H. P. Schnebli, private communication). This observation is also consistent with the tight binding of HL elastase to elastin.

Porcine pancreatic elastase in contrast does not exhibit any clear preference for the cross-link model residues. In a few cases (P_2-substituted substrates), high reactivity is observed, but with the majority of substrates, either little change or low reactivity is observed. This indicates to us that PP elastase would just as soon cleave in non-cross-linked regions of elastin.

The conclusions drawn in this paper concerning elastin must be tempered by our lack of knowledge of either the complete amino acid sequence of elastin or its three-dimensional structure. A few peptides containing desmosine, isodesmosine, or dehydrolysinonorleucine cross-links have been isolated from elastin digests and sequenced (Foster et al., 1973, 1974, 1976). In general, the arms of the cross-linking desmosine or isodesmosine residues are linked by two or three alanine residues. However, the amino acid sequences C-terminal to desmosine residues exhibit much more variety (Gerber & Anwar, 1974; Anwar, 1977). These sequences contain some aromatic residues but are still primarily composed of Ala and Gly residues with a few valines. These few desmosine peptides which have been characterized may, of course, not be representative of the complete elastin molecule. However, in the tropoelastin sequences which have been determined, there are usually similar residues (Gly, Ala, and Pro) following the lysine residues which must form the elastin cross-links (Gray et al., 1973; Sandberg et al., 1977). In a few cases there is a valine residue ideally located for cleavage, and in the majority of the sequences there are properly located alanine residues. Even though HL elastase hydrolyzes P_1 Val synthetic substrates 8–14 times faster than those with P_1 Ala, it is quite an effective protease with both residues (McRae et al., 1980; Zimmerman & Ashe, 1977). Thus the available sequence data are consistent with our hypothesis.

The reactivity of peptide chains near cross-links will also depend on the accessibility of the cross-linking amino acid residues and the nature of the residue itself. An elastase approaching an accessible or partially accessible cross-linking residue might see a hydrophobic structure or primarily a charged structure, depending on which of the four peptide chains linked to the desmosine or isodesmosine residue the enzyme bound to. And as we have seen, a change from a charged to a hydrophobic residue can significantly alter the reactivity of HL elastase.

In conclusion, the results of this study have probably posed as many questions as have been answered. But we believe that the hypothesis that HL elastase is a cross-link selective enzyme is worthy of testing with native elastin.

Acknowledgments

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Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (8 pages). Ordering information is given on any current masthead page.

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