Catalysis by Human Leukocyte Elastase: Mechanistic Insights into Specificity Requirements†

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ABSTRACT: Steady-state kinetic parameters were determined for the human leukocyte elastase catalyzed hydrolysis of a series of peptide-based thiobenzyl esters and *p*-nitroanilides. The peptide units are MeO-Suc-Val, MeOSuc-Ala$_n$-Pro-Val (n = 0–2), and MeOSuc-Ala$_n$-Pro-Ala (n = 1 or 2). The results of this study suggest five important mechanistic features for HLE. (1) Few important remote subsite contacts are established in the Michaelis complex. (2) Full recognition and tight binding of the substrate occurs in the transition state for acylation. (3) The P_3–S_3 interaction is critical during acylation. (4) Subsite contacts are unimportant in deacylation. (5) P_1 specificity is regulated by peptide length. An important steady-state kinetic consequence of this specificity is that the rate-limiting step of k_c for *p*-nitroanilide hydrolysis changes from acylation to deacylation as the peptide chain is lengthened.

Explorations of the substrate specificity[1] of human leukocyte elastase have been conducted by several investigators (Harper et al., 1984; Lesteine & Bieth, 1980; McRae et al., 1980; Stein, 1985a; Wenzel & Tschesche, 1981; Zimmerman & Ashe, 1977) and suggest three important generalities: (1) Although a preference for small, hydrophobic groups at P_1 is clearly evident, the P_1 specificity for HLE[2] is dependent on substrate length, becoming broader with decreasing chain length. For tetrapeptide-based substrates, HLE exhibits an almost absolute requirement for Val at P_1, while for reactive monomeric substrates its specificity becomes broad enough to include even Z-Phe-ONP and Z-Gly-ONP. (2) P_1 specificity is dependent on both the leaving group and the steady-state kinetic parameter being correlated. For specificity correlations using k_c, Val is preferred for the hydrolyses of amides and tripeptide *p*-nitroanilides, while Ala is preferred for esters, thiobenzyl esters, and tetrapeptide *p*-nitroanilides. On the other hand, if k_c/K_m is used as the measure of specificity, Val is preferred regardless of the leaving group. (3) Both k_c and k_c/K_m increase with increasing peptide chain length of *p*-nitroanilide substrates.

An important aspect of HLE's substrate specificity not yet addressed is how it is manifested in individual reaction steps. Our current view of the kinetic mechanism of HLE appears in Scheme I (Stein, 1985b) and involves the three steps of substrate binding, acylation, and deacylation. Acylation is thought to proceed through an initial physical step, possibly a conformational change of the E:S complex, followed by the actual chemical steps of serine acylation (Stein, 1985b). We wish to know how the rate of each reaction step of the mechanism in Scheme I responds to systemic variation of substrate structure. A previous study (Stein, 1985b), based on a small number of substrates, suggested that binding and deacylation are generally insensitive to substrate structure, while the chemical step of acylation (k_b) is keenly sensitive to substrate chain length.

Scheme I

binding

$$\text{E–OH} + \text{R–C(=O)–X} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E–OH:R–C(=O)–X}$$

acylation

$$\text{E–OH:R–C(=O)–X} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} [\text{E–OH:R–C(=O)–X}]' \xrightarrow{k_b} \text{E–O–C(=O)–R} + \text{X}$$

deacylation

$$\text{E–O–C(=O)–R} \xrightarrow{k_3} \text{E–OH} + \text{R–C(=O)–OH}$$

$$k_c = k_2k_3/(k_2 + k_3) \quad (1)$$

$$K_m = K_sk_3/(k_2 + k_3) \quad (2)$$

$$k_c/K_m = k_1k_2/(k_{-1} + k_2) \quad (3)$$

$$K_s = (k_{-1} + k_2)/k_1 \quad (4)$$

$$k_2 = k_ak_b/(k_{-a} + k_b) \quad (5)$$

To explore this aspect of HLE's specificity in greater depth, we have determined the steady-state kinetic parameters, K_m

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[1] In this paper, the term "substrate specificity" refers to the correlation between substrate structure and an enzyme kinetic parameter. Although it has been demonstrated by Fersht (1974) that the most appropriate measure of specificity is the kinetic parameters k_c/K_m, it is convenient for the purposes of discussion to extend the use of this phrase to the individual steady-state constants, K_m and k_c, as well as to the mechanistic constants themselves, K_s, k_2, and k_3 (see Scheme I).

[2] Abbreviations: MeOSuc, methoxysuccinyl; pNA, *p*-nitroanilide; SBzl, thiobenzyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ONP, *p*-nitrophenyl ester; Abu, α-aminobutyric acid; Nva, norvaline; TLC, thin-layer chromatography; CI-MS, chemical ionization mass spectrometry; HLE, human leukocyte elastase.

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and k_c (see eq 1–5), for the HLE-catalyzed hydrolyses of a series of corresponding *p*-nitroanilides and thiobenzyl esters. The identity of k_c with k_3 for thiobenzyl ester hydrolysis allowed us to calculate mechanistic kinetic parameters, K_s, k_2, and k_3, for the *p*-nitroanilides. These values indicate that HLE's substrate specificity is manifested entirely in acylation.

Materials and Methods

HLE was prepared as previously described (Stein, 1985b; Viscarello et al., 1983). Buffer salts and Me_2SO were of analytical grade from several sources.

Peptide Synthesis. All melting points are uncorrected. The purity of all compounds was checked by thin-layer chromatography on Merck silica gel plates by using the following solvent system: chloroform–methanol (9:1 v/v). NMR spectra were consistent with the assigned structures and were determined by using a Varian A-60 and a Bruker WM 300 instrument. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA. All amino acids are of the L configuration unless indicated. The chemical ionization mass spectra were obtained on a Varian MAT 112S. Isobutyl chloroformate, monomethyl succinate, and most other reagents were products of the Aldrich Chemical Co.; blocked amino acid derivatives were products of Bachem or Chemical Dynamics, Inc.

Mixed-Anhydride Procedure. (*A*) *MeO-Suc-Ala-Ala-Pro-Ala-SBzl.* This compound was prepared by the following mixed-anhydride procedure. MeO-Suc-Ala-Ala-Pro-OH (1.1 g, 2.78 mmol) was dissolved in methylene chloride (20 mL) and cooled to −15 °C. Triethylamine (0.42 mL, 2.78 mmol) and isobutyl chloroformate (0.43 mL, 2.78 mmol) were added to this solution. After stirring for 30 min at −15 °C, a cooled solution of H-Ala-SBzl·HCl (640 mg, 2.78 mmol) in methylene chloride was added. After being stirred for 2.5 h at −15 °C and overnight at room temperature, the reaction mixture was washed with 10% citric acid, 4% sodium bicarbonate, and water successively. The methylene chloride solution was dried over $MgSO_4$, filtered, and evaporated to yield a white solid (1.38 g, 91%), which was recrystallized from ethyl ether (900 mg): single spot on TLC, R_f 0.48; mp 162–163 °C. Anal. Calcd for $C_{26}H_{36}O_7N_4S$: C, 56.92; H, 6.61; N, 10.21. Found: C, 56.81, H, 6.65; N, 10.20.

(*B*) *MeO-Suc-Ala-Ala-Pro-Ala-pNA.* MeO-Suc-Ala-Ala-Pro-OH and H-Ala-pNA·HCl were coupled by the mixed-anhydride method, and the product was obtained in 62% yield: single spot on TLC, R_f 0.33; mp 224–226 °C. Anal. Calcd for $C_{25}H_{34}O_9N_6$: C, 53.37; H, 6.09; N, 14.94. Found: C, 53.44; H, 6.12; N, 14.93.

(*C*) *MeO-Suc-Ala-Pro-Ala-pNA.* MeO-Suc-Ala-Pro-OH and H-Ala-pNA·HCl were coupled by the mixed-anhydride method, and the product was obtained in 78% yield: single spot on TLC, R_f 0.63; mp 159–160 °C. Anal. Calcd for $C_{22}H_{29}O_8N_5$: C, 53.76; H, 5.95; N, 14.25. Found: C, 53.63; H, 5.57; N, 14.24.

(*D*) *MeO-Suc-Ala-Pro-Ala-SBzl.* MeO-Suc-Ala-Pro-OH and H-Ala-SBzl·HCl were coupled by the mixed-anhydride method, and the product was obtained in 89% yield: single spot on TLC, R_f 0.52; mp 128–129 °C. Anal. Calcd for $C_{23}H_{31}O_6N_3S$: C, 57.84; H, 6.54; N, 8.80. Found: C, 57.74; H, 6.54; N, 8.79.

(*E*) *MeO-Suc-Ala-Pro-Val-pNA.* MeO-Suc-Ala-Pro-OH and H-Val-pNA were coupled by the mixed-anhydride method, and the product was obtained in 76% yield: single spot on TLC, R_f 0.52; mp 105.5–107 °C. Anal. Calcd for $C_{24}H_{33}O_8N_5 \cdot {}^1/_2H_2O$: C, 54.43; H, 6.66; N, 13.23. Found: C, 54.58; H, 6.51; N, 13.21.

(*F*) *MeO-Suc-Ala-Pro-Val-SBzl.* MeO-Suc-Ala-Pro-OH and H-Val-SBzl·HCl were coupled by the mixed-anhydride method, and the product was obtained in 93% yield: single spot on TLC, R_f 0.52; mp 183–185 °C. Anal. Calcd for $C_{25}H_{35}O_6N_3S$: C, 59.38; H, 6.98; N, 8.31. Found: C, 59.44; H, 7.00; N, 8.29.

(*G*) *MeO-Suc-Pro-Val-pNA.* MeO-Suc-Pro-OH and H-Val-pNA were coupled by the mixed-anhydride method, and the product was obtained in 89% yield: single spot on TLC, R_f 0.50; mp 91–93 °C; CI-MS m/z 449 (M + 1). Anal. Calcd for $C_{21}H_{28}O_7N_4 \cdot {}^2/_3H_2O$: C, 54.77; H, 6.13; N, 12.17. Found: C, 54.65; H, 6.29; N, 11.94.

(*H*) *MeO-Suc-Pro-Val-SBzl.* MeO-Suc-Pro-OH and H-Val-SBzl·HCl were coupled by the mixed-anhydride method, and the product was obtained as an oil in 92% yield: single spot on TLC, R_f 0.54; CI-MS m/z 435 (M + 1). Anal. Calcd for $C_{22}H_{30}O_5N_2S$: C, 60.80; H, 6.96; N, 6.45. Found: C, 60.60; H, 6.99; N, 6.43.

(*I*) *MeO-Suc-Val-pNA.* MeO-Suc-OH and H-Val-pNA were coupled by the mixed-anhydride method, and the product was obtained in 67% yield: single spot on TLC, R_f 0.57; mp 160–161 °C; CI-MS m/z 352 (M + 1). Anal. Calcd for $C_{16}H_{21}O_6N_3$: C, 54.69; H, 6.03; N, 11.96. Found: C, 54.51; N, 6.03; N, 11.90.

(*J*) *MeO-Suc-Val-SBzl.* MeO-Suc-OH and H-Val-SBzl·HCl were coupled by the mixed-anhydride method, and the product was obtained as an oil in 65% yield: single spot on TLC, R_f 0.66; CI-MS m/z 338 (M + 1). Anal. Calcd for $C_{17}H_{23}O_4NS \cdot {}^1/_3H_2O$: C, 59.45; H, 6.75; N, 4.08. Found: C, 59.41; H, 6.93; N, 4.04.

(*K*) *MeO-Suc-Ala-Ala-Pro-Abu-pNA.* This compound was prepared by the mixed-anhydride method and isolated by a column chromatography on silica gel in CH_2Cl_2–MeOH (19:1), and the product was obtained in 28% yield: single spot on TLC, R_f 0.58; mp 209–210 °C. Anal. Calcd for $C_{26}H_{36}O_9N_6 \cdot {}^3/_2H_2O$: C, 51.74; H, 6.51; N, 13.92%. Found: C, 51.78; H, 6.25; N, 13.93.

Kinetic Procedures. Reaction progress for hydrolyses of *p*-nitroanilides was measured spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm. Thiobenzyl ester hydrolysis was measured in a coupled assay (Farmer & Hageman, 1975) that uses the reagent 5,5′-dithiobis(2-nitrobenzoic acid). By this method, benzylthiol, released during the course of thiobenzyl ester hydrolysis, reacts with DTNB to produce the colored 3-carboxy-4-(nitrothio)phenoxide (ϵ_{412} = 13 600).

In a typical experiment, either 100 μL of a *p*-nitroanilide solution in Me_2SO or 50 μL each of a thiobenzyl solution and a 10 mM DTNB solution in Me_2SO was added to a cuvette containing 2.88 mL of buffer (100 mM phosphate, 500 mM NaCl, pH 7.4). The cuvette was placed in a jacketed holder in the cell compartment of a Cary 210 spectrophotometer and allowed to reach thermal equilibrium (5–10 min). The temperature was maintained at 25 ± 0.1 °C by water circulated from a Lauda K-2/RD bath. Injection of 20 μL of enzyme solution initiated the reaction. Absorbances were continuously measured, digitized, and stored in a Digital Electronics Corp. PDP 11/23 minicomputer. The interface to the Cary used its digital interface port. Initial velocities were calculated by a fit of the experimental data to a linear dependence on time by linear least-squares analysis.

Data Analysis. Values of k_c, K_m, and k_c/K_m and their error estimates were derived from two to three separate kinetic experiments. Each experiment consisted of determining initial velocities, in duplicate or triplicate, at four to six substrate

Table I: Steady-State Kinetic Parameters for the HLE-Catalyzed Hydrolysis of Peptide-Derived Substrates[a]

	k_c (s^{-1})	K_m (μM)	k_c/K_m (M^{-1} s^{-1})
MeOSuc-Val-pNA	0.06	788	75
MeOSuc-Pro-Val-pNA	0.4	696	575
MeOSuc-Ala-Pro-Val-pNA	10	189	56 000
MeOSuc-Ala-Ala-Pro-Val-pNA	10	55	182 000
MeOSuc-Val-SBzl	10	15	680 000
MeOSuc-Pro-Val-SBzl	11	12	880 000
MeOSuc-Ala-Pro-Val-SBzl	13	2.3	5 600 000
MeOSuc-Ala-Ala-Pro-Val-SBzl	13	2.3	5 600 000
MeOSuc-Ala-Pro-Ala-pNA	7	1500	4 500
MeOSuc-Ala-Ala-Pro-Ala-pNA	22	833	26 900
MeOSuc-Ala-Pro-Ala-SBzl	46	12	3 840 000
MeOSuc-Ala-Ala-Pro-Ala-SBzl	53	13	4 100 000

[a] Conditions: 100 mM phosphate, 500 mM NaCl, pH 7.4, 3.3% Me_2SO, 25 ± 0.1 °C. For reactions of thiobenzyl esters, [DTNB] = 0.17 mM. Standard deviations for the kinetic parameters are less than 10% in all cases.

concentrations. For each kinetic experiment, k_c and K_m were calculated by nonlinear least-squares fit of the initial velocity data to the Michaelis–Menten equation. Double-reciprocal plots were linear in all cases.

Results

Steady-state kinetic parameters were determined for the HLE-catalyzed hydrolyses of a series of peptide *p*-nitroanilides and thiobenzyl esters. These values are summarized in Table I. The sensitivity of the magnitude of these parameters to peptide chain length is dependent on the leaving group and P_1 amino acid residue. For *p*-nitroanilides with Val at P_1, extending the monomeric substrate MeOSuc-Val-pNA to the tetrapeptide MeOSuc-Ala-Ala-Pro-Val-pNA is accompanied by a 200-fold increase in k_c, a 14-fold decrease in K_m, and a 2400-fold increase in k_c/K_m. Interestingly, the most dramatic incremental change in all three kinetic parameters is observed upon addition of the P_3 Ala residue.

The hydrolysis kinetics of *p*-nitroanilides with Ala at P_1 show a similar dependence on substrate chain length. Although this compound class is represented by only two members in Table I, the sensitivity of the kinetic parameters to substrate chain length is clear.

Thiobenzyl ester hydrolysis is much less sensitive to substrate chain length. For example, we observe only an 8-fold increase in k_c/K_m and essentially no increase in k_c as MeOSuc-Val-SBzl is extended to the tetrapeptide.

Unfortunately, these steady-state kinetic parameters do not give a clear picture of HLE's specificity. It has previously been reported that k_c for the HLE-catalyzed hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-pNA is at least partially rate limited by deacylation (Stein et al., 1984; Stein, 1985b). In contrast, k_c is entirely rate limited by acylation during Suc-Ala-Ala-Ala-pNA and Z-Val-pNA hydrolyses. It appears then that the relative magnitudes of k_2 and k_3 are acutely sensitive to structural features of the substrate. k_c, which is a composite of these mechanistic parameters (see eq 1), cannot provde an accurate estimate of this sensitivity and is therefore an inappropriate measure of specificity. K_m is also a composite parameter, equal to K_s divided by the term $(k_2 + k_3)/k_3$ (see eq 2). If substrate specificity is manifested in binding, K_m cannot accurately reveal it. Clearly, the only way to get an accurate picture of HLE's substrate specificity is through the determination of the mechanistic kinetic parameters K_s, k_2, and k_3.

The calculation of these constants for the hydrolyses of the *p*-nitroanilides relies on the fact that k_c for the hydrolysis of thiobenzyl esters is equal to k_3, the first-order rate constant for hydrolysis of the acyl-enzyme.[3] Since thiobenzyl ester and *p*-nitroanilide derivatives of thee same peptide acid will react with HLE to produce the same acyl-enzyme, k_3 for these substrates must be identical. k_c for the anilide together with k_3 allows calculation of k_2 according to eq 6. Having both

$$k_2 = k_c k_3/(k_3 - k_c) \quad (6)$$

k_2 and k_3 allows us to calculate K_s according to

$$K_s = K_m/[k_3/(k_2 + k_3)] \quad (7)$$

The mechanistic kinetics parameters calculated according to eq 6 and 7 are given in Table II.

Table II: Mechanistic Kinetic Parameters for the HLE-Catalyzed Hydrolysis of Peptide *p*-Nitroanilides[a]

	K_s[b]	k_2[c]	k_3[d]	k_c/k_3[e]
MeOSuc-Val-pNA	788	0.06	10	0.006
MeOSuc-Pro-Val-pNA	722	0.42	11	0.036
MeOSuc-Ala-Pro-Val-pNA	810	43	13	0.77
MeOSuc-Ala-Ala-Pro-Val-pNA	240	43	13	0.77
MeOSuc-Ala-Pro-Ala-pNA	1800	8	46	0.15
MeOSuc-Ala-Ala-Pro-Ala-pNA	1400	38	53	0.42

[a] Derived from steady-state kinetics conducted in 0.1 M phosphate, 0.5 M NaCl, pH 7.4, and 3.3% Me_2SO at 25 ± 0.1 °C. k_c, k_2, and k_3 are expressed as s^{-1}. K_s is expressed as μM. [b] $K_s = K_m/[k_3/(k_2 + k_3)]$. [c] $k_2 = k_c k_3/(k_3 - k_c)$. [d] k_3 is assumed to equal k_c for the hydrolysis of the corresponding thiobenzyl ester. [e] Contribution of deacylation to the rate determination of k_c.

These results reveal an intriguing specificity for *p*-nitroanilides that have Val at P_1. It is clear that specificity is manifested entirely in acylation: while k_2 increases by nearly 3 orders of magnitude as MeOSuc-Val-pNA is extended to MeOSuc-Ala-Ala-Pro-Val-pNA, K_s decreases by only a factor of 3 and k_3 remains unchanged. The largest increase in k_2 occurs upon extending MeOSuc-Pro-Val-pNA to MeOSuc-Ala-Pro-Val-pNA and suggests a critical role for the P_3–S_3 interaction during acylation.

Also given in Table II are values of the ratio k_c/k_3, the fractional contribution of deacylation to the rate determination of k_c. For MeOSuc-Val-pNA, k_c/k_3 is equal to 0.006 and indicates that acylation is entirely rate limiting for the HLE-catalyzed hydrolysis of this substrate. However, for MeO-Suc-Ala-Ala-Pro-Val-pNA, k_c/k_3 equals 0.77, indicating that deacylation almost entirely limits the rate. This dependence of rate-limiting step on peptide length is a direct result of the marked sensitivity of acylation and insensitivity of deacylation to peptide chain length.

To better understand P_1 specificity, steady-state kinetic parameters were determined for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Abu-pNA and are compared to the kinetic parameters for the hydrolyses of the P_1 Ala and P_1 Val analogues in Table III. Also included are estimates of mechanistic kinetic parameters for the Abu substrate. P_1 specificity is manifested in deacylation and binding; acylation is quite insensitive to the P_1 residue. Also, as P_1 is changed from Ala to Abu to Val, k_c is increasingly rate limited by deacylation. This trend results from a progressively slower deacylation rate.

[3] Two lines of evidence indicate that k_c is rate-limited by deacylation for the HLE-catalyzed hydrolyses of thiobenzyl esters. (1) k_c values are identical for acyl-similar thiobenzyl and *p*-nitrophenyl esters, the latter which are known to hydrolyze with rate-limiting deacylation (Stein, 1984). (2) K_m values for thiobenzyl esters are much smaller than K_m and K_s values for corresponding *p*-nitroanilides.

Table III: Kinetic Parameters for the HLE-Catalyzed Hydrolysis of *p*-Nitroanilide Substrates of General Structure MeOSuc-Ala-Ala-Pro-AA-pNA[a]

amino acid	k_c	K_m	k_c/K_m	k_2[b]	k_3[c]	K_s[d]	k_c/k_3
Ala	22	833	27 000	38	53	1400	0.42
Abu	20	240	80 000	(40)[e]	(40)[e]	(480)[e]	(0.50)[e]
Val	10	55	180 000	43	13	240	0.77

[a] Derived from steady-state kinetics conducted in 0.1 M phosphate, 0.5 M NaCl pH 7.4, and 3.3% Me_2SO at 25 ± 0.1 °C. k_c, k_2, and k_3 are expressed as s^{-1}. K_m and K_s are expressed as μM. k_c/K_m is expressed as M^{-1} s^{-1}. [b] $k_2 = k_c k_3/(k_3 - k_c)$. [c] k_3 is assumed to equal k_c for the hydrolysis of the corresponding thiobenzyl ester. [d] $K_s = K_m/[k_3/(k_2 + k_3)]$. [e] Values in parentheses for the hydrolysis of the Abu derivative were obtained by first assuming that k_2 lies between k_2 values for the Ala and Val derivatives. With this assumption, $k_3 = k_c k_2/(k_2 - k_c)$ and $K_s = K_m/[k_3/(k_2 + k_3)]$.

Discussion

HLE's substrate specificity requirements, as revealed by this and earlier studies [see Table II, Stein (1985a), and Harper et al. (1984)], suggest several important mechanistic features for HLE (Stein, 1983, 1985b).

(1) Few Important Remote Contacts Are Established When Substrates Bind to HLE To Form Michaelis Complexes. K_s values are relatively large and insensitive to substrate structural features other than the P_1 residue and thus suggest that the substrate is rather loosely bound in the Michaelis complex with the interaction with the P_1 residue being of predominant importance.

(2) Full Recognition and Tight Binding of the Substrate Occurs in the Transition State for Acylation. The dramatic dependence of k_2 on substrate chain length indicates that in the transition state for acylation intimate and precise contacts at remote subsites are established. It is these favorable transition-state interactions that pay the energetic price for catalysis (Fersht, 1974; Jencks, 1975).

(3) The P_3–S_3 Interaction Plays a Critical Role during Acylation. The acylation rate undergoes a 100-fold increase upon addition of a P_3 residue and does not change upon addition of an additional residue.

(4) Remote Subsite Contacts Play a Minor Role during Deacylation. In the acyl-enzyme, the substrate is bound to HLE via a covalent ester bond. Relatively weak secondary interactions are expected to have little influence on the spatial arrangement of the reacting atoms of the acyl-enzyme and its rate of hydrolysis. This is, of course, in contrast to acyl-enzyme formation, during which remote interactions work together with interactions at the specificity pocket to align the acyl group of the substrate for efficient transfer to serine.

(5) P_1 Specificity Is Regulated by Peptide Length. This was first suggested in an earlier study (Stein, 1985a) where it was found that very bulky P_1 residues, such as Phe, are tolerated by HLE only in reactive monomeric substrates. Extended substrates with Phe at P_1 were found to be unreactive toward HLE. To explain this, it was proposed that binding at remote subsites changes the size of the S_1 "specificity pocket" (Stein, 1985a). Presumably, the "communication" between remote subsites and the primary binding site is mediated through subtle changes in the three-dimensional structure of HLE. An alternate explanation for the apparent dependence of P_1 specificity on substrate length is that the P_1 residue of monomeric substrates may be able to bind in catalytically productive modes that are unavailable to larger, peptide-based substrates.

Subtilisin BPN′, another member of the serine protease family, also appears to have alternate productive binding modes. Peptides containing P_1 aromatic amino acid residues bind normally with the P_1 side chain in the hydrophobic S_1 site of the enzyme. In contrast, Bz-Arg-OH binds with its carboxyl group near the catalytic residues but with its arginyl side chain far removed from S_1 (Wright et al., 1972). Subtilisin will slowly hydrolyze Bz-Arg-OEt and peptidyl nitroanilides (Pozsgay et al., 1979) but will leave arginyl bonds in peptides infrequently (A. D. Harley, E. Myers, and J. C. Powers, unpublished observations).

HLE's substrate specificity has important steady-state kinetic consequences in that it sets an upper limit on k_c/K_m and determines which reaction step rate limits k_c (Stein et al., 1984; Stein, 1985b). Concerning the limitation of k_c/K_m, the results of Table I indicate that acylation of HLE by the four tri- and tetrapeptide thiobenzyl esters of this study occurs at very similar rates. The average value of k_c/K_m is 5 ± 1 μM^{-1} s^{-1} and is similar to the value of 2.6 μM^{-1} s^{-1} for reaction of MeOSuc-Ala-Ala-Pro-Val-ONP with HLE (Stein, 1984). For substrates such as these, with very reactive leaving groups, the step representing the actual acylation of serine, k_b (Scheme I), occurs much faster than k_{-a} (Stein, 1985b) and k_c/K_m equals k_a/K_s (see eq 1–5). Now, if k_a and K_s are estimated to be about 200 s^{-1} (Stein, 1985b) and 100 μM, respectively, then their ratio, k_a/K_s, equals 2 μM^{-1} s^{-1} and represents the approximate upper limit on k_c/K_m for substrates of this peptide structural type. This limitation on k_c/K_m is a consequence of the insensitivity of both substrate binding and the physical step to substrate structure and an acylation process that is keenly sensitive to substrate structure.

The rate limitation of k_c is also the direct result of specificity. We have seen that tri- and tetrapeptide-based substrates, because of their ability to interact at remote subsites, will acylate HLE very efficiently. Deacylation, which is not influenced by subsite interactions, will rate limit k_c for these substrates. On the other hand, truncated substrates that are unable to bind at remote subsites will acylate HLE much less effectively. Acylation of HLE by these nonspecific substrates will become slower than deacylation and will finally rate limit k_c.

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Catalysis by Human Leukocyte Elastase: Proton Inventory as a Mechanistic Probe†

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ABSTRACT: Proton inventories (rate measurements in mixtures of H_2O and D_2O) were determined for the human leukocyte elastase catalyzed hydrolyses of thiobenzyl esters and *p*-nitroanilides of the peptides MeOSuc-Val, MeOSuc-Ala$_n$-Pro-Val (n = 0–2), and MeOSuc-Ala$_n$-Pro-Ala (n = 1 or 2). The dependencies of k_2/K_s on mole fraction of solvent deuterium for the *p*-nitroanilides are "dome-shaped" and were fit to a model that incorporates the mechanistic features of generalized solvent reorganization when substrate binds to enzyme and partial rate limitation of k_2/K_s by physical and chemical steps [Stein, R. L. (1985) *J. Am. Chem. Soc. 107*, 7768–7769]. The proton inventories for the deacylation of MeOSuc-Val-HLE and MeOSuc-Pro-Val-HLE are linear while those for the deacylation of MeOSuc-Ala-Pro-Val-HLE and MeOSuc-Ala-Ala-Pro-Val-HLE are "bowl-shaped" and could be fit to a quadratic dependence of rate on mole fraction of deuterium. These results are interpreted to suggest that the correct operation of the catalytic triad is dependent on substrate structure. Minimal substrates, which cannot interact with elastase at remote subsites, are hydrolyzed via a mechanism involving simple general-base catalysis by the active site histidine and transfer of a single proton in the rate-limiting transition state. In contrast, tri- and tetrapeptide substrates, which are able to interact at remote subsites, are hydrolyzed by a more complex mechanism of protolytic catalysis involving full functioning of the catalytic triad and transfer of two protons in the rate-limiting transition state. Finally, the proton inventories for the deacylation of MeOSuc-Ala-Pro-Ala-HLE and MeOSuc-Ala-Ala-Pro-Ala-HLE are dome-shaped and suggest that the chemical events of acyl-enzyme hydrolysis are only partially rate limiting for these reactions and that some other physical step is also partially rate limiting.

The proton inventory technique is an extension of the more routinely used solvent isotope effect; the latter is determined by measuring reaction rates in H_2O and D_2O and expressed as a ratio of rate constants, typically k_H/k_D and abbreviated here as ^Dk, while the former is determined by measuring reaction rates in mixtures of the isotopic waters and expressed graphically as the dependence of rate on the mole fraction of solvent deuterium n. The size of the solvent isotope effect and shape of the proton inventory are diagnostic of reaction mechanism [Schowen and Schowen (1982), especially pp 559–567; Venkatasubban & Schowen, 1985] and, upon detailed analysis, often lead to novel mechanistic insights (Harmony et al., 1975; Hunkapiller et al., 1976; Pollock et al., 1973; Matta & Toenjes, 1985; Stein, 1985c).

As probes of enzymic mechanisms, the solvent isotope effect and proton inventory have found their greatest use with serine hydrolases (Venkatasubban & Schowen, 1985). These enzymes catalyze the hydrolysis of amide and ester bonds via the double-displacement mechanism of Scheme I, in which E is enzyme, S is amide or ester substrate, E:S is the Michaelis complex, E-acyl is the acyl-enzyme, P_1 is the first product released (amine or alcohol), and P_2 is the second product released (carboxylic acid).

Scheme I: Acyl-Enzyme Mechanism for Reactions of Serine Hydrolases

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}S \xrightarrow{k_2} E\text{-acyl} \xrightarrow{k_3} E + P_2$$
$$\downarrow P_1$$

$$k_c = k_2k_3/(k_2 + k_3) \quad (1)$$

$$K_m = K_sk_3/(k_2 + k_3) \quad (2)$$

$$K_s = (k_{-1} + k_1)/k_1 \quad (3)$$

$$k_c/K_m = k_1k_2/(k_{-1} + k_2) \quad (4)$$

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