Catalysis by Human Leukocyte Elastase. 4.[1] Role of Secondary-Subsite Interactions

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Abstract: Steady-state and pre-steady-state kinetic constants, solvent deuterium isotope effects, and proton inventories were determined for the (HLE) human leukocyte elastase-catalyzed hydrolyses of the *p*-nitrophenyl esters and *p*-nitroanilides of *N*-(carbobenzoxy)-L-amino acids and *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val, an HLE-specific peptide. The results of these experiments indicate that remote subsite interactions between protease and substrate have a significant effect on four mechanistic features: (i) *Catalytic Efficiency*. Changing the carbobenzoxy group of Z-Val-X (X = *p*-nitrophenol or *p*-nitroaniline) to MeOSuc-Ala-Ala-Pro resulted in large increases in both k_c and k_c/K_m and confirmed studies by other investigators documenting the effect that peptide chain length has on catalytic efficiency. Pre-steady-state kinetic experiments further demonstrated that subsite interactions have a greater effect on acylation than on deacylation: while k_2 (first-order rate constant for reaction within the Michaelis complex to form the acyl-enzyme) increased 50 000-fold upon changing Z-Val-*p*-nitroanilide to MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide, k_3 (first-order rate constant for hydrolysis of the acyl-enzyme) increased only 6-fold. (ii) *P_1 Specificity*. The effect of remote interactions on P_1 specificity during deacylation was assessed by comparing ratios of k_3 for pairs of substrates having either Ala or Val at P_1. k_3-Ala/k_3-Val varied with substrate structure past P_1 as follows: Z-AA, 13; *t*-Boc-Ala-Ala-AA, 7; MeOSuc-Ala-Pro-AA, 3.5; and MeOSuc-Ala-Ala-Pro-AA, 2. (iii) *Rate-Determining Step*. k_c $[=k_2k_3/(k_2 + k_3)]$ was found to be rate-limited by k_2 for the *p*-nitroanilides of Z-Val and other minimal acyl portions. In contrast, very specific peptide-derived anilides which afford the enzyme extensive opportunities for subsite contacts acylate HLE quite efficiently and are hydrolyzed with rate-limiting k_3. Furthermore, the process governed by k_2 appears to have a "virtual transition state" composed of the transition state for acylation, which is rate-limiting for minimal substrates, and the transition state for some physical step, which becomes partially rate-limiting for specific substrates. (iv) *Charge-Relay Catalysis*. Despite only a 6-fold difference in deacylation rates between Z-Val-HLE and MeOSuc-Ala-Ala-Pro-Val-HLE, the nature of the protonic catalysis involved in these processes appears to be different. The data are consistent with a mechanism for Z-Val-HLE hydrolysis in which only the active-site His acts as the general catalyst. Hydrolysis of MeOSuc-Ala-Ala-Pro-Val-HLE, on the other hand, proceeds with full involvement of the charge-relay system. The catalytic role of the charge-relay system is discussed in light of these observations.

Human leukocytes contain an endopeptidase capable of digesting elastin and a variety of other connective tissue proteins.[2] This enzyme is a glycoprotein of molecular weight 30 000 and exists as four major isozymes that are resolvable by cation-exchange chromatography.[2] Irreversible inhibition by such agents as diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, and α-1-proteinase inhibitor indicate that this enzyme, human leukocyte elastase, is a serine protease.[2]

Like other serine proteases,[3-5] HLE[6] employs a kinetic mechanism involving the formation and subsequent hydrolysis of an acyl-enzyme intermediate [see Scheme I and eq 1–4].

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

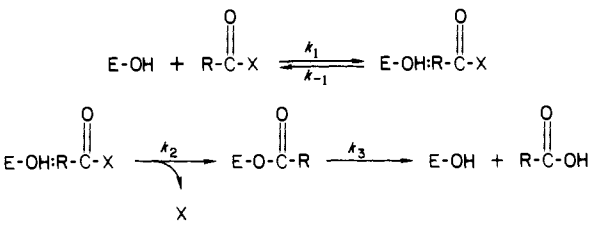
$$K_m = K_sk_3/(k_2 + k_3) \quad (2)$$

$$K_s = (k_{-1} + k_2)/k_1 \quad (3)$$

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

The intermediacy of such a species during HLE catalysis is supported by (i) observation of "burst" kinetics during the hydrolysis of aza amino acid esters,[7] (ii) identical k_c values for the hydrolyses of the thiobenzyl ester, *p*-nitrophenyl ester, ethyl ester, *p*-nitroanilide, and dialanyl amide of the specific peptide MeO-Suc-Ala-Ala-Pro-Val,[8] (iii) nucleophile competition experiments,[8] and (iv) pre-steady-state kinetics (see below).

Scheme I



Another feature common to leukocyte elastase and other serine proteases is the use of general acid/general base catalysis to assist acyl transfer to and from the active-site serine. For HLE, the evidence supporting general catalysis is the observation of large solvent deuterium isotope effects.[9] For several other serine proteases, there is this evidence[3-5,10-14] as well as X-ray[15,16] and

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(6) Abbreviations: HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; CT, chymotrypsin; Ac, acetyl; Z, carbobenzoxy; ONP, *p*-nitrophenyl; MeOSuc, methoxysuccinyl; *p*-NA, *p*-nitroanilide.
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neutron[17] structures consistent with an active-site histidine acting as the general catalyst. In addition, proton inventory experiments have revealed that during the reaction of HLE with specific substrates, there appears to be transfer of not one but two protons.[9] This has been interpreted to suggest operation of the enzyme's "charge-relay system", an assembly of active-site amino acid residues (Asp-102, His-57, Ser-195, chymotrypsin numbering) which functions to enhance the nucleophilicity of Ser-195 during acylation and water during deacylation. Structural evidence for the existence of such a system has come from X-ray and neutron diffraction studies of pancreatic proteases, while its actual operation during catalysis by these enzymes is provided by proton inventories.[11-14]

Finally, HLE and other serine proteases have substrate specificity requirements that not only include that portion of the substrate directly bonded to the targeted acyl linkage but also portions of peptide-derived substrates removed from this bond.[18] Observations of such substrate specificity have been interpreted as supporting the existence of sites on the enzyme surface that are complementary to and can bind the various regions of the substrate.[15] Thus, the amino acid side chain of the P_1 residue[19] binds into the "specificity pocket", while the rest of the substrate binds at more remote subsites.

The object of this paper is to explore the mechanistic consequences of remote subsite interactions for HLE. This is accomplished through the determination of steady-state and pre-steady-state kinetic constants, solvent isotope effects, and proton inventories for the HLE-catalyzed hydrolyses of *p*-nitroanilides and *p*-nitrophenyl esters of simple, *N*-acyl amino acids and the HLE-specific peptide, MeOSuc-Ala-Ala-Pro-Val. I will show that subsite interactions have a direct effect on four mechanistic features: (i) catalytic efficiency; (ii) P_1 specificity; (iii) rate-limiting step; and (iv) charge-relay catalysis.

Material and Methods

Ac-ONP, Z-Ala-ONP, Z-Val-ONP, Z-Phe-ONP, and Ac-$(Ala)_3$-*p*-NA were obtained from Sigma Chemical Co. Z-Val-*p*-NA was synthesized by Mara Specialty Chemical Co., Philadelphia. Ac-$(Ala)_3$-ONP was a generous gift of Prof. James C. Powers. MeOSuc-Ala-Ala-Pro-Val-*p*-NA and MeOSuc-Ala-Ala-Pro-Val-ONP were available from previous studies.[8,9] Buffer salts, acetonitrile, and Me_2SO were analytical grade from several sources. D_2O (99%) was purchased from Sigma Chemical Co.

HLE Solutions. Human leukocyte elastase was purchased from Elastin Products, Pacific, MO. The material was purified from purulent sputum as previously described[21] and supplied as a salt-free lyophilized powder. Stock solutions of HLE (0.1-8.0 mg/mL) were prepared in 50 mM acetate, 500 mM NaCl, pH 5.5 buffer and were found to be stable for at least 7 days if stored at 4 °C.[22] Concentration of active enzyme was determined from established kinetic parameters for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-*p*-NA.[9]

Determination of Kinetic Parameters. Steady-state kinetic parameters for HLE-catalyzed hydrolyses were determined as previously described.[1,9] Solvent deuterium isotope effect determinations and proton inventories for the reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-ONP and Z-Val-ONP were conducted according to established methods.[9] Pre-steady-state kinetic parameters were determined essentially as described previously.[23]

Analysis of Pre-Steady-State Kinetic Data. Pre-steady-state kinetic parameters, K_s and k_2, for the HLE-catalyzed hydrolysis of *N*-(carbobenzoxy)-L-amino acid *p*-nitrophenyl esters were determined from enzyme acylation experiments[24,25] in which reaction progress is monitored under conditions of $[E]_0 \gg [S]_0$.

The kinetics of such reactions predict saturation of substrate by the enzyme, which leads to a limiting velocity at high enzyme concentration. If $k_2 \gg k_3$ (see Scheme I), it may be shown that

$$d[X]/dt = \{k_2[E]_0/(K_s + [E]_0)\}[S] \quad (5)$$

where X is *p*-nitrophenolate anion, E is HLE, S is the *N*-acyl amino acid ester, and $[S] = [S]_0 - [X]$. Thus, these reactions will be first-order in substrate with observed first-order rate constants given by

$$k_{obsd} = k_2[E]_0/(K_s + [E]_0) \quad (6)$$

Values for k_2 and K_s are found by determining k_{obsd} at several concentrations of enzyme and then fitting the data to eq 6 by nonlinear least squares. Note that eq 6 is analogous to the Michaelis-Menton equation. Thus, if the experimental data adhere to the mechanism from which this expression comes, double-reciprocal plots of $1/k_{obsd}$ vs. $1/[E]_0$ must be linear. For such plots, *x*-axis intercepts will be equal to $-1/K_s$, *y*-axis intercepts equal to $1/k_2$, and slopes equal to K_s/k_2.

Values of k_{obsd} are conveniently calculated by the method of half-lives from semilog plots of $(A_{00} - A_t)$ vs. time where A_{00} is the absorbance at the completion of the reaction and A_t is the absorbance at time t.

For the HLE-catalyzed hydrolyses of Ac-ONP, MeOSuc-Ala-Ala-Pro-Val-ONP, and MeOSuc-Ala-Ala-Pro-Val-*p*-NA, the experimental condition $[E]_0 \ll [S]_0$ at very high concentrations of enzyme was set up with a view toward monitoring "burst" kinetics[24,26] for these reactions. Under this condition, the time course of leaving group release in the early stages of reaction when $[S] = [S]_0$ obeys the expression

$$[X] = vt + a[E]_0\{1 - \exp(-bt)\} \quad (7)$$

where v is the steady-state velocity given by

$$v = k_c[E]_0[S]_0/(K_m + [S]_0) \quad (8)$$

and

$$a = \left[\frac{\frac{k_2}{k_2 + k_3}}{1 + K_m/[S]_0}\right]^2 \quad (9)$$

$$b = (k_2 + k_3)[S]_0/(K_s + [S]_0) \quad (10)$$

When k_2 is similar to or greater than k_3, these equations predict a pre-steady-state "burst" of product release of amplitude A ($=a[E]_0$) with first-order rate constant b. This phase is followed by the steady state where product production is linearly dependent on time and is described by vt.

Pre-steady-state kinetic parameters k_2, k_3, and K_s can be determined from the above relationships as follows. Progress curves are recorded at several concentrations of substrate, and values of a, b, and v are extracted from each curve. The observed substrate concentration dependence of a and b is then fit, by nonlinear least-squares, to eq 9 and 10 to generate values of $k_2/(k_2 + k_3)$ and K_m and $(k_2 + k_3)$ and K_s, respectively. Similarly, the substrate concentration dependence of the steady-state velocities, v, are fit to eq 8 to yield values of k_c and K_m. Finally, algebraic manipulation of these composite parameters will yield the individual pre-steady-state parameters.

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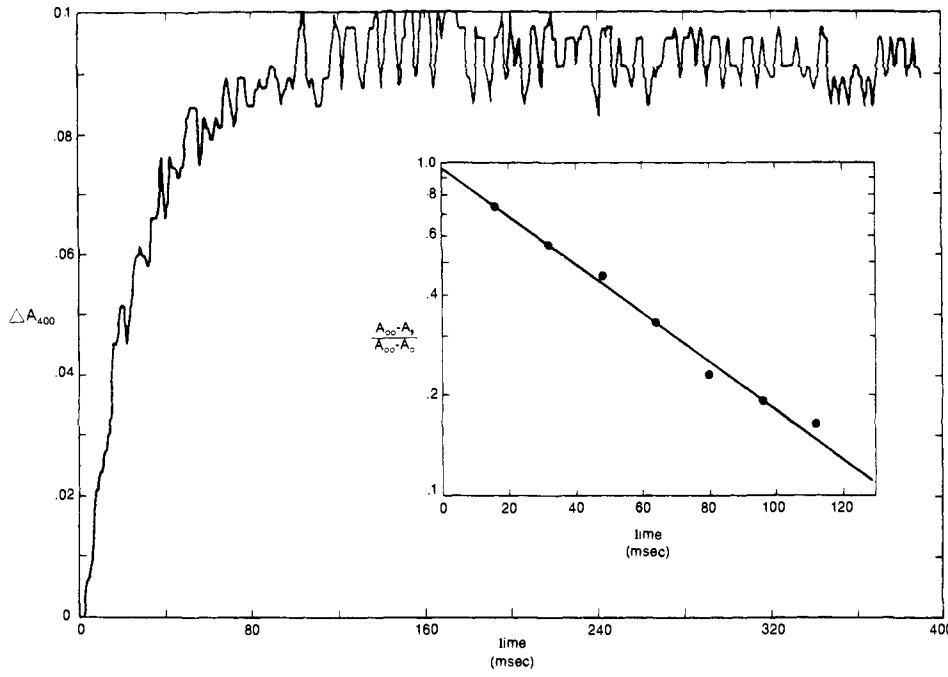

Figure 1. Progress curve for acylation of HLE by Z-Val-ONP: [Z-Val-ONP] = 7.5 μM, [HLE] = 106 μM, 100 mM phosphate, 20 mM acetate, 500 mM NaCl, pH 7.4, 5% acetonitrile, 29 °C. Insert is first-order plot of progress curve data.

The rate constant b can be calculated by a graphical method in which the steady-state zero-order curve is extrapolated into the pre-steady-state, and differences between it and the actual experimental curve are plotted vs. time on semilog paper. b is then determined from this plot by the method of half-lives.

Results

***N*-(Carbobenzoxy)-L-amino Acid *p*-Nitrophenyl Ester Hydrolysis.** Acylation experiments were performed to determine pre-steady-state kinetic parameters for the reaction of HLE with three *N*-(carbobenzoxy)-L-amino acid *p*-nitrophenyl esters. Figure 1 is a typical kinetic run showing the reaction of 7.5 μM Z-Val-ONP and 106 μM HLE. As demonstrated here, the data were generally of excellent quality and cleanly first-order for at least 85% of the reaction. Acylation experiments such as this one generated first-order rate constants, k_{obsd}, that were dependent on enzyme concentration according to eq 6.

Table I contains steady-state and pre-steady-state kinetic parameters for the reaction of HLE with Z-Ala-ONP, Z-Val-ONP, and Z-Phe-ONP. The steady-state parameters k_c and K_m were determined in an independent study by initial velocity experiments.[1] The pre-steady-state parameters k_2 and K_s for Z-Ala-ONP and Z-Val-ONP were determined as outlined above. k_3 was calculated according to

$$k_3 = k_c k_2/(k_2 - k_c) \qquad (11)$$

Acylation kinetics for Z-Phe-ONP were not determined over a wide enough range of HLE concentration to detect saturation. However, even with the limited data set, approximate values can still be assigned to the kinetic parameters. At $[\text{Z-Phe-ONP}]_0$ = 2.2 μM, observed first-order acylation rate constants were found to be linearly dependent on $[\text{HLE}]_0$ from 8 to 33 μM. The slope of the line constructed from this dependence has a value of 66 000 M^{-1} s^{-1} and is equal to k_2/K_s. Now, a conservative estimate of K_s is equal to about 3-fold the highest substrate concentration: 100 μM. This lower limit for K_s and the experimentally determined value of k_2/K_s of 66 000 M^{-1} s^{-1} allows a lower limit of 7 s^{-1} to be set on k_2. Furthermore, since $k_2 \gg k_c = 0.3$ s^{-1}, k_3 can be set equal to k_c (see eq 11).

For all three esters, values of $K_s k_3/(k_2 + k_3)$ are identical with the steady-state parameter K_m (see eq 2). Similarly, values of k_2/K_s are nearly equal to k_c/K_m. The agreement between these pre-steady-state and steady-state parameters indicates a reasonable level of accuracy for the pre-steady-state data and argues for the usefulness of acylation kinetics in the study of HLE catalysis.

Table I. Steady-State and Acylation Kinetics for the Hydrolysis of *N*-(Carbobenzoxy)-L-amino Acid *p*-Nitrophenyl Esters by Human Leukocyte Elastase

	amino acid		
kinetic param	Ala	Val	Phe
k_c,[a] s^{-1}	30 ± 4	3.5 ± 0.3	0.3 ± 0.1
K_m,[a] μM	80 ± 4	5.6 ± 0.4	3.5 ± 0.7
k_c/K_m,[a] M^{-1} s^{-1}	330 000	625 000	86 000
k_2,[b] s^{-1}	83 ± 7	65 ± 5	>7[d]
K_s,[b] μM	200 ± 25	100 ± 8	>100[d]
k_2/K_s, M^{-1} s^{-1}	415 000	650 000	66 000
k_3,[c] s^{-1}	47	3.7	0.3
$K_s k_3/(k_2 + k_3)$, μM	72	5.4	4.2

[a] Steady-state kinetics: 100 mM phosphate, 500 mM NaCl, pH 7.4, 3.3% Me_2SO. [b] Acylation kinetic data; i.e., $[E]_0 \gg [S]_0$: 100 mM phosphate, 20 mM acetate, 500 mM NaCl, pH 7.4, 5% acetonitrile, 29 °C. For Z-Ala-ONP and Z-Val-ONP, $[S]_0$ = 6 μM, while $[\text{HLE}]_0$ ranged from 26 to 106 μM. [c] $k_3 = k_c k_2/(k_2 - k_c)$. [d] At $[\text{Z-Phe-ONP}]_0$ = 2.2 μM, observed first-order acylation rate constants were linearly dependent on $[\text{HLE}]_0$ from 8 to 33 μM and allowed these lower limits on k_2 and K_s to be set.

The pre-steady-state data of Table I indicate that deacylation is rate-limiting for all three substrates ($k_2 > k_3$). This contrasts with results of similar experiments with PPE,[26] where acylation was found to be rate-limiting ($k_2 < k_3$) during the hydrolysis of Z-Ala-ONP.

Hydrolyses of Ac-(Ala)$_3$-*p*-NA and Ac-(Ala)$_3$-ONP. Steady-state kinetic parameters were determined for the HLE-catalyzed hydrolyses of the *p*-nitroanilide and *p*-nitrophenyl ester of Ac-(Ala)$_3$ in 0.1 M phosphate, 0.5 M NaCl, pH 7.4 containing 3.3% cosolvent (Me_2SO and acetonitrile for the anilide and ester, respectively). For the anilide k_c, K_m, and k_c/K_m are 0.27 s^{-1}, 910 μM, and 297 M^{-1} s^{-1}, respectively, while for the ester these constants are 47 s^{-1}, 58 μM, and 810 000 M^{-1} s^{-1}. Spontaneous rates of Ac-(Ala)$_3$-ONP hydrolysis were subtracted from enzymatic rates before analysis.

At pH 6.1 (0.1 M citrate, 3.3% acetonitrile) the hydrolysis of Ac-(Ala)$_3$-ONP had values of k_c, K_m, and k_c/K_m equal to 22 s^{-1}, 105 μM, and 212 000 M^{-1} s^{-1}, respectively. These values are consistent with both the known pH dependence of k_c for HLE-catalysis[9] and kinetic constants for Suc-(Ala)$_3$-ONP hydrolysis at this pH (ref 27; k_c = 12 s^{-1}, K_m = 200 μM, and k_c/K_m = 60 000

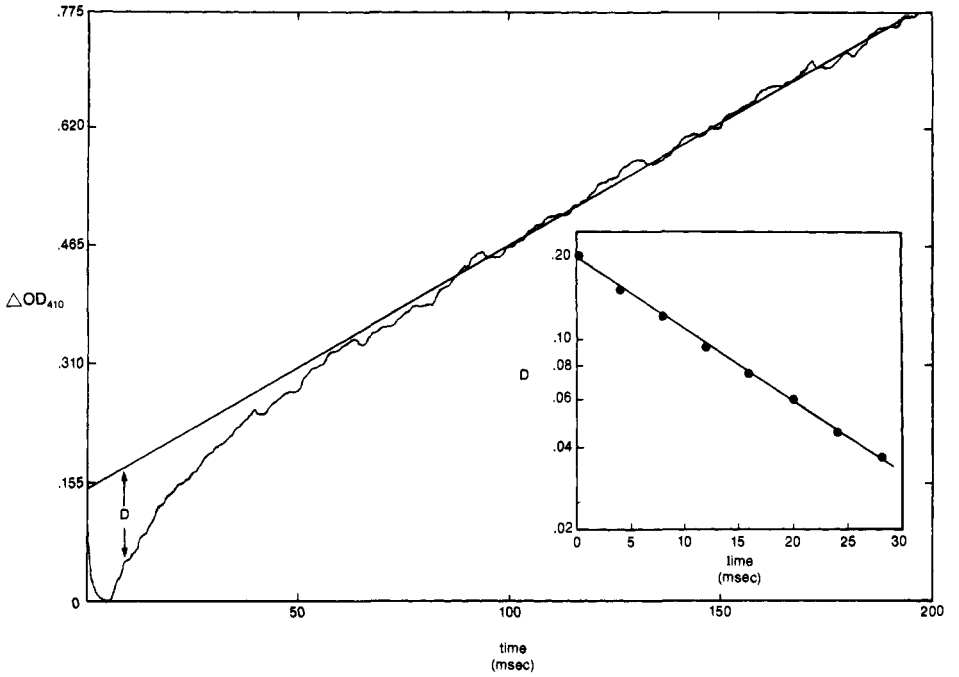

Figure 2. Burst kinetics for reaction of HLE and MeOSuc-Ala-Ala-Pro-Val-*p*-NA: [HLE] = 42 μM, [S] = 850 μM, 100 mM phosphate, 20 mM acetate, 500 mM NaCl, pH 7.4, 5% Me_2SO, 29 °C. The insert is a semilog plot of the differences between the extrapolated steady-state data and the pre-steady-state data vs. time.

Table II. Steady-State and Burst Kinetics for the Reaction of HLE with the *p*-Nitrophenyl Ester and *p*-Nitroanilide of MeOSuc-Ala-Ala-Pro-Val[a]

kinet param	anilide	ester
k_c, s^{-1}	13.3 ± 0.8[b]	23 ± 1[c]
K_m, μM	55 ± 6[b]	3.4[b]
k_c/K_m, M^{-1} s^{-1}	240 000	6 800 000
k_2, s^{-1}	30 ± 4[e]	200 ± 15[f]
k_3, s^{-1}	23 ± 4[e]	26 ± 4[f]
K_s, μM	115 ± 7[e]	34 ± 6[g]
k_2/K_s, M^{-1} s^{-1}	260 000	5 800 000
$K_s k_3/(k_2 + k_3)$, μM	50	2.8

[a] 100 mM phosphate, 20 mM acetate, 500 mM NaCl, pH 7.4, 5% Me_2SO (anilide) or acetonitrile (ester), 29 °C. [b] Determined from a fit of the steady-state velocities to the Michaelis–Menten equation. [c] Determined from steady-state velocities at $[S]_o/K_m = 63$. [d] Previous work. [e] Determined from the substrate concentration dependence of the burst rate constants. [f] Determined from burst amplitudes at $[S]_o/K_m = 63$. [g] $K_s = \{[(k_2 + k_3)/b] - 1\}[S]_o$.

M^{-1} s^{-1}) but disagree with kinetic constants recently reported by Powers and co-workers (ref 28; pH 6, $k_c = 83$ s^{-1}, $K_m = 660$ μM, and $k_c/K_m = 130\,000$ M^{-1} s^{-1}). The origin of this discrepancy is presently unknown.

Hydrolysis of MeOSuc-Ala-Ala-Pro-Val-*p*-NA. Figure 2 contains a progress curve for the reaction of 42 μM HLE and 850 μM MeOSuc-Ala-Ala-Pro-Val-*p*-NA. A burst of *p*-nitroaniline release is clearly evident and is consistent with previously reported steady-state experiments, suggesting that $k_2 > k_3$. The inset of Figure 2 illustrates the graphical method described earlier that is used to determine the burst rate constant, *b*.

Reaction progress curves were recorded at several substrate concentrations ($[HLE]_0 = 42$ μM). Values of burst rate constant, *b*, and steady-state velocity, *v*, were extracted from these curves. By using the methods described above, pre-steady-state and steady-state kinetic parameters were determined; these are given in Table II. Figure 3 contains the double-reciprocal plots $1/b$ and $[E]_0/v$ vs. $1/[S]_0$. The solid lines were drawn using the kinetic parameters of Table II.

When one takes into account the temperature differences, the steady-state kinetic parameters determined here are within experimental error of previously determined values[9] of $k_c = 10$ s^{-1}, $K_m = 54$ μM, and $k_c/K_m = 185\,000$ M^{-1} s^{-1}. The accuracy of

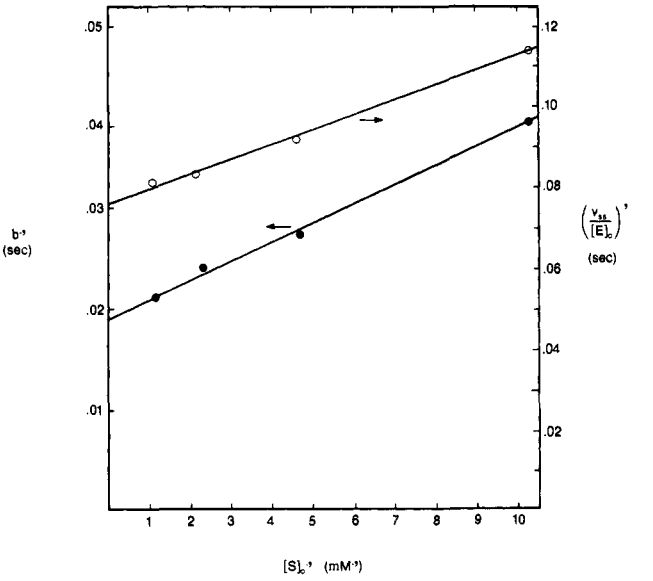

Figure 3. Plots of reciprocal burst and steady-state rate constants vs. reciprocal substrate concentrations. Solid lines were drawn with kinetic parameters of Table II.

the pre-steady-state data is demonstrated by the agreement between (a) the calculated value $K_s k_3/(k_2 + k_3)$ and the experimentally determined steady-state parameter K_m and (b) k_2/K_s and k_c/K_m.

Hydrolysis of MeOSuc-Ala-Ala-Pro-Val-ONP. Progress curves were recorded at 34 μM HLE and 214 μM MeOSuc-Ala-Ala-Pro-Val-ONP. Burst kinetics for *p*-nitrophenol release were observed in all cases. The level of substrate used in these experiments is about $63K_m$ and allows the expressions for the steady-state velocity, *v*, and burst amplitude, $a[E]_0$, of eq 8 and 9 to be simplified to

$$v = k_c[E]_0 = \{k_2k_3/(k_2 + k_3)\}[E]_0 \quad (12)$$

$$a = \{k_2/(k_2 + k_3)\}^2 \quad (13)$$

Using these expressions and the experimentally determined values of *v* and *a*, the pre-steady-state kinetic parameters k_2 and k_3 were

Table III. Summary of Kinetic Parameters for HLE-Catalyzed Reactions[a]

	k_c, s^{-1}	K_m, μM	k_c/K_m, M^{-1} s^{-1}	k_2, s^{-1}	k_3, s^{-1}	K_s, μM	k_2/K_s, M^{-1} s^{-1}
Z-Val-*p*-NA[b]	0.002	410	5	0.002	4	410	5
MeOSuc-Ala-Ala-Pro-Val-*p*-NA[c]	13	55	240 000	30	23	115	260 000
Ac-ONP[d]	0.008	64	125	0.008	0.023	74	110
Z-Val-ONP[e]	3.5	5.6	625 000	65	4	100	650 000
MeOSuc-Ala-Ala-Pro-Val-ONP[c]	23	3.4	6 800 000	200	26	34	5 800 000

[a] 0.1 M phosphate, 0.5 M NaCl, 3–5% Me_2SO or acetonitrile, 29 °C. [b] k_c/K_m was determined from initial velocities with [HLE] = 4 μM and [S] = 61 μM. K_m was determined as the K_i value for the inhibition of MeOSuc-Ala-Ala-Pro-Val-*p*-NA hydrolysis by HLE. $k_c = K_m(k_c/K_m)$. [c] See Table II. [d] Kinetic parameters for the hydrolysis of *p*-nitrophenyl acetate were determined from "burst" kinetics observed with [HLE] = 3.3 μM and [Ac-ONP] = 0.05–0.25 mM. [e] See Table II.

calculated and are given in Table II. From these same progress curves, a value for the burst rate constant, b, of 182 s^{-1} was determined and along with values for k_2 and k_3 allowed calculation of K_s according to a rearranged form of eq 10:

$$K_s = \{[(k_2 + k_3)/b] - 1\}[S]_0 \qquad (14)$$

Again, the accuracy of the pre-steady-state data is reflected in the agreement between $K_s k_3/(k_2 + k_3)$ and K_m and k_2/K_s and k_c/K_m.

Hydrolysis of *p*-Nitrophenyl Acetate. When conventional spectrophotometric methods were used, burst kinetics could be observed for the reaction of 3.3 μM HLE and concentrations of Ac-ONP ranging from 50 to 250 μM. Steady-state and pre-steady-state rate constants were determined essentially as described above for the peptide anilide and are given in Table III. The parameter $K_s k_3/(k_2 + k_3)$ is equal to 55 μM and is in fair agreement with the observed K_m of 64 μM. Unlike the acyl amino acid esters, *p*-nitrophenyl acetate is hydrolyzed by HLE with rate-limiting acylation.

Solvent Deuterium Isotope Effects.[29] Initial velocities were determined in H_2O and D_2O for the reaction of HLE and 114 μM MeOSuc-Ala-Ala-Pro-Val-ONP ($30K_m$). An isotope effect of 3.19 ± 0.09 was calculated and reflects the isotope effect on k_c. Likewise, first-order rate constants were determined in light and heavy water at $[S]_0$ = 0.9 μM ($0.27K_m$). An isotope effect of 1.09 ± 0.03 was calculated and reflects, predominantly, the isotope effect on k_c/K_m.

Proton Inventories.[29] Initial velocities at $[S]_0$ at 17 μM ($3K_m$) and first-order rate constants at $[S]_0$ = 0.97 μM ($0.16K_m$) were determined in mixtures of light and heavy water for the HLE-catalyzed hydrolysis of Z-Val-ONP. These data, constituting proton inventories of k_c and k_c/K_m, respectively, are displayed in Figure 4. These proton inventories are both linear with overall effects of 2.40 ± 0.08 and 1.98 ± 0.06.

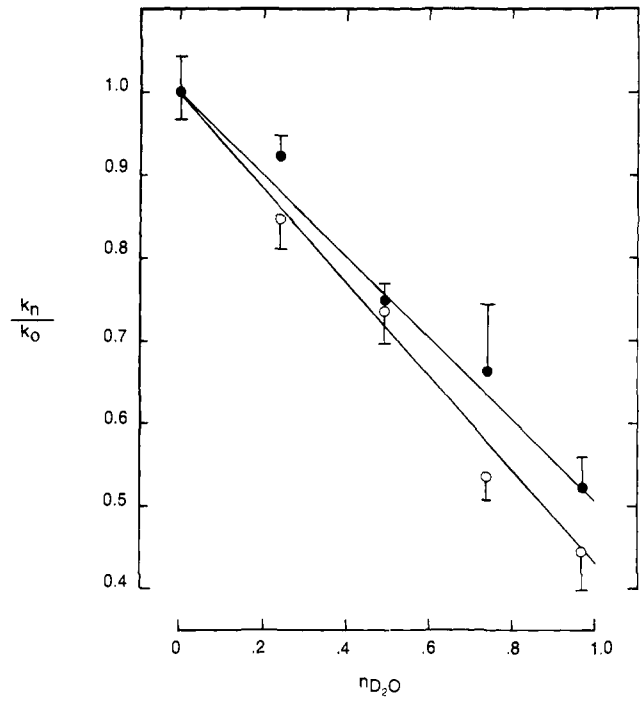

Figure 4. Proton inventories for the HLE-catalyzed hydrolysis of Z-Val-OMP. Filled circles correspond to initial velocities determined at $[S]_0$ = 17 μM ($3K_m$). Best-fit line drawn as $k_n/k_0 = 1 - n + n/2.40$. Circles correspond to first-order rate constants determined at $[S]_0$ = 0.97 μM ($0.16K_m$). Best-fit line drawn as $k_n/k_0 = 1 - n + n/1.98$.

Discussion

Catalytic Efficiency. Lestienne and Bieth[27] were the first to demonstrate the pronounced effect that peptide chain length has on the efficiency of HLE catalysis. For a series of *p*-nitroanilides of general structure Suc-$(Ala)_n$-*p*-NA (n = 2–4), they reported increases in k_c and k_c/K_m of 30- and 300-fold, respectively. Significant but smaller rate enhancements were noted by Wenzel and Tschesche,[30] who reported increases in k_c and k_c/K_m of 8- and 40-fold, respectively, for the HLE-catalyzed hydrolysis of substrates of structure Suc-$(Ala)_n$-Val-*p*-NA (n = 1–3). The results of the present study, summarized in Table III, are consistent with these data and demonstrate the importance of remote subsite interactions in determining catalytic efficiency.

Large differences in the steady-state parameters, k_c and k_c/K_m, for the *p*-nitroanilides derived from Z-Val and MeOSuc-Ala-Ala-Pro-Val are clearly evident. Furthermore, examination of the pre-steady-state rate constants, k_2 and k_3, reveals that subsite interactions have their most significant effects on acylation: as features of peptide structure are introduced into the substrate, k_2 increases by a factor of 14 000 while k_3 increases by only a factor of 6. Interestingly, this structural change produces only a 3-fold decrease in K_s and suggests that subsite interactions play a relatively minor role in binding.

The situation for the HLE-catalyzed hydrolyses of *p*-nitrophenyl esters is similar to this: relative values of k_3 for acetyl-HLE, Z-Val-HLE, and MeOSuc-Ala-Ala-Pro-Val-HLE are 1, 170, and 1000, while relative k_2/K_s values for the corresponding *p*-nitrophenyl esters are 1, 6000, and 53 000. k_2 varies as 1, 8000, and 25 000, and we once again find that K_s remains relatively constant. As with the anilides, these results suggest that remote contacts have a major role in determining the efficiency with which HLE is acylated but a relatively minor role during binding and deacylation.

The interpretation presented above will, of course, become more complex if either of the substrates bind to HLE in nonproductive modes. Nonproductive binding[4] is said to occur when substrate binds to the enzyme in modes that are thermodynamically significant, but kinetically incompetent, and has the effect of reducing the observed value of k_2 and K_s. However, since nonproductive binding can have no influence on k_2/K_s and probably has none on k_3, the major conclusion drawn here, that remote interactions play a more important role in acylation than in deacylation, is still valid.

The absence of a large effect on deacylation is not surprising. In the acyl-enzyme, the substrate is bound to HLE via a covalent

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(30) Wenzel, H. R.; Tschesche, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1981**, *362*, 829–831.

Table IV. Comparison of Kinetic Properties for the HLE-Catalyzed Hydrolyses of Substrates with P_1 Ala or Val

substrate	k_3, s^{-1}	Ala/Val[a]	k_2/K_s, M^{-1} s^{-1}	Ala/Val[b]	K_s, μM	Ala/Val[c]	ref
MeOSuc-Ala-Ala-Pro-Ala-SBZ	53	2.3	4 100 000	0.73			38
MeOSuc-Ala-Ala-Pro-Val-SBZ	23		5 600 000				
MeOSuc-Ala-Pro-Ala-SBZ	46	3.5	3 840 000	0.69			38
MeOSuc-Ala-Pro-Val-SBZ	13		5 600 000				
Boc-Ala-Ala-Ala-SBZ	47	7	730 000	0.24			35
Boc-Ala-Ala-Val-SBZ	9		3 000 000				
Z-Ala-ONP	47	13	420 000	0.65	200	2	*d*
Z-Val-ONP	3.7		650 000		100		
MeOSuc-Ala-Ala-Pro-Ala-*p*-NA	53	2.3	27 000	0.15	1 400[e]	6	38
MeOSuc-Ala-Ala-Pro-Val-*p*-NA	23		180 000		240[e]		
MeOSuc-Ala-Pro-Ala-*p*-NA	46	3.5	4 600	0.09	1 800[e]	2	38
MeOSuc-Ala-Pro-Val-*p*-NA	13		53 000		810[e]		
Suc-Ala-Ala-Ala-*p*-NA			450	0.09	1 300[f]	<2	*d*
Suc-Ala-Ala-Val-*p*-NA			4 800		>700[g]		
Ac-Ala-Ala-Pro-Ala-NH_2			28	0.07	12 000[f]		33
Ac-Ala-Ala-Pro-Val-NH_2			400		6 000[f]		

[a] Ratio of k_3 values for substrate pairs R-P_1-X, where P_1 is Ala or Val. [b] Ratio of k_2/K_s for substrate pairs R-P_1-X, where P_1 is Ala or Val. [c] Ratio of K_s for substrate pairs R-P_1-X, where P_1 is Ala or Val. [d] This work. [e] Calculated from the expression $K_s = K_m[(k_2 + k_3)/k_3]$, where k_3 was assumed equal to k_c for the corresponding thiobenzyl ester and k_2 calculated according to $k_2 = k_c k_3/(k_c - k_3)$. [f] K_s assumed equal to K_m. [g] $K_s = K_m[(k_2 + k_3)/k_3]$; K_m = 700 μM, k_2 and k_3 are unknown.

ester bond and the introduction of relatively weak secondary interactions would be expected to have little influence on the spatial arrangement of the reacting atoms of the acyl-enzyme and its rate of hydrolysis. During formation of the acyl-enzyme, however, remote interactions must work together with interactions at the specificity pocket to align the acyl group of the substrate for efficient transfer to serine.

P_1 Specificity. In a previous publication,[1] I reported that HLE possesses a broad substrate specificity for *p*-nitrophenyl esters of *N*-(carbobenzoxy)-L-amino acids. Substrates that were efficiently hydrolyzed by HLE were found to include not only Z-Ala-ONP and Z-Val-ONP but also Z-Gly-ONP, Z-Phe-ONP, and Z-Tyr-ONP. These results were contrasted with studies of peptide amide and anilide hydrolysis[31-34] which suggested an almost absolute requirement for Val at P_1 and with results from investigations of peptide-thioester[35] and aza-peptide ester[27] hydrolysis, which, while supporting a more relaxed specificity, still indicated that peptides with Gly or Phe at P_1 were totally inactive as HLE substrates. Together, these results were interpreted to suggest that interactions between enzyme and substrate remote from the scissle bond regulate P_1 specificity. It was also found in this investigation that k_c and k_c/K_m did not exhibit the same dependence on substrate structure. This suggested significant differences in P_1 specificity between acylation and deacylation for HLE-catalyzed reactions.

The breadth of substrate specificity displayed by HLE toward monomeric substrates is reflected here in the pre-steady-state kinetic data (Table I) as in the steady-state data just described. We also learn from the present work that discrimination among these minimal substrates is more fully expressed in deacylation than in acylation:

Z-AA-ONP	$(k_2/K_s)_{rel}$	$(k_3)_{rel}$
Phe	1	1
Val	10	12
Ala	6	160

The relative values of k_2/K_s and k_3 show that as the P_1 specificity requirements of HLE are fulfilled by replacing the α-benzyl substituent of Phe with smaller side chains, deacylation responds more dramatically than acylation.

These results, which relate to interactions with the specificity pocket of the enzyme, are in direct contrast to the situation discussed earlier for the role of interactions at remote subsites, which were found to exert more influence on the rate of acylation. Together these results suggest that while the efficiency with which a substrate acylates HLE is determined in great measure by the availability of favorable interactions remote from the scissle bond, deacylation is more influenced by a proper fit of the P_1 side chain into the specificity pocket.

Another aspect of P_1 specificity concerns its disproportional expression in acylation and deacylation. It was noted earlier in this section that previously reported steady-state data suggest a difference in specificity between acylation and deacylation. This is supported by the data of Table I, where it is shown that the HLE-catalyzed hydrolyses of Z-Ala-ONP and Z-Val-ONP proceed with similar acylation rate constants but very different deacylation rate constants. This observation may not only hold for simple amino acid esters but may pertain to HLE catalysis in general. Values of k_3 and k_2/K_s for several P_1 Ala/Val pairs are collected in Table IV and demonstrate the difference in P_1 specificity for acylation and deacylation. Note that this comparison is only valid if substrates with Val at P_1 bind to HLE no tighter than the corresponding Ala analogues. Unfortunately, there are only a limited number of K_s values available for HLE-catalyzed reactions but these are summarized in Table IV and suggest that generally there is about a 2-fold difference between K_s for P_1 Val and P_1 Ala substrates. If this trend is carried over to other Ala/Val substrate pairs, the argument that acylation and deacylation have different P_1 specificity requirements is still valid.

We would, of course, also like to know how P_1 specificity is influenced by subsite interactions. Experimentally, this problem could be approached by determining kinetic parameters for the HLE-catalyzed hydrolyses of series of substrates related to those of Table I in which the N-terminal carbobenzoxy groups would be replaced by peptidic groups of increasing length. No systematic study of this sort has been conducted, but pertinent data collected in several laboratories are available and are summarized in Table IV. If we compare the four P_1 Ala/Val ester pairs, it appears that as the peptide chain is extended from Z-AA- to MeOSuc-Ala-Ala-Pro-AA, the role played by the P_1 residue in determining k_3 decreases. On the other hand, the data for k_2/K_s suggest that

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remote interactions may play a relatively minor role in determining P_1 specificity during acylation.

Rate-Limiting Step. Shown in Scheme I of the Introduction section is the minimal mechanism that describes reactions of serine proteases. According to this mechanism, initial formation of the Michaelis complex is followed by attack of the active-site serine on the carbonyl carbon of the substrate with formation of an acyl-enzyme intermediate and release of the first product. In the final stage of reaction, the acyl-enzyme undergoes hydrolysis to liberate free enzyme and second product. Although Scheme I adequately describes both the kinetic mechanism and the chemical transformations used by serine proteases, it fails to account for the rate enhancements observed during the hydrolyses of substrates that are able to interact with proteases at remote subsites.

In general, one can imagine two distinct strategies by which an enzyme can bring about this sort of rate enhancement: generalized stabilization of all transition states along the reaction pathway or stabilization of only select transition states. If the latter strategy is used, rate-limiting steps will be dependent upon structural features of the substrate and we would anticipate regular changes in the rate-limiting step to accompany rate enhancement as systematic structural variations are introduced into the substrate. On the other hand, if all transition states are stabilized equally, the same reaction step will limit the rate regardless of substrate structure. In this section, we will examine the substrate structural dependence of the rate-limiting step of k_c and k_c/K_m for the HLE-catalyzed hydrolyses of esters and anilides with a view to explaining how this enzyme takes advantage of remote interactions during catalysis.

It is generally believed that acylation rate-limits k_c during amide and anilide hydrolysis. This notion is well supported in the serine protease literature[36] and is in accord with a large body of chemical literature on nonenzymatic acyl-transfer reactions, which documents that nucleophilic attack of an alcohol on the carbonyl carbonyl of an amide to form an ester will have a much larger energy of activation than hydrolysis of that ester. Recently, however, Christensen and Ipsen suggested that the k_c may in fact be limited by deacylation for reactions of certain serine proteases with highly specific peptide *p*-nitroanilides.[37] This suggestion gained experimental validation in our laboratory with the demonstration of rate-limiting deacylation for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-*p*-NA[8] and partial rate-determining deacylation for MeOSuc-Ala-Ala-Pro-Ala-*p*-NA.[38]

These conclusions, however, are based on observations made during the steady state and therefore cannot constitute proof of rate-determining deacylation. The ability to dissect k_c into its constituent parameters and unambiguously demonstrate that acylation proceeds more rapidly than deacylation lies with pre-steady-state kinetic experiments of the sort reported here. The results of these experiments indicate that acylation of HLE by MeOSuc-Ala-Ala-Pro-Val-*p*-NA proceeds from the Michaelis complex with a first-order rate constant of 30 s^{-1}, while the hydrolysis of the acyl-enzyme formed from this reaction occurs with a rate constant of 23 s^{-1}. It appears that acylation and deacylation make similar contributions to the rate-determination of k_c. This is in contrast to the earlier steady-state studies which suggested that k_c is entirely rate-limited by deacylation (i.e., $k_2/k_3 > 10$). This discrepancy may be due to differences in reaction conditions or may simply be due to the greater experimental error of stopped-flow experiments. In any case, the results indicate that k_c for the HLE-catalyzed hydrolyses of specific anilides is at best only partially rate-limited by acylation.

For the HLE-catalyzed hydrolyses of less specific nitroanilides, acylation does entirely limit k_c. For example, Lesteine and Beith demonstrated rate-limiting acylation during the hydrolysis of Suc-$(Ala)_3$-*p*-NA.[29] Similarly, steady-state kinetics reported here for the hydrolyses of the *p*-nitroanilides and *p*-nitrophenyl esters of Ac-$(Ala)_3$ and Z-Val suggest that k_c for the hydrolyses of the anilides is again limited by acylation. This was confirmed for the reaction of Z-Val-*p*-NA by the determination of k_2 and k_3 (Table III).

Table V. Ratios of Acylation Rate Constants for the Hydrolyses of Corresponding *p*-Nitrophenyl Esters and *p*-Nitroanilides

catalyst		kinet param	k(ONP)/k(*p*-NA)	ref
HO^-	Ac	k_{II}[a]	1900	39, 40
CT	Ac-Tyr	k_2	121000	41
PPE	Ac-Ala-Pro-Ala	k_2	1000	12
HLE	Z-Val	k_2	15000	*b*
HLE	MeOSuc-Ala-Ala-Pro-Val	k_2	7	*b*
CT	Ac-Phe	k_c/K_m	130000	42, 43
PPE	Ac-Ala-Pro-Ala	k_c/K_m	1600	12
HLE	Z-Val	k_c/K_m	140000	*b*
HLE	Suc-Ala-Ala-Ala	k_c/K_m	2000	29
HLE	Ac-Ala-Ala-Ala	k_c/K_m	2700	*b*
HLE	MeOSuc-Ala-Ala-Pro-Val	k_c/K_m	22	*b*

[a] For both *p*-nitrophenyl acetate and *p*-nitroacetanilide, k_{II} represents the second-order rate constant for attack of hydroxide.[39,40] [b] This work.

Table VI. Solvent Isotope Effects for HLE-Catalyzed Reactions

	k_c/K_m, M^{-1} s^{-1}	^Dk_c	$^D(k_c/K_m)$	ref
Suc-Ala-Ala-Val-*p*-NA	4800	2.7	2.2	9
Z-Val-ONP	625000	2.4	2.0	*a*
MeOSuc-Ala-Ala-Pro-Val-*p*-NA	240000	3.3	1.4	9
MeOSuc-Ala-Ala-Pro-Val-ONP	6800000	3.2	1.1	*a*

[a] This work.

These results clearly indicate that the rate-limiting step of k_c is dependent on the availability of subsite interactions. Substrates, such as MeOSuc-Ala-Ala-Pro-Val-*p*-NA, which offer opportunities for extensive subsite contacts with HLE, will acylate the enzyme with great facility. Deacylation, which is not so dependent on remote interactions, will experience little rate enhancement and will be rate-limiting. On the other hand, substrates which are truncated and provide fewer remote contacts will acylate HLE more sluggishly and will be hydrolyzed with rate-determining acylation.

It is generally true that k_c/K_m provides information concerning the transition state for the chemical steps of acylation. In relatively rare instances of very reactive substrates where acylation proceeds faster than dissociation of the Michaelis complex, k_c/K_m will be equal to k_1 and will instead provide information about substrate binding. While these statements may hold for many serine protease-catalyzed reactions, the results presented in this report suggest that the situation is not so straightforward for the HLE-catalyzed hydrolyses of specific substrates. For these reactions, it appears that the transition state for k_c/K_m corresponds to neither binding nor the chemical steps of acylation but rather reflects some physical step that occurs after formation of the Michaelis complex but prior to acylation. As detailed below, this conclusion is based on two sets of observations: (i) relative values of k_2 or k_c/K_m for serine protease-catalyzed hydrolyses of corresponding *p*-nitroanilides and *p*-nitrophenyl esters and (ii) solvent isotope effects on HLE-catalyzed reactions.

It is well documented that rates of serine protease acylation, as reflected in either k_2 or k_c/K_m, are much faster for *p*-nitrophenyl esters than for the corresponding *p*-nitroanilides.[11,29,39-43] A sampling of the available data, expressed as ratios of rate constants

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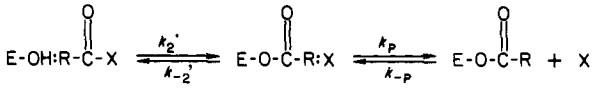
Scheme II



for the anilide and ester, is shown in Table V and serves to demonstrate this point. Significantly, for the reaction of HLE with the anilide and ester derived from MeOSuc-Ala-Ala-Pro-Val, the ratios for both k_2 and k_c/K_m are extremely small. Such small reactivity ratios are not consistent with enzyme acylation being the sole rate-determining step but rather suggest that acylation only partially rate-limits the processes governed by k_2 and k_c/K_m. Some reaction step that is insensitive to the electronic nature of the leaving group must also be partially rate-limiting.

The suggestion that k_2 and k_c/K_m are only partially rate-limited by acylation is also supported by a comparison of solvent isotope effects for HLE-catalyzed hydrolyses of several substrates (Table VI). For all the substrates examined, the solvent isotope effect on k_c (^Dk_c) is large, falling between 2.4 and 3.4. These isotope effects are similar to values of ^Dk_c determined for other serine protease-catalyzed reactions and suggest rate-determining general acid/general base assisted acylation or deacylation. Similarly, $^D(k_c/K_m)$ values for the HLE-catalyzed hydrolysis of Suc-Ala-Ala-Val-*p*-NA and Z-Val-ONP are also large, again consistent with the involvement of some form of protolytic catalysis in rate-limiting transition states.

In contrast are isotope effects of 1.4 and 1.1 on k_c/K_m for the HLE-catalyzed hydrolyses of MeOSuc-Ala-Ala-Pro-Val-*p*-NA and MeOSuc-Ala-Ala-Pro-Val-ONP, respectively. These isotope effects are consistent with a mechanism[9] in which the process governed by k_c/K_m has at least two sequential reaction steps: a step that is relatively insensitive to the isotopic composition of the solvent and enzyme acylation.[44]

We now consider what step, in addition to acylation, limits k_c/K_m. Possible rate-determining steps that are insensitive to both the electronic nature of the leaving group and solvent isotopic composition are (i) binding of substrate (included in k_1), (ii) conformational changes of free enzyme or some enzyme–substrate complex (included in k_1 or k_2, respectively), and, (iii) release of first product (included in k_2). Although it is not possible at the present time to unambiguously demonstrate which of these steps limits k_c/K_m, the available data tend to exclude substrate binding and product release.

We can exclude substrate binding and other reaction steps contained in k_1 as rate-limiting because the reactivity ratio for hydrolysis of the anilide and ester of MeOSuc-Ala-Ala-Pro-Val is unusually small not only for k_c/K_m but also for k_2 (Table V). If some reaction steps included in k_1 were rate-limiting, the reactivity ratio for k_c/K_m would be small but that for k_2 would be large, similar to the other k_2–reactivity ratios of Table V.

For product release to be rate-limiting, it must occur slower than the reaction of product with the acyl-enzyme. According to Scheme II, k_p must be less than k_{-2}'. This is difficult to imagine for X equal to *p*-nitroaniline or *p*-nitrophenolate. These leaving groups are not likely to form specific interactions with HLE at S_1' and are not nucleophilic. Together these conditions are consistent with k_{-2}' being very much smaller than k_p and thus favor rapid release of X from E–O–C(O)–R:X.

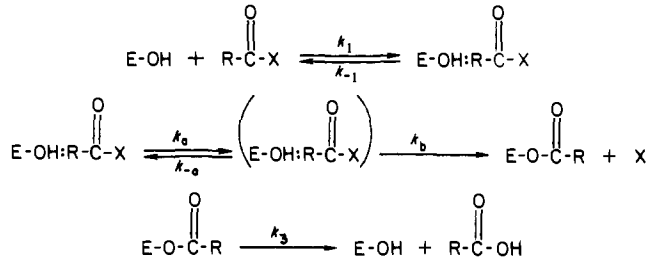
It is clear from the previous discussion that the mechanism of Scheme I cannot fully account for HLE-catalyzed reactions. To accommodate these new findings, the mechanism of Scheme III is proposed.

Equations 1–4 still hold but now

$$k_2 = k_a k_b/(k_{-a} + k_b + k_a) \qquad (15)$$

(44) An alternate explanation for the very small values of $^D(k_c/K_m)$ observed for these substrates is that the acylation transition state is in fact fully expressed in k_c/K_m but is simply characterized by an unusually small degree of proton reorganization. This would be expected, for instance, if the transition states for acylation were exceptionally early. While this possibility cannot be ruled out at the present time, the view invoking partially rate-limiting acylation seems more likely, especially in light of the small differences in k_c/K_m between the anilide and ester.

Scheme III



According to Scheme III, the Michaelis complex, E–OH:R–C(=O)–X, reversibly forms a second noncovalent complex, {E–OH:R–C(=O)–X}′, through reaction steps governed by k_a and k_{-a}. It is through reactions occurring within this latter complex that the acyl-enzyme, E–O–C(=O)–R, is formed. These reaction steps are governed by k_b and at least for anilides probably include formation and decomposition of a tetrahedral addition complex.

The substrate structural dependence of the rate-limiting step in k_2, and thus k_c/K_m, can now be explained as follows. When substrates possess certain structural features, transition states for the chemical steps of acylation (k_b) are stabilized relative to the physical step (k_a). If sufficient stabilization occurs, k_b will be very fast compared to both k_{-a} and k_a, and k_2 will be rate-limited by the nonchemical physical step k_a (see eq 15). MeOSuc-Ala-Ala-Pro-Val evidently possesses the required structural features. As judged by similar acylation rates and values of $^Dk_c/K_m$ near unity, the *p*-nitroanilide and *p*-nitrophenyl ester of this peptide react with HLE with at least partially rate-limiting k_a. On the other hand, when the substrate does not have these structural requirements, transition states for the chemical step of k_2 are of high energy relative to the physical step. In these situations, k_b is less than both k_{-a} and k_a, and the chemical step, k_b, is rate-limiting. For reaction of these substrates, values k_2 and k_c/K_m will be greatly different for corresponding *p*-nitrophenyl esters and *p*-nitroanilides, and large solvent isotope effects on k_c/K_m will be observed.

Charge-Relay Catalysis. X-ray diffraction studies of serine proteases[15,16,45] have demonstrated the existence of an active site structural unit composed of Asp-102, His-57, and Ser-195 (chymotrypsin numbering). In these structures, N^2 of His-57 is within hydrogen-bonding distance of the Ser-195 hydroxyl while Asp-102, buried in a hydrophobic pocket, is within hydrogen-bonding distance of N^1 of His-57.[3-5] Such an arrangement has led several investigators to speculate upon the possible catalytic role this ensemble might play. It has been suggested that Asp-102 and His-57 may operate through some form of protolytic catalysis involving the concerted transfer of protons to promote first the attack of serine on the carbonyl carbon of substrate molecules and then the hydrolysis of the acyl-enzyme. Due to its presumed catalytic activity, this structural unit has become known as the "charge-relay system"[45] or, more recently, the "catalytic triad".[15]

The static pictures provided by X-ray diffraction studies cannot prove or disprove any catalytic function possessed by these residues. Although the results of chemical modification and pH-rate studies suggest that Ser-195 is acylated during catalysis and that His-57 acts as a general catalyst, it was not until the "proton inventory" technique[29] was applied to serine proteases that evidence was available, supporting the view that Asp-102 and His-57 act concertedly as protolytic catalysts.

Proton inventories have now been determined for hydrolytic reactions catalyzed by several serine proteases[9,11-14] and suggest that "minimal" substrates hydrolyze via a mechanism involving one-proton catalysis and involvement of only the active-site His, while "specific" substrates hydrolyze with two-proton catalysis and full functioning of the charge-relay system. The present study is entirely consistent with this view. The proton inventory of k_c reported here for the HLE-catalyzed hydrolysis of Z-Val-ONP

(45) Blow, D. M.; Birktoft, J. J.; Hartley, B. S. *Nature (London)* **1970**, *221*, 337–339.

is linear and indicates that a single proton is transferred in the rate-limiting transition state. In contrast is the curved proton inventory of k_c for the HLE-catalyzed hydrolysis of the specific substrate MeOSuc-Ala-Ala-Pro-Val-*p*-NA[9] which indicates two-proton catalysis. For both reactions, k_c is rate-limited by deacylation.[46]

Curiously, despite this significant difference in catalytic mechanism, the deacylation rates for Z-Val-HLE and MeO-Suc-Ala-Ala-Pro-Val-HLE differ by only a factor of 6. If the function of the charge-relay system is to enhance catalytic efficiency by stabilizing transition states for acyl-transfer to and from the active-site serine, we might have expected a more dramatic rate enhancement. For deacylation, it appears that the charge-relay system makes only a minor contribution to determining overall catalytic efficiency. The situation for acylation may be quite different. As discussed earlier, remote interactions between enzyme and substrate have a profound influence on rates of acylation, and it may well be that the enhanced rates observed for peptide-derived substrates originate at least in part from charge-relay catalysis.

Summary—A Mechanism of Action for Human Leukocyte Elastase. In this paper, I have tried to demonstrate the mechanistic impact resulting from remote subsite interactions between HLE and peptide substrates. My discussion has focused on individual mechanistic features and how they are influenced by these remote interactions. Specifically, four features were considered and shown to be dependent on subsite interactions: catalytic efficiency, P_1 specificity, rate-limiting step, and charge-relay catalysis. In the remainder of this paper, I will try to reassemble and integrate these features and what we know of their dependence on remote interactions into a single, unified mechanism of action for HLE.

The first stage of reactions catalyzed by HLE involves the noncovalent and reversible binding of substrate at the active-site to form the Michaelis complex. This process displays rather little specificity for substrate structure and therefore suggests that few important contacts between enzyme and substrate are established here. The transition state for binding is of lower energy relative to subsequent reactions steps and is characterized by significant solvent reorganization.[47]

Reaction of the Michaelis complex to form the acyl-enzyme is a multistep process consisting of an initial physical step, possibly a conformational change of E:S, followed by the actual chemical steps of serine acylation, probably involving the intermediacy of a tetrahedral addition adduct. The activation energy of the physical step is relatively low and displays little dependence on substrate structure. The energy barrier for serine acylation, on the other hand, is quite sensitive to substrate structure: for minimal substrates that make few subsite contacts with HLE, this barrier is high, while for more specific substrates the barrier for acylation is lower and in some cases similar in magnitude to that of the physical step. The stabilization of the transition states for serine acylation presumably results from the ability of HLE to utilize the free energy released as remote subsite contacts are established with specific polypeptide substrates.[48] As we have seen, the stabilization of acylation transition states for specific substrates can be sufficient to cause this step to be partially rate-limiting with the physical step.

Among the mechanisms used by HLE for transition-state stabilization is charge-relay catalysis. The molecular machinery of the charge-relay system is operative and works to stabilize the transition states of acyl transfer only for those peptide-based substrates that fulfill exacting transition-state structural requirements imposed by HLE. While we have clearly achieved some understanding of the circumstances under which the charge-relay system becomes functional,[9,11-14] it is not yet clear how the enzyme translates the physical event of a substrate binding at its subsites into a signal that calls this system into operation.

As mentioned above, the dramatic stabilization of acylation transition states enjoyed by specific substrates will not be shared by substrates lacking critical structural determinants. These minimal substrates will be unable to establish remote contacts with HLE and therefore will not be able to enlist the charge-relay system or other mechanisms of transition state stabilization that are operative for more specific substrates. For these minimal substrates, chemical steps of acyl-enzyme formation will entirely rate-limit their reaction with HLE.

The role played by subsite interactions during hydrolysis of the acyl-enzyme is not altogether clear. Several observations are disturbing. Despite the involvement of charge-relay catalysis during the hydrolysis of acyl-enzymes derived from specific substrates, the rate enhancement for these reactions over deacylation of minimal substrates is quite small and calls into question the catalytic function of the charge-relay system during deacylation. Also perplexing is the P_1 specificity observed for deacylation. Recall that for acylation, Val is preferred over Ala at P_1 for all substrate types. This is in marked contrast to deacylation for which Ala is the preferred residue. Furthermore, this discrimination between Val and Ala during deacylation *decreases* as elements of peptide structure are introduced into the substrate.

Despite the mechanistic elusiveness of deacylation, an important point does emerge. Rates of deacylation are, in general, not responsive to substrate structure. This observation, coupled with the keen sensitivity of acylation rates to substrate structure, explains the change from rate-limiting acylation to deacylation that k_c experiences as *p*-nitroanilide substrate structure is made more specific.

The mechanistic picture presented here for human leukocyte elastase is still incomplete. Problems that remain to be resolved include (i) the catalytic role played by the charge-relay system during deacylation, (ii) dependence of P_1 specificity on remote subsite interactions, (iii) the elucidation of P′ specificity, and (iv) dependence of mechanistic features on substrate binding at S′ subsites.

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(46) k_c for the hydrolysis of the peptide anilide is only partially rate-limited by k_3. However, keeping in mind that (i) the major contributor to determining the properties of the virtual transition state of k_c is k_3 and (ii) k_2 is itself associated with a virtual transition state involving little proton transfer, we can conclude that the two-proton catalysis indicated by the proton inventory for MeOSuc-Ala-Ala-Pro-Val-*p*-NA reliably reflect the hydrolysis of the acyl-enzyme, MeOSuc-Ala-Ala-Pro-Val-HLE. This conclusion is supported by recent unpublished data of Stein and Powers for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-thiobenzyl ester. k_c for this reaction is equal to k_3, and the proton inventory for this reaction is consistent with charge-relay catalysis.

(47) Stein, R. L. *J. Am. Chem. Soc.*, in press.

(48) Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 219-410.