# Catalysis by Human Leukocyte Elastase: Substrate Structural Dependence of Rate-Limiting Protolytic Catalysis and Operation of the Charge Relay System

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**Abstract:** The catalytic mechanism of human leukocyte elastase (HLE) has been probed by the determination of solvent isotope effects (calculated as the ratio of rate constants $k_{H_2O}/k_{D_2O}$) for the hydrolysis of three substrates of varying reactivity toward HLE: *N*-succinylalanylalanylalanine *p*-nitroanilide (I; $k_c/K_m$ = 450 $M^{-1}$ $s^{-1}$), *N*-succinylalanylalanylvaline *p*-nitroanilide (II; $k_c/K_m$ = 4800 $M^{-1}$ $s^{-1}$), and *N*-methoxysuccinylalanylalanylprolylvaline *p*-nitroanilide (III; $k_c/K_m$ = 185 000 $M^{-1}$ $s^{-1}$). For all three substrates, the isotope effect on $k_c$ is near 3 and suggests that the rate-limiting step involves proton transfer. $k_c$ [$=(k_2k_3)/(k_2 + k_3)$] is a complex first-order rate constant reflecting both the conversion of enzyme–substrate complex to acylated enzyme ($k_2$) and the subsequent hydrolysis of this intermediate to free enzyme and product ($k_3$). The observed isotope effects on $k_c$ for the three substrates are consistent with a mechanism for transition-state stabilization involving some form of general-acid/general-base, or protolytic, catalysis. For substrates I and II the isotope effect on $k_c/K_m$ is again large and near 2.5. $k_c/K_m$ is the second-order rate constant for reaction of free enzyme and substrate and can reflect transition-state properties of all steps up to and including the release of the first product, *p*-nitroaniline. The solvent isotope effects on this process for I and II suggest a transition state involving proton transfer and are consistent with rate-limiting protolytically catalyzed acylation. In contrast, the isotope effect on $k_c/K_m$ for the specific substrate III is small, equal to 1.4, and suggests that acylation is now only *partially* rate limiting. The transition state we observe here is a "virtual" transition state, a composite having a structural feature of the several rate-limiting transition states. The small observed isotope effect originates from dilution of a large isotope effect for acylation by a near unit isotope effect for the other partially rate-limiting step(s). Proton inventories (rate measurements in mixtures of $H_2O$ and $D_2O$) for the reactions of substrates I and II gave linear dependences of reaction velocity on mole fraction of solvent deuterium, *n*, and suggest that the isotope effects observed for these reactions originate from fractionation at a single hydrogenic site in the catalytic transition state. Presumably this site is the proton bridge between the active-site serine hydroxyl and histidine imidazole. For the hydrolysis of III by HLE a quadratic dependence of reaction rate on *n* was observed, suggesting that the isotope effect seen for this reaction originates from coupled transfer of *two* protons in the catalytic transition state and implying the involvement of a general-acid/general-base functionality beyond the active site histidine. This observation is consistent with a mechanism for HLE in which specific substrates such as III can fully engage the catalytic machinery of the charge-relay system.

The serine proteases are mechanistically among the best characterized enzymes.[1-3] The hydrolytic reactions they catalyze follow the mechanism of Scheme I involving the reversible formation of an encounter complex (E—OH·R—C(=O)—X) followed by the production and subsequent hydrolysis of an acyl-enzyme intermediate (E—O—C(=O)—R). The steady-state kinetic parameters measured at [E] ≫ [S] are expressed in terms of their constituent microscopic rate constants in eq 1-4. For

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

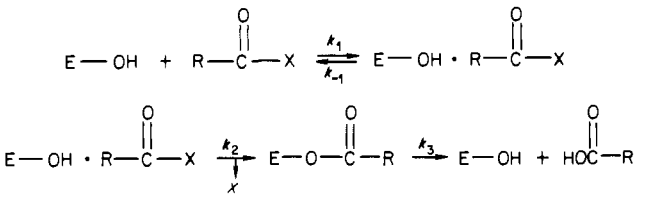
$$K_m = K_sk_3/(k_2 + k_3) \quad (2)$$

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

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

amides and anilides it is generally true that acylation ($k_2$) proceeds much slower than deacylation ($k_3$) and thus, $k_c = k_2$, while for esters acylation is more rapid and $k_c = k_3$. In certain limiting cases $K_m$ can also take on a simpler form. If we assume that formation of the initial encounter complex is at thermodynamic equilibrium during the steady state (i.e., $k_{-1} \gg k_2$), then for amides $K_m$ becomes a dissociation constant equal to $k_{-1}/k_1$, and for esters $K_m = (k_{-1}/k_1)(k_3/k_2)$. Still assuming $k_{-1} \gg k_2$, the second-order rate constant $k_c/K_m$ becomes $k_2/(k_1/k_{-1})$ and reflects enzyme acylation.

Scheme I



Similarities among the serine proteases may extend beyond the intermediacy of an acyl-enzyme to mechanisms for transition-state stabilization.[1,2] Indeed, the hallmark of these enzymes is the so-called "charge-relay system", an array of active-site amino acid residues (Asp-102, His-57, Ser-195; chymotrypsin numbering) whose structure appears to be conserved among all serine proteases and which presumably functions to enhance the nucleophilicity of Ser-195 during acylation and water during deacylation through some form of general-acid/general-base catalysis. Much of our understanding of the mechanism of serine proteases has come from study of the pancreatic enzymes. Recently, however, interest in proteases of cellular origin[4-6] has increased due to discoveries of their involvement in the control and regulation of many important physiologic functions[5,6] and their implication in the pathogenesis of several connective tissue disorders.[5,7] It is thought, for example,

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Table I. pL Dependence of the Reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-*p*NA[a]

| | | rate constant | $pK_a$ |
|---|---|---|---|
| $(k_c)_{lim}$ | $H_2O$ | 13.6 ± 0.3 $s^{-1}$ | 6.14 ± 0.05 |
| | $D_2O$ | 4.1 ± 0.1 $s^{-1}$ | 6.51 ± 0.07 |
| | | $^{D}(k_c)_{lim}$ = 3.3 ± 0.1 | $\Delta pK_a$ = 0.37 ± 0.01 |
| $(k_c/K_m)_{lim}$ | $H_2O$ | 295000 ± 5300 $M^{-1}$ $s^{-1}$ | 7.18 ± 0.04 |
| | $D_2O$ | 182000 ± 2000 $M^{-1}$ $s^{-1}$ | 7.70 ± 0.03 |
| | | $^{D}(k_c/K_m)_{lim}$ = 1.62 ± 0.03 | $\Delta pK_a$ = 0.52 ± 0.01 |

[a] Kinetics determined in $H_2O$ or $D_2O$ solutions containing 50 mM succinate/50 mM Tricine, 500 mM NaCl, and 3.3% $Me_2SO$ at 25.1 ± 0.05 °C.

that leukocyte elastase is the principle destructive agent in the development of pulmonary emphysema,[7,8] a disease characterized by destruction of lung connective tissue. Inhibition of human leukocyte elastase (HLE) has been recognized for some time as an appealing strategy for pharmacologic intervention in emphysema. Although the design of potent, selective inhibitors of HLE would be facilitated by a detailed understanding of its catalytic mechanism, many features of mechanism are unclear. For example, it is not known if leukocyte elastase uses general-acid/general-base catalysis, possibly involving a "charge-relay" system, to stabilize transition states for ester and amide hydrolysis. Should such catalytic mechamisms be found to operate in HLE the elucidation of the substrate structural dependence of their involvement in rate-limiting processes becomes essential.

These problems have been addressed here by the determination of solvent isotope effects and proton inventories for the hydrolysis of three peptide *p*-nitroanilide substrates: *N*-succinylalanylalanylalanine *p*-nitroanalide[9] (Suc-Ala-Ala-Ala-*p*NA; $k_c/K_m$ = 450 $M^{-1}$ $s^{-1}$), *N*-succinylalanylalanylvaline *p*-nitroanalide[10] (Suc-Ala-Ala-Val-*p*NA; $k_c/K_m$ = 4800 $M^{-1}$ $s^{-1}$), and *N*-methoxysuccinylalanylalanylprolylvaline *p*-nitroanalide[11] (MeOSuc-Ala-Ala-Pro-Val-*p*NA; $k_c/K_m$ = 185 000 $M^{-1}$ $s^{-1}$). These substrates fulfill to a greater or less extent structural requirements set by HLE for efficient catalysis. Kinetic solvent isotope effects were determined to gain an understanding of the underlying mechanistic basis for these reactivity differences.

In general, kinetic isotopic effects have proven to be especially useful in enzyme mechanistic studies.[12-14] Unlike other mechanistic probes, isotopic substitution does not change the potential energy surface of the reaction and thus allows a single unperturbed reaction mechanism and transition state to be studied. For investigation of serine proteases, the solvent isotope effect should provide an additional advantage in being able to observe and report on the proton transfers accompanying protolytically catalyzed reaction steps. An extension of the solvent isotope effect used in this study is the proton inventory.[15] These experiments involve the measurement of reaction rates in mixtures of light and heavy water and are used to estimate the number of protonic sites generating the isotope effect and the magnitude of the isotope effects produced at each site. We see then that this technique may be useful as an indicator of charge-relay catalysis where its operation should be manifested in proton inventories reflecting isotope effects originating at two protonic sites. It was hoped that the application of these isotopic probes to several HLE-catalyzed reactions would provide insight into mechanistic features dependent on substrate structure.

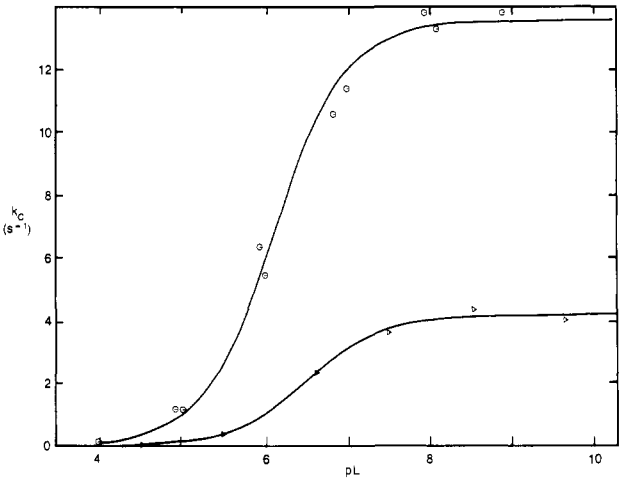


**Figure 1.** Dependence of $k_c$ on pL (pH in $H_2O$ upper curve; pD in $D_2O$, lower curve) for the reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-*p*NA. 50 mM succinate/50 mM Tricine, 500 mM NaCl, 3.3% $Me_2SO$, 25.1 ± 0.05 °C.

## Experimental Section

**Materials.** Human leukocyte elastase was purified from purulent sputum as described previously.[16] MeOSuc-Ala-Ala-Pro-Val-*p*NA was synthesized by R. Wildonger of the Department of Medicinal Chemistry, ICI Americas. Suc-$(Ala)_3$-*p*NA, Suc-Ala-Ala-Val-*p*NA, deuterium oxide (99%), and buffer salts were obtained from Sigma Chemical Co., St. Louis, MO. $Me_2SO$ (Gold Label) Analytical Grade) was from Aldrich.

**Buffer Solutions.** Buffers for the pH/pD studies were prepared by titrating $H_2O$ or $D_2O$ solutions containing 50 mM succinic acid, 50 mM Tricine (*N*-[*tris*(hydroxymethyl)methyl]glycine), and 500 mM NaCl to the desired pH or pD with 1 N NaOH in $H_2O$ or $D_2O$. pD values were calculated from the relation pD = meter reading + 0.4.

All other kinetic runs were conducted in solutions of 0.100 M sodium phosphate, 0.50 M NaCl, and pH 7.37 or equivalent pD.[15] Solutions in $H_2O$ and $D_2O$ having the same buffer ratio[15] were prepared by diluting to the same volume, with either $H_2O$ or $D_2O$, identical amounts of mono- and dihydrogen phosphate and NaCl. Solutions having different mole fractions of deuterium oxide were prepared gravimetrically from the stock $H_2O$ and $D_2O$ buffer solutions.

**Kinetic Procedures.** All reaction rates were measured spectrophotometrically by monitoring the release of 4-nitroaniline at 410 nm. In a typical experiment, a cuvette containing buffer and 100 μL of the appropriate dilution of a substrate stock solution (final [$Me_2SO$] = 3.3%) was brought to thermal equilibrium (20 min) in a jacketed holder in the cell compartment of a Cary 210 spectrophotometer. The temperature was maintained 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, averaged, and stored in an Apple II mini-computer. The initial velocities were fit to a linear dependence on time by linear least squares.

## Results

**pH and pD Rate Profiles.** Kinetic parameters at several values of pH and pD were determined for the reaction of HLE and MeOSuc-Ala-Ala-Pro-Val-*p*NA by nonlinear least-squares fit of

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Table II. Kinetic Parameters[a,b] of Reactions of Human Leukocyte Elastase in $H_2O$ and $D_2O$

| substrate | | $k_c$, $s^{-1}$ | $K_m$, mM | $k_c/K_m$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|---|
| Suc-Ala-Ala-Ala-*p*Na | $H_2O$ | 0.61 ± 0.01 | 1.36 ± 0.06 | 449 ± 21 |
| | $D_2O$ | 0.200 ± 0.004 | 1.13 ± 0.05 | 177 ± 10 |
| Suc-Ala-Ala-Val-*p*Na | $H_2O$ | 3.71 ± 0.18 | 0.77 ± 0.04 | 4810 ± 235 |
| | $D_2O$ | 0.68 ± 0.03 | 0.62 ± 0.04 | 2200 ± 92 |
| MeOSuc-Ala-Ala-Pro-Val-*p*Na | $H_2O$ | 10.0 ± 0.2 | 0.054 ± 0.004 | 185000 ± 14000 |
| | $D_2O$ | 2.96 ± 0.08 | 0.022 ± 0.003 | 135000 ± 16000 |

[a] Experiments conducted in 0.100 M phosphate, 0.50 M NaCl, pH 7.37 and equivalent pD, 3.3% $Me_2SO$ at 25.0 ± 0.1 °C. [b] Kinetic parameters determined by nonlinear least-squares fit of initial velocity data to the Michaelis–Menton equation. Error limits are standard deviations derived from the nonlinear fit.

Table III. Substrate Concentration Dependence of the Solvent Isotope Effect for Reactions of Human Leckocyte Elastase[a]

| substrate | substrate concentration [isotope effect][b] |
|---|---|
| Suc-Ala-Ala-Ala-*p*NA | 429 [2.69 ± 0.07], 600 [2.80 ± 0.08], 1000 [2.83 ± 0.06], 3000 [2.97 ± 0.04] |
| Suc-Ala-Ala-Val-*p*NA | 67 [2.22 ± 0.05], 380 [2.36 ± 0.08], 760 [2.48 ± 0.06], 6070 [2.64 ± 0.11] |
| MeOSuc-Ala-Ala-Pro-Val-*p*NA | 20.5 [1.96 ± 0.05], 50.6 [2.34 ± 0.04], 84.3 [2.49 ± 0.12], 253 [3.00 ± 0.09], 410 [3.14 ± 0.09] |

[a] Kinetics were determined in 0.100 M sodium phosphate, 0.50 M NaCl, pH 7.37 and equivalent pD, and 3.3% $Me_2SO$ at 25.00 ± 0.05 °C. [b] Substrate concentrations are expressed as $10^{-6}$ M.

Table IV. Solvent Isotope Effects for Reactions of Human Leukocyte Elastase[a]

| substrate | $^D(k_c)$ | $^D(K_m)$ | $^D(k_c/K_m)$ | $\phi_{T1}$ | $\phi_{T2}$ |
|---|---|---|---|---|---|
| Suc-Ala-Ala-Ala-*p*NA[e] | 3.15 ± 0.07 | 1.22 ± 0.04 | 2.59 ± 0.05 | 0.34[b] | 1.00[b] |
| Suc-Ala-Ala-Val-*p*NA[f] | 2.71 ± 0.04 | 1.24 ± 0.03 | 2.19 ± 0.04 | 0.45[c] | 1.00[c] |
| MeOSuc-Ala-Ala-Pro-Val-*p*NA[g] | 3.34 ± 0.05 | 2.34 ± 0.11 | 1.43 ± 0.07 | 0.55[d] | 0.55[d] |

[a] Isotope effects on kinetic parameters were determined by nonlinear least-square fit of $(v_0)^D$ vs. $[S]_0$ to eq 1. [b] Calculated from proton inventory data with $[S]_0 = 3K_m$. [c] Calculated from proton inventory data with $[S]_0 = 0.05K_m$. [d] Calculated from proton inventory data with $[S]_0 = 10K_m$. [e] [HLE] = $1.1 \times 10^{-7}$ M. [f] [HLE] = $4.0 \times 10^{-8}$ M. [g] [HLE] = $1.0 \times 10^{-8}$ M.

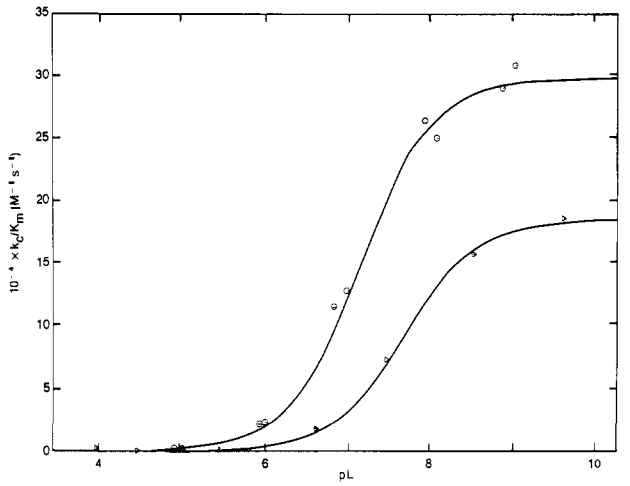


**Figure 2.** Dependence of $k_c/K_m$ on pL (pH in $H_2O$, upper curve; pD in $D_2O$ lower curve) for the reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-*p*NA. 50 mM succinate/50 mM Tricine, 500 mM NaCl, 25.1 ± 0.05 °C.

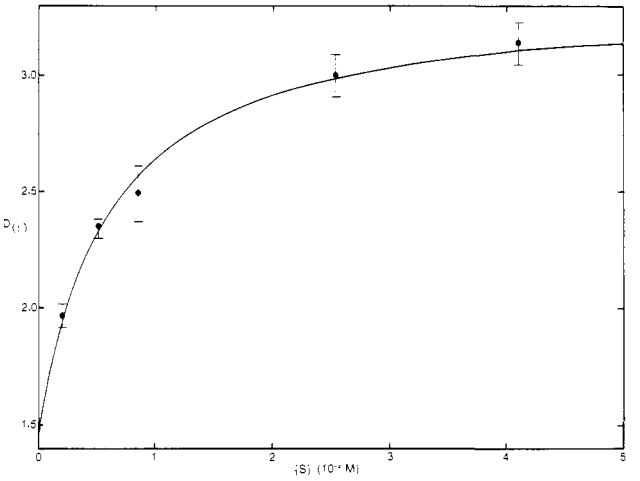


**Figure 3.** Plot of the dependence of the observed solvent isotope effect on substrate concentration for the HLE-catalyzed hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-*p*NA. Solid line is best-fit curve according to eq 6 with $^D(k_c) = 3.34$, $^D(k_c/K_m) = 1.43$, and $K_m = 5.3 \times 10^{-5}$ M.

initial velocity data to the Michaelis–Menton equation. The dependence of $k_c$ and $k_c/K_m$ on pL (Figures 1 and 2, respectively) can be fit to the expression of eq 5, where $k_o$ is the observed rate

$$k_o = k_{lim}[K_a/(K_a + a_H)] \quad (5)$$

constant, $k_{lim}$ is the limiting value in basic solution, $K_a$ is an apparent ionization constant, and $a_H$ is the hydrogen ion activity expressed in molar units, for both $H_2O$ and $D_2O$. Values of $(k_c)_{lim}$, $(k_c/K_m)_{lim}$, and $pK_a$ determined by nonlinear least-squares fit of the experimental data to eq 5 appear in Table I together with the isotope effects and $pK_a$ values calculated from these parameters.

The $pK_a$ values of 7.18 ± 0.04 ($H_2O$) and 7.70 ± 0.03 ($D_2O$) determined from the dependence of $k_c/K_m$ on pL are for ionization of free enzyme.[2] A $\Delta pK_a$ of 0.52 suggests that this enzyme behaves as a "normal acid". Interestingly, $pK_a$ values determined from the dependence of $k_c$ on pL are a full pH unit smaller than those for $k_c/K_m$, and furthermore, $\Delta pK_a$ for $k_c$ is only 0.37. These results are consistent with several mechanistic interpretations including a pH-dependent change in rate-determining step for $k_c$.[2,28] This aspect of HLE catalysis is currently being investigated in this laboratory. Isotope effects determined from the limiting values of the kinetic parameters agree with more accurate isotope effects determined at a single pH and pD equivalent (see below).

**Solvent Isotope Effects.** Kinetic parameters for the hydrolysis of Suc-Ala-Ala-Ala-*p*NA, Suc-Ala-Ala-Val-*p*NA, and MeO-Suc-Ala-Ala-Pro-Val-*p*NA in $H_2O$ and $D_2O$ appear in Table II and were determined from nonlinear least-squares fit of initial velocity data to the Michaelis–Menton equation. Double-reciprocal plots of the data were linear in all cases.

Isotope effects on $k_c$ and $k_c/K_m$ [expressed as $^D(k_c)$ and $^D(k_c/K_m)$, respectively] were determined by a nonlinear least-squares fit of the experimental data (Table III) consisting of values of observed isotope effects, $^D(v_o)$, and corresponding substrate concentrations, [S], to eq 6. This procedure can be seen to provide

$$^D(v_o) = [^D(k_c) - ^D(k_c/K_m)]\frac{[S]}{[S] + K_m} + ^D(k_c/K_m) \quad (6)$$

*direct* estimates of $^D(k_c)$ and $^D(k_c/K_m)$. As discussed elsewhere,[17,18]

Table V. Proton Inventories of Reactions of Human Leukocyte Elastase

| reaction and conditions | mole fraction of deuterium [$10^2 v_n$, OD/min] |
|---|---|
| Suc-Ala-Ala-Ala-*p*NA, $3.0 \times 10^{-3}$ M; HLE, $1.1 \times 10^{-7}$ M; 0.100 M sodium phosphate; 0.50 M NaCl; 3.3% $Me_2SO$; pH 7.37, equiv pD, 24.6 ± 0.05 °C | 0.00 [1.96, 1.98, 1.98, 2.03]; 0.19 [1.75, 1.77]; 0.24 [1.68]; 0.39 [1.46, 1.50]; 0.49 [1.32, 1.29]; 0.58 [1.23, 1.22]; 0.73 [1.00]; 0.78 [0.970, 0.953]; 0.97 [0.674, 0.685, 0.674, 0.703] |
| Suc-Ala-Ala-Val-*p*NA, $3.8 \times 10^{-5}$ M; HLE, $4.0 \times 10^{-8}$ M; 0.100 M sodium phosphate; 0.50 M NaCl; 3.3% $Me_2SO$; pH 7.45, equiv pD; 25.2 ± 0.05 °C | 0.00 [2.44, 2.49]; 0.19 [2.23, 2.18]; 0.39 [2.00, 1.97]; 0.58 [1.66, 1.66]; 0.78 [1.47, 1.42]; 0.97 [1.11, 1.16] |
| MeOSuc-Ala-Ala-Pro-Val-*p*NA, $6.0 \times 10^{-4}$ M; HLE, $1.0 \times 10^{-8}$ M; 0.100 M sodium phosphate; 0.50 M NaCl; 3.3% $Me_2SO$; pH 7.37, equiv pD 24.8 ± 0.05 °C | 0.00 [5.60, 5.74]; 0.19 [4.78, 4.67]; 0.39 [3.78, 3.58]; 0.58 [3.06, 2.93]; 0.78 [2.34, 2.36]; 0.97 [1.72, 1.82] |

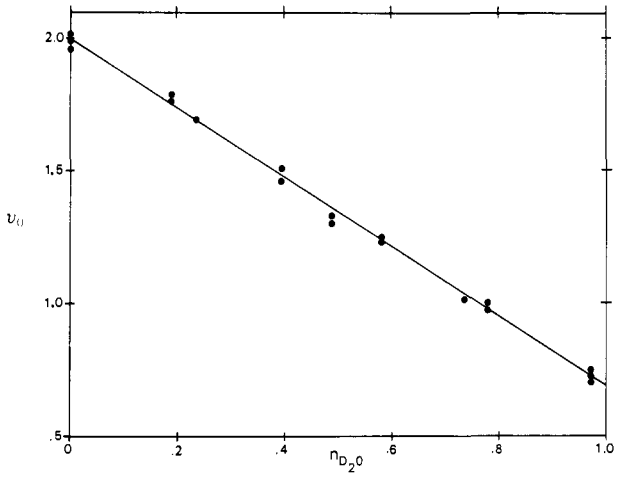


**Figure 4.** Proton inventory of the HLE-catalyzed hydrolysis of Suc-Ala-Ala-Ala-*p*NA. Initial velocities ($10^2 \times v_0$ OD/min) were determined at a substrate concentration of $3.00 \times 10^{-3}$ M ($3K_m$). The solid line was calculated by least-squares fit and is described by $v_0 = 2.00 \times 10^{-2}$ OD/min $(1 - n + n/2.94)$.

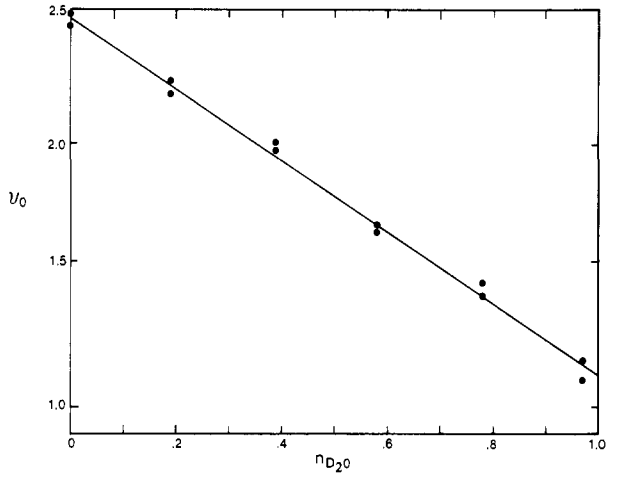


**Figure 5.** Proton inventory of the HLE-catalyzed hydrolysis of Suc-Ala-Ala-Val-*p*NA. Initial velocities ($10^2 \times v_0$ OD/min) were determined at a substrate concentration of $3.8 \times 10^{-5}$ M ($0.05K_m$). The solid line was calculated by least-squares fit and is described by $v_0 = 2.47 \times 10^{-2}$ OD/min $(1 - n + n/2.11)$.

isotope effects determined in this way are more accurate and precise than those calculated by division of the parameters determined by kinetic analyses of the two isotopic reactions. Figure 3 is a plot of the observed isotope effects vs. substrate concentration for the reaction of MeOSuc-Ala-Ala-Pro-Val-*p*NA with HLE and includes the best-fit curve according to eq 6. Isotope effects on $k_c$ and $k_c/K_m$ for the three substrates are collected in Table IV.

**Proton Inventories.** Initial velocities were determined in mixtures of light and heavy water for the HLE-catalyzed hydrolysis of Suc-Ala-Ala-Ala-*p*NA ($[S]_o = 3K_m$), Suc-Ala-Ala-Val-*p*NA ($[S]_o = 0.05K_m$), and MeOSuc-Ala-Ala-Pro-Val-*p*NA ($[S]_o = 10K_m$). The data, constituting proton inventory experiments, are collected in Table V and displayed graphically in Figures 4–6.

In general, for proton inventory experiments,[15,19] $k_n$, the observed rate constant measured at mole fraction of deuterium $n$, is equal to a term times $k_0$, the rate constant measured in pure $H_2O$ ($n = 0$), and obeys the Gross–Butler equation[15,19] shown below:

$$k_n = k_0 \frac{\prod_i^{v_T}(1 - n + n\phi_{Ti})}{\prod_j^{v_R}(1 - n + n\phi_{Rj})} \quad (7)$$

where $v_T$ and $v_R$ are the number of exchangeable protons in the transition state and reactant state, respectively, and $\phi_{Ti}$ and $\phi_{Rj}$ are the corresponding deuterium fractionation factors for the exchangeable protonic sites relative to bulk water. Equation 7 can be simplified if we consider that most protons do not change state on activation, resulting in cancellation of their reactant and transition state terms, and that exchangeable protons of proteins

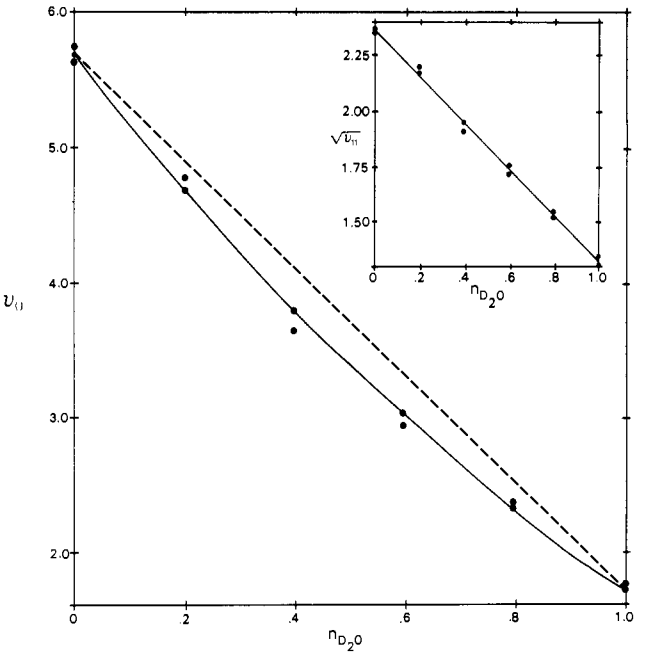


**Figure 6.** Proton inventory of the HLE-catalyzed hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-*p*NA. Initial velocities at ($10^2 \times v_0$ OD/min) were determined at a substrate concentration of $6.00 \times 10^{-4}$ M ($10K_m$). The solid line was calculated by least-squares fit and is described by $v_0 = 5.64 \times 10^{-2}$ OD/min $(1 - n + n/0.55)^2$. The dashed, straight line connects the points in pure light and heavy water. The insert demonstrates the linear dependence of square root of the initial velocity on the mole fraction of solvent deuterium.

(except for those of sulfur) should have $\phi_R = 1$.[15,19] These conditions lead to eq 8, which relates the number of isotope-effect-

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$$k_n = k_o \prod_i (1 - n + n\phi_{Ti}) \quad (8)$$

producing hydrogenic sites and the magnitude of the isotope effect that each site generates. A single contributing site produces a linear dependence of $k_n$ on $n$, as predicted by eq 9, where $\phi_T$ gives

$$k_n = k_o(1 - n + n\phi_T) \quad (9)$$

the inverse isotope effect for the single site. Two contributing sites generate the quadratic function of eq 10, where $\phi_{T1}$ and $\phi_{T2}$

$$k_n = k_o(1 - n + n\phi_{T1})(1 - n + n\phi_{T2}) \quad (10)$$

measure the isotope effects at the two sites. More complex mechanisms will of course generate more complex dependencies of $k_n$ on $n$.

The proton inventories for the reactions of HLE with Suc-Ala-Ala-Ala-*p*NA (Figure 4) and Suc-Ala-Ala-Val-*p*NA (Figure 5) are linear ($\phi_T^{-1} = 2.94$ and 2.22, respectively) and suggest that in each case a single protonic interaction in the catalytic transition state is responsible for the solvent isotope effect, that is, one-proton catalysis. For the reaction of MeOSuc-Ala-Ala-Pro-Val-*p*NA on the other hand, a "bulging down"[15] proton inventory is observed (Figure 6) suggesting multiproton catalysis. The solid line through the data represents the best-fit curve for two-proton catalysis according to eq 9 with $\phi_{T1}^{-1} = \phi_{T2}^{-1} = 1.82$.

By Fisher's $F$ test the quadratic term is significant at the 99.9% confidence level, while inclusion of a cubic term, corresponding to three-proton catalysis, is significant at less than the 80% level.

## Discussion

**Solvent Isotope Effects.** The solvent isotope effects reported here for HLE catalysis (Table IV) reveal major mechanistic differences among the three substrates investigated. For the hydrolyses of Suc-Ala-Ala-Ala-*p*NA and Suc-Ala-Ala-Val-*p*NA the isotope effects agree with our expectations for catalysis by serine proteases.[1,20-24] $^D(k_c)$ and $^D(k_c/K_m)$, both around 3, reflect processes having transition states involving proton transfer or reorganization and are consistent with a mechanism involving rate-limiting protolytic catalysis for both $k_c$ and $k_c/K_m$. The small isotope effects on the Michaelis constants, $^D(K_m)$, suggest that the $K_m$ for these substrates may be true dissociation constants and that the effects arise simply due to the greater solubility of the substrates in light water.[24]

The solvent isotope effects for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-*p*NA (Table IV) reflect an entirely different situation. Comparison of $^D(k_c)$ and $^D(k_c/K_m)$ reveals that the transition states for these two processes are very different, with the transition state of the reaction governed by $k_c/K_m$ characterized by significantly less proton reorganization. The observed solvent isotope effect on $k_c$ is again consistent with this process involving rate-limiting protolytic catalysis. However, for $k_c/K_m$ a very small isotope effect is seen, suggesting that protolytic catalysis is probably not entirely rate-limiting and that some step that has little or no isotope effect is partially rate determining. The large value for $^D(K_m)$ suggests that the Michaelis constant for this substrate is probably not a simple dissociation constant but rather contains kinetic terms for reaction steps involving protolytic catalysis.

The solvent isotope effects measured for the reactions of the three anilide substrates with HLE are consistent with a mechanism for this enzyme in which substrate structure plays a significant role in determining which step is rate limiting. Reactions of substrates possessing few of the structural determinants required by HLE will proceed with some "chemical" step setting the rate for both $k_c$ and $k_c/K_m$. "Chemical" steps include all of the bond making and breaking that occurs during enzyme acylation and deacylation. For $k_c/K_m$ these steps can only be those involved in acylation (see eq 4). It is tempting to speculate that acylation is also rate limiting for $k_c$. This has in fact been found to be the case for several serine protease-catalyzed anilide hydrolyses[1-3] including the reaction of HLE with Suc-Ala-Ala-Ala-*p*NA.[9] However, there is no evidence suggesting that this is true for the more specific substrate Suc-Ala-Ala-Val-*p*NA. Indeed, the recent studies of Christensen and Ipsen[25] raise the possibility that *deacylation* may be rate limiting for reactions of certain serine proteases and peptide *p*-nitroanilides. Studies are presently underway in this laboratory to address just this problem for reactions of HLE.

Introduction of specificity into the substrate appears to bring about stabilization of transition states for chemical steps. For certain highly specific substrates, such as MeOSuc-Ala-Ala-Pro-Val-*p*NA, these chemical transition states have been stabilized to the point that they are of similar energy to the transition state(s) of some "physical process", such as a conformational change, substrate binding, or product release, and can now only *partially* set the rate for $k_c/K_m$. The transition state for $k_c/K_m$ will no longer reflect a single structure but rather will be a "virtual transition state",[17,26] whose structure is a composite of the transition-state structures of partially rate-limiting physical and chemical steps. Thus, the small isotope effect on $k_c/K_m$ for reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-*p*NA is the result of dilution of a large isotope effect on acylation by a near unit isotope effect on the physical step.

**Proton Inventories.** With the solvent isotope effects just discussed we have some idea now about which reaction steps are rate limiting and about the dependence of the rate-determining step on substrate structure. We will now consider the operation of the charge-relay system in HLE, specifically asking what the substrate structural requirements are for the correct functioning of the system. Recent kinetic investigations of the well-characterized pancreatic proteases by the proton inventory technique have provided evidence supporting the view that the charge-relay system becomes fully operative only during the hydrolysis of specific (i.e., oligopeptide) substrates.[27-29] Consistent with these transition-state structural studies are investigations of protease *stable*-state structures[30,31] by neutron and X-ray diffraction and NMR spectroscopy which indicates that the amino acid residues comprising the charge-relay system behave independently of each other. Thus, as anticipated, the charge-relay system is uncoupled in ground states. Full functioning of the catalytic machinery possessed by serine proteases is reserved for transition-state stabilization and, furthermore, only for those transition states fulfilling exacting structural requirements.

The results of the present investigation of HLE are in complete agreement with this view of serine protease catalysis. The proton inventories of the hydrolyses of Suc-Ala-Ala-Ala-*p*NA and Suc-Ala-Ala-Val-*p*NA (Figures 4 and 5) are linear with overall isotope effects of about 3 and indicate one-proton catalysis in each case. The observed isotope effect presumably arises from general catalysis by the imidizole ion of His-57 and due to its small magnitude may reflect "solvation catalysis"[32] rather than catalytic mechanisms in which proton motion is part of the reaction coordinate.[32-35] In contrast to the previous results is the proton

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Table VI. Solvent Isotope Effects for Reactions of Porcine Pancreatic and Human Leukocyte Elastase

| enzyme | substrate | $k_{H_2O}/k_{D_2O}$ | $\phi_{T_1}$[f] | $\phi_{T_2}$[f] | ref |
|---|---|---|---|---|---|
| PPE | Ac-ONP[a] | 2.45 | 0.47 | 1.00 | 27 |
| PPE | (Z)-Gly-ONP[a] | 2.45 | 0.47 | 1.00 | 29 |
| PPE | (Z)-Ala-ONP[b] | 1.75 | 0.57 | 1.00 | 28 |
| HLE | Suc-Ala-Ala-Ala-pNA[c] | 3.15 | 0.32 | 1.00 | g |
| HLE | Suc-Ala Ala-Val-pNA[d] | 2.22 | 0.45 | 1.00 | g |
| PPE | Ac-Ala-Pro-Ala-pNA[c] | 2.16 | 0.46 | 0.46 | 36 |
| HLE | MeOSuc-Ala-Ala-Pro-Val-pNA[e] | 3.34 | 0.55 | 0.55 | g |

[a] Kinetics of deacylation measured. [b] Kinetics of acylation measured ($[S]_0 < K_m; k_2 >> k_3$). [c] Kinetics of acylation measured ($[S]_0 > K_m; k_2 << k_3$). [d] Kinetics of acylation measured ($[S]_0 < K_m$). [e] Kinetics measured at $[S]_0 > K_m$. [f] Results of proton inventory experiments. [g] This study.

inventory for the HLE-catalyzed hydrolysis of the specific substrate MeOSuc-Ala-Ala-Pro-Val-*p*NA (Figure 6), which suggests *two* catalytically important protons. According to this view, the observed isotope effect of 3.3 arises from coupling of the charge-relay system's two protonic sites, with each generating an isotope effect of about 1.8. Proper functioning of HLE's charge-relay system is observed during the hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-*p*NA as a consequence of this substrate's ability to form specific interactions with HLE in the catalytic transition state.

As observed during catalysis by other serine proteases,[27-29,36] the catalytic machinery of the charge-relay system is called into action only when quite specific substrate structural requirements are met, thus permitting an optimum transition-state fit. When substrates possess structural inadequacies a tight transition-state fit cannot be achieved, and the charge-relay system uncouples resulting in one-proton catalysis. Substrate structural requirements for the operation of the charge-relay system appear to vary with the protease. For example, chymotrypsin's requirements are not strict,[27,29] requiring only an aromatic amino acid residue at $P_1$ or peptide-like structural elements past $P_1$. In contrast, both porcine pancreatic elastase (PPE) and HLE impose more severe requirements on their substrates.[27-29,36] As shown in table VI, simply fulfilling the $P_1$ requirement for PPE with an alanine residue, achieved with (Z)-Ala-ONP, is not sufficient to couple the charge-relay system. PPE requires an "extended" substrate, such as Ac-Ala-Pro-Ala-*p*NA, to engage its catalytic machinery. For HLE, substrate requirements not only include an extended peptide structure but also the proper residue at $P_1$ (valine) and possibly the presence of a proline at $P_2$. Although we can *define* for some cases substrate structural requirements, at this time we are still unable to predict the structural features that will result in the intimate and precise transition-state interactions necessary for coupling and full functioning of the charge-relay system.

**Registry No.** I, 52299-14-6; II, 61043-47-8; III, 70967-90-7; HLE, 9004-06-2.

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# Equilibrium Species in Cobalt(II) Carbonic Anhydrase

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*Contribution from the Institutes of General and Inorganic Chemistry, University of Florence, Florence, Italy.* *Received December 14, 1982*

**Abstract:** By measurement of the $^1H$ NMR $T_1$ values of the 4 H proton of the coordinated histidine-119 of cobalt(II)-substituted human carbonic anhydrase B and bovine carbonic anhydrase B as a function of pH, it is proposed that the coordination number around the cobalt(II) ion in bovine carbonic anhydrase B is always four, whereas it is largely five in human carbonic anhydrase B in the low pH limit. This hypothesis is consistent with the different spectral properties and $pK_a$ values of the two isoenzymes.

A major difference between the high-activity (human C and bovine B) and the low-activity (human B) isoenzymes of carbonic anhydrase as native zinc enzymes is the $pK_z$ value of the group regulating the catalytic activity, which can be set around 6.5 and 7.5 for the two kinds of isoenzymes, respectively,[1-3] although there is not a single acid-base equilibrium.[4,5] If the cobalt(II) derivatives are considered, the difference in $pK_a$ is maintained;[4] additionally, while the high pH forms of both isoenzymes have very similar electronic spectra[4] and water proton relaxation capabilities,[6-9] the low pH form of the human B isoenzyme (CoHCAB hereafter) has sensibly lower molar absorbance in the visible region[4] and much lower water proton relaxing capability[4,6-9] than the corresponding high-activity bovine enzyme (CoBCAB hereafter). Recently a thorough nuclear magnetic relaxation dispersion (NMRD) study on this[10] and related systems,[11,12] showed that the dispersion curve is of the type expected on the basis of the Solomon theory:[13]

$$T_1^{-1} = KGf(\tau_c) \qquad (1)$$

$$K = \frac{[E]}{111} g^2\beta^2\gamma_I^2 S(S+1) \qquad G = \sum_i \frac{1}{r_i^6}$$

$$f(\tau_c) = \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}$$

where $[E]/111$ is the enzyme/water proton molar ratio, $r_i$ is the distance of the $i$th proton from the metal, and $\tau_c$ is a correlation

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