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# Catalysis by Human Leukocyte Elastase. Aminolysis of Acyl-Enzymes by Amino Acid Amides and Peptides†


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ABSTRACT: Acyl-enzymes of human leukocyte elastase (HLE) were generated in situ during the hydrolysis of peptide thiobenzyl esters and served as substrates for aminolysis by a variety of amino acid amides and short peptide nucleophiles. For amino acid amides, there is a positive correlation between nucleophilic reactivity toward *N*-methoxysuccinyl (MeOSuc)-Ala-Ala-Pro-Val-HLE and the hydrophobicity of the side chain. For peptides, nucleophilicity toward MeOSuc-Ala-Ala-Pro-Val-HLE decreases dramatically with increasing chain length. Combined, these results suggest that (i) substrate specificity for the $P_1'$ residue may be more dependent on side chain hydrophobicity than on specific, structural features of the side chain and (ii) there may be no important binding interactions available past $S_1'$. Kinetic parameters were also determined for the nucleophilic reactions of $PheNH_2$ and $TyrNH_2$ with MeOSuc-Pro-Val-HLE, MeOSuc-Ala-Pro-Val-HLE, MeOSuc-Ala-Ala-Pro-Val-HLE, and MeOSuc-Ala-Ala-Pro-Ala-HLE. Reactivity of these acyl-enzymes toward nucleophilic attack displays no dependence on peptide chain length but does increase significantly for the substrate with Ala at $P_1$. This same correlation between reactivity and acyl-enzyme structure is also seen for nucleophilic attack by water.


Serine proteases catalyze the hydrolyses of esters and amides by a mechanism involving the intermediacy of an acyl-enzyme. An important consequence of this mechanism is that it allows nucleophiles, of appropriate structure, to compete with water for the acyl-enzyme and provides a second pathway for deacylation. This mechanism is illustrated in Scheme I. According to this mechanism, the acyl-enzyme, formed from reaction within the Michaelis complex, can react with either

† For part 7 of this series, see Stein (1987b).


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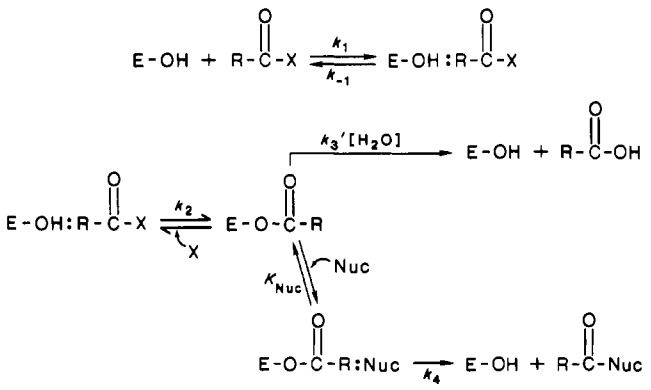
Scheme I



water, to produce free enzyme and carboxylic acid, or a nucleophile, Nuc, to produce free enzyme and covalent addition adduct, R–C(O)–Nuc. This mechanism, as depicted in Scheme I, demands that a binary complex of acyl-enzyme and nucleophile be formed prior to attack of nucleophile on the acyl-enzyme.

This reaction has been the object of a number of studies. Generally, these investigations were conducted either to demonstrate the existence of an acyl-enzyme intermediate (Bender et al., 1964; Berezin et al., 1971; Fastrez & Fersht, 1973; Fersht et al., 1973) or to examine the utility of serine proteases as catalysts of amide bond formation in peptide synthesis (Jakubke et al., 1985; Martinek et al., 1974; Petkov & Stoineva, 1984; Riechmann & Kasche, 1985).

Our interest is in the aminolysis of acyl-enzymes of human leukocyte elastase (Stein et al., 1985) by amino acid amides and short peptides. This reaction is the microscopic reverse of acyl-enzyme formation and thus may serve to probe the $P'$[1] specificity of human leukocyte elastase (HLE).[2] In this paper, we report results of experiments designed to explore this aspect of HLE's mechanism. Two important findings emerge from this work: (i) HLE's $P_1'$ specificity is dependent on the hydrophobicity of the amino acid side chain and not on structural features of this residue, and (ii) HLE may offer no important opportunities for binding beyond $S_1'$.

## Materials and Methods

*Materials.* HLE was prepared as previously described (Stein, 1983; Viscarello et al., 1983). Thiobenzyl ester substrates for HLE were available from previous studies (Stein et al., 1987a,b). Amino acid and peptide derivatives were purchased from Bachem Biochemicals or Sigma Chemical Co. Phe-AlaNH$_2$ was a generous gift of Dr. Donald Wolanin of the Department of Medicinal Chemistry, Stuart Pharmaceuticals. Buffer salts and Me$_2$SO were analytical grade from several sources.

*Kinetic Methods.* The kinetics of aminolysis of acyl-enzymes of HLE were determined by a method in which initial velocities for thiobenzyl ester hydrolysis were measured in buffers of increasing concentration of the amine nucleophile. High concentrations of thiobenzyl ester substrates were used so that observed initial velocities were equal to $k_c$[E]. Since $k_c = k_3$ for these substrates (Stein et al., 1987a), the dependence of observed values of $k_c$ on amine concentration can be expressed as in eq 1, which is based on Scheme I.

$$(k_c)_{obsd} = k_3 + \frac{k_4[Nuc]}{K_{Nuc} + [Nuc]} \quad (1)$$

These reactions were conducted in solutions of 0.1 M CHES, buffered at a pH of 9.0 with the final ionic strength adjusted to 0.5 M with NaCl. At this pH, the $\alpha$-amine of the amino acid amides and peptides used in this study exists as the free, unprotonated base to an extent in excess of 90%.

Thiobenzyl ester hydrolysis was measured in a coupled assay (Farmer & Hageman, 1975) using the reagent 5,5′-dithiobis(2-nitrobenzoic acid). According to this method, benzyl mercaptan, released during the course of thiobenzyl ester hydrolysis, reacts with DTNB to produce the colored product 5-mercapto-2-nitrobenzoic acid ($\epsilon_{412}$ = 13 600).

In a typical experiment, 0.05 mL each of a thiobenzyl ester solution and a 10 mM DTNB solution in Me$_2$SO was added to a cuvette containing 2.88 mL of buffer. The cuvette was placed in a jacketed holder in the cell compartment of a Cary 210 spectrophotometer and allowed to reach thermal equilibrium (5 min). The temperature was maintained at 25 ± 0.1 °C by water circulated from a Lauda K-2/RD bath. Injection of 0.02 mL of enzyme solution initiated the reaction. Absorbances were continuously measured, digitized, and stored in a Digital Electronics Corp. PDP 11/73 minicomputer. The interface to the Cary used the spectrophotometer's digital interface port. Initial velocities were calculated by a fit of the experimental data to a linear dependence on time by linear least-squares analysis.

## Results

*Validation of Method.* In this investigation, we wanted to study the aminolysis of acyl-enzymes of HLE using an experimental method in which acyl-enzymes were generated in situ during the course of HLE-catalyzed thiobenzyl ester hydrolysis. Furthermore, these reactions had to be conducted at pH 9 to allow the amine nucleophile to exist as the unprotonated base. Despite the apparent simplicity of this experimental protocol, it is associated with several potential complications. These problems could be resolved with the appropriate control experiments. The results of these experiments are as follows: (1) The thiobenzyl esters used in this study were not observed to spontaneously hydrolyze at pH 9.0 during a period of 10 min. Concentrations of esters used in these experiments are the same as those found in footnote *a* of Table III. (2) These esters are also not subject to aminolysis by the amines of this study at amine concentrations of 150 mM. (3) The increases in $k_c$ observed with increasing concentrations of amino acid amides are due to the availability of an additional route for deacylation and not to some allosteric effect of amides and peptides. This was demonstrated in an earlier study in which the peptide MeOSuc-Ala-Ala-Pro-Val-PheNH$_2$ was shown to be formed from the reaction of PheNH$_2$ with MeOSuc-Ala-Ala-Pro-Val-HLE (Stein et al., 1984).

*Kinetics of Acyl Transfer as a Function of Nucleophile Structure.* Figure 1 contains plots of the dependence of $(k_c)_{obsd}$, for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-SBzl (Castillo et al., 1979), on the concentration of the amino acid amides PheNH$_2$ and HisNH$_2$. As described above, $k_c = k_3$ for this reaction and allows the data of Figure 1 to be described in the context of Scheme I and analyzed according to eq 1. The data for PheNH$_2$ were fit to this equation by nonlinear, least-squares analysis and yield the following

[1] The nomenclature for the amino acid residues of substrates ($P_n$, ..., $P_2$, $P_1$, $P_1'$, $P_2'$, ..., $P_n'$) and corresponding protease subsites to which they bind ($S_n$, ..., $S_2$, $S_1$, $S_1'$, $S_2'$, ..., $S_n'$) is that of Schecter and Berger (1967).

[2] Abbreviations: MeOSuc, *N*-methoxysuccinyl; SBzl, thiobenzyl ester; HLE, human leukocyte elastase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Me$_2$SO, dimethyl sulfoxide; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

Table I: Nucleophilic Competition of L-Amino Acid Amides for MeOSuc-Ala-Ala-Pro-Val-HLE[a]

| L-amino acid amide | $k_4$ ($s^{-1}$) | $K_{Nuc}$ (mM) | $k_4/K_{Nuc}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| Gly | | | 0 |
| Ala | | | 0 |
| Leu | 20 | 130 | 151 |
| His | | | 60 |
| Met | | | 207 |
| Tyr | | | 41 |
| Phe | 45 | 164 | 270 |
| Trp | 25 | 82 | 305 |
| Pro | | | 0 |
| Asn | | | 29 |
| Arg | 18 | 115 | 157 |
| Ser | | | 0 |
| Thr | | | 0 |

[a] 0.1 M CHES, ionic strength = 0.50 M (NaCl), pH 9.0, 25 °C. In all cases nucleophile concentration was varied from 10 to 150 mM. HLE and MeOSuc-Ala-Ala-Pro-Val-SBzl concentrations were 5 nM and 100 μM, respectively. At this substrate concentration, $[S]/K_m \simeq 50$.

Table II: Nucleophilic Competition of Derivatives of L-Phe for MeOSuc-Ala-Ala-Pro-Val-HLE[a]

| nucleophile | $k_4/K_{Nuc}$ ($M^{-1}$ $s^{-1}$) | nucleophile | $k_4/K_{Nuc}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| Phe | 0 | Phe-Ala | 0 |
| $PheOCH_3$ | 10 | $Phe\text{-}AlaNH_2$ | 50 |
| $PheNH_2$ | 270 | Phe-Gly-Gly | 38 |
| Phe-Gly | 0 | | |

[a] 0.1 M CHES, ionic strength = 0.50 M (NaCl), pH 9.0, 25 °C. In all cases, nucleophile concentration was varied from 10 to 150 mM. HLE and MeOSuc-Ala-Ala-Pro-Val-SBzl concentrations were 5 nM and 100 μM, respectively. At this substrate concentration, $[S]/K_m \simeq 50$.

values: $k_3 = 13.6 \pm 0.6$ $s^{-1}$, $K_{Nuc} = 144 \pm 36$ mM, $k_4 = 39 \pm 5$ $s^{-1}$, and $k_4/K_{Nuc} = 273 \pm 36$ $M^{-1}$ $s^{-1}$.

In contrast to the data for $PheNH_2$, the dependence of $(k_c)_{obsd}$ on the concentration of $HisNH_2$ is linear and suggests that the concentrations of $HisNH_2$ used in this experiment are all well below $K_{Nuc}$. Concentrations of $HisNH_2$ above 0.15 M could not be used due to this nucleophile's limited solubility. The condition that [Nuc] $\ll K_{Nuc}$ leads to the simplification of eq 1 shown in eq 2.

$$(k_c)_{obsd} = k_3 + (k_4/K_{Nuc})[Nuc] \quad (2)$$

The data for $HisNH_2$ were fit to eq 2 by linear least-squares analysis and yield the following values: $k_3 = 12.8 \pm 0.4$ $s^{-1}$ and $k_4/K_{Nuc} = 59 \pm 4$ $M^{-1}$ $s^{-1}$.

Several other amino acid amides and peptide derivatives were studied in this way. Hyperbolic dependencies of $(k_c)_{obsd}$ on [Nuc] were analyzed according to eq 1 while linear dependencies were analyzed according to eq 2. For each nucleophile, two to four separate kinetic experiments were conducted and analyzed independently. Average values for the kinetic parameters were calculated and are summarized in Tables I and II. In all cases, error estimates for the parameters are less than 10% of the mean.

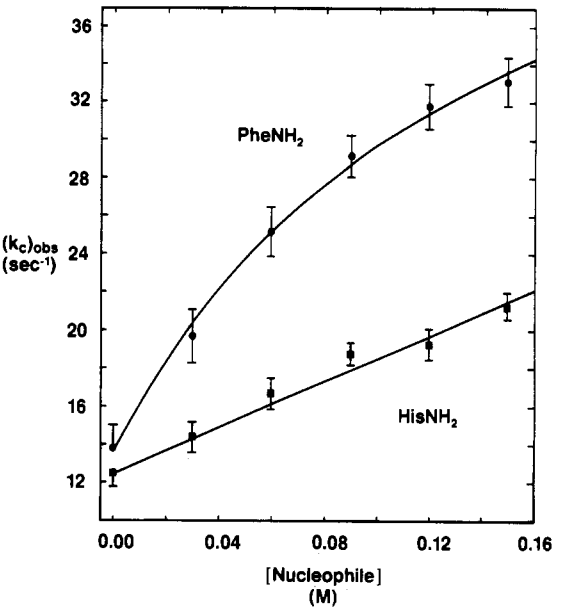



FIGURE 1: Aminolysis of MeOSuc-Ala-Ala-Pro-Val-HLE by amino acid amides. Values of $k_c$ were determined for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-SBzl as a function of the concentration of $PheNH_2$ and $HisNH_2$ as outlined under Materials and Methods. The solid line through the $(k_c)_{obsd}$ vs. [$PheNH_2$] data was drawn by using the best-fit parameters according to eq 1, while the line through the $HisNH_2$ data is according to eq 2.

*Kinetics of Acyl Transfer as a Function of Acyl Structure.* For the two nucleophiles $PheNH_2$ and $TyrNH_2$, the kinetics of acyl transfer were determined for four acyl-enzymes: MeOSuc-Pro-Val-HLE, MeOSuc-Ala-Pro-Val-HLE, MeOSuc-Ala-Ala-Pro-Val-HLE, and MeOSuc-Ala-Ala-Pro-Ala-HLE. The experimental results are summarized in Table III. Several observations are noteworthy: (i) for the same acyl-enzyme, $TyrNH_2$ is always less reactive than $PheNH_2$; (ii) binding of the nucleophile to HLE, as reflected in $K_{Nuc}$, becomes measurable only for tetrapeptide-based acyl-enzymes; and (iii) both $k_3'$ and $k_4/K_{Nuc}$ display similar sensitivities to structural changes in the acyl-enzyme.

## DISCUSSION

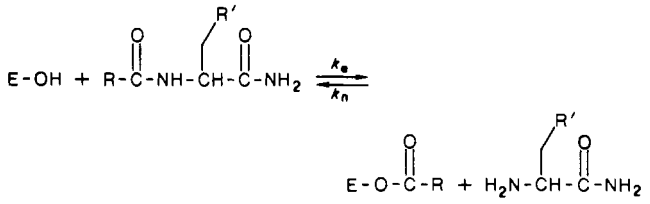
Although a number of studies have probed the S region of HLE's substrate binding site (Harper et al., 1984; Lesteine & Bieth, 1980; Stein, 1985; Stein et al., 1987a; Wenzel & Tschesche, 1981; Zimmerman & Ashe, 1977), there is only a single investigation that explores the S′ region[3] (McRae et

Table III: Nucleophilic Competition for Acyl-Enzymes Derived from Human Leukocyte Elastase[a,b]

| R | Nuc | $k_4$ ($s^{-1}$) | $K_{Nuc}$ (M) | $k_4/K_{Nuc}$ ($M^{-1}$ $s^{-1}$) | $k_3'$[c] ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|
| MeOSuc-Pro-Val | $PheNH_2$ | >100[e] | >0.45[d] | 233 | 0.21 |
| MeOSuc-Ala-Pro-Val | $PheNH_2$ | >100 | >0.45 | 242 | 0.26 |
| MeOSuc-Ala-Ala-Pro-Val | $PheNH_2$ | 45 | 0.16 | 272 | 0.24 |
| MeOSuc-Ala-Ala-Pro-Ala | $PheNH_2$ | 104 | 0.08 | 1330 | 1.2 |
| MeOSuc-Pro-Val | $TyrNH_2$ | >30 | >0.45 | 63 | 0.20 |
| MeOSuc-Ala-Pro-Val | $TyrNH_2$ | >35 | >0.45 | 77 | 0.24 |
| MeOSuc-Ala-Ala-Pro-Val | $TyrNH_2$ | >20 | >0.45 | 41 | 0.25 |
| MeOSuc-Ala-Ala-Pro-Ala | $TyrNH_2$ | 107 | 0.12 | 860 | 1.1 |

[a] 0.1 M CHES, pH 9.0, ionic strength = 0.50 M (NaCl), 25 °C. In all cases, nucleophile concentration was varied from 10 to 150 mM. In all reactions, [HLE] = 5 nM. Substrate concentrations and $[S]/K_m$ ratios are the following, respectively: MeOSuc-Pro-Val-SBzl, 120 μM, 10; MeOSuc-Ala-Pro-Val-SBzl, 100 μM, 50; MeOSuc-Ala-Ala-Pro-Val-SBzl, 100 μM, 50; and MeOSuc-Ala-Ala-Pro-Ala-SBzl, 280 μM, 20. [b] Error estimates are less than 15% for all kinetic parameters. [c] $k_3' = k_3/[H_2O]$. [d] The lower limit on $K_{Nuc}$ is set equal to 3 times the highest nucleophile concentration used. [e] The lower limit on $k_4$ is equal to $K_{Nuc}$ $(k_4/K_{Nuc})$.

Scheme II



al., 1980). In that study, McRae et al. (1980) investigated the hydrolysis of peptides that are structurally related to the amino acid sequence of the reactive site of $\alpha$1-protease inhibitor and demonstrated that, like other serine proteases (Kraut, 1977), HLE has an S′ binding region.

We felt that it was important to explore HLE's S′ region in more detail and initiated our investigations with this study of acyl-enzyme aminolysis. We hoped that these data would serve as a probe of HLE's P′ substrate specificity and the enzyme's S′ binding region.

The ability of aminolysis kinetics to report P′ specificity relies on the fact that acyl-enzyme aminolysis is the microscopic reverse of acyl-enzyme formation (see Scheme II, where $k_e$ corresponds to $k_c/K_m$ for peptide hydrolysis and $k_n$ corresponds to $k_4/K_{Nuc}$ for acyl-enzyme aminolysis). This was pointed out by Fersht et al. (1973) and this general approach successfully used to predict P′ substrate specificity for chymotrypsin (Fersht et al., 1973; Riechmann & Kasche, 1984).

We should be aware, however, that only under certain circumstances will this approach work. For the forward and reverse reactions of Scheme II to display similar sensitivities to structural variation at R′, the magnitude of $K_{eq}$ (eq 3) must

$$K_{eq} = k_e/k_n = [\text{E–acyl}][\text{Nuc}]/[\text{E}][\text{S}] \qquad (3)$$

have no dependence on R′ structure. Stated another way, structural variation of R′ must only perturb the stability of the rate-limiting transition state common to both $k_e$ and $k_n$. If the structure of R′ significantly influences the relative stabilities of the two ground states, $k_e$ and $k_n$ will not display the same correlation with R′ structure.

These principles are illustrated in the free energy reaction progress diagram of Figure 2. This figure depicts reversible acyl-enzyme formation for a series of four peptide substrates in which only R′ varies. For these reactions, we see that only the free energies of the transition states are influenced by variations in R′ and that these same variations have no influence on $\Delta G_{eq}$. In such situations, $k_e$ and $k_n$ will display the same dependence on R′, and in fact, a plot of $\Delta G^{\ddagger}_e$ vs. $\Delta G^{\ddagger}_n$

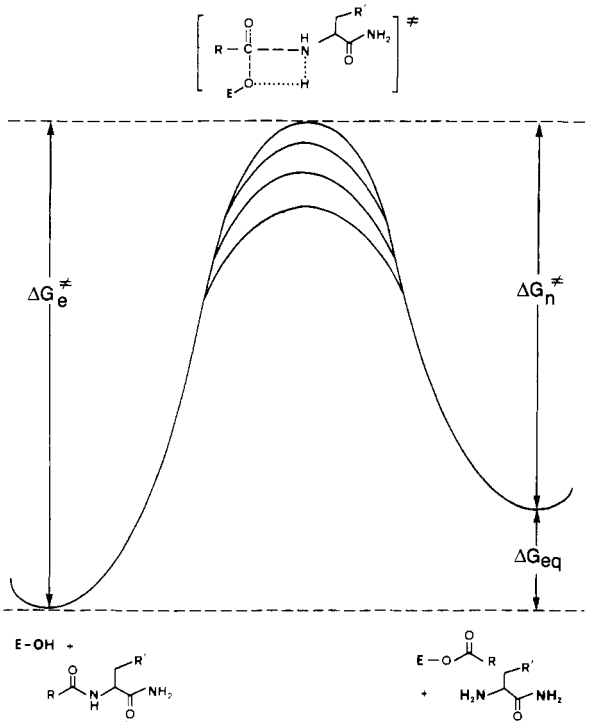



FIGURE 2: Free energy reaction progress diagram for acyl-enzyme formation. This diagram corresponds to a situation in which [peptide] $\ll K_m$ and [Nuc] $\ll K_{Nuc}$ and the only structural feature that varies is R′.

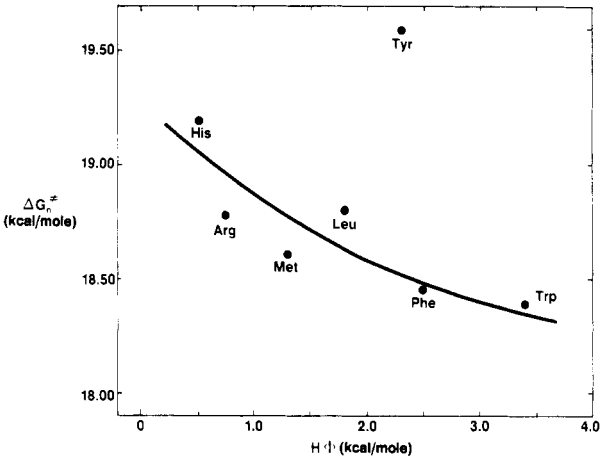



FIGURE 3: Dependence of aminolysis rate on amino acid amide hydrophobicity. Energies of activation for the aminolysis of MeO-Suc-Ala-Ala-Pro-Val-HLE were calculated according to $\Delta G^{\ddagger} = -RT \ln [(k_4/K_{Nuc})[\text{Nuc}](h/kT)]$ with a standard-state nucleophile concentration of $10^{-3}$ M and a temperature of 298 K and were plotted vs. Tanford's hydrophobicity constants (Nozaki & Tanford, 1971; Tanford, 1962). The solid line through the data is to guide the eye and represents no model.

will be linear with a slope of 1 and an intercept of $\Delta G_{eq}$.

In the present case, it is clear that structural variations in R′ will have no effect on $K_{eq}$. First, R′ can have no influence on the equilibrium concentrations of either E or E–acyl, since the amino acid amide containing R′ is not part of these molecules. Second, the electronic and steric features of the R′ structures examined in this study should have little influence on the equilibrium concentration of the peptide substrate due to the remote location of R′ relative to the scissile bond. Third, due to the small influence that R′ is anticipated to have on the nucleophilicity of the $\alpha$-amine, it should have little influence on the equilibrium concentration of the nucleophile. This is reflected in the very small differences in $pK_a$ values that are

[3] Another study also claimed to have explored the S′ region of HLE. Renaud et al. (1983) studied a series of trifluoroacetyl peptides as inhibitors of HLE. For porcine pancreatic elastase, it was known (Hughes et al., 1982) that at least one mode of binding for these compounds has the trifluoroacetyl group at $S_1$ and the rest of the peptide binding to the S′ region. On the basis of analogy to porcine elastase, Renaud et al. interpreted the $K_i$ values determined for inhibition of HLE to reflect binding to the S′ region. However, two important kinetic considerations make this interpretation questionable: (1) $K_i$ values reflect all binding modes that are available to the inhibitor. Trifluoroacetyl peptide *p*-nitroanilides interact with HLE as substrates and are hydrolyzed to release *p*-nitroaniline (Lestienne et al., 1981). This indicates that in at least one of their binding modes, these compounds do not interact with the S′ region of HLE. (2) Kinetic parameters that reflect binding will frequently display a substrate specificity that is different from the specificity observed for parameters reflecting catalysis (Stein, 1985; Stein et al., 1987a). Therefore, even if the $K_i$ values for the inhibition of HLE by trifluoroacetyl peptides reflected binding in a single mode in S′, we could still not be sure if the observed specificity would be reflected during catalysis.

observed among amino acid amides (Fersht et al., 1973). For the typical situation [S] $\simeq$ [Nuc] $\gg$ [E], the second and third considerations are of no real importance, since the equilibrium concentrations of substrate and nucleophile will be equal to their initial concentrations.

The foregoing discussion suggests that this approach may be a valid probe of HLE's P′ specificity, at least for the amino acid amides of this study. Assuming this, we can now describe several important features of this specificity.

(1) The results of Table I suggest a positive correlation between $k_4/K_{Nuc}$ and hydrophobicity of the amino acid amide nucleophiles. This correlation is illustrated in Figure 3, where the free energy of activation, calculated from $k_4/K_{Nuc}$ values, is plotted vs. the hydrophobicity constant, $H\Phi$ (Tanford, 1962; Nozaki & Tanford, 1971), and not only suggests that $S_1'$ is hydrophobic but also suggests that this subsite may not impose strict structural requirements on its ligands. That is, the ability of a substrate to bind at $S_1'$ may not depend so much on the presence of a specific amino acid residue at $P_1'$ but rather may only depend on this residue's hydrophobic character. However, the correlation of Figure 3 also shows that $TyrNH_2$ is about 6 times more reactive than might be predicted from its hydrophobicity and suggests that $S_1'$ may possess some degree of structural specificity.

(2) Various derivatives of Phe were also studied as nucleophiles, and these results are given in Table II. Again, there is at least a casual correlation between hydrophobicity and reactivity, as reflected in the data for PheOH, $PheOCH_3$, and $PheNH_2$. This is consistent with our proposition that binding at $S_1'$ is governed more by the hydrophobicity than by specific structural features of the ligand.

(3) Lengthening $PheNH_2$ to the level of di- and tripeptides results in greatly decreased values of $k_4/K_{Nuc}$. This is a surprising result. We anticipated that di- and tripeptide derivatives of Phe would be able to establish favorable interactions with HLE at subsites $S_1'$–$S_3'$ and enter into nucleophilic reactions with the acyl-enzyme with great facility. Our anticipation was clearly not realized, suggesting either that there are no important binding interactions past $S_1'$ or that the predominant mode of binding at $S_1'$–$S_3'$ is nonproductive for catalysis.

The results we have just discussed correlate nucleophilic reactivity with nucleophile structure and are true reflections of P′ specificity. In contrast, the results of Table III relate acyl-enzyme reactivity toward nucleophilic attack with acyl-enzyme structure and are a reflection of P specificity. Several features of these data should be noted.

(1) Reactivity toward nucleophilic addition, as reflected in $k_4/K_{Nuc}$, is not dependent on the peptide chain length of the substrate from which the acyl-enzyme is derived. This is in contrast to the strong dependence that $k_4/K_{Nuc}$ has on the $P_1$ residue of the acyl-enzyme. An identical trend is seen in the nucleophilic reactions of these acyl-enzymes with water and confirms that the dependence of reactivity of acyl-enzymes toward aminolysis on acyl-enzyme structure reflects P specificity.

Similar results were reported by Petkov and Stoineva (1984), who observed no dependence of reactivity of acyl-chymotrypsins toward aminolysis on acyl-enzyme structure.

(2) Binding of nucleophiles to acyl-enzymes, as reflected in $K_{Nuc}$, can only be observed for the tetrapeptide-based acyl-enzymes; acyl-enzymes derived from shorter peptides bind amino acid amides very poorly. This suggests that there is some sort of communication between S and S′ subsites: the occupation of $S_4$ is transmitted to $S_1'$ with the result that amino acid amides are then allowed to bind at this subsite. This transmission of information is presumably mediated by subtle conformational changes of the acyl-enzyme.

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