at the two pH values and also since the X-ray studies with indole-α-chymotrypsin clearly showed indole binding to the dimer within the asymmetric unit.[4]

The aromatic moiety on Met-192 was definitely involved in interactions occurring in the active center and specificity pocket. The direct implications to catalytic rate acceleration (or depression) and substrate binding equilibria (K_S, K_m) are discussed in the following paper in this issue.

Experimental Section

Materials. α-Chymotrypsin (lots CDI2KD, CDI2LX, and CDI34D632) was purchased from Worthington Biochemical Corp. *p*-(Trifluoromethyl)aniline was purchased from J. T. Baker Laboratory Chemicals, and *o*- and *m*-(trifluoromethyl)aniline were obtained from Matheson, Coleman & Bell. Bromoacetyl bromide, Ac-Tyr-OEt, and trifluoroacetic acid were purchased from Aldrich Chemical Co.

Indole (Eastman Organic Chemicals) was recrystallized from methanol/water. *p*-Nitrophenyl acetate (AcONP), purchased from Sigma Chemical Co., was dried overnight in vacuo before use. CM-52 ion-exchange resin was from H. Reeve Angel, Inc., Clifton, N.J. Dow cellulose acetate hollow fibers (Bio-fiber 80 beaker) were obtained from Bio-Rad Laboratories. The (trifluoromethyl)bromoacetanilide alkylating reagents were prepared by the method of Bittner and Gerig.[7b]

Procedures. Sedimentation velocities were measured on a Beckman Model E analytical centrifuge (Schlieren Optics) with an An-D rotor at 220000*g*.

Enzyme Assays. α-Chymotrypsin activity was assayed by the spectrophotometric method of Schwert and Takenaka,[15] with a Unicam SP1800 spectrophotometer at 25 °C.

Enzyme concentration was determined on the basis of active-site titrations using *p*-nitrophenyl acetate at pH 5.0, 0.10 M acetate, 6.9% (v/v) acetonitrile.[16] The *p*-nitrophenol liberated was observed at 347.8 nm ($\Delta\epsilon = 5.06 \times 10^3$ M^{-1} cm^{-1}). Protein was measured by its absorbance at 280 nm using a $\epsilon_{280nm} = 5.0 \times 10^4$ M^{-1} cm^{-1}.[17]

Alkylation. A 3- to 15-fold excess of alkylating reagent A was added in ethanol (final cosolvent concentration was 9% v/v) to an approximately 10 mg/mL solution of α-chymotrypsin at pH 5.0, 0.1 M acetate.[7b] When the Ac-Tyr-OEt activity reached a limiting value (see Table I), the solution was dialyzed vs. pH 3 HCl and then deionized water, and finally lyophilized.

Purification of Alkylated Chymotrypsin Derivatives. Alkylated enzyme was purified by a modification of the method of Kosman.[18] Approximately 150 to 200 mg of the lyophilized alkylated enzyme was dissolved at 10–20 mg/mL in 0.01 M phosphate buffer, 0.100 M total potassium, at pH 5.7. The solution was centrifuged to remove undissolved protein and applied to a CM-52 column (25 × 2.5 cm) and eluted with the same buffer (1.5 to 2.0 mL/min). Following a small peak at the void volume, protein absorbing at 280 nm eluted in the following order: unreacted enzyme, monoalkylated enzyme, and a very minor dialkylated enzyme peak which was significant only with acetamidochymotrypsin.[19] The fractions corresponding to monoalkylated enzyme were combined, the pH was reduced to 4 with 1 M HCl, and the solution was concentrated by (Biofiber 80) hollow fibers and then dialyzed against water and lyophilized. The acetamidochymotrypsin was subsequently reapplied to the CM-52 column to enhance removal of the dialkylated material. Based on previous reports of the specificity of these reagents we can confidently conclude that Met-192 was the modified residue.[5b]

^{19}F NMR. ^{19}F Fourier transform NMR spectra were taken with a Bruker HX-90 resonance spectrometer operating at 84.67 MHz. Trifluoroacetate (10–15 mM) at pH 5.7 (0.009 M phosphate, 0.08 M KCl, 20% D_2O) was used as an external reference and occasionally as an internal reference. Protein solutions for NMR were prepared by dissolving about 20 mg of freeze-dried, chromatographed (CM-52) protein into 0.6 to 1.7 mL of buffer (0.009 M phosphate, 0.08 M KCl, 20% D_2O), adjusting the pH to 4.2 or 5.7, and centrifuging to remove undissolved material.

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Structural Models for the Rate-Altering Effects of Alkylated [Methionine-192]-α-chymotrypsins[1]

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Abstract: The (trifluoromethyl)acetanilido[Met^{192}]-α-chymotrypsin derivatives prepared in the preceding paper (B. H. Landis and L. J. Berliner, *J. Am. Chem. Soc.*, preceding paper in this issue) and acetanilido- and acetamido[Met^{192}]chymotrypsins were examined kinetically with the nonspecific ester substrate *p*-nitrophenyl acetate and the specific substrates acetyltyrosine ethyl ester and acetyl-L-alanylglycylglycine methyl ester. Rate accelerations of 1.5- to 14.3-fold were observed with nitrophenyl acetate for the acetanilido[Met^{192}] derivatives relative to the acetamido[Met^{192}] derivative. Contributions from pK_a shifts or nonproductive substrate binding, due to an acetanilido moiety situated solely in the tosyl pocket, were insufficient to account for these rate accelerations. The binding of 10 mM indole to acetamido-α-chymotrypsin or native α-chymotrypsin resulted in a 1.5- to 2-fold rate acceleration with nitrophenyl acetate, while no additional accelerations were observed with the aromatic (acetanilido) derivatives under the same conditions. In kinetic studies with the specific substrate acetyltyrosine ethyl ester, K_m(app) was essentially unaltered, while k_{cat} was reduced for most derivatives. Kinetically this was accounted for by reductions in both k_2 and k_3. The tripeptide substrate, described above, which contains no side chain to bind in the specificity pocket, also displayed rate enhancements with most derivatives, while indole was generally inhibitory. Analysis of the combined kinetic results points to a secondary interaction site (other than the tosyl pocket) between the aromatic alkylating moiety and the enzyme (or substrate) molecule. These considerations, along with model-building studies, suggested the peptide subsite S_2 (near the acylamido site) as the possible locus of interaction.

The previous paper in this issue described a physical approach to the structural role of an alkylating moiety attached to Met-192.[3] The work presented here examines the kinetic behavior of several Met-192 alkylated α-chymotrypsin derivatives with three ester

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substrates: a "nonspecific substrate", *p*-nitrophenyl acetate; a specific substrate, *N*-acetyltyrosine ethyl ester (Ac-Tyr-OEt); and an oligopeptide substrate (having no interactions with the tosyl pocket), *N*-acetyl-L-alanylglycylglycine methyl ester (*N*-Ac-Ala-Gly-Gly-OMe). There are three general models which have been suggested or inferred from previous work on Met-192 modified α-chymotrypsin: (a) the alkylating moiety at Met-192 resides *in* the tosyl pocket—for example, Foster's discovery of the indole activation of nitrophenyl acetate hydrolysis,[4] X-ray crystallographic data which found indole to bind in the tosyl pocket;[5] (b) the moiety resides in the bulk solvent environment—dictated by the increased polarity of the modified thiol ether linkage (carboxamidomethylation or oxidation to the sulfoxide); and (c) the moiety resides at a secondary active site locus—in general, there are several possibilities. In past work, the acylamido site was suggested by Kēzdy et al.[6] for [methionine-*S*-[[*N*-(2-carboxyisopropyl)carbamyl]methyl]sulfonium-192]-α-chymotrypsin.

Results

Preparation of Derivatized Enzyme. The alkylation and purification procedures were essentially identical with those reported in the literature for all modification reagents examined.[3,7-9] The *o*-, *m*- and *p*-(trifluoromethyl)acetanilide moieties are designated *o*-, *m*-, and *p*-A, respectively.[3]

***p*-Nitrophenyl Acetate Kinetics.** Steady-state kinetics were measured spectrophotometrically at pH 7.16 for each derivative in the absence and presence of 10 mM indole, where the latter was saturating in most cases. The rate acceleration, k_{cat}(10 mM indole)/k_{cat}, of 1.5- to 2-fold reported by Foster[4] with the native enzyme was observed here for native and acetamido derivatives but was generally depressed or absent for the aromatic derivatives (Table I). Throughout this study we have compared these derivatives to the acetamido ($-CH_2CONH_2$) modified enzyme, which also contains a positively charged Met-192 sulfonium group but lacks the large aromatic moiety. It was also of interest to find qualitatively similar rate enhancements in the presence of dioxane (7% v/v), another tosyl pocket ligand.[5,12]

In order to assess possible changes in k_{cat} due to pK_a differences arising from the introduction of a positive charge at Met-192, pH-rate profiles were determined over the range pH 5.91 to 8.49 at 0.51 mM *p*-nitrophenyl acetate. The results are listed in Table II. It was assumed in these experiments that K_m(app) was relatively insensitive to pH, since Bender et al. have shown that K_s was relatively pH independent.[11] The pH-independent rate constants, k_{ind}, were calculated based on the k_{cat} values from Table I and the determined pK_a values (Table II). Since the rate profiles determined here were actually related to k_{cat}/K_m(app), it must be emphasized that calculated k_{ind} values are lower limits. Therefore, the pH-corrected k_{cat} ratios, $k_{ind}/k_{ind(-CH_2CONH_2)}$, which are thus lower limits (Table II), are greater than unity, implicating additional mechanism(s) for the k_{cat} enhancement.

***N*-Acetyltyrosine Ethyl Ester Kinetics.** For each derivative, k_{cat} and K_m(app) were measured as well as the competitive inhibition behavior of indole (in all cases) over the concentration range 0 to 10.0 mM. The results are listed in Table III. While the K_m(app) was increased twofold by the introduction of a positive charge on the thio ether in acetamido[Met[192]]chymotrypsin, the additional aromatic moiety of the acetanilido derivatives caused a general decrease in k_{cat} while affecting K_m(app) only slightly.[13]

***N*-Ac-Ala-Gly-Gly-OMe Kinetics.** This substrate, which was synthesized by standard methods, was designed as a substrate which would bind similarly to the oligopeptide binding model from X-ray results[14] but which lacked a tosyl pocket binding side chain by virtue of its carboxyl terminal glycyl moiety. Due to both the high K_m(app) values for this and other glycyl ester substrates (0.014–1.0 M)[15] and to its solubility limit of <50 mM, we were unable to evaluate accurate k_{cat} or K_m(app) values for any of these derivatives, since the rates increased essentially linearly with substrate concentrations up to 30 mM; thus for $[S_0] \ll K_m$(app), $v = V_m[S]/K_m$(app). A tabulation of $k_{obsd} = v/[E_0]$ is reported at 30 mM *N*-Ac-Ala-Gly-Gly-OMe with and without indole (Table IV). These data, while limited in kinetic information, qualitatively supported the conclusions drawn from the other two substrates above (see Discussion).

Discussion

Nonspecific Substrates. *p*-Nitrophenyl Acetate. Since all of the derivatives examined in Table I displayed rate accelerations in k_{cat} as did the specificity pocket directed reversible ligand, indole, it would first appear that an alkylating moiety on Met-192 which binds in the tosyl hole would contribute to such an activation. Furthermore, the ^{19}F NMR results showed that the (trifluoromethyl)acetanilido moieties were shifted upon indole binding and that a partition coefficient was derivable between *in* and *out* of pocket binding.[3,16] However, the measured k_{cat} enhancements greatly exceeded that of saturating indole with almost every acetanilido derivative.

Second, the pH-rate profiles for each derivative showed a lowering in the pK_a of the rate constant, k_{ind}, of 0.3 to 0.6 pH unit by the introduction of a positively charged sulfonium group at Met-192 (see Table II). Again correcting for these pK_a differences, the rate enhancements for the acetanilido derivatives still exceeded those due to simple tosyl pocket (indole binding) effects.

Since *p*-nitrophenyl acetate is potentially capable of adopting nonspecific binding modes with α-chymotrypsin, we must also address those kinetic consequences. For the case where the effect of the alkylating agent (or indole) is solely to block nonproductive binding, the relationship, k_{cat}/K_m(app) = k_2/K_s holds,[6] inferring that the ratio k_{cat}/K_m(app) should be identical for both native and derivatized enzymes. Comparison of k_{cat}/K_m(app) ratios for the aromatic-alkylated (acetanilido) derivatives with this same ratio for acetamidochymotrypsin shows fairly close agreement. In the presence of 10 mM indole, the agreement is slightly better (Table I). However, if one calculates the maximum k_{cat} for acetamidochymotrypsin at saturating indole concentrations, the value for acetanilidochymotrypsin is still 6.3 times greater. Also, if the aromatic moieties were bound solely at the tosyl pocket, one would expect that at saturating indole concentrations the aromatic-alkylated enzymes should appear kinetically identical with acetamidochymotrypsin in saturating (10 mM) indole. Since indole has essentially no effect on these k_{cat} values, rather than "normalizing" them all to $k_{cat(-CH_2CONH_2)}^{(10\ mM\ indole)}$ we must conclude that these acetanilido moieties influence *p*-nitrophenyl acetate kinetics

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Table I. p-Nitrophenyl Acetate Kinetics[a]

derivative	$k_{cat} \times 10^3$, s^{-1}	K_m(app) $\times 10^5$, M	k_{cat}(10 mM indole)/k_{cat}	$k_{cat}/k_{cat(-CH_2CONH_2)}$	k_{cat}(10 mM indole)/$k_{cat(-CH_2CONH_2)}$(10 mM indole)	k_{cat}/K_m(app) $\times 10^{-2}$, s^{-1} M^{-1}
native + 10 mM indole	3.25 ± 0.10	0.77 ± 0.13		0.80		4.22
	6.27 ± 0.17	4.67 ± 0.41	1.93		0.96	1.34
$-CH_2CONH_2$ + 10 mM indole	4.05 ± 0.13	1.74 ± 0.18		1.00		2.3
	6.48 ± 0.56	6.8 ± 0.92	1.60		1.00	0.95
o-A- + 10 mM indole	10.6 ± 0.2	8.16 ± 0.48		2.62		1.30
	12.1 ± 0.5	12.6 ± 1.4	1.14		1.86	0.96
m-A- + 10 mM indole	11.0 ± 0.2	4.98 ± 0.28		2.72		2.21
	13.5 ± 0.8	12.4 ± 1.6	1.23		2.08	1.09
p-A-[b] + 10 mM indole	(10.7 ± 1.0)[c]	6.40 ± 0.92		2.64		1.67
	11.3 ± 0.5	22.6 ± 2.1	1.06		1.74	0.50
acetanilido[d] + 10 mM indole	57.9 ± 1.4	16.3 ± 0.7		14.3		3.55
	63.3 ± 2.4	23.4 ± 1.5	1.09		9.77	2.71

[a] pH 7.16, 0.10 M potassium phosphate, 6.9% (v/v) CH_3CN, 25 °C. [b] The complete chromatographic resolution of native enzyme from this derivative was virtually impossible; the resultant k_{cat} and K_m(app) values are lower limits. [c] Highest k_{cat} at this pH derived from the pH-rate profile studies (Table II). [d] The derivative obtained by modification with bromoacetanilide (i.e., this derivative resembles *o*-, *m*-, and *p*-A but contains no substituent on the ring); see ref 8.

Table II. pK_as and pH-Independent Rate Constants for the Hydrolysis of p-Nitrophenyl Acetate[a]

derivative	pK_a	$k_{ind} \times 10^3$,[b] s^{-1}	$k_{ind}/k_{ind(-CH_2CONH_2)}$	derivative	pK_a	$k_{ind} \times 10^3$,[b] s^{-1}	$k_{ind}/k_{ind(-CH_2CONH_2)}$
native	7.32 ± 0.06	8.0 ± 1.1		*m*-A-	7.08 ± 0.15	20 ± 7	3.17
$-CH_2CONH_2$	6.90 ± 0.13	6.3 ± 1.9	1.00	*p*-A-	6.62 ± 0.03	12.3[c]	1.95
o-A-	6.78 ± 0.10	15 ± 5	2.38	acetanilido	6.78 ± 0.02	82 ± 8	13.0

[a] The substrate concentration was 0.51 mM. [b] Error limits calculated according to Bevington.[10] [c] Maximum rate constant obtained from pH-rate profile; if extrapolated to saturating substrate, $k_{ind} \approx 13.8$.

Table III. Ac-Tyr-OEt Kinetics[a]

derivative	k_{cat}	K_m'(app), mM	$k_{cat}/k_{cat(-CH_2CONH_2)}$	K_I,[b] mM	K_A[c]	tosyl pocket binding, %	$K_s/(1 + k_2'/k_3')$, mM	$(K_s/k_2) \times 10^5$	$K_s/k_{2(-CH_2CONH_2)}/K_s/k_2$
native	164 ± 5	1.27 ± 0.05[d]	0.89	0.66 ± 0.37					
$-CH_2CONH_2$	184 ± 19	3.93 ± 0.29[d]	1.00	1.97 ± 0.29			3.93 ± 0.29[d]	2.1 ± 0.3	1.00
o-A	60.0 ± 4.7	3.99 ± 0.22	0.33	2.57 ± 0.08	3.3 ± 0.6	23 ± 4	3.06 ± 0.57	5.1 ± 1.0	0.41
m-A	122 ± 8	6.80 ± 0.34	0.66	4.01 ± 0.51	0.96 ± 0.19	51 ± 10	3.34 ± 0.67	2.7 ± 0.6	0.78
p-A	136 ± 5	3.50 ± 0.09	0.74	2.57 ± 0.31	3.3 ± 0.6	23 ± 4	2.68 ± 0.51	2.0 ± 0.4	1.05
acetanilido	21.0 ± 1.6	3.16 ± 0.17	0.11	2.93 ± 0.71	2.1 ± 0.6	32 ± 9	2.13 ± 0.61	10 ± 3	0.21

[a] pH 8.0, 0.100 M KCl, 25 °C. [b] Determined graphically by the Dixon method.[18] [c] Intramolecular partitioning constant, $K_A = [E_{out}]/[E_{in}]$, where $[E_{in}]$ and $[E_{out}]$ are labeled enzyme species with the aromatic moiety *in* or displaced *out* of the tosyl pocket.[3] This term shows up in the expression $K_m'(\text{app}) = [K_s/(1 + k_2'/k_3')][1 + 1/K_A]$. [d] By definition, $K_m(\text{app}) = K_s(1 + k_2'/k_3')$, or $K_A = \infty$ (see footnote *c*.

Table IV. Ac-Ala-Gly-Gly-OMe Kinetic Data

derivative	k_{obsd},[a] min^{-1}	$k_{obsd}/k_{obsd(-CH_2CONH_2)}$	k_{obsd}(6.1 mM indole)/k_{obsd}	derivative	k_{obsd},[a] min^{-1}	$k_{obsd}/k_{obsd(-CH_2CONH_2)}$	k_{obsd}(6.1 mM indole)/k_{obsd}
native + 6.1 mM indole	2.65	0.62		*m*-A- + 6.1 mM indole	30.0	7.04	
	1.71		0.64		18.3		0.61
$-CH_2CONH_2$ + 6.1 mM indole	4.26	1.00		*p*-A- + 6.1 mM indole	5.03	1.18	
	2.53		0.59		4.82		0.95
o-A- + 6.1 mM indole	0.5	0.12		acetanilido + 6.1 mM indole	215	50.5	
	1.26		2.52		212		0.99

[a] Observed rates, $k_{obsd} = v/[E_o]$, at a substrate concentration of 30 mM (pH 8.0, 0.100 M KCl, 25 °C) which was near the solubility limit. Estimated uncertainty in k_{obsd} values is approximately 5%.

at a site *other* than the tosyl pocket. Assuming that $k_3 \gg k_2$ for the alkylated derivatives, it seems likely that this rate acceleration is manifested in k_3 (deacylation rate constant), as was also the case for indole complexes with native enzyme.[4]

Specific Substrates. Ac-Tyr-OEt. This "specific" substrate is presumably bound in the acyl enzyme complex with the tyrosyl group in the specificity pocket and the acylamido moiety in a hydrogen bond with the carbonyl group of Ser-214.[14] Again, if the sole effect of the aromatic moieties were to competitively block substrate binding in the tosyl pocket, the k_{cat} values in Table III should all be identical, save that of native enzyme which does not carry a positively charged (modified) Met-192 sulfonium moiety. In fact, k_{cat} for acetamido– relative to native enzyme is only slightly changed, suggesting that this introduction of a positive charge has little effect on the catalytic rate constant, whereas the bulkier aromatic moieties definitely affect k_{cat}. There is an effect on binding, however, as is most clearly demonstrated by the threshold increase in K_I(indole) for acetamidochymotrypsin vs. the native enzyme.

In order to examine in more detail the possible effects on the individual kinetic parameters for Ac-Tyr-OEt hydrolysis, we note that K_m(app) is given by $K_m(\text{app}) = K_s/(1 + k_2/k_3)$, where k_2 and k_3 are the acylation and deacylation rate constants, respectively. We note from Table III that K_m(app) for all of the alkylated derivatives varies only slightly within the group. Ignoring for the moment the equilibrium described in the previous paper[3] by K_A, then for K_m(app) unchanged, if one compares the aromatic alkylated derivatives with acetamidochymotrypsin, where K_s(app) denotes an aromatic alkylated derivative, one of the following conditions holds: (i) **K_s is unaffected by alkylation. K_s(app) = K_s**; k_2'/k_3' for one alkylated derivative is equal to k_2/k_3 for the acetamido– derivative. (ii) **K_s(app) < K_s.** Since K_m(app) for acetamidochymotrypsin vs. native chymotrypsin increased threefold and since the K_I values for indole increased almost identically, a decrease in K_s for the Ac-Tyr-OEt seems very unlikely. Furthermore, Kēzdy et al.[6] showed that k_2 was unaffected by Met-192 alkylation with N-(bromoacetyl)-2-aminoisobutyrate. Since their ratio $[K_m(\text{app})/k_{cat}]^{\text{alk}}/[K_m(\text{app})/k_{cat}]^{\text{native}}$ increased, K_s must have increased. With this as a precedent, it seems ever more unreasonable that $K_s(\text{app}) < K_s$. (iii) **K_s(app) > K_s.** This is excluded if one assumes that substrate binding and binding of the aromatic alkylating moiety in the pocket are mutually exclusive.

Therefore condition (i) holds where $K_s(\text{app}) = K_s$. In the preceding paper[3] an intramolecular partition constant, K_A, was derived describing the equilibrium of any aromatic alkylating moiety between the tosyl pocket and other mutually exclusive site(s) (see Table III). This shows up as a term in the expression for K_m'(app) for these derivatives

$$K_m'(\text{app}) = \frac{K_s\left(1 + \dfrac{1}{K_A}\right)}{1 + k_2'/k_3'}$$

If each K_m'(app) is adjusted by dividing by the corresponding $(1 + 1/K_A)$ term, one obtains the $K_s/(1 + k_2'/k_3')$ values in Table III. These should be used in place of K_m'(app) to calculate $K_m(\text{app})/k_{cat} = K_s/k_2$. If one notes that for Ac-Tyr-OEt $k_2 \gg k_3 = k_{cat}$[17] and allows that K_s is relatively unchanged amongst these alkylated derivatives, the value of K_s/k_2 relative to the value for the acetamido– derivative, $[K_s/k_{2(\text{-CH}_2\text{CONH}_2)}]/(K_s/k_2)$ in Table III, is roughly the same as $(\approx k_3/k_{3(\text{-CH}_2\text{CONH}_2)})$ in Table III. Thus it may be generally concluded that each alkylating moiety, displaced from the tosyl pocket, alters (decreases) k_2 and k_3 to about the same extent, the acetanilido and o-A derivatives effecting the greatest changes. However, as indicated before, the kinetic effects on k_2 and k_3 cannot be solely due to pocket binding by the aromatic moiety; intramolecular competitive inhibition by this moiety describes only binding phenomena, such as the differences in K_I for indole with each derivative.

N-Ac-Ala-Gly-Gly-OMe. Since the observed rate constants, k_{obsd}, in Table IV contain a term for incomplete saturation, $[S]/K_m$(app), we can assume that K_m(app) for each aromatic alkylated derivative, if changed at all, has increased relative to the acetamido– derivative. Thus, the $k_{obsd}/k_{obsd(\text{-CH}_2\text{CONH}_2)}$ ratios in Table III should probably be construed as *lower limits*. Examining these data, we note that, in general, the binding of indole had little effect in reducing these (underestimated) rate constants to values comparable to the $-CH_2CONH_2$ derivative under the same conditions. With one exception, the rate reduction in the presence of indole found for native or acetamidochymotrypsin was also found for the aromatic alkylated derivatives. Yet a net rate acceleration was still present, the principal effect of indole binding amounting to *intramolecular competition*[3] with the alkylating moiety. Therefore, as concluded for nitrophenyl acetate (Table I), these alkylating moieties must (also) bind, and are kinetically most effective, at an active-site locus other than the tosyl pocket.

Model Building. To assist in the evaluation of models for noncovalent interactions between these alkyl moieties and substrate binding or active-site residue loci, each alkylating moiety was examined in a three-dimensional skeletal model of the active site of α-chymotrypsin using the coordinates from tosylchymotrypsin.[19]

Each aromatic moiety could not be bound fully into the tosyl pocket since bending the Met-192 side chain and carboxamido arm into a cisoid "U" shape allowed for too many van der Waals overlaps. However, partial insertion into the tosyl pocket at an orientation parallel to that for, e.g., N-formyl-L-tryptophan, was possible.[5]

A secondary binding mode for the aromatic moieties crossed the peptide binding region at roughly right angles to the chain direction of a bound peptide substrate. The aromatic moiety was located near C_α-NH of the penultimate residue (P_2) and the side chains Trp-215 and Ile-99 in the protein subsite S_2, entering the apolar binding site proposed by Segal et al.[14] at S_2. The carboxamido side chain of the alkylating moiety was completely transoid. Furthermore, this conformation did not block access to the tosyl pocket, thus not hindering Ac-Tyr-OEt binding nor nonproductive tosyl pocket binding for p-nitrophenyl acetate, while sterically interacting with the tripeptide substrate binding at S_2.

The most striking kinetic consequence of the position of the $-CF_3$ group on the aromatic ring is for o-A which displays a significantly reduced k_{cat} with Ac-Tyr-OEt (Table III) and k_{obsd} for Ac-Ala-Gly-Gly-OMe (Table IV). The model-building studies above found the $-CF_3$ group of o-A sterically crowding the O_γ of Ser-195. In the secondary binding mode discussed above, the o-CF_3 moiety sterically prevented a completely planar transoid configuration of the carboxamido side chain of the alkylating moiety which would suggest here that o-A must adopt a binding mode different from m- or p-A. In this regard, one would not be surprised to find altered kinetic effects for o-A as a result of its different binding orientation.

Conclusions

The models for rate alteration by acetanilido-type moieties at Met-192 cannot be totally accounted for by binding strictly in the tosyl product. While tosyl pocket binding by these aromatic moieties has been shown by the NMR studies in the preceding paper,[3] kinetic and NMR evidence suggest that the moiety must, in general, interact at a second site on the enzyme surface, quite possibly the S_2 subsite of the peptide binding site. The aromatic moieties do not bind exclusively in one or the other site but rather partition between them. The kinetic modifications of both the deacylation (k_3) and acylation (k_2) rate constants for the ester substrates examined here may be of a steric nature, centering near site S_2 and perturbing the alignment of substrate with active-site residues. This would also be adjacent to the S_1 acylamido site, proposed by Kēzdy et al.[6] for the kinetic alterations by [N-(2-carboxyisopropyl)carbamyl]sulfonium[Met[192]]-α-chymotrypsin.

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Experimental Section

Materials. Enzymes, substrates, and alkylating agents were purchased or synthesized as described in the preceding paper in this issue.[3] Bromoacetanilide was a generous gift of Dr. W. B. Lawson, New York State Department of Health, Albany.

Synthesis of N-Ac-Ala-Gly-Gly-OMe. To 775 mg (3.8 mmol) of L-alanylglycylglycine (Ala-Gly-Gly, Nutritional Biochemicals Corp., Cleveland, Ohio) slurried in 125 mL of dry methanol at −60 °C was added 10.0 mL (139 mmol) of thionyl chloride in 50 mL of $CHCl_3$. The solution was allowed to warm to room temperature with constant stirring, after which the solvents were removed in vacuo, followed by redissolution of the residue in 50 mL each of pyridine and water. At 0 °C, 4.0 mL (116 mmol) of acetic anhydride was added with vigorous stirring, followed by a second 4.0 mL 15 min later. The reaction proceeded 30 min longer, followed by evaporation and chromatography on silica gel (14.5 × 3.8 cm) in 300-mL steps of 5, 10, and 15% methanol–chloroform. The acetylated tripeptide methyl ester, *N*-Ac-Ala-Gly-Gly-OMe, eluted in the last two steps: yield 530 mg (54%); mp 133–135 °C; MS parent peak at 259 ($A + 1/A = 0.124 \pm 0.004$; calcd., 0.124), fragmentation peaks at *m/e* 171, 144, 114, and 87 for $CH_3CONHCHCH_3CONHCH_2CO^+$, $^+NCH_2CONHCH_2CO_2CH_3$, $CH_3CONHCHCH_3CO^+$, and $^+NCH_2CO_2CH_3$, respectively; ^1H NMR (^2H_2O, DSS internal reference) 4.28 (quartet, C_α-H of Ala), 4.04 and 3.96 (2s, α-CH_2 of each Gly), 3.75 (s, methyl ester CH_3), 2.03 (s, CH_3 of acetyl group), 1.39 ppm (doublet β-CH_3 of Ala).

Procedures. Enzyme alkylation, assay, and purification have been described in the preceding paper.[3] *p*-Nitrophenyl acetate kinetics were measured spectrophotometrically on a Unicam SP 1800 instrument at pH 7.16 (0.09 M phosphate), 25 °C, 6.9% CH_3CN (to dissolve the substrate), at 347.8 nm where an isosbestic point for nitrophenol was used: $\Delta\epsilon = 5.06 \times 10^3$ M^{-1} cm^{-1}. The pH–rate profile was measured in phosphate buffers as well.

Ac-Tyr-OEt and *N*-Ac-Ala-Gly-Gly-OMe kinetics were measured with a Radiometer pH-stat system at pH 8.0, 0.1 M, KCl, 25 °C. All kinetic data were processed on a Data General Nova 1220 minicomputer by either linear least squares or a nonlinear regression analysis written by M. H. Klapper based on CURFIT by Bevington.[10]

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Protonic Reorganization in Catalysis by Serine Proteases: Acylation by Small Substrates[1]

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Abstract: The pH (pD)–rate profiles for acylation of α-lytic protease in protium and deuterium oxides by *p*-nitrophenyl acetate show p*K* values of 5.92 and 6.60, well below the enzyme ionization pK values of 6.70 and 7.35. This is attributed to a pH-induced change in the rate-determining step. The data are consistent with an initial acylation of active-site histidine (protolytically assisted, $k_H/k_D = 2.4$), followed by an intramolecular N → O acyl shift to active-site serine by parallel specific-acid-catalyzed ($k_H/k_D = 0.5$) and general-acid-catalyzed ($k_H/k_D = 2$) routes. The magnitude of $pK(D_2O) - pK(H_2O)$ and a proton inventory of the general-acid-catalyzed N → O acyl shift both suggest that deprotonation of α-lytic protease generates an unusual protonic site with a "loosely bound" proton. The β-deuterium isotope effect, $k_{3H}/k_{3D} = 0.98$, for the same step confirms nucleophilic interaction at carbonyl in the transition state. An abbreviated proton inventory for acylation of α-chymotrypsin by *p*-nitrophenyl acetate is consistent with a "loosely bound" proton there also. A proton inventory for acylation of elastase by *N*-(carbobenzyloxy)-L-alanine *p*-nitrophenyl ester is linear, suggesting one-proton catalysis and indicating that if "loosely bound" reactant-state protons are present, they are catalytically silent. The general picture, from this work and that of others, is that the catalytic response of serine proteases to small, "unnatural" substrates is highly variable, both in site of nucleophilic attack and involvement of protolytic catalysis. Probably mutual transition-state interactions over an extended region of both enzyme and natural-substrate structure are required to bring into active function the full catalytic capability with which the serine proteases have been endowed by biological evolution.

Enzymes of the serine hydrolase class[2] catalyze the hydrolysis of acyl substrates, such as peptides, other amides, and esters, by the double-displacement (acylation–deacylation) mechanism of eq 1. Their catalytic power is generally supposed to derive, at

$$\text{E-OH} + \text{RCOX} \rightarrow \text{EOCOR} + \text{HX} \qquad (1a)$$

$$\text{EOCOR} + H_2O \rightarrow \text{E-OH} + RCO_2^- + H^+ \qquad (1b)$$

least in part, from general acid–base catalysis[3] because (a) their active sites contain an assembly of carboxylate and histidine units[4] which are chemically suited for such a purpose[5] and which are biologically conserved from bacteria to mammals[6] and because (b) their catalytic rates are commonly reduced by two- to fourfold in deuterium oxide.[7]

The carboxylate–histidine assembly (the "charge-relay system";[4,5] hereafter ABCE, for "acid–base catalytic entity") has been examined in stable states of the enzymes by NMR techniques.[8–10] One of the protons associated with this assembly displays an unusual chemical shift,[8] but NMR titrations of the bacterial enzyme α-lytic protease in which the histidine has been isotopically labeled at carbon[9] or nitrogen[10] are in disagreement

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