molecules dissolved in a glass at low temperature. Their studies yield rates of reaction as a function of distance (edge to edge) between donor and acceptor down to about 14 Å. Their data appear to be in good accord with the quantum-mechanical model for a nonadiabatic system.[31] The edge-to-edge distance between aromatic rings in $I^{-}\cdot/K^{+}$ is about 7 Å. Miller's results for electron transfer from acridine radical anion to ethylanthraquinone in methyltetrahydrofuran glass may be extrapolated to the same distance to give 5×10^{9} s^{-1}.[35] That particular intermolecular reaction is slightly exothermic and believed to proceed with negligible activation energy; therefore, the rate may be directly compared with the preexponential factor for intramolecular transfer in $I^{-}\cdot/K^{+}$ (4.6×10^{7} s^{-1}). The 100-fold discrepancy may be due to differences in either H_{if} or the vibrational overlap terms or both. In Miller's experiments, the rate represents an average for various different relative orientations of the two components. Among these may be some for which electronic coupling is somewhat stronger than in $I^{-}\cdot/K^{+}$. A possible difference in the vibrational overlap terms may stem from the fact that there are four high-frequency nitrile stretching modes for $I^{-}\cdot/K^{+}$ but only two comparable carbonyl stretching modes in the quinone of Miller's example.

Note Added in Proof. Solutions of $II^{-}\cdot$, in the presence of a large excess of II, gave rise to ESR signals substantially broadened due to intermolecular electron transfer. These spectra differ qualitatively from those of dilute solutions of $I^{-}\cdot/K^{+}$ in DME, further substantiating an intramolecular mechanism for the latter.

Acknowledgments. This work was supported by grants from the National Science Foundation and the U.S. Army Research Office (CHE77-13302), and the Swiss National Science Foundation (Project 2.013.078). The authors wish to acknowledge the valuable technical assistance of Drs. J. Heinzer,[37] G. Plattner,[1c] and P. Fürderer[1c] and Mr. P. Share.[1b] We are also grateful to Dr. John Miller for several enlightening comments and for providing results of his experiments prior to publication.

(35) J. R. Miller, personal communication.
(36) Optical spectra are uncorrected for background absorption from cell.
(37) Laboratorium für Organische Chemie der Eidgen, Technischen Hochschule, Zürich.

Active-Site Interactions in Fluorine-19 Labeled [Methionine-192]-α-chymotrypsin[1]

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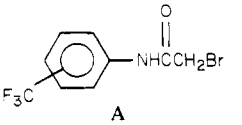
Abstract: Fluorine-labeled derivatives of α-chymotrypsin were prepared by specific alkylation at the active-site Met-192 by *o*-, *m*-, and *p*-(trifluoromethyl)bromoacetanilides (*o*-, *m*-, and *p*-A), respectively. Their chemical shifts were sensitive to the pH-dependent oligomerization equilibria at pH 5.7 and to the binding of the specificity pocket directed competitive inhibitor indole. In all cases an upfield shift was observed with indole that approached the (pH independent) chemical-shift values in 6 M urea. The K_{dissoc} (= K_D) for indole binding at pH 4.2 from the ^{19}F NMR titrations were as follows: *o*-A, 5.3 ± 2.3 mM; *m*-A, 3.2 ± 0.7 mM; *p*-A, 3.7 ± 0.5 mM. These latter constants were in reasonably close agreement with the K_I values obtained for indole vs. the substrate Ac-Tyr-OEt (B. H. Landis and L. J. Berliner, *J. Am. Chem. Soc.*, following paper in this issue). The extrapolated chemical shifts for infinite indole binding were indicative of a shift of the CF_3 moiety toward the solvent environment but still retained a significant degree of nonsolvated character as compared with the corresponding 6 M urea denatured derivative. A partition coefficient or "intramolecular competitive inhibition constant" was derived for the equilibrium between binding by the trifluoromethylacetanilido moiety in the "tosyl pocket" and a second environment outside this pocket where tosyl pocket binding was 23, 51, and 23% for *o*-, *m*-, and *p*-A, respectively.

Previous structural and chemical modification studies have indicated a role for the side chain of Met-192 in both the activation of α-chymotrypsin from its zymogen, chymotrypsinogen, and in the binding and orientation of α-chymotrypsin substrates during catalysis.[3] Particularly for smaller substrates, the Met-192 side chain acts as a "lid" over the hydrophobic side chain binding pocket, the "tosyl hole".[4] Of particular interest has been the kinetic consequences of specific alkylation of this side chain with α-halo ketones or amides as shown, for example, by Schramm and Lawson[5] and Kézdy et al.[6] Furthermore, it has seemed reasonable that an aromatic moiety on Met-192 might, in fact, bind favorably in the "tosyl pocket".

Fluorine NMR studies of biological systems are advantageous from two principal viewpoints: the high sensitivity of ^{19}F and the simplicity of no more than a few lines in the NMR spectrum. The broad chemical-shift range and sensitivity of ^{19}F can render an appropriately designed active-site probe a sensitive indicator of active-center interactions.[7a] In particular, Bittner and Gerig[7b,c] first reported the synthesis of the *o*-, *m*-, and *p*-(trifluoromethyl)bromoacetanilides, *o*-, *m*-, *p*-A, and ^{19}F relaxation times



A

for these reagents as their Met-192 sulfonium derivatives on native and denatured α-chymotrypsin. The transverse relaxation times (T_2) of the labeled alkylated derivatives decreased in the order *o*-A > *m*-A > *p*-A-chymotrypsin, respectively, which they sug-

(1) Portions of this work were presented at the 11th Jerusalem Symposium on Quantum Chemistry and Biochemistry, Apr 3-7, 1978.
(2) Armour Pharmaceuticals Co., Kankakee, IL 60901.
(3) D. M. Blow, *Enzymes, 3rd Ed.*, **3**, 185-212 (1971).
(4) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337-348 (1969).
(5) (a) H. J. Schramm and W. B. Lawson, *Z. Physiol. Chem.*, **332**, 97-100 (1963). (b) W. B. Lawson and H. J. Schramm, *Biochemistry*, **4**, 377-386 (1965).
(6) F. J. Kézdy, J. Feder, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 1009-1016 (1967).
(7) (a) J. T. Gerig, *Biol. Magn. Reson.*, **1**, 139-203 (1978). (b) E. W. Bittner and J. T. Gerig, *J. Am. Chem. Soc.*, **92**, 2114-2118 (1970). (c) *ibid.*, *92*, 5001-5003 (1970).

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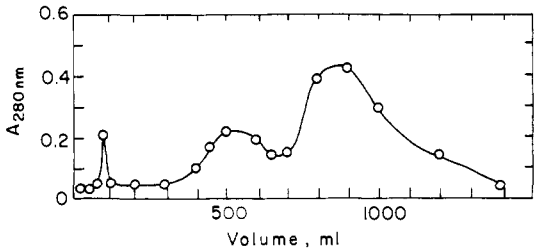

Figure 1. Elution profile for *o*-A-chymotrypsin on Whatman CM-52 (2.5 × 25 cm), pH 5.7, 0.10 M phosphate (0.10 M KCl), flow rate 1.5 to 2.0 mL/min. The peaks eluted in the following order: void peak (inactive material), native enzyme, Met-192 alkylated enzyme, and occasionally higher charged species.

Table I. Time Course of Alkylation[a]

derivative	molar excess	incubation, days	final Ac-Tyr-OEt act.,[b] % of initial
o-A-chymotrypsin	5–16	4–11	5–30
m-A-chymotrypsin	5–17	2–11	1–10
p-A-chymotrypsin	4–9	16–29	20–29

[a] Conditions were pH 5.0, 0.1 M acetate, 9% (v/v) ethanol, ambient temperature. Each result summarizes several experiments. [b] Ac-Tyr-OEt, acetyltyrosine ethyl ester.

gested was a reflection of the decreasing motional freedom of the respective trifluoromethyl groups on the enzyme. Thus, they believed that all were bound in the "tosyl pocket"; however, the *o*-CF_3 group was more "exposed" than the *p*-CF_3 moiety. Unfortunately, their equipment did not enable them to measure accurate ^{19}F chemical shifts for these derivatives nor to assess the homogeneity of the labeled derivatives. We report here an extensive study of these ^{19}F-labeled derivatives, their purification, and their sensitivity to interactions at the active site as one part of a structural–kinetic study of the effects of these and other Met-192 alkylating agents on α-chymotrypsin action.[8]

Results

Chromatography of Met-192 Alkylated α-Chymotrypsin. A typical elution profile for a CM-52 column at pH 5.7 is depicted in Figure 1. Except for *p*-A-chymotrypsin, all of these derivatives, as well as the several other alkylated derivatives discussed in the following paper in this issue,[8] separated clearly into the native (unalkylated) enzyme, the Met-192 alkylated derivative, and a minor component containing higher charged species. The *p*-A-chymotrypsin derivative essentially coeluted with unlabeled, native enzyme over the several pH and ionic strength gradients which were attempted. However, this *p*-A-chymotrypsin peak was at least 70–80% pure even assuming zero activity for the fully alkylated enzyme, which is unlikely (see Table I). Of course, a small impurity of unalkylated chymotrypsin was of no consequence for the ^{19}F NMR experiments, since only alkylated enzyme was detected. A more realistic estimate, based on the kinetic results discussed in the following paper, placed *p*-A-chymotrypsin between 90 and 100% pure. Modification of Met-192 was assumed from previous reports with these reagents and their analogues.[5a,b]

NMR. Figure 2 presents ^{19}F NMR spectra for *m*-A-chymotrypsin under varying conditions in solution at pH 4.2, 0.008 M phosphate, 0.08 M KCl, 20% (v/v) D_2O. Addition of indole (spectrum b) or denaturation with urea (spectrum c) produced an upfield shift in the resonance signal relative to external F_3AcOH (trifluoroacetic acid). In addition to the major protein peak in these spectra, there was a smaller peak upfield (denoted by arrows). This peak was unaffected by indole and was not resolved from the major peak in 6 M urea. Only over periods of several days did this peak increase in size, concomitant with a decrease

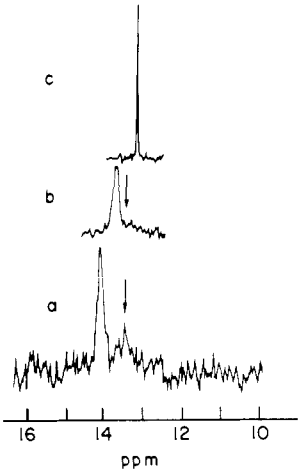

Figure 2. ^{19}F spectra of *m*-A-chymotrypsin. Chemical shifts are defined downfield from external trifluoroacetate in the same buffer. Spectra were taken in the Fourier transform mode at 84.67 MHz, 3.8-μs pulse width, 9025-Hz offset, 4000-Hz sweep with a repetition rate of 0.5 s. Each spectrum represents 1000–2000 scans of a typically 0.7 to 1.5 mM protein solution. Other conditions were pH 4.2, 0.008 M phosphate buffer, 0.08 M KCl, 20% (v/v) D_2O, 25 °C: (a) "native" enzyme, (b) in the presence of 7.5 mM indole, (c) in 6 M urea. Chemical shifts were insensitive to protein concentrations as low as 0.3 mM.

in active sites as measured from *p*-nitrophenyl acetate burst titrations. This minor peak was most probably due to a small amount of partially autolyzed or denatured protein.[9] Since this second peak predominated in unchromatographed alkylated enzyme, it was apparently overlooked in the previous relaxation experiments.[7c]

Since only one line was observed upon addition of indole, slow exchange of the CF_3 moieties between two (or more) sites was ruled out. A temperature study with *p*-A-chymotrypsin showed that the line width was essentially independent of temperature (over the range 5 to 35 °C), suggesting that the exchange rate was indeed rapid and not a borderline rate between fast and slow exchange. For fast exchange between two sites, A and B

$$\nu_{obsd} = \chi_A\nu_A + \chi_B\nu_B \tag{1}$$

where ν_{obsd} is the observed frequency, ν_A is the resonance frequency in site A, ν_B is the resonance frequency in site B, and χ_A and χ_B are the respective populations in sites A and B. If in the presence of zero indole $\nu_{obsd} = \nu_A$ and all chemical shifts are expressed relative to ν_A, then

$$\Delta\nu_{obsd} = \chi_B\Delta\nu_B = \frac{[EI]}{[E_t]}\Delta\nu_B \tag{2}$$

$$\Delta\nu_B = \nu_B - \nu_A$$

$$K_D = \frac{[E][I]}{[EI]}$$

Assuming $[I_0] - [EI] \approx [I_0] = [I]$, then

$$\frac{K_D}{[I_0]} = \frac{[E_t](1 - \Delta\nu_{obsd}/\Delta\nu_B)}{[E_t]\Delta\nu_{obsd}/\Delta\nu_B} = \frac{\Delta\nu_B}{\Delta\nu_{obsd}} - 1$$

and

$$\Delta\nu_{obsd} = \frac{\Delta\nu_B[I_0]}{K_D + [I_0]} \tag{3}$$

Analysis by nonlinear regression analysis of $\Delta\nu_{obsd}$ vs. indole data yielded K_D and $\Delta\nu_B$, the enzyme–indole dissociation constant and the maximum theoretical change in chemical shift, respectively.

Titration with indole was initially attempted at pH 5.7 in order to approximate conditions under which the enzyme was active,

(8) B. H. Landis and L. J. Berliner, *J. Am. Chem. Soc.*, following paper in this issue.

(9) Least likely was alkylation at Met-180, which normally situates in the enzyme interior; alkylation would necessitate its removal from these environs and produce (partially) denatured enzyme.

Table II. Summary of ^{19}F NMR Chemical-Shift Data and Related Dissociation Constants

derivative	conditions	pH 4.2	pH 5.7
o-A-chymotrypsin	native, ppm[a]	16.08 ± 0.03	15.87 ± 0.03
	6 M urea, ppm	14.68 ± 0.02	14.66 ± 0.02
	K_D, mM[b]	5.3 ± 2.3	15 ± 5
	ν_B, ppm[c]	15.61 ± 0.13	15.00 ± 0.15
	K_I, mM (pH 8.0)[d]	2.6 ± 0.1	
	K_A[e]	3.3 ± 0.6	
	tosyl pocket binding, %	23 ± 4	
	ν_o, ppm[f]	17.62 ± 0.49	
m-A-chymotrypsin	native, ppm	14.13 ± 0.03	14.07 ± 0.03
	6 M urea, ppm	13.19 ± 0.02	13.19 ± 0.02
	K_D, mM	3.2 ± 0.7	19 ± 3
	ν_B, ppm	13.54 ± 0.07	12.94 ± 0.15
	K_I, mM (pH 8.0)	4.0 ± 0.5	
	K_A	0.96 ± 0.19	
	tosyl pocket binding, %	51 ± 10	
	ν_o, ppm	14.61 ± 0.19	
p-A-chymotrypsin	native, ppm	15.66 ± 0.03	15.41 ± 0.03
	6 M urea, ppm	13.70 ± 0.02	13.68 ± 0.02
	K_D, mM	3.7 ± 0.5	16 ± 3
	ν_B, ppm	13.84 ± 0.11	13.05 ± 0.30
	K_I, mM (pH 8.0)	2.6 ± 0.3	
	K_A	3.3 ± 0.6	
	tosyl pocket binding, %	23 ± 4	
	ν_o, ppm	21.76 ± 1.32	

[a] All chemical-shift data are expressed in parts per million downfield from F_3AcOH (external) at pH 5.7. No corrections were made of bulk susceptibility. Conditions were 0.008 M phosphate buffer, 0.08 M KCl, 20% (v/v) D_2O, 25 °C, unless noted otherwise. [b] Best fit (nonlinear regression analysis) to the indole binding chemical-shift data as exemplified in Figure 3. [c] Theoretical chemical shift at saturating (infinite) indole concentration. [d] Competitive inhibition constant for indole vs. Ac-Tyr-OEt at pH 8.0, 0.1 M KCl, 25 °C. See ref 8. [e] Partition coefficient between *in* tosyl pocket and *out* of tosyl pocket binding as derived in the text. [f] Theoretical chemical shift for 100% binding of the trifluoromethylacetanilido moiety *in* the tosyl pocket.

without getting too far into the pH range where autolysis might occur. These K_D values for indole binding at pH 5.7, which are listed in Table II, were much higher than those found from competitive inhibition studies with Ac-Tyr-OEt as substrate,[8] also listed in Table II. Qualitatively similar results were obtained with dioxane, a ligand also known to bind in the tosyl hole.[4]

It was well known that native chymotrypsin underwent dimerization at pH 4 and oligomerization at pH 6.[11a,b] Neet[11c] had also shown that chymotrypsin modified at Met-192 with *p*-nitrophenacyl bromide oligomerized at pH 6.2 but did not dimerize at pH 4.0. To examine whether oligomerization occurred with the acetanilidochymotrypsins, sedimentation velocity studies were performed at pH 5.7 and 4.2 wiith *o*-A-chymotrypsin. At pH 5.7, the sedimentation coefficients ($s_{20,w}$) were 3.72 ± 0.10 S at 4.7 mg/mL and 3.48 ± 0.12 S at 1.9 mg/mL. At pH 4.2, the sedimentation coefficient was found to be essentially independent of concentration and more consistent with the values reported for the monomeric enzyme, 2.61 ± 0.07 S at 6 mg/mL and 2.63 ± 0.06 S at 2 mg/mL vs. 2.6 S for native chymotrypsin[11b] and 2.5 S for monomeric [Met[192]]-*S*-(*p*-nitrophenacyl)chymotrypsin.[11c] The seemingly high K_D values observed at pH 5.7 by ^{19}F NMR were probably the result of oligomerization.

(10) Since the assumption that $[I] \approx [I_0]$ was imperfect ($[E_0] \approx [I_0] \approx K_D$), once K_D was determined it was possible to calculate [I] and refit the data. This gave a slightly improved fit and did not greatly change K_D. More than one iteration changed K_D by less than 10%, so K_D values reported herein represent only one such iteration.

(11) (a) K. C. Aune and S. N. Timasheff, *Biochemistry*, **10**, 1609–1616 (1971). (b) K. C. Aune, L. C. Goldsmith, and S. N. Timasheff. *ibid.*, **10**, 1617–1622 (1971). (c) K. E. Neet, K. M. Sackrison, G. R. Ainslee, and L. C. Barritt, *Arch. Biochem. Biophys.*, **160**, 569–576 (1974).

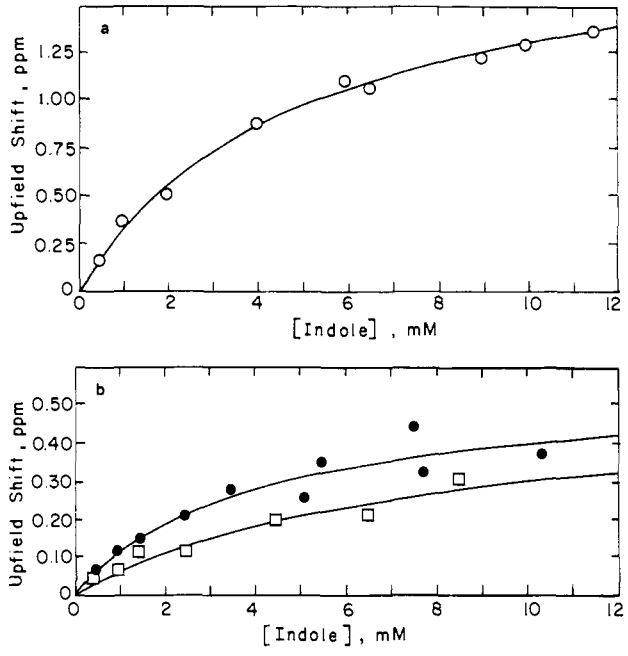

Figure 3. Upfield shift for derivatized chymotrypsins with increasing indole concentration. Conditions were as in Figure 2. The solid line represents the theoretical fit to a single dissociation constant, K_D, listed in Table II: (a) *p*-A-chymotrypsin, ○; (b) *m*-A-chymotrypsin, ●; *o*-A-chymotrypsin, □.

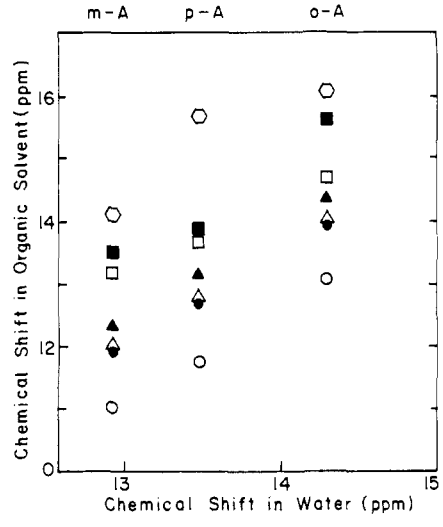

Figure 4. Chemical shifts of the trifluoroacetanilide moieties vs. their shift in water. The free bromoacetanilide reagents were at 10 mM; the labeled proteins were at pH 4.2, 1 mM. No bulk susceptibility corrections were included. All solutions were 20% (v/v) D_2O, 25 °C: ○, methanol; ●, acetone; △, acetonitrile; ▲, dioxane; □, proteins, 6 M urea; ■, proteins, theoretical maximum chemical shift at infinite indole concentration; ○, "native" labeled proteins. The free bromoacetanilide reagents were dissolved in water with 5% (v/v) CH_3CN cosolvent.

At pH 4.2, where oligomerization did not occur, titration with indole yielded the typical hyperbolic binding curve shown in Figure 3. Here the derived K_D values were in much closer agreement with those obtained from kinetic measurements (see Table II or the following paper in this issue).

In order to assess "solvent" character of the chymotrypsin active site, each alkylating agent was examined in several solvents and then their relative chemical shifts were plotted vs. the shift in a common solvent (water) after the analysis of Weiner and Malinowski.[12] This analysis predicts that for a family of substituent types on a solute (e.g., *o*-, *m*- and *p*-CF_3 groups in A) a family

(12) P. H. Weiner and E. R. Malinowski, *J. Phys. Chem.*, **75**, 1207–1210 (1971).

of parallel lines should be obtained if the observed chemical shifts were simply "solvent induced chemical shifts". For the moiety on the protein, the "solvent" here is construed to be the immediate protein–solvent environment in the active-site region where the trifluoromethylacetanilido moiety resides. The data of Figure 4 clearly show that a significant degree of nonuniform solvation existed for the native proteins, while the free reagents, model compounds, or urea-denatured enzymes experienced generally uniform solvent interactions.

Discussion

Since the binding of indole altered the ^{19}F chemical shift of the trifluoromethyl-alkylated derivatives, it must have effected a change in the environment(s) of those aromatic moieties. Since indole had been shown to bind in the tosyl pocket, while furthermore causing no apparent conformational changes in the native enzyme,[4] a reasonable model for the observed NMR chemical-shift changes at pH 4.2 is a (competitive) direct displacement of a CF_3 alkyl moiety by indole. This is corroborated quantitatively by the similarity in K_D for the NMR and K_I values derived from competitive inhibition measurements with acetyltyrosine ethyl ester (Ac-Tyr-OEt) as substrate for each derivative (Table II). Since the observed chemical shift of each derivative is under fast chemical exchange conditions, this (observed) shift may represent that of the aryl moiety (1) bound completely in the tosyl pocket, (2) in an equilibrium between "in pocket" binding and exposed to the solvent, or (3) an even more complex model involving a secondary binding mode in the active center as well. Figure 4 and Table II both indicate that upon total saturation with indole the chemical shift approaches but does not equal that of the urea-denatured enzyme, implying, not surprisingly, that the moiety does not experience a totally solvated, aqueous environment. This was further shown from solvent isotope shifts of the urea-denatured derivatives where it was estimated that the fluorine moiety was at best 80% solvated by the external solvent.[13] Thus, while the observed NMR spectrum represented an average of an equilibrium between binding in the "tosyl pocket" and a second "environment", it was difficult from these studies alone to determine whether another discrete binding site was contained in this second environment.

In order to ascertain the extent to which the aromatic moieties bind in the tosyl pocket, we must examine the Ac-Tyr-OEt competitive inhibition data (Table II). Since an aromatic substrate or inhibitor cannot bind to the enzyme when an aromatic moiety is already bound in the pocket, the groups on Met-192 are exhibiting an intramolecular competitive inhibition. For these alkylated derivatives with Ac-Tyr-OEt, if one assumes that K_S or K_I, respectively, is identical for species with the aromatic moiety in (E_{in}) or with the aromatic moiety displaced from the tosyl pocket (E_{out}), the contribution from this intramolecular inhibition, K_A, may be calculated (eq 4–8) where I is indole. These rate equations

$$E_{in} \overset{K_A}{\rightleftharpoons} E_{out} \qquad K_A = [E_{out}]/[E_{in}] \tag{4}$$

$$E_{out} + S \overset{K_S}{\rightleftharpoons} E_{out}S \overset{k_2'}{\longrightarrow} E_{out}S' \overset{k_3'}{\longrightarrow} E_{out} + \text{products}$$

$$E_{out} + I \overset{K_I}{\rightleftharpoons} E_{out}I$$

$$v/[E_t] = \frac{k_2'k_3'}{k_2' + k_3'}[S]/[K_S/(1 + k_2'/k_3')](1 + 1/K_A) + [S]$$

$$k_{cat}' = \frac{k_2'k_3'}{k_2' + k_3'} \tag{5}$$

$$K_m'(\text{app}) = [K_S/(1 + k_2'/k_3')][1 + 1/K_A] \tag{6}$$

$$K_m'(\text{app})/k_{cat}' = K_S(1 + 1/K_A)/k_2' \tag{7}$$

$$K_I(\text{app}) = K_I(1 + 1/K_A) \tag{8}$$

differ from those of the unalkylated enzyme insofar as the partition coefficient (K_A) is nonexistent (approaches infinity) for the unalkylated enzyme. The acylation (k_2) and deacylation (k_3) rate constants are primed to indicate that while the aromatic moieties (displaced from the "tosyl pocket") may not alter binding significantly, they may change these rate constants. Thus knowledge of K_I, the dissociation constant for indole to a species where the alkyl moiety is not situated in the tosyl pocket, and the apparent inhibition constants, K_I(app), for the three aromatic trifluoromethyl derivatives *o*-, *m*-, and *p*-A-chymotrypsin, allow us to calculate the intramolecular partition coefficient, K_A. The derivative chosen for the species E_{out}, which most clearly models a "control", is [Met^{192}]acetamidochymotrypsin, which differs from the *o*-, *m*-, or *p*-A-chymotrypsin species by an aromatic group. Taking the K_I for acetamidochymotrypsin from the indole inhibition of Ac-Tyr-OEt hydrolysis,[8] the partition coefficients, K_A, were derived as listed in Table II, together with the theoretical chemical shift, ν_0, of each alkyl moiety for 100% binding in the tosyl pocket, assuming that K_A is unchanged at pH 4.2.[14] First, the calculated chemical shift for binding in the pocket is substantially downfield from that for the 6 M urea-denatured enzyme. That is, the physicochemical environment of the ^{19}F moiety in the apolar pocket was significantly deshielded compared with the moiety in the aqueous medium in the denatured case. This has been observed frequently with earlier ^{19}F protein labeling studies, as contributions such as van der Waals forces, diamagnetic and paramagnetic anisotropy, hydrogen bonding, charge transfer, and other interactions may originate from an "hydrophobic pocket" or adjacent active-center environment (for a review, see ref 7a).

The strikingly large chemical-shift difference for *p*-A-chymotrypsin between total tosyl pocket binding (ν_0 = 21.76 ppm) and urea-denatured protein (13.7 ppm) must be due to specific contributions from residues lining the aromatic specificity pocket. This 8-ppm downfield shift for this para derivative (*p*-A) is to be compared with the 1.4- to 3-ppm downfield shifts for the meta and ortho derivatives, *m*- and *o*-A, respectively. While it is more difficult to speculate without the X-ray structure of this derivative, some interactions seem possible from our model building studies which would contribute to a major downfield shift. The bottom of the tosyl hole is lined with two serine residues (189 and 190), one or both of which might potentially hydrogen bond with a fluorine on the *p*-(trifluoromethyl) group. In the case of the *o*- or *m*-A derivatives, the $-CF_3$ group would not be properly oriented for such an interaction. Interestingly, Gerig has reported a 7-ppm downfield shift for *p*-fluoro-*trans*-cinnamoylchymotrypsin, while the meta and ortho analogues were shifted significantly less.[7a]

The extreme sensitivity of ^{19}F NMR was displayed quite convincingly here not only in the monitoring of indole binding but also in the detection of inactivated species and the manifestation of protein oligomerization. The secondary narrower line peaks which occasionally appeared near the denatured protein region of the spectrum (see Figure 2) were frequently of significant intensity in the impure alkylated derivative *prior* to chromatography on CM-52. Such (presumably autolyzed) derivatives could contribute appreciably to errors in correct assignments, assessment of active species concentrations, or T_1 and T_2 measurements.

The dimerization phenomena occurring at pH 5.7 were directly linked to active-site conformation, as judged by the differences in the chemical shift of the monomer at pH 4.2 (Table II). For the subsequent binding of indole, while in general describable as binding only to monomer forms

$$(E)_n \rightleftharpoons E + I \overset{K_D}{\rightleftharpoons} E{\cdot}I \qquad n \geq 2 \tag{9}$$

or to the dimer (oligomer)

$$(E)_n + I \overset{K_D'}{\rightleftharpoons} (E)_n{\cdot}I$$

the latter expression was probably more realistic, since ν_B values for 100% indole saturated species (see Table II) were different

(13) P. C. Lauterbur, B. V. Kaufman, and M. K. Crawford in "Biomolecular Structure and Function", P. F. Agris, B. Sykes, and R. Loeppky, Eds., Academic Press, New York, pp 329–351 (1978).

(14) This is not without precedent, for indole binding to α-chymotrypsin was essentially unchanged over the range pH 4.5–7.8. See A. R. Fersht and Y. A. Requena, *J. Am. Chem. Soc.*, **93**, 7079–7087 (1971).

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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