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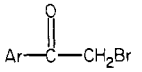
Reactions of α-Chymotrypsin with 4-(Trifluoromethyl)-α-bromoacetanilide†

M. E. Ando and J. T. Gerig*

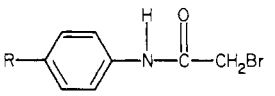
ABSTRACT: 4-(Trifluoromethyl)-α-bromoacetanilide is structurally similar to a large number of compounds that inactivate α-chymotrypsin by alkylating the methionine-192 residue or occasionally serine-195. Fluorine nuclear magnetic resonance (NMR) experiments suggest that this material reacts with the enzyme at two distinct loci. One of these involves alkylation of methionine while reaction at a second site, which does not appear to be near the active site, diminishes the proclivity for reaction at methionine. Solvent effects (H_2O/D_2O) and fluorine–proton Overhauser experiments indicate that the reporter group attached to methionine closely contacts the protein surface and is thereby shielded from solvent while the CF_3 group at the second site is more accessible to solvent.

Selective chemical modification of amino acid residues remains an important tool for structural studies of proteins, especially when the reagent used for the modification has a feature that is easily detected spectroscopically. In an early study Schramm & Lawson (1963) demonstrated that molecules having the general structure I are effective affinity labels



I

for α-chymotrypsin, and more recent work with a greatly expanded series of inactivators has illuminated those features of I that lead to rapid and selective reactions with this enzyme (Lawson & Rao, 1980; Lawson, 1980). In most cases alkylation at a methionine residue, presumed to be Met-192, of the protein was observed. However, a few structures were found to react at a serine residue, which likely was Ser-195. The Ar group of structure I is usually derived from a substituted aniline. Although substituents on the aromatic ring of Ar have some influence on the rate of the protein modification reaction, a diverse collection of para substituents on α-bromacetanilide (II) was found to have only relatively minor



II, R = H, CH_3, F, CH_3O, NO_2

effects on the reaction rate; in all cases these compounds were found to rapidly alkylate methionine (Lawson & Rao, 1980).

With the goal of introducing a fluorine NMR[1] reporter group into chymotrypsin, we earlier prepared a set of trifluoromethyl-substituted analogues of II and examined the inactivation of chymotrypsin by these structures (Bittner & Gerig, 1970). At the ortho and meta positions the CF_3 derivatives reacted 8–9 times more slowly than the corresponding CH_3-substituted α-bromoacetanilides. However, at the para position the CF_3 substituent slowed the reaction nearly 2 orders of magnitude relative to the rate when the para substituent was CH_3. Landis & Berliner (1980a,b) have reported additional studies of these fluorine-labeled derivatives of chymotrypsin and showed via fluorine chemical shifts that indole, a good competitive inhibitor of the enzyme known to bind in the "tosyl pocket" (Steitz et al., 1969), is able to displace the trifluoromethylaryl group from its normal location in or on the enzyme structure. Alkylation of the enzyme by these reagents was also demonstrated to have substantial effects on the kinetics of the hydrolysis of specific and nonspecific substrates (Landis & Berliner, 1980b).

Upon starting high-resolution fluorine NMR studies of the trifluoromethyl-labeled enzyme derivatives, we were perplexed by the appearance of two major signals in the spectrum. Landis & Berliner (1980a) have reported similar observations and have assigned one of the resonances to partially autolyzed or denatured protein. In the case of the ortho and meta CF_3-substituted derivatives, this explanation may well be correct, for chromatographic procedures substantially reduce the contribution of one signal to the spectrum. However, the same methods do not appreciably remove the second component from the fluorine spectrum of the 4-(trifluoromethyl)-

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[1] Abbreviations: NMR, nuclear magnetic resonance; GPNA, *N*-glutarylphenylalanine *p*-nitroanilide; fid, free induction decay; ^{19}F NMR, fluorine-19 nuclear magnetic resonance; NOE, nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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α-bromoacetanilide derivative of α-chymotrypsin; evidence is presented here that suggests that the extra signal corresponds to reaction of this inactivator at one or more amino acids of the protein that are *not* Met-192 or Ser-195. Our observations also indicate that modification of these unidentified amino acids can influence the rate of the inactivation reaction at Met-192. Thus, to conclude that molecules with general structure I invariably react at Met-192 or Ser-195 of this enzyme may be erroneous, especially if the rate of loss of enzyme activity during the modification reaction is slow.

Experimental Procedures

Materials

4-(Trifluoromethyl)-α-bromoacetanilide was prepared according to the procedure reported previously (Bittner & Gerig, 1970). 4-Fluoro-α-bromoacetanilide was prepared by a similar procedure and had NMR spectral data consistent with the expected structure and a melting point of 135–137 °C (lit. mp 138–139 °C; Lawson & Rao, 1980). *N*-Glutarylphenylalanine *p*-nitroanilide (GPNA) was purchased from Sigma while *p*-nitrophenyl acetate was obtained from Aldrich. All buffers and salts were of reagent grade, and the water used was distilled from glass after passage through an ion exchanger. α-Chymotrypsin was supplied by Worthington Biochemicals or Miles and showed 0.89 ± 0.04 active site/molecule of enzyme as determined by the method described below. Indistinguishable kinetic and NMR results were obtained with enzymes from either source.

Methods

Preparation of Inactivated Chymotrypsin. The appropriate α-bromoacetanilide (1.4 mmol) was dissolved in 100 mL of absolute ethanol. Aqueous phosphate buffer (0.1 M, 830 mL, adjusted to the desired pH) was added slowly to the ethanol solution. Chymotrypsin (2 g, 0.07 mmol) was dissolved in 70 mL of the same buffer and the solution added slowly to the inhibitor solution. The mixture was allowed to stand at room temperature (~25 ± 1 °C), and small aliquots were occasionally drawn for an activity assay with GPNA as the substrate. When the reaction was complete, the mixture was dialyzed extensively against water at 4 °C, filtered through paper, and lyophilized. In some experiments dimethyl sulfoxide replaced ethanol as the cosolvent.

Activity Assays. Activity of the enzyme derivatives toward the specific substrate GPNA was determined as described previously (Bittner & Gerig, 1970). Activity toward the nonspecific substrate *p*-nitrophenyl acetate (PNPA) was assayed with 0.1 M Tris-HCl buffer, pH 7.5, at 25 °C. A 0.1-mL aliquot of enzyme solution was added to 3.0 mL of buffer in a cuvette and the absorbance at 280 nm measured to determine the concentration of dissolved enzyme. Then 0.1 mL of PNPA stock solution (64.3 mg of PNPA dissolved in 10 mL of acetonitrile) was added and the rate of increase in absorbance at 400 nm due to production of *p*-nitrophenol measured with a Gilford 2000 spectrophotometer operating with a full-scale deflection of 2.5 absorbance units. The rate of increase of absorbance due to spontaneous hydrolysis was always measured at the same time in a separate cuvette. After correction for spontaneous hydrolysis, the rates of absorbance increase were divided by the absorbance at 280 nm to correct for variations in enzyme concentration. Activities for modified enzymes were expressed as a percentage of the activity so observed for the native enzyme used to prepare the modified form.

Active Site Concentrations. Active site concentrations of the native and modified enzymes were determined by the initial-burst method with *p*-nitrophenyl acetate as the substrate, as described by Landis & Berliner (1980a). The liberation of *p*-nitrophenol was followed at 347.5 nm with solutions containing 0.1 M acetic acid and 6.1% acetonitrile at pH 5 by comparison to blanks containing all components but the substrate. Typically, 10–20 min was sufficient for the linear, steady-state region of the process to be well-defined, and extrapolation of this linear region back to zero time defined the initial burst in the absorbance. This absorbance change was converted to concentration with $\epsilon_{347.5} = 5060\ M^{-1}\ cm^{-1}$.

Protein concentrations were determined from the absorbance at 280 nm with the extinction coefficient $\epsilon_{280} = 50000$ (Wilcox, 1970).

Isoelectric focusing was carried out as described previously (Ando et al., 1980).

Ion-exchange chromatography employed a 3 cm × 55 cm column of Whatman CM-32 carboxymethylcellulose, equilibrated according to suppliers directions. Approximately 0.15 g of enzyme was applied to the column and eluted at ambient temperature with 0.1 M KCl, adjusted to pH 7 with KOH solution, at a flow rate of 60 mL/h. The eluent was monitored at 280 nm with an ISCO UA-5 monitor and was partitioned into 8-mL fractions. A typical trace from such an experiment is shown in Figure 2.

Fluorine NMR Spectra. Fluorine spectra were recorded at 94.13 MHz on a Varian Associates XL-100. Sample temperatures were controlled at 25 ± 1 °C. Protein solutions for ^{19}F NMR spectroscopy were ca. 1 mM in protein and contained 0.1 M KCl, 0.5 mM EDTA, 0.5 mM sodium trifluoroacetate, and 10% deuterium oxide. Usually the sample pH was adjusted to 6 or 7. Fluorine T_1 and $^{19}F\{^1H\}$ nuclear Overhauser experiments were carried out as described previously (Gerig et al., 1979). Fluorine spectra at 470.38 MHz were obtained with the Bruker WM-500 instrument of the Southern California Regional NMR Facility housed at the California Institute of Technology.

Number of Reporter Groups per Mole of Protein. Fluorine NMR spectroscopy was used to determine the number of 4-(trifluoromethyl)aryl or 4-fluoroaryl groups present per mole of modified protein. A known amount of a reference compound, usually sodium 2-(trifluoromethyl)-4-nitrobenzenesulfonate (Gerig & Reinheimer, 1975) or *p*-fluorobenzoic acid (Aldrich), was introduced into samples of modified protein for which the protein concentration was known from the UV absorption at 280 nm. Urea (8 M) or sodium dodecyl sulfate (5%) was present so that all fluorine reporter groups of a given type were represented by a single sharp resonance at the chemical shift of the denatured protein. Enough transients were collected in the Fourier transform mode to give a good signal-to-noise ratio with a delay between transients equal to at least 5 times the spin–lattice relaxation rate for the most slowly recovering signal in the spectrum, so that saturation effects were negligible. Integration of the transformed fids by routines in the Nicolet software was carried out and the ratio of the intensity of the reporter group signal to that of the reference compound determined. Such assays were reproducible to ±0.2 reporter group/mol of protein and are assumed to be accurate within the same uncertainty.

Amino acid analyses were carried out by AAA Laboratories, Mercer Island, WA.

Results

4-Fluoro-α-bromoacetanilide Derivative. Lawson and Rao have reported that 4-fluoro-α-bromoacetanilide rapidly inactivates α-chymotrypsin by alkylating a methionine residue. Under our conditions the loss of activity of the enzyme toward

the specific substrate GPNA is rapid and follows a pseudo-first-order rate law. With a 20-fold excess of inhibitor the half-life for activity loss was 15 min, in good agreement with the value given by Lawson & Rao (1980). After dialysis to remove excess reagent the alkylated enzyme showed no detectable activity toward GPNA. However, activity toward the nonspecific substrate *p*-nitrophenyl acetate increased 460% relative to activity of the native enzyme.

The fluorine NMR signal for the native modified enzyme appears at 40.2 ppm upfield from internal trifluoroacetate. Solutions used for ^{19}F NMR spectroscopy were stable for at least 1 week, and no detectable changes in the fluorine spectra were observed over this time interval. Denaturation of the protein by heat shifts the resonance to 40.7 ppm upfield, a chemical shift that is similar to the fluorine shift of 4-fluoro-α-bromoacetanilide under the same conditions. Protein from several preparations showed 1.0 ± 0.1 mol of fluorinated reporter group/mol of protein. Finally, the effect of changing the solvent from water to deuterium oxide on the fluorine shift of the fluorine shift was investigated. A small but detectable upfield shift of the fluorine resonance is expected if the fluoroaryl group is exposed to solvent protons (Lauterbur et al., 1978). With an external reference, no solvent effect on the fluorine chemical shift of the inactivated enzyme was observed.

The activity toward specific and nonspecific substrates and the fluorine NMR results are in complete accord with Lawson and Rao's conclusions regarding the orientation of the aromatic ring of the inactivator during alkylation and suggest that this group remains in the active site depression after alkylation.

4-(Trifluoromethyl)-α-bromoacetanilide Derivative. Treatment of the enzyme with the *p*-CF_3-substituted inhibitor was carried out by the same procedure as used for the *p*-F case. The trifluoromethylated inhibitor is less soluble under these conditions, and it was occasionally difficult to maintain a 20-fold concentration excess of reagent over protein as indicated by the appearance of a small amount of precipitated inhibitor. The course of the inactivation was again followed by assays for activity toward GPNA. At pH 4 and 25 °C a half-life for inactivation of approximately 72 h was observed. After 300 h activity approached a plateau. However, residual activity never reached zero, and after dialysis, the protein obtained in these reactions retained 29% activity toward the specific substrate. Some autolysis over this period is expected, and indeed activity toward *p*-nitrophenyl acetate declined 50%. The dialyzed material isolated at the completion of the reaction was 130% active toward this substrate. At pH 7 the loss of activity toward GPNA was slightly more rapid, requiring about 200 h ($t_{1/2} \sim 47$ h) to reach a plateau. The isolated product in this case was 27% active toward GPNA. Landis & Berliner (1980a) report similar results when the modification reaction with 4-(trifluoromethyl)-α-bromacetanilide is carried out at pH 5 under slightly different conditions and *N*-acetyltyrosine ethyl ester is used in the activity assay.

In some of our experiments dimethyl sulfoxide was used as a cosolvent. The solubility of the inactivator is improved under these conditions although the reaction kinetics are not greatly changed. This procedure was abandoned when it became clear that it was very difficult to remove dimethyl sulfoxide from the modified protein by extended dialysis. This solvent also favored the undesired reaction with the protein described below.

Fluorine NMR Studies of the p-CF_3 Derivative. The ^{19}F spectrum of α-chymotrypsin that has been treated with 4-(trifluoromethyl)-α-bromoacetanilide shows three resonances

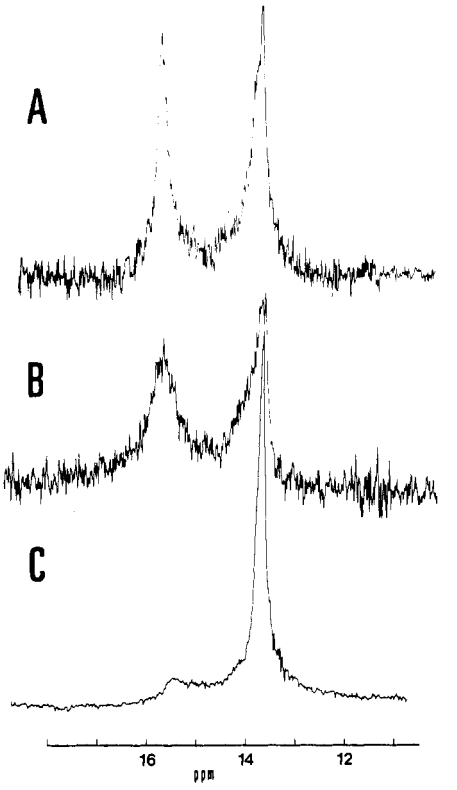


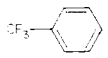
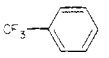
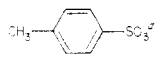
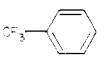
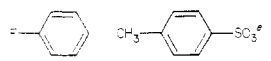
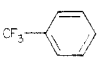
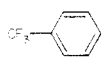
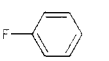
FIGURE 1: Fluorine NMR spectra of α-chymotrypsin inactivated with 4-(trifluoromethyl)-α-bromoacetanilide. (A) Spectrum recorded at 94.13 MHz, sample pH of 3, 25 °C. The protein was prepared at pH 4.4 with ethanol as a cosolvent. (B) Spectrum recorded at 470.38 MHz; same protein preparation as for trace A with the NMR sample at pH 5.5. (C) Spectrum recorded at 94.13 MHz, sample pH of 7, 25 °C. The protein was prepared at pH 7 with ethanol as the cosolvent. The chemical shift scale uses internal trifluoroacetate (0.5 mM) as a reference; the signals shown appear downfield of the reference.

at 13.6, 13.7, and 15.6 ppm downfield from the signal of trifluoroacetate (Figure 1). The exact position of these signals relative to the trifluoroacetate reference were slightly (±0.1 ppm) pH dependent but there were no large effects of pH on chemical shifts over the range pH 3–8. Samples appeared to be indifinitely stable as judged by the lack of change in the ^{19}F NMR spectra with time. In particular, samples of modified enzyme prepared at pH 7, which were incubated in 0.1 M acetic acid at pH 3.8, showed no change in their spectra over periods up to 15 days. Landis & Berliner (1980a) have noted that ^{19}F NMR spectra of this enzyme derivative are independent of concentration down to 0.3 mM, and we have confirmed this observation. The signals at 13.6 and 13.7 ppm overlap substantially; attempts to better resolve these signals by operating at a higher magnetic field were unsuccessful (Figure 1, trace B) presumably due to an increased contribution of the chemical shift anisotropy mechanism to the line widths (Hull & Sykes, 1975).

Enzyme that had been denatured by dissolving in 5% sodium dodecyl sulfate or 8 M urea showed a single sharp line at 13.6 ppm. Thus, the resonances observed at 15.6 and 13.7 ppm must arise because of the presence of native protein structure.

The intensity of the signal at 13.6 ppm in the spectra of native enzyme could be reduced by extended dialysis, ultrafiltration through UM-10 membranes, and various chromatographic steps, as described below. Similar observations have been made with [[4-(trifluoromethyl)phenyl]-sulfonyl]-α-chymotrypsin (Ando et al., 1980), and in both cases we believe that the species represented by the fluorine chemical shift identical with that of the denatured enzyme represent a collection of autolysis products and possibly unreacted

Table I: Double Modification Experiments

first group[a]	second group[a]	pH[b]	chemical shifts (ppm)[c] CF_3	chemical shifts (ppm)[c] F
	none	5.0		−40.2 (1.0)
	none	4.4	15.6, 13.7 (0.8)	
		7.0	15.6, 13.7 (1.3)	
		4.4	13.7 (0.2)	−40.2 (1.1)
		7.0	13.7 (1.2)	
		7.0	13.7, 13.6 (~5)	−40.8 (1.0)
		4.4	15.6, 13.7 (0.6)	−40.2, −41.1 (0.6)
		7.0	15.6, 13.7 (1.2)	−40.2, −41.1 (0.3)

[a] The first reporter group was placed on the enzyme by reaction of the native enzyme with the appropriate reagent. The second reporter group was placed on this modified enzyme with the conditions specified under Experimental Procedures. [b] pH of the reaction medium used in attaching the second group to the enzyme. [c] Fluorine chemical shifts relative to internal trifluoroacetate. The number in parentheses is the moles of reporter group of a given type per mole of doubly modified protein. [d] Tosylchymotrypsin in which the serine-195 residue is modified (Steitz et al., 1969) was used. [e] Chymotrypsin was first treated with 4-fluoro-α-bromoacetanilide and then with tosyl fluoride. The doubly modified enzyme showed no activity toward GPNA *or* PNPA.

modification reagent. On the premise that the 13.7-ppm peak represents unreacted bromide that is tightly bound to the protein but in an unreactive position, we attempted to "chase" such material by extended dialysis of the modified protein against 10 mM D-phenylalanine. This treatment produced no change in the fluorine spectrum.

Solvent isotope effects on the fluorine chemical shifts were examined. There was no shift of the 15.6-ppm peak relative to external trifluoroacetate when the solvent was changed from H_2O (containing 9% deuterium oxide for a spectrometer lock) to D_2O (>90%). However, the signals at 13.6 and 13.7 ppm shifted approximately 0.2 ppm upfield with the same solvent change, implying that the CF_3 groups represented by these signals are exposed to solvent.

Some initial fluorine–proton nuclear Overhauser experiments were carried out at 94.13 MHz. Upon saturation of the proton resonances of the enzyme, the intensity of the fluorine resonance at 15.6 ppm was reduced to 17% of its value in the absence of such irradiation. The intensity changes in the peaks at 13.6 and 13.7 ppm were difficult to quantitate since there is appreciable overlap; in these cases the $^{19}F\{^1H\}$ NOEs were much less, with integrated intensities being reduced to ca. 70% of their values in the absence of irradiation. In the same experiments, the intensity of the trifluoroacetate signal was enhanced slightly (~5%).

Attempted Purifications. The fluorine NMR spectra show that the product of reaction between α-chymotrypsin and 4-(trifluoromethyl)-α-bromoacetanilide is heterogeneous, and various attempts were made to resolve the crude reaction product into its components chromatographically. Affinity chromatography according to the procedure of Wilkinson et al. (1976) appeared to resolve modified from native enzyme but was ineffective in further resolution of the modified enzyme(s).

Isoelectric focusing of the modified (and native) enzyme showed several components of different isoelectric points, and so efforts were made to develop an ion-exchange system for separation of the modified chymotrypsins. A typical chromatogram when carboxymethylcellulose (Whatman CM-32) was the stationary phase is shown in Figure 2. Early fractions were inactive toward *p*-nitrophenyl acetate or GPNA, and since similar bands are observed in chromatograms of the native enzyme used in the modification reaction, these were

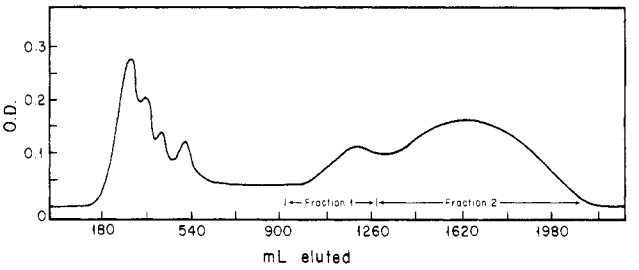


FIGURE 2: Typical eluton profile for 4-(trifluoromethyl)-α-bromoacetanilide-modified α-chymotrypsin chromatographed according to the method given under Experimental Procedures. Properties of fractions 1 and 2 are described in the text. The modified enzyme was prepared at pH 4.4.

ignored. We were never able to resolve the remaining part of the chromatograms into more than two broad, overlapping peaks. Subfractions of the eluent were pooled into fractions 1 and 2 as indicated in Figure 2 and examined for activity and the presence of CF_3 reporter groups. Fraction 1 contained only 0.2 mol of CF_3 group/mol of enzyme but retained appreciable activity toward GPNA (~60% that of the native enzyme) and *p*-nitrophenyl acetate. Fraction 2 contained 0.8 mol of CF_3 groups/mol of protein and had a reduced activity toward GPNA (~25% that of native enzyme) but an augmented activity toward *p*-nitrophenyl acetate (~120%). Fraction 1 thus appears to represent largely native (nonalkylated) enzyme while fraction 2 represents modified protein. The ^{19}F NMR spectrum of fraction 2 was similar to trace A in Figure 1; that is, this fraction contained protein(s) that placed the CF_3 group in at least two distinct environments.

Other Observations. Further evidence regarding the nature of the modified enzymes formed when α-chymotrypsin is treated with 4-(trifluoromethyl)-α-bromoacetanilide was sought by examining the products obtained when a previously modified enzyme was used in the reaction. Table I summarizes the results. When the native enzyme is modified with 4-fluoro-α-bromoacetanilide and then treated with the 4-(trifluoromethyl) analogue, an appreciable reaction with the second reagent takes place even though all methionine-192 residues have been presumably alkylated by the 4-fluoro compound. All CF_3 groups in this enzyme resonate at the upfield (13.7-ppm) position. Treatment of tosylchymotrypsin

with the 4-(trifluoromethyl) reagent likewise leads to alkylation of the enzyme even though the active site serine is esterified and access to the active site region is blocked by the tosyl group. The introduced CF_3 groups appear at 13.7 ppm in the fluorine spectrum. When both Met-192 is alkylated by the 4-fluoro compound and the active site serine is esterified, reaction with 4-(trifluoromethyl)-α-bromoacetanilide still takes place to give a protein with CF_3 NMR signals at the upfield position. This doubly modified enzyme appears to have enhanced reactivity toward the third reagent, and about five trifluoromethyl groups per mol of enzyme are introduced by the standard reaction conditions. The chemical shift of the aromatic fluorine of the multiply modified protein is somewhat perturbed. When enzyme that has been modified with 4-(trifluoromethyl)-α-bromoacetanilide is treated further with the 4-fluoro analogue, detectable reaction with the second reagent takes place. On the basis of chemical shifts and peak intensities, the 4-fluoro reporter group still reacts with methionine-192 (peak at −40.2 ppm) and also gives another reaction product represented by a peak at −41.1 ppm with roughly equal probability.

The pH dependence of the reaction between the 4-(trifluoromethyl) inactivator and the enzyme was explored. At both pH 3.0 and 4.4 under our standard reaction conditions approximately 0.8 mol of CF_3 group/mol was introduced into the protein. Of these groups, roughly half were characterized by the low-field (15.6-ppm) fluorine chemical shift. Some variations in the proportion of total fluorine signal intensity found at the low-field position was observed, and the reasons for these are not yet clear. An analysis of the material formed at pH 4 for the number of enzymic active sites present indicated 0.8 ± 0.1 active site/molecule of protein when protein concentration was determined by absorbance at 280 nm. A similar value is reported by Landis (1976) for protein modified at pH 5. At pH 7.0, the reaction leads to incorporation of 1.3 mol of CF_3 reporter group, but now about 85% of the total fluorine intensity is found at the high-field position. The number of active sites was found to be (0.7 ± 0.1)/molecule in this case.

Thus, at pH values between 3 and 7, the number of moles of low-field CF_3 groups introduced per mole of protein by our reaction conditions stays relatively constant at about 0.3. Significantly more reaction to give products characterized by the high-field signal appears at pH 7.

Amino acid analysis of enzyme that had been modified at pH 4.4 with 4-(trifluoromethyl)-α-bromoacetanilide was carried out and compared to an analysis of the native enzyme used in preparing the derivative. The amino acid composition of both protein samples generally fell within the range of values reported in previous analyses [data from several laboratories cited in Lawson & Schramm (1965)]. There was a significant reduction in the methionine content of the alkylated protein, corresponding to about 0.6 residue/mol.

Discussion

4-Fluoro-α-bromoacetanilide provides a rapid and specific alkylation of α-chymotrypsin. Our results show a simple 1:1 stoichiometry of moles of fluorinated reporter groups introduced to moles of protein. "Initial-burst" kinetics with *p*-nitrophenyl acetate as the substrate indicate 0.7 ± 0.1 mol of active sites is present per mol of modified protein. While the derivatized enzyme is reactive toward this substrate, activity toward the specific substrate GPNA is completely lost. The lack of a solvent isotope effect on the chemical shift of the para fluorine nucleus is consistent with the model-building studies of Lawson & Rao (1980), which suggest that the fluoroaryl ring of the inactivator can reside in the tosyl pocket.

With the 4-(trifluoromethyl) inhibitor, the alkylation reaction does not reach completion even though an excess of substituted α-bromoacetanilide is present, and the reaction can eventually incorporate more than one 4-(trifluoromethyl)aryl ring per mol into the protein that is isolated from the reaction mixture, depending upon reaction conditions. Activity toward the specific substrate is diminished but never completely lost. While amino acid analysis suggests that appreciable alkylation at methionine has taken place with this reagent, subsequent treatment with the rapidly reacting and methionine-specific 4-fluoro reagent indicates that unalkylated methionine-192 residues remain. Fluorine NMR spectra of the CF_3-containing enzyme suggest that 4-(trifluoromethyl)aryl groups are introduced into at least two distinguishable regions of the protein structure.

We propose that 4-(trifluoromethyl)-α-bromoacetanilide is, in fact, able to alkylate methionine-192 of the enzyme, although at a very slow rate. Alkylation at this position results in loss of activity toward the specific substrate and enhancement of reactivity toward *p*-nitrophenyl acetate. We assign the fluorine resonance at 15.6 ppm to the reporter groups attached to Met-192; the substantial downfield shift away from the position for CF_3-aryl groups attached to denatured enzyme suggests close contact between the trifluoromethyl group and the enzyme (Hull & Sykes, 1976). The lack of a solvent isotope effect on the shift and the $^{19}F\{^1H\}$ NOE, which approximates the theoretical limit for relaxation dominated by proton–fluorine dipolar interactions, also suggest an environment for the CF_3 group that places it away from solvent and in close proximity to the amino acids of the protein. Thus, the 4-(trifluoromethyl)aryl groups attached to Met-192 could, like the 4-fluoroaryl group, largely reside inside the tosyl pocket. Landis & Berliner (1980a) have shown that indole, a good competitive inhibitor of the enzyme that binds in this location, alters the fluorine chemical shift of the CF_3 reporter group in this system, presumably by displacing the 4-(trifluoromethyl)aryl group from its preferred orientation within the tosyl pocket.

Since methionine-192 alkylation by the 4-(trifluoromethyl)-substituted reagent is so slow, other reactions of this material with enzyme have a chance to come to the fore. The second fluorine NMR signal (13.7 ppm) must represent reaction at one or more other amino acids of the protein. Reaction at these secondary sites places the reporter group in an environment in which the CF_3 group is largely exposed to solvent, as indicated by the near-normal chemical shift, an observable solvent isotope effect on the chemical shift, and the reduced magnitude of the $^{19}F\{^1H\}$ NOE.

The available data suggest that a second site of reaction cannot be serine-195. When modified at pH 7, about 0.3 mol of low-field CF_3 groups is introduced, representing, if the conclusion above is correct, modification of Met-192. Another ~0.8 mol of CF_3 groups, resonating at higher field, is present per mol of protein. However, active site determination shows that this protein still possesses about 0.7 mol of reactive sites/mol of protein. On the presumption that our initial-burst kinetics measure the number of reactive (unmodified) serine-195 residues in the protein, the CF_3 groups not attached to Met-192 also cannot be attached to Ser-195. In addition, covalent modification with 4-(trifluoromethyl)-α-bromoacetanilide takes place even when serine-195 is tosylated or when both serine-195 and methionine-192 are blocked by previous reactions. In both of these cases, the CF_3 groups introduced give NMR signals at the high-field position.

FIGURE 3: A sketch of the orientation of the substituents in the nitroaryl and trifluoromethylaryl rings, viewed end-on. With the nitro substituent, as well as fluoro, methyl, and methoxyl, conformations can be found in which the electron density of the substituent is located mostly within the "thickness" of the aromatic ring. The size of the fluorine atom and the length of the C–F bond combine in the CF_3 group to make the substituent occupy a volume whose diameter is appreciably larger than the thickness of the aromatic ring so that the electron density from the substituent appears well above and below the "top" and "bottom" of the aromatic ring.

We suggest that there may be some "communication" between the secondary reaction sites and the region containing Met-192 and that alkylation of these secondary sites may inhibit alkylation at Met-192. Thus, material that is alkylated first at Met-192 can subsequently also be alkylated at the secondary site. However, protein that is alkylated first at the secondary site appears not to be readily alkylated at Met-192 by the 4-(trifluoromethyl) reagent; enzyme activity persists in this form, and some of the unreacted Met-192 groups can still be alkylated with the 4-fluoro reagent even after the loss of activity in the 4-(trifluoromethyl) modification reaction has reached a plateau. Thus, the product of reaction between 4-(trifluoromethyl)-α-bromoacetanilide and α-chymotrypsin may be a mixture of at least three modified proteins, which are distinguished by alkylation at methionine-192, alkylation at one or more secondary sites, or reaction at both centers. All of the proteins would retain normal or enhanced activity toward *p*-nitrophenyl acetate, but only the second would remain active with specific substrates such as GPNA. The relative amounts of the various alkylated enzymes formed upon exposure to this reagent are pH sensitive, but at all pH values alkylation at both sites takes place. Regrettably, neither the NMR nor the chromatographic experiments provide a means to quantitate the exact amounts of the various alkylated proteins that appear to be formed in this reaction system.

It has been noted that chymotrypsin modified with α-bromoacetophenone can be isolated at two conformational isomers, depending upon the solution pH used in preparation of the derivative (Mariano et al., 1978). In this work attempts to detect equilibration between 4-(trifluoromethyl)-α-bromoacetanilide-modified proteins prepared at pH 4.4 and 7 by fluorine spectroscopy produced no evidence for conformational isomerization. It is difficult to rationalize the incomplete loss of activity in reactions at either pH strictly in terms of conformational differences, and we believe that conformational effects play a minor role in the observations reported here.

Why does the 4-(trifluoromethyl)-substituted inhibitor react so slowly? The answer probably does not lie in the electronic nature of the CF_3 group for, while the nitro (NO_2) group is more electron withdrawing (Alder et al., 1971), 4-nitro-α-bromoacetanilide reacts with the enzyme with a half-life of 12 min at pH 5 or 7 (Lawson & Rao, 1980). Consideration of Corey–Pauling–Koltun models suggests that, of the many para substituents examined in this system, trifluoromethyl is the bulkiest. The electron density of this substituent would appear above and below the density of the planar aromatic ring, as indicated in Figure 3. A particular mode of binding α-bromoacetanilide inactivators to the binding pocket at the active site of α-chymotrypsin for subsequent attack by methionine-192 has been proposed (Lawson & Rao, 1980). The present results and our earlier ones suggest that this interaction is susceptible to rather subtle steric effects especially when a substituent like CF_3 on the aromatic ring appreciably "thickens" this structure. While the experiments described here suggest that α-bromoacetanilides can react at a second site on the enzyme when reaction at Met-192 or Ser-195 is slow, the identity of this site is not revealed by our experiments, and it remains for future studies to provide these details.

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