Motion at the Active Site of [(4-Fluorophenyl)sulfonyl]chymotrypsin†

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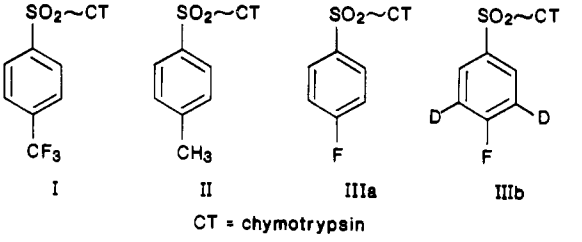
Received December 18, 1985; Revised Manuscript Received April 15, 1986

ABSTRACT: Fluorine and deuterium NMR relaxation studies have been used to examine the motion of the 4-fluorophenyl ring attached to the active site of [(4-fluorophenyl)sulfonyl]-α-chymotrypsin at pH 4. Analysis of the results indicates that rotation about the 2-fold axis of this ring is reasonably rapid, though not as fast as in tosylchymotrypsin. Two-dimensional (2D) nuclear Overhauser effects (NOEs) were used to suggest the shifts of those protons of the enzyme close enough to the fluorine nucleus to lead to relaxation; important proton–fluorine dipolar relaxation contributions arise from protons with shifts of 7.4 ± 0.3 ppm and between 4.0 and 5.4 ppm. Specific deuteration permits the assignment of the first of these to the protons ortho to the fluorine while serine-189, cysteines-191 and -220, and methionine-192 are suggested as possible bearers of the other protons. The fluorine chemical shift effect observed for the native conformation of this protein is 9 ppm downfield of the shift observed with the denatured protein; this large shift may be the result of van der Waals interactions between the fluorine and one or more of the protons whose signals appear in the 2D NOE experiments.

Arylsulfonyl fluorides have been widely used in the design of active site directed inactivators of serine proteases and dihydrofolate reductase (Glazer et al., 1975; Baker, 1967, 1969; Blow, 1971; Wong et al., 1978). The mechanism for action of serine proteases involves, transiently, the formation of an enzyme species in which a covalent bond is developed between a serine at the active site of the enzyme and the acyl group of the substrate (Fersht, 1977). Sulfonyl fluorides are reactive with the same serine residue, forming analogues of acylated enzymes that are stable for relatively long periods of time at neutral pH and thereby opening the possibility of spectroscopic or crystallographic examination of these species (Weiner et al., 1966; Birktoft & Blow, 1972; Berliner & Wong, 1974; Vaz & Schoellmann, 1976; Steitz & Shulman, 1982). In order to utilize the advantages of fluorine NMR spectroscopy (Gerig, 1978) in structural studies of the active site of α-chymotrypsin, we prepared a series of fluorine-substituted benzenesulfonyl fluorides and examined the inactivation of the enzyme by these materials (Gerig & Roe, 1974). Fluorine relaxation studies of [[4-(trifluoromethyl)phenyl]sulfonyl]-chymotrypsin (I) (see Chart I) indicated that the trifluoromethyl group is rapidly rotating in this protein but provided no information on the mobility of the aromatic ring to which the CF_3 is attached (Gerig et al., 1979). Experiments with a similar structure (II) suggest that both the CH_3 and the aromatic ring are undergoing rapid rotation in this enzyme derivative (Ando et al., 1982). The present paper describes the results of studies of the 4-fluorobenzenesulfonyl derivative of chymotrypsin (III), a system that would be expected to have a somewhat different dynamical situation at active site compared to I or II because of the reduced steric demands of the fluorine substituent relative to those of the methyl or trifluoromethyl. We have found that the 4-fluorophenyl ring of another acylated chymotrypsin appears to rotate slowly, and the factors at work in that system may also operate in derivative III. We describe here evidence from nuclear relaxation rates in IIIa and a deuterated analogue, IIIb, which indicates that motion of the aromatic ring takes place more slowly in III than in II. Interactions with protons of the protein appear to play a significant role in relaxation of fluorine in this structure, and experiments that may aid in eventual identification of these protons are described.

Chart I



While this manuscript was in preparation, Liao and Berlin (1985) reported fluorine NMR studies of the reactions of 4-fluorobenzenesulfonyl chloride with chymotrypsin. Derivatization of the enzyme with this reagent is much less specific than is the case when the corresponding sulfonyl fluoride is used, although judging from the published spectral data, the species examined here was likely present in their experiments.

Experimental Procedures

Materials. All water was deionized and distilled in an all-glass apparatus. Deuterium oxide (99.8 atom % D), sulfuric acid-d_2 (99.5% atom % D), and deuterium-depleted water were purchased from Aldrich. 4-Fluorobenzenesulfonyl fluoride was prepared as described previously (Gerig & Roe, 1974). α-Chymotrypsin (3× recrystallized, salt free) was obtained from Worthington Biochemicals or Sigma and was used as received. Titrations by the methods described in an earlier paper (Gerig & Roe, 1974) showed 78–83% of the active sites expected on a gravimetric basis.

Deuterated 4-fluorobenzenesulfonyl fluoride was prepared from 4-fluoroacetanilide (Fairfield Chemical Co.) by the following series of reactions. 4-Fluoroacetanilide (7 g) was dissolved in 20 mL of sulfuric acid-d_2 and heated with stirring to 95–100 °C for 7 h. The material was isolated by pouring

† This work was supported by the U.S. Public Health Service through National Institutes of Health Grant GM-25975. The National Science Foundation also supported this work through a grant for the purchase of the 300-MHz NMR instrument (CHE 80-018438) and support of the Southern California Regional NMR Facility (CHE 79-16324A1).

0006-2960/86/0425-4772$01.50/0

the mixture into ice, followed by extraction with ethyl acetate. The organic layer was dried over $MgSO_4$ and the solid recovered by evaporation of the solvent in vacuo. The exchange was repeated, with 4.5 g of the acetanilide being recovered. While the predominant product of this reaction is the one in which the protons ortho to the fluorine are replaced by deuterium, other deuterated species were present. For convenience, this material is referred to as the dideuterated product. 4-Fluoro-3,5-dideuterioaniline hydrochloride was obtained by heating 1.6 g of the deuterated fluoroacetanilide with 10 mL of 6 N HCl at reflux for 2 h. The solvent was removed in vacuo. 4-Fluoro-3,5-dideuteriobenzenesulfonyl chloride was obtained by dissolving 1.5 g of the deuterated fluoroaniline hydrochloride in a mixture of 3.5 mL of concentrated HCl and 1 mL of glacial acetic acid. The solution was placed in an ice bath, and when it was thoroughly chilled, an ice-cold solution of sodium nitrite (0.9 g in 2 mL of water) was added over 2–3 min. This solution was then added, with vigorous stirring, to 13 mL of glacial acetic acid containing 0.33 g of $CuCl_2$ (suspended) that had been saturated with SO_2 gas. Stirring was continued while the mixture was warmed to 45 °C for 30 m. The reaction mixture was poured into ice and extracted with ether. The ether extracts were cautiously treated with saturated $NaHCO_3$ solution until CO_2 evolution ceased, then dried over $MgSO_4$, and evaporated. Conversion of the sulfonyl chloride to 4-fluoro-3,5-dideuteriobenzenesulfonyl fluoride followed the procedure described previously (Gerig & Roe, 1974). The aromatic portion of the fluorine spectrum of this product consisted of three signals in the ratio, from low to high field, of 8:38:54 and separated by 0.262 ppm. The signals arise from species containing 0, 1, and 2 deuterium atoms ortho to the fluorine (Gerig & Hammond, 1984). In combination with deuterium spectra of the same material, the results indicated the presence of 1.5 deuterium atoms per molecule at the ring positions ortho to the fluorine and 0.7 deuterium per molecule at the meta positions.

[(4-Fluorophenyl)sulfonyl]-α-chymotrypsin was prepared by dissolving 1.14 g of $K_2HPO_4 \cdot H_2O$, 5 mL of 2-propanol, and 1 g of α-chymotrypsin in 100 mL of water. The pH was adjusted to 7, and 50 mg of 4-fluorobenzenesulfonyl fluoride was added. The mixture was stirred for 1.5 h at room temperature [approximately 10 half-lives for the inactivation reaction (Gerig & Roe, 1974)] and then dialyzed against 0.05 M acetic acid. The mixture was lyophilized and the solid stored at 4 °C until used. An assay using the hydrolysis of *N*-glutarylphenylalanine-*p*-nitroanilide indicated less than 5% activity. It has been shown earlier that the stoichiometry of this modification reaction is 1:1, with one fluorophenyl ring attached per molecule of enzyme (Gerig & Roe, 1974).

Instrumentation. Melting points were measured on a Thomas-Hoover capillary melting point apparatus. A Radiometer PHM52 meter was used to record pH. Visible and UV spectra were recorded on a Cary 15 spectrometer. Routine ^1H NMR spectra were recorded on Varian T-60, EM360A, or Nicolet NT300 instruments. Routine ^{13}C spectra were obtained with a Varian CFT20.

Procedures. Fluorine-19 spectra at 94 MHz were accumulated with a Varian XL-100 as described previously, using 12 mm o.d. sample tubes (Gerig et al., 1979; Gerig & Hammond, 1984). Fluorine spectra at 282 MHz were recorded with the Nicolet NT300 using 10 mm o.d. samples. A capillary of 0.1 M sodium 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O was used to provide a reference signal for proton spectroscopy. Experiments at 470 MHz used 5 mm o.d. tubes and were carried out on the Bruker WM-500 at the Southern California Regional NMR Facility, California Institute of Technology. In all cases, the temperature controllers supplied with the instruments were used to maintain the sample temperature at 25 °C, unless otherwise stated.

Deuterium spectroscopy at 46 MHz was done by using a broad-band probe on the NT-300, which was run unlocked. Protein solutions for deuterium spectroscopy were made up in deuterium-depleted water (Aldrich).

Preparation of samples for NMR spectroscopy and the methods used to obtain T_1, T_2, NOE,[1] and line shape data were the same as described previously (Gerig & Hammond, 1984). Determinations of T_1 and the NOE were reproducible to within 5–10% as were T_2 determinations by measurement of line widths. T_2 values determined by Hahn spin-echo or Carr–Purcell experiments were reproducible to within 10–15%. The reproducibility of determinations of the deuterium line width was ±15%. In each case, the reproducibility of these experiments is assumed to be a reasonable estimator of their accuracy. An effort was made to detect nonexponential spin–lattice fluorine relaxation behavior at 282 MHz, but T_1 for the native enzyme was well represented by a single-exponential process within the errors of the experiment. Two-dimensional $^{19}F[^1H]$ NOE data were collected by using the pulse sequence of Rinaldi (1983) as described for a related system (Hammond, 1984).

Results

Initial Observations. The fluorine NMR spectrum of the modified α-chymotrypsin III consists of a single broad line appearing 24.62 ppm upfield of the signal from a trace of trifluoroacetate present as an internal reference. The chemical shift did not appear to be pH-dependent over the range 3.6–6 and does not depend on the isotopic composition of the solvent (H_2O vs. D_2O). When the protein is dissolved in 8 M urea or 1% sodium dodecyl sulfate to bring about denaturation, the resonance was much sharper and appeared at 33.51 ppm. The chemical shifts of 4-fluorobenzenesulfonic acid and of 4-fluorobenzenesulfonyl fluoride (aromatic fluorine) are close to the latter value. A freshly prepared sample of III exhibits only the low-field signal initially, but over a period of time that appears to depend upon sample pH, a signal begins to appear at the high-field position, presumably because of hydrolysis or elimination of the sulfonate group from the enzyme or possibly because of enzyme denaturation (Figure 1). At pH 4 there is no noticeable decomposition over a period of 5 days at room temperature; at pH 6 the process seems to be somewhat faster although the extent of this change was not quantitated. Fluorine spectra of samples of the modified enzyme stored as a lyophilized powder at 4 °C indicated that the decomposition processes also take place in the solid and, after storage for 1 year in this manner, more than half of the total fluorine signal intensity from such samples was due to the upfield signal.

Relaxation Rates. Fluorine spin–lattice relaxation rates ($R_1 = 1/T_1$), transverse relaxation rates ($R_2 = 1/T_2$), and the $^{19}F[^1H]$ nuclear Overhauser effect on the intensity of the fluorine signal upon saturation of all protons of the system were determined at several frequencies. Figure 1 illustrates results of the NOE experiments. Note that only the low-field signal, presumably representing native enzyme, shows an appreciable effect. There were some indications that the relaxation parameters are dependent on protein concentration over the range

[1] Abbreviations: CSA, chemical shift anisotropy; NOE, nuclear Overhauser effect; fid, free induction decay; rf, radio frequency.

Table I: Relaxation Parameters for [(4-Fluorophenyl)sulfonyl]chymotrypsin[a]

aromatic deuterium present	rf (MHz)	$w_{1/2}$ (Hz)	R_1 (s^{-1})	R_2 (s^{-1})	NOE
none	94.1	17	53 (16)	6.7 (6.7)	−0.53 (−0.58)
	282.3	38	119 (18)	2.1 (2.6)	−0.79 (−0.74)
	470.6	54	170 (28)	(1.8)	(−0.94)
d_1	94.1	15	47 (14)	4.2 (5.6)	
	282.3		[b]	1.7 (2.2)	
d_2	94.1	16	56 (12)	2.9 (4.5)	
	282.3		125[c] (16)	1.6 (1.8)	
	46.1[d]	215	675 (748)		

[a] At 25 °C, 1 mM protein, 0.1 M KCl in D_2O, and pH 4. Numbers in parenthesis are those calculated by using the model for relaxation described in the text. [b] Not reliably determined. [c] Obtained by a Hahn 90°–τ–180° spin-echo experiment. [d] Line width of deuterium resonance.

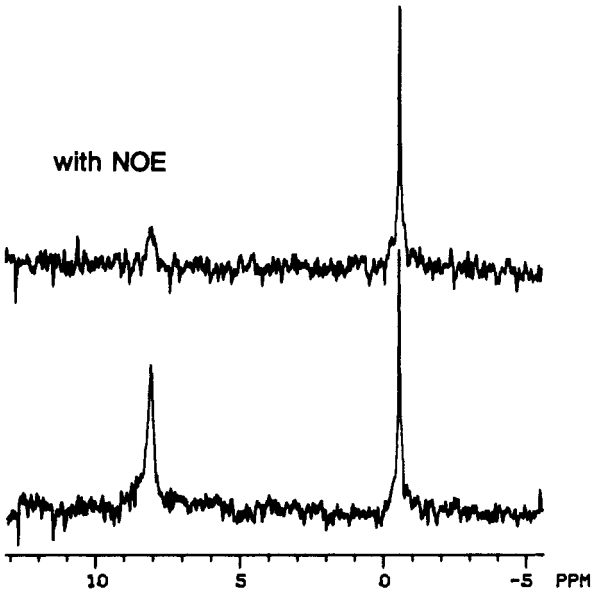


FIGURE 1: Fluorine NMR spectra of [(4-fluorophenyl)sulfonyl]-chymotrypsin at 282 MHz, pH 4, 25 °C, and 1 mM protein concentration. The signal from the degradation product at high field is described in the text. The top trace shows the steady-state fluorine–proton nuclear Overhauser effect when the proton signals are saturated.

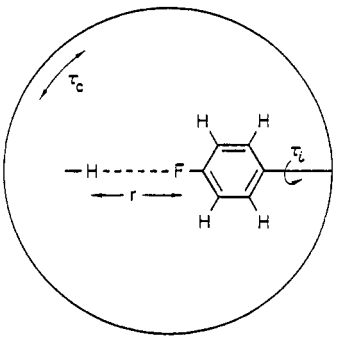


FIGURE 2: Dynamical model used to analyze the relaxation data. The fluorophenyl ring is assumed to rotate about an axis that is attached to a sphere undergoing reorientation characterized by the correlation time τ_c. A single proton at a distance r from the fluorine is used to represent the various protons of enzyme enzme that might alter relaxation by dipole–dipole interactions.

0–3 mM, presumably because of the known propensity of chymotrypsin to associate (Horbett & Teller, 1973; Neet & Brydon, 1970). To minimize the effects of aggregation, relaxation experiments were done at pH 4. A protein concentration of 1 mM was used; this represented a compromise between the requirements for reasonable signal strengths and the desirability of working at very low concentrations to minimize association. The data obtained under these conditions are summarized in Table I. Transverse relaxation rates estimated from the line widths according to the relation $w_{1/2} = \pi R_2$ were in reasonable accord with determinations made by Hahn 90°–τ–180° spin-echo experiments.

The temperature dependence of the $^{19}F[^1H]$ NOE and the signal line width was examined at 282 MHz. The Overhauser effect was observed not to change significantly over the temperature range 5–25 °C while the line width of the fluorine signal varied smoothly from 30 Hz at 35 °C to 73 Hz at 5 °C.

Deuterated enzyme IIIb exhibited a set of three overlapping fluorine signals which were separated by 0.28 ppm and appeared in the same intensity ratios as the fluorine signals of the corresponding sulfonyl fluoride, as described above. It was possible, with some loss of accuracy due to the incompletely resolved signals, to determine relaxation and NOE parameters for the signals corresponding to the enzyme species with two deuterium atoms ortho to the fluorine (designated the d_2 species) and with a single deuterium in this position (designated d_1). Data for these systems are also collected in Table I.

The deuterium spectrum of IIIb exhibited a sharp peak adjacent to a broad line. The sharp signal ($w_{1/2} \sim 1$–2 Hz) is due to residual deuterium in the solvent, and if this is assigned a chemical shift of 4.7 ppm, the chemical shift of the broad resonance is about 7.8 ppm. A second narrow line, superimposed on the broad component, appeared in deuterium spectra of aged samples and presumable arises from fluorobenzenesulfonyl groups released during hydrolysis/decomposition reactions. Line shape fittings of the deuterium spectra were used as described previously (Gerig & Hammond, 1984) to determine R_2 (Table I).

A small amount of relaxation data for the protein in H_2O were collected but not enough for a detailed analysis. Qualitatively, it appears that the R_1 relaxation rates are slightly increased in this solvent, implying that a solvent-derived proton (or protons) comes close enough to the fluorine in [(4-fluorophenyl)sulfonyl]chymotrypsin to participate in relaxation.

Analysis of Relaxation Data. In order to extract information about the dynamics of the fluorophenyl ring in [(4-fluorophenyl)sulfonyl]chymotrypsin, it is necessary to assume a theoretical model for the dynamics of the system. [A "model-free" approach to the description of relaxation of side chains in proteins is available but, as published, appears to be strictly applicable only in the limit of rapid side-chain motion (Lipari & Szabo, 1982).] In previous work with a protein-bound 4-fluorophenyl ring (Gerig & Hammond, 1984) we adopted the model depicted in Figure 2, and this was used for the present case as well. The correlation time τ_i describes the diffusive rotation of the fluorophenyl ring about its symmetry axis, which is attached to a sphere representing the protein that tumbles isotropically with a correlation time τ_c. The protons of the fluorophenyl ring are explicitly included in the calculation by using F–H distances and angles based on the structure of fluorobenzene (Nygaard et al., 1968). The re-

Table II: Fluorine Spin–Spin Relaxation[a]

enzyme	rf (MHz)	τ (ms)[b]	line width		Hahn echo		Carr–Purcell	
			R_2(obsd)	R_2(calcd)	R_2(obsd)	R_2(calcd)	R_2(obsd)	R_2(calcd)
IIIa	94.1		53	53				
	282.3		119	121				
	470.6		170	146				
IIIb	282.3				125	119		
	282.3	0.8					81	79
	282.3	2.4					83	110
	282.3	6.0					111	116

[a] Calculated values were obtained for the two-site exchange situation described in the text. [b] Time between refocusing 180° pulses in Carr–Purcell sequence.

laxation due to protons of the protein and solvent was accounted for by assuming the presence of one other proton at a distance r from the fluorine along an exentsion of the symmetry axis of the fluorophenyl ring.

Proton–fluorine dipolar interactions and chemical shift anisotropy (CSA) were the only relaxation mechanisms considered for the fluorine nucleus. The parameters used to compute the contributions of the CSA mechanism were the same as those used previously and were derived from preliminary results of a study of the fluorine chemical shift anisotropy of (4-fluorophenyl)alanine (Hiyama et al., 1986). Spin–lattice relaxation by the CSA mechanism is computed to make a minor contribution to R_1, and the analysis of R_1 and NOE data described is not very sensitive to the choice of CSA parameters.

Deuterium relaxation rates were computed by using the equations described earlier (Ando et al., 1982) and by using a value of 178 kHz for the quadrupolar coupling constant (Gerig & Hammond, 1984). The asymmetry parameter was neglected.

Initially, an attempt was made to find a single set of values for τ_i, τ_c, and r that would reproduce all the experimental fluorine and deuterium relaxation rates and the NOEs given in Table I. When it became clear that this would not be possible, we excluded the fluorine R_2 data from the analysis and were then able to fit the fluorine R_1 and NOE results as well as the deuterium line width within the estimated experimental errors by using the values $\tau_c = 15 \pm 1$ ns, $\tau_i = 2 \pm 0.5$ ns, and $r = 0.23 \pm 0.05$ nm. The computed data are given in parentheses in Table I; the fit is reasonably "tight" and is largely constrained by the values of the NOE and the deuterium line width. The same parameters lead to computed relaxation rates for the ortho-deuterated species that are in reasonable agreement with experiment, although the change in R_1 at 94 MHz upon successive deuteration is predicted better than the absolute value of this parameter. The experimental errors for the relaxation rates of the deuterated enzyme are larger, as noted above.

Attempts were made to fit the relaxation time and NOE data in Table I to a model in which the fluoroaromatic ring rotates by 180° flips rather than diffusively (London, 1980). Although it was possible to fit the frequency dependence of T_1 with this model, we could not find a set of parameters that would accomplish this *and* simultaneously fit the NOE and deuterium line width data.

Transverse relaxation is much more sensitive to the parameters chosen to characterize the CSA contribution, but any reasonable adjustment of these did not significantly improve the agreement between the calculated and experimental line widths in the analysis described above. A selective excitation ("hole burning") experiment was carried out by using the DANTE sequence (Morris & Freeman, 1978) in order to check for the possibility that several enzyme species are represented by the single (broad) fluorine resonance present in the spectrum of III, each with similar but unresolved chemical shifts. As in an earlier study (Gerig & Hammond, 1984), the effect of the selective irradiation of the signal from [(4-fluorophenyl)sulfonyl]chymotrypsin was simply saturation of the entire signal; there was no indication from this experiment that the observed line does not represent a single species.

Chemical Exchange. If we presume that the relaxation analysis is essentially correct, then another factor must be responsible for the disparity between the observed and calculated fluorine line widths. Post et al. (1984) have recently made similar observations in the case of 5-fluorotryptophan-labeled histidine-binding protein J and suggest that their results can be explained by the existence of several conformational forms of the protein, each characterized by different fluorine chemical shifts. Exchanges between these forms were apparently rapid enough to average chemical shifts but slow enough that an appreciable exchange contribution to the line width was present. Chemical exchange has also been invoked to explain line widths in fluorine-containing dihydrofolate reductase (Kimber et al., 1977) and in a chymotrypsin derivative examined in our laboratory (Gerig & Hammond, 1984). An indication that chemical exchange may account for the difference between calculated and observed line widths in fluorine spectra of III was the observation (Table II) that R_2 observed in Carr–Purcell experiments appeared to depend on the time between refocusing pulses (Carver & Richards, 1972). To explore the parameters that might characterize an exchange process responsible for the line broadening observed and this dependence, a computer simulation was done in which an exchange of the fluorine nucleus of III between two environments, A and B, was assumed. These differed in chemical shift by an amount δ ppm, while the fraction of molecules in the low abundance form is x_B, and the half-life of the system in this form is τ_B. It was also assumed that R_2 for both sites in the absence of chemical exchange has the value calculated by using the correlation times and distance r found in the analysis of the other relaxation data. The parameters δ, x_B, and τ_B were adjusted until agreement with the experimental R_2 values was obtained. A good fit to all experimentally observed R_2 data, collected at several frequencies, was obtained when $\delta = 2.3 \pm 0.3$ ppm, $\tau = 0.5 \pm 0.1$ ms, and $x_B = 0.06 \pm 0.01$. Thus, an explanation for the unexpectedly broad fluorine lines can reasonably be couched in terms of an exchange process.

The fluorine line widths observed with III and with the fluorine-labeled histidine-binding protein J were found to increase by roughly a factor of 2 when the sample temperature was changed from 5 to 35 °C. The change in the viscosity of water over the same temperature range is approximately a factor of 2 (Daniels et al., 1956), and although the temperature dependence of the viscosity of the protein solutions has not been determined nor has linkage between macroscopic viscosity and the correlation time for overall tumbling been

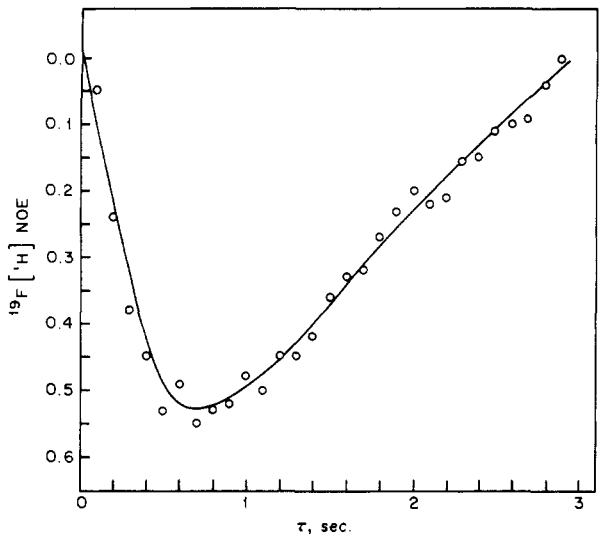


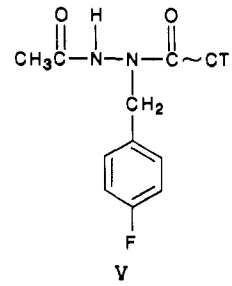
FIGURE 3: Nonselective transient $^{19}F[^1H]$ NOE generated in enzyme III by the sequence $180°_H$–t–$90°_F$–fid at 282 MHz. About 200 fids were averaged to obtain each point.

demonstrated, it is possible that the observed line width changes merely reflect viscosity changes. That the fluorine–proton NOE of III is not affected by temperature indicates that the dominant motion for relaxation at the active site is not the overall tumbling rate of the protein. However, given the value of the NOE observed (–0.79), an appreciable change in tumbling rate (by at least a factor of 3) would be needed to alter the observed NOE beyond experimental uncertainty if the dynamical model developed above is correct.

Two-Dimensional $^{19}F[^1H]$ Overhauser Effects. The analysis of relaxation data indicates that the fluorine nucleus of [(4-fluorophenyl)sulfonyl]chymotrypsin is relaxed in part by dipolar interactions with protons other than those that are adjacent to it on the fluorophenyl ring. A heteronuclear two-dimensional (2D) Overhauser experiment was attempted in order to learn more about these protons. A nonselective transient NOE experiment produced by the pulse sequence $180°_H$–t_1–$90°_F$–fid gave the data shown in Figure 3. On the basis of this result, mixing times of 0.4–0.8 s were employed in the 2D experiments to minimize the effects of spin diffusion. Projection of contour maps generated in the 2D NOE experiments to the proton frequency axis gives the chemical shifts of those protons close enough to the fluorine to take part in dipolar relaxation. It was found that in enzyme III a dominant proton–fluorine interaction involves protons with a chemical shift of 7.4 ± 0.3 ppm, where the accuracy of the shift determination is limited by the digital resolution along the proton axis. This shift is assignable to the protons on the aromatic ring that are ortho to the fluorine since this feature disappears in an experiment using the deuterated analogue, IIIb. For protein III additional proton–fluorine dipolar interactions occur with protons having shifts in the range 4–6 ppm.

Discussion

Aromatic rings of phenylalanine and tyrosine residues in proteins undergo rotational motion about their local symmetry axes at widely variable rates (Wuthrich & Wagner, 1984; Cohen, 1983: Jardetzky & Roberts, 1981). We previously reported indications that in solution rotation of the aromatic ring in tosylchymotrypsin (II) is rapid. To the extent the model indicated by Figure 2 is correct, that rotation appears to be diffusive and characterized by τ_s = 0.14 ns at pH 4 (Ando et al., 1982). In contrast, rotation of the fluoroaromatic ring of the carbazoate derivative of the enzyme represented by V appears to be very slow, and it is likely that this ring is essentially immobilized within the active site depression (Gerig & Hammond, 1984). The results reported herein for [(4-



V

fluorophenyl)sulfonyl]chymotrypsin, a structure that is formally quite similar to that of tosylchymotrypsin, indicate that aromatic ring rotation at the active site of this enzyme, while still diffusive, is of an intermediate nature with τ_i = 2 ns. The immobilization of the aromatic ring of acylenzyme V may be a result of the more extended nature of the acyl group in this system—four covalent bonds separate the active site serine residue from the aromatic ring in V while only two covalent bonds intervene in tosylchymotrypsin and the fluorobenzenesulfonyl derivatives—permitting stabilizing interactions to occur more deeply within the active site pocket. The greater steric requirements of the methyl group relative to fluorine may interfere with interactions between the tosyl group and the protein. Alternatively, some interaction peculiar to fluorine may stabilized III sufficiently to hinder ring rotation. A van der Waals interaction with groups of the protein may be strong enough to provide such stabilization and could also account for the very large effect of the native enzyme on the chemical shift of the fluorine in III.

The 4-fluorobenzenesulfonylated enzyme is the second modified chymotrypsin where it has not been possible to account for transverse relaxation without invoking broadening of the fluorine resonance by an exchange process. Athough a two-site exchange process is consistent with our results, there is, of course, nothing in the analysis that precludes a more complex situation. It is, however, striking that the parameters x_B and τ_B that would have to characterize a two-site exchange in III are close to those that arose from a similar analysis of transverse relaxation in V.

Any exchange process present in III might also influence spin–lattice relaxation, the fluorine–proton NOE, and the line shape of the deuterium resonance from IIIb. Deuterium on an immobile aromatic ring attached to a protein the size of chymotrypsin would have a line width of about 800 Hz (R_2 = 2800 s^{-1}) while a rapidly rotating ring would be characterized by a deuterium line width of about 20 Hz. The line width observed with III could thus be consistent with rapid exchange in a mixture of two structures, one of which has the fluoroaromatic ring highly immobilized and the other having rapid rotation, if the mixture consisted of about 26% of the form with the immobilized ring. However, as indicated by Figure 4, within the confines of the model system shown (Figure 1), the fluorine–proton NOE is dependent on the rate of internal rotation and the fluorine resonance frequency in such a way that essentially the same NOE (about –0.9) would be observed in both the fast and slow internal rotation limits. Although the exact NOE observed experimentally for the exchanging mixture of species would depend on the rate of exchange (Borzo & Maciel, 1981; Clore & Gronenborn, 1982), it is unlikely conditions would exist where the (averaged) NOE would be significantly different from this. The NOE data in Table I constrains the situation with regard to ring dynamics

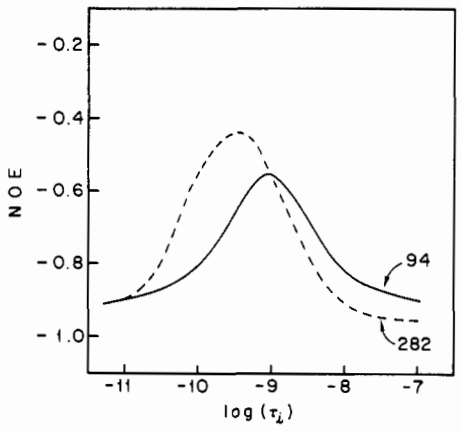


FIGURE 4: Computed fluorine–proton Overhauser effects as a function of τ_i when τ_c is fixed at 16 ns and $r = 0.23$ nm, calculated by using the model given in Figure 2.

that can exist at the active site of [(4-fluorophenyl)-sulfonyl]chymotrypsin, and the exchange process(es) suggested by the line width data most probably involves (involve) species that have similar structure and dynamics of ring rotation.

With the denatured form of the protein as a reference point, there is a very large enzyme-induced chemical shift effect on the fluorine resonance of III, nearly 9 ppm. Effects of a similar magnitude have been observed in the 4-fluorobenzoyl (Amshey & Bender, 1983) and 4-fluorocinnamoyl (Gerig & Halley, 1981) derivatives of chymotrypsin. Such massive shift changes probably arise from the influence of van der Waals interactions on the fluorine shielding parameter (Rummens, 1975) and involve intimate contacts between the fluorine nucleus and other atoms of the enzyme. The van der Waals effect depends on the sixth power of the distance between interacting atoms and, thus, even small alterations in protein structure could produce an appreciable change in chemical shift. It is, therefore, a reasonable possibility that the exchange event or events that take place at the active site of the 4-fluoro-benzenesulfonyl derivative could involve a structureal change that alters the chemical shift parameter of the fluorine nucleus by perhaps several parts per million but is sufficiently localized that the dynamics of the interactions between the fluoro-aromatic ring and the enzyme are not greatly altered.

The chemical shifts of the protons of the enzyme that are close enough to contribute to fluorine relaxation and, presumable, van der Waals effects on shielding are approximately 5.4, 4.7, and 4.0 (?) ppm. (The latter shift is rendered doubtful by the poor signal-to-noise ratio in the two-dimensional map.) The widths of the signals from these protons are around 150 Hz. Hydrogen atoms attached to C_α of cysteine-220, C_β of serine-189, C_α of cysteine-191, and the peptide N of serine-217 and glycine-216 are within 0.36 nm of the methyl carbon atom of the tosyl group in the crystal structure of tosylchymotrypsin (Birktoft & Blow, 1972). The unperturbed chemical shifts of the carbon-bound protons in this set would be expected to be between 4.5 and 4.7 ppm (Jardetzky & Roberts, 1981). However, enzyme protons within the van der Waals distance of the fluorine nucleus would likely experience a ring current effect from the fluorinated aromatic ring and could easily be shifted by up to 1 ppm in either direction (Becker, 1980).

It remains for additional experimentation to identify more completely the amino acids involved in fluorine relaxation at the active site of [(4-fluorophenyl)sulfonyl]chymotrypsin.

Registry No. *p*-FC_6H_4NHAc, 351-83-7; *p*-$FC_6H_4SO_2F$, 368-85-4; D_2, 7782-39-0; F_2, 7782-41-4; 4-fluoro-3,5-dideuteriobenzenesulfonyl fluoride, 103368-16-7; 4-fluoro-3,5-dideuterioacetanilide, 103368-13-4; 4-fluoro-3,5-dideuterioaniline hydrochloride, 103368-14-5; 4-fluoro-3,5-dideuteriobenzenesulfonyl chloride, 103368-15-6.

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A Cysteine Residue (Cysteine-116) in the Histidinol Binding Site of Histidinol Dehydrogenase†

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ABSTRACT: *Salmonella typhimurium* L-histidinol dehydrogenase (EC 1.1.1.23), a four-electron dehydrogenase, was inactivated by an active-site-directed modification reagent, 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl). The inactivation followed pseudo-first-order kinetics and was prevented by low concentrations of the substrate L-histidinol or by the competitive inhibitors histamine and imidazole. The observed rate saturation kinetics for inactivation suggest that NBD-Cl binds to the enzyme noncovalently before covalent inactivation occurs. The UV spectrum of the inactivated enzyme showed a peak at 420 nm, indicative of sulfhydryl modification. Stoichiometry experiments indicated that full inactivation was correlated with modification of 1.5 sulfhydryl groups per subunit of enzyme. By use of a substrate protection scheme, it was shown that 0.5 sulfhydryl per enzyme subunit was neither protected against NBD-Cl modification by L-histidinol nor essential for activity. Modification of the additional 1.0 sulfhydryl caused complete loss of enzyme activity and was prevented by L-histidinol. Pepsin digestion of NBD-modified enzyme was used to prepare labeled peptides under conditions that prevented migration of the NBD group. HPLC purification of the peptides was monitored at 420 nm, which is highly selective for NBD-labeled cysteine residues. By amino acid sequencing of the major peptides, it was shown that the reagent modified primarily Cys-116 and Cys-377 and that the presence of L-histidinol gave significant protection of Cys-116. The presence of a cysteine residue in the histidinol binding site is consistent with models in which formation and subsequent oxidation of a thiohemiacetal occurs as an intermediate step in the overall reaction.

L-Histidinol dehydrogenase (EC 1.1.1.23) catalyzes the terminal step in the microbial biosynthesis of histidine, the four-electron oxidation of the α-amino alcohol L-histidinol to histidine. Such four-electron dehydrogenases (three are known) are extremely interesting mechanistically because a single active site catalyzes two distinct types of oxidation, from the substrate alcohol to an intermediate aldehyde, and then to the product acid. *Salmonella* L-histidinol dehydrogenase is of additional interest because it is the product of the *hisD* gene, mutations in which are used in the Ames mutagenicity assay (McCann et al., 1975). Although L-histidinol dehydrogenase has been known for 30 years (Adams, 1955), and the homogeneous enzyme is available in large quantities (Yourno & Ino, 1968), little is known about the enzyme mechanism. Most recent speculation has focused on a proposed mechanistic similarity between L-histidinol dehydrogenase and UDP-glucose dehydrogenase, which utilizes a lysine-derived imine and a cysteine thiohemiacetal–thiol ester in its mechanism (Ordman & Kirkwood, 1977a). Like UDP-glucose dehydrogenase, L-histidinol dehydrogenase uses 2 mol of NAD^+ to oxidize its substrate and also proceeds via a tightly or covalently bound form of the intermediate aldehyde (Eccleston et al., 1979). In addition, both enzymes follow ordered mechanisms in which substrate binds before coenzyme, in contrast to nearly all other known dehydrogenases (Ordman & Kirkwood, 1977b; Gorisch, 1979; Burger & Gorisch, 1981a). However, the published stereochemistry of NAD^+ reduction is S(B) for both steps in the mammalian liver UDP-glucose dehydrogenase reaction (Krakow et al., 1963) but R(A) for both steps in the L-histidinol dehydrogenase reaction catalyzed by extracts of *Neurospora* (Davies et al., 1972). Since the fungal L-histidinol dehydrogenase is known to be homologous with that of *Salmonella* (Donahue et al., 1982), it is most likely that the latter enzyme also shows R(A) specificity.

Since thiohemiacetal intermediates are employed in the oxidation of aldehydes by liver aldehyde dehydrogenases (Feldman & Weiner, 1972) and glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976), cysteine is a likely catalytic site residue for the second oxidation step of the L-histidinol dehydrogenase reaction. Earlier work on L-histidinol dehydrogenase identified a single cysteine sulfhydryl on the enzyme whose complete modification by *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide resulted in partial

† This research was supported by grants to C.T.G. from the Department of Biology, the New York University Challenge Fund (NIH/BRSG 2507 RR07062-16), and the National Science Foundation (PCM-8409256) and to W.R.G. from the U.S. Public Health Service (GM24686).

0006-2960/86/0425-4778$01.50/0