Selective Fluorine–Proton Nuclear Overhauser Effects in 4-Trifluoromethylbenzenesulfonyl-α-chymotrypsin

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Abstract: Selective $^{19}F\{^1H\}$ nuclear Overhauser effects have been used to explore the nature of the protons which contribute to relaxation of the trifluoromethyl group in *p*-trifluoromethylbenzenesulfonylchymotrypsin. Spin diffusion is shown to play a role in decreasing the specificity of these effects, but its influence is not so strong as to completely obliterate structural information. The Overhauser effects were determined as a function of irradiation power level (γH_2) and a structural model for the environment around the CF_3 group in the modified enzyme was developed. The model is consistent with observed spin–lattice relaxation rates for the protons and fluorine nuclei in the system and the variation in the $^{19}F\{^1H\}$ Overhauser effects with the frequency and intensity of the irradiating field.

Following the seminal observations of Anet and Bourn[1] on the utility of the nuclear Overhauser effect (NOE) in the elucidation of organic structures, a wide variety of applications of this technique to macromolecular systems of biological interest has been reported.[2,3] In using the NOE for structural studies of large molecules, several considerations conspire to limit the quality of information obtainable. One of these is resolution of the resonances being observed and irradiated from the broad massifs which often characterize the proton or carbon spectra of a macromolecule such as a protein. Assignment of the observed and irradiated resonances may also be a problem. Finally, the phenomenon of spin diffusion arises in large molecules as the molecular tumbling rates become comparable to or less than the Larmor frequencies of the nuclei involved; spin diffusion has the effect that irradiation of one nucleus may excite other nuclei in the system with the result that specificity is lost in the NOE experiment.[3d,h]

One means to circumvent the resolution–assignment problem is the introduction of fluorine reporter groups into the structure under consideration. The range of fluorine chemical shifts observed in biological systems is large[4] and, since the only fluorine in the system is that introduced by the experimenter, the assignment of the observed resonance(s) is made easier. However, the complications arising from spin diffusion effects are not so easily overcome and calculations show that for molecules larger than ~20 000 daltons these effects may be so serious as to vitiate completely the $^{19}F\{^1H\}$ NOE experiment as a useful tool.[5] We describe here attempts to provide some details of the structure of the inactivated enzyme *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin by means of fluorine–proton Overhauser effects. This enzyme is a "fluorine-labeled" analogue of tosylchymotrypsin in which the CF_3 NMR reporter group replaces the CH_3 of the toluenesulfonyl moiety. An X-ray crystallographic study of tosylchymotrypsin is available[6] and it will thus be possible to compare our results to the structure of a related, modified enzyme in the solid state.

Experimental Section

p-Trifluoromethylbenzenesulfonyl-α-chymotrypsin was prepared by treating native α-chymotrypsin (Worthington, three times recrystallized) with *p*-trifluoromethylbenzenesulfonyl fluoride[7] as described previously.[8] For much of the work reported here, material purified by chromatography on Whatman CM-32 carboxymethylcellulose was used,[8b] but the results obtained with the crude modified enzyme preparation were essentially identical. 3,5-Dideuterio-4-trifluoromethylbenzenesulfonyl-α-chymotrypsin was prepared as described previously.[8a]

Samples for proton and fluorine magnetic resonance spectroscopy were prepared as for the previous paper;[8a] the protein concentration for all experiments reported here was approximately 25 mg/mL (1 mM). Solutions in D_2O were prepared using deuterium oxide as supplied by Stohler Isotope Chemicals.

^{19}F and ^1H NMR spectra were obtained at 94.13 and 100.05 MHz, respectively, with a Varian Associates XL-100 spectrometer interfaced to a Nicolet Technology Corp. TT-100A Fourier transform accessory. Double irradiation experiments used a General Radio 1061 synthesizer as the frequency source. The 42-MHz signal from this synthesizer was downconverted to 15.4 MHz by an Alto Engineering device and this signal was used in place of the deuterium "master oscillator" of the XL-100. Thus, all frequencies for irradiation and observation were locked to one source. An interface to the General Radio synthesizer, supplied by Nicolet, in conjunction with locally developed emendations to the Nicolet operating software, permitted computer control of the irradiation frequency to a resolution of 0.1 Hz. The irradiation and observation frequencies were coupled to the V-4412 probe using specially designed matching networks.[9] The intensity of the irradiating field in the $^{19}F\{^1H\}$ experiments was calibrated using a 0.3 M solution of α-fluorocinnamic acid (Aldrich) in basic D_2O. The nuclei attached to the vinyl group in this compound define an AX system and changes in the intensities or positions of components of the AX pattern depend upon the strength of the irradiating field and its frequency, relative to the chemical shift of one of the nuclei.[10,11] Calculations based on observations of relative line intensities or position gave results which usually agreed within 5% over the range $30 < \gamma_H H_2 < 200$ rad/s ($1 < H_2 < 8$ mG).

Fluorine relaxation times at 22.8, 36, and 51 MHz were obtained with a Bruker BKR-321S spectrometer as described previously.[12] An external sample of D_2O provided a lock signal for operation at the two highest frequencies while the ^7Li resonance of a saturated LiBr sample was used for locking at 22.8 MHz.

Sample temperatures were controlled for all experiments at 25 ± 1 °C by Varian controllers and were checked routinely with a 5-mm o.d. thermometer supplied by Kontes Glass Co.

Spin–lattice relaxation rates (R_1) were obtained using the 180–τ–90 sequence.[13] Implementation of these experiments on the Bruker instrument has been described earlier.[14] At 94.1 MHz, the observed line width, less the instrumental line width represented by the width of a trifluoroacetate reference peak, was used to estimate the fluorine spin–spin relaxation rate (R_2). At the lower frequencies, the Carr–Purcell–Meiboom–Gill experiment was used to estimate R_2.[13,14] Data at the lower frequencies was obtained before it was realized that the enzyme samples can be heterogeneous; in these cases the magnetization decay curves are observed to be nonexponential. A nonlinear least-squares program based on the Marquardt algorithm[15] or a Fourier transform deconvolution program[16] were used to estimate the decay constants for the major components in these systems. The estimated uncertainties in the relaxation data obtained at 94.1 MHz are ±10% and at the lower frequencies ±20%.

R_2 values for the protons of the protein were estimated by the Carr–Purcell–Meiboom–Gill method at 100 MHz. Modulation of echo amplitudes by homonuclear spin coupling[17] was not taken into account when analyzing the data and the resulting apparent relaxation rates can only be regarded as approximate.

In acquiring the $^{19}F\{^1H\}$ NOE data a waiting time of at least 2 s (~6–10T_1) between each accumulation was used.[18] The Overhauser

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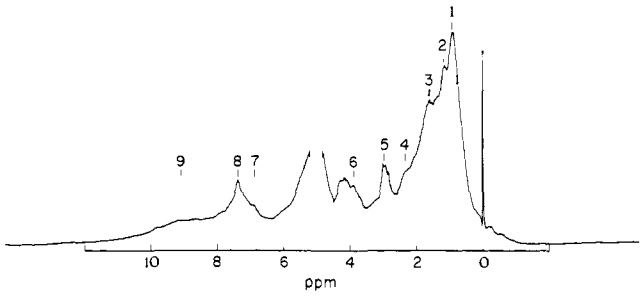

Figure 1. The ^1H NMR spectrum of *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin at 100 MHz at 25 °C in D_2O. The sample pH was approximately 5 and the protein concentration was 2 mM. The reference peak at 0 ppm arises from a trace of added DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). Relaxation rates were determined for the resonances labeled, as indicated in the text.

effects are expressed as $(F_z - F_0)/F_0$, where F_z is the observed fluorine signal intensity in the presence of the irradiating field and F_0 is the equilibrium intensity in its absence. The Overhauser effects are judged to be reliable to within 10–20%, although the errors are likely larger when the effects are small.

Results

Proton Relaxation. In large molecules the spin-lattice relaxation rates (R_1) tend to become equal as the efficiency of spin diffusion increases; Kalk and Berendsen have suggested that rapidly rotating methyl groups act as relaxation sinks in proteins such that R_1 values for other protons become leveled to the relaxation rates for these groups.[3d] Figure 1 shows the ^1H NMR spectrum of the modified enzyme at 2.348 T. The inversion-recovery method was used to obtain an apparent relaxation rate constant (R_1) for spin-lattice relaxation at the nine positions in the spectrum that are indicated. The signal intensity at each of these spectral regions represents many amino acids, yet the relaxation curve at most points appeared to be a simple exponential function within the errors of the experiment. Table I gives the R_1 values calculated from the initial slopes of these plots.

Spin-spin relaxation rates (R_2) for the protons of the inhibited enzyme were estimated using a spin-echo technique. The apparent R_2 values for the nine spectral regions indicated in Figure 1 are also given in Table I. The echo amplitudes observed in these experiments may be modulated by spin-coupling interactions;[17] this considerable complication was not taken into account in obtaining the R_2 data in Table I. Sykes and co-workers have reported R_1 and R_2 data for 1 mM native α-chymotrypsin at pH 7.8 (30 °C)[18] and our results with the derivatized enzyme, obtained at 25 °C, are in reasonable accord with theirs.

Fluorine Relaxation. We have previously reported determinations of the fluorine R_1 and R_2 relaxation rates for *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin, including results for systems in which the protons ortho to the trifluoromethyl group or protons derived from the solvent are replaced by deuterium.[8a] These studies have been extended by determining these relaxation rate constants as a function of radio frequency and are summarized in Table II. The instrumental sensitivity available to us at the lower fluorine frequencies was not high and no attempt was made to determine the concentration dependence of R_1 and R_2; the data reported are for samples approximately 1 mM in protein. We have previously noted that the fluorine R_1 for the enzyme derivative is not detectably concentration dependent up to 3 mM protein but that R_2, as reflected in the resonance line width, does depend upon concentration.

$^1H\{^{19}F\}$ Nuclear Overhauser Effects. There is evidence that relaxation of the CF_3 group in *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin is dominated by proton-fluorine interactions.[8a] If spin diffusion effects are overwhelmingly important in the relaxation of the protons and the fluorine nuclei of this material, irradiation (saturation) of the fluorine signal may perturb the ^1H NMR spectrum of the enzyme. This experiment was carried out on both specifically deuterated *p*-trifluoromethylbenzenesulfonylated enzyme and the fully protonated form. Difference spectra obtained by subtracting the ^1H NMR spectrum obtained with fluorine irradiation from that obtained without irradiation, or vice versa, showed that changes in spectral intensities throughout the ^1H NMR spectral region did not exceed 1 ± 1% when the CF_3 resonance was saturated. No reliable changes beyond experimental errors were detected.

Table I. Apparent Proton Spin-Lattice (R_1) and Spin-Spin (R_2) Relaxation Parameters of *p*-Trifluoromethylbenzenesulfonyl-α-chymotrypsin[a]

signal[b]	chemical shift, ppm[c]	R_1, s^{-1}	R_2, s^{-1}
1	0.91	2.7	58
2	1.16	2.6	70
3	1.52	2.9	70
4	2.32	2.5	68
5	2.92	2.8	69
6	3.89	2.6	64
7	6.92	1.8	72
8	7.36	1.8	61
9	9.04	2.2	87

[a] In D_2O at 24–25 °C, pH ~6, protein concentration 0.9 mM. [b] Labeled as indicated in Figure 1. [c] In parts per million, relative to internal DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate).

$^{19}F\{^1H\}$ Nuclear Overhauser Effects. We have previously indicated that saturation of all proton resonances of the modified enzyme leads to a substantial decrease in fluorine signal intensity, corresponding to an NOE of about −0.8.[8a] Here we describe selective $^{19}F\{^1H\}$ effects, obtained by irradiating a discrete frequency in the enzyme ^1H NMR spectrum at a known rf power level.

Figure 2 records some representative results. Each solid point represents the $^{19}F\{^1H\}$ NOE obtained when the enzyme sample is irradiated at the proton frequency and power level indicated. In order to obtain each point, 1000–2000 fid's were accumulated and about 12 h was required for such a scan through the ^1H NMR spectrum. The enzyme samples are stable for this time period as indicated by kinetic experiments and the invariance of the fluorine spectrum over this period.[8b] There are clearly some structural features apparent in the NOE curves given in Figure 2 and the characteristics of these were found to depend upon the magnitude of the irradiating field (H_2).

When *p*-trifluoromethylbenzenesulfonylchymotrypsin was examined under denaturing conditions (8 M urea or 5% sodium dodecyl sulfate), the $^{19}F\{^1H\}$ Overhauser effects were essentially zero and showed no dependence on proton frequency when examined in the kind of experiments described above. Thus, the native structure of the protein is necessary for the development of these effects.

For a single fluorine nucleus relaxed by a single proton, the dependence of the $^{19}F\{^1H\}$ NOE on the magnitude of H_2 is given by[20]

$$\frac{1}{\eta} = \frac{F_0}{F_z - F_0} = \frac{\gamma_F}{\gamma_H}\frac{R_F}{\sigma_{FH}}\left\{1 + \frac{R_1^H R_2^H}{(\gamma_H H_2)^2}\right\} \quad (1)$$

where η is the observed NOE, γ_F and γ_H are the gyromagnetic ratios of fluorine and protons, respectively, R_F is the initial spin-lattice relaxation rate of the fluorine spin, and σ_{FH} is the proton-fluorine dipole-dipole cross-relaxation contribution. Equation 1 predicts that $1/\eta$ will vary linearly with $1/H_2^2$, and

Table II. Comparison of Observed and Calculated Fluorine Relaxation Parameters[a]

enzyme derivative	solvent	MHz	R_1, s^{-1}	R_2, s^{-1}	NOE[e]
4-trifluoromethyl	H_2O[b]	94.1[c]	5.1 (5.7)		
		94.1	4.4 (4.5)	35. (34.)	−0.82 (−0.86)
		51.0	5.2 (5.4)	52. (34.)	
		36.0	8.8 (8.1)	39. (35.)	
		22.8	(14.)	42. (41.)	
4-trifluoromethyl	D_2O	94.1[c]	3.3 (3.8)		
		94.1	3.6 (3.3)	47. (28.)	−0.81 (−0.80)
3,5-dideuterio-4-trifluoromethyl	H_2O[d]	94.1[c]	4.2 (4.8)		
		94.1	4.3 (3.8)	26. (31.)	−0.84 (0.84)
3,5-dideuterio-4-trifluoromethyl	D_2O	94.1[c]	2.7 (2.9)		
		94.1	2.7 (2.6)	49. (26.)	−0.76 (−0.76)

[a] All data collected at 25 ± 1 °C. Solutions contained 0.05 M KCl, about 1 mM protein and were adjusted to pH or pD 6–7. Quantities in parentheses are those calculated using the model system described in the text. Data at 94.1 MHz are taken from ref 8a. [b] Solvent was 80% H_2O/20% D_2O. [c] R_1 obtained with full proton decoupling. [d] Solvent was 96% H_2O/4% D_2O. [e] $^{19}F\{^1H\}$ NOE when all protons are saturated.

Table III. Extrapolated $^{19}F\{^1H\}$ Overhauser Effects in *p*-Trifluoromethylbenzenesulfonyl-α-chymotrypsin[a]

solvent position irradiated,[b] ppm	20% D_2O/80% H_2O η_{max}	D_2O η_{max}
0.90	−0.42	−0.60
2.70	−0.72	−0.62
4.10	−0.73	−0.67
4.70	−0.68	−0.52
6.50	−0.43	−0.42
7.70	−0.47	−0.42

[a] At pH 6–7, 25 °C. Estimated errors of slope and intercepts are ±20%. Five to ten data points were used for each extrapolation. [b] Irradiation position relative to internal DSS.

by extrapolating $1/H_2^2$ to zero one can estimate the maximum obtainable Overhauser effect ($\gamma_F R_F/\gamma_H \sigma_{FH}$) that results from a particular fluorine–proton interaction. Equation 1 and the extrapolation procedure should be valid in multispin systems as long as spin-diffusion effects are negligible. However, low H_2 powers should be used, for with large H_2 the "bandwidth effect" [5] guarantees that NOEs obtained at any proton frequency will have the same value.

The dependence of the $^{19}F\{^1H\}$ NOE on the magnitude of the proton field (H_2) was examined in detail at several frequencies in the proton spectrum of *p*-trifluoromethylbenzenesulfonylchymotrypsin and the data analyzed by means of eq 1. Although scattered, double reciprocal plots were reasonably linear and permitted an extrapolation to infinite H_2 power at a given frequency. Table III gives the extrapolated maximum NOEs (η_{max}) obtained in this way and it is clear that these limiting values for the NOE depend upon the frequency of irradiation and, to a lesser extent, the isotopic composition of the solvent.

Equation 1 indicates that the slopes of plots of $1/\eta$ vs. $1/(H_2)^2$ might provide an estimate of R_1R_2 for the proton irradiated. While the slopes of our double-reciprocal plots were the correct order of magnitude (corresponding to $40 \leq R_1R_2 \leq 400$), they cannot be equated to the product of the relaxation rates for a particular proton in a spin system as complex as the one defined by the enzyme, especially when spin diffusion is present.

Toward a Quantitative Interpretation. The proton–fluorine dipole–dipole contribution to the fluorine relaxation rates of *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin, the selective $^{19}F\{^1H\}$ nuclear Overhauser effects observed, and the dependence of these effects on the intensity of the irradiating field all derive from the properties of the proton matrix which surrounds the CF_3 group in the modified enzyme. We have sought the simplest geometrical model which would reproduce as closely as possible the experimentally observed fluorine and proton relaxation rates as well as the features of the various selective $^{19}F\{^1H\}$ NOE data obtained with both the fully protonated and specifically deuterated forms of the modified enzyme in water and deuterium oxide solvents. With considerable trial-and-error calculations, a model system has been developed which substantially meets these specifications. The model consists of a trifluoromethyl group surrounded by an array of 13 protons; two of these represent the protons on the phenyl ring of the inhibitor molecule while a single proton was used to represent solvent or solvent-exchangeable protons. The Cartesian coordinates for the nuclei in the model system are given in Table IV and a sketch of the structure appears in Figure 3. Some proton spins in the model potentially represent *sets* of equivalent nuclei such as those of a methyl or methylene group. Possible internal motions of these groups introduce additional complications (and parameters) into any theoretical treatment of the model and it was felt that the best initial approach in model building would be to represent these groups

Table IV. Properties of Model System

Cartesian coordinates, Å			chemical shift,		calcd[b]	
X	Y	Z	ppm	$\bar{r}_F$, Å[a]	R_1, s^{-1}	assignment
			Fluorine			
−0.623	1.079	0.0			3.3	
−0.623	1.079	0.0			3.3	
1.246	0.0	0.0			3.3	
			Hydrogen[c]			
0.	2.4	−2.44	7.55	3.6	2.2	ortho H[d]
0.	2.4	−4.92	6.85	5.6	2.2	meta H[d]
0.	5.6	−2.44	3.68	6.2	2.6	CH
0.	6.2	−0.10	4.00	6.3	2.7	CH_2
0.	4.7	1.90	0.85	5.2	2.8	CH_3
0.	3.1	3.90	2.10	5.1	3.2	CH
1.1	0.6	3.35	1.00	3.8	4.2	CH_3
−1.1	0.6	3.20	2.80	3.6	4.2	CH_2
0.	−1.3	3.07	4.10	3.5	4.2	CH
0.	−3.0	5.55	7.00	6.4	2.7	tyrosine
0.	−3.0	8.03	7.20	8.6	2.3	tyrosine
−2.5	5.6	−2.44	8.50	6.6	2.4	NH
−2.5	0.	2.00	4.60	3.4		solvent[e]

[a] Average distance to the fluorine nuclei. [b] Calculated initial R_1 relaxation rate for the fully protonated system in D_2O. [c] R_{xi}, the spin–lattice relaxation contribution from possible intragroup interactions, was set to 2 s^{-1} for each proton in the system; R_{xi} was added to ρ_i. R_{2_i}, the spin–spin relaxation rate, was set to 250 s^{-1} for each proton. [d] The protons ortho and meta to the CF_3 group on the benzenesulfonyl moiety were represented by single protons in the model since the rate of internal rotation of the aromatic ring about its symmetry axis was unknown. [e] A single proton was used to represent all solvent or solvent-derived protons.

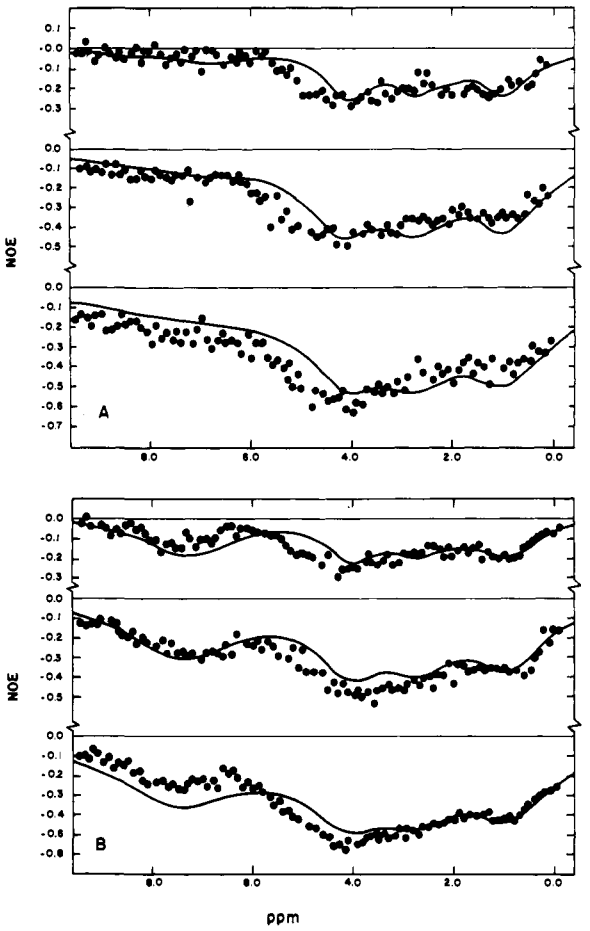

Figure 2. Typical $^{19}F\{^1H\}$ nuclear Overhauser effects for 3,5-dideuterio-4-trifluoromethylbenzenesulfonyl-α-chymotrypsin (top series) and 4-trifluoromethylbenzenesulfonyl-α-chymotrypsin (bottom series). The solvent in both cases was deuterium oxide. Samples were ~1 mM in protein at pD 6–7 (25 °C); solutions contained 0.05 M KCl but no buffer. The bottom axis refers to the chemical shifts characteristic of the ^1H NMR spectrum (Figure 1). The solid lines represent the NOEs calculated using the model system described in the text while the points are experimental values. The proton rf power levels were (top to bottom in each series) 6.39, 12.8, and 17.5 Hz, respectively, corresponding to H_2 = 1.5, 3.0, and 4.1 mG.

by composite nuclei rigidly imbedded in the protein structure. Equivalent spins within a group can relax each other and an adjustable parameter, R_{1x}, was used to take this into account.

Using the computational methods outlined in the Appendix, the dependence of the selective fluorine–proton Overhauser effects on proton irradiation frequency and power level were computed. Some of these calculations are compared to experimental data in Figure 2. For most cases that we have examined, the model system reproduces fairly well the observed effects, especially when experimental errors at low H_2 power levels are taken into consideration. The agreement was best when the protons ortho to the CF_3 group were replaced by deuterium and when D_2O was the solvent; in H_2O the disparities between observed and computed NOEs were somewhat higher in certain regions of the proton spectrum. This likely indicates that representing the solvent or solvent-exchangeable protons near the CF_3 group by a single nucleus is too crude an approximation. The model also leads to calculated $^{19}F\{^1H\}$ NOEs in the system where the protons adjacent to the trifluoromethyl group are present that are in reasonable agreement with observation, correctly predicting an increased magnitude for the NOE when the aromatic portion of the proton spectrum is irradiated.

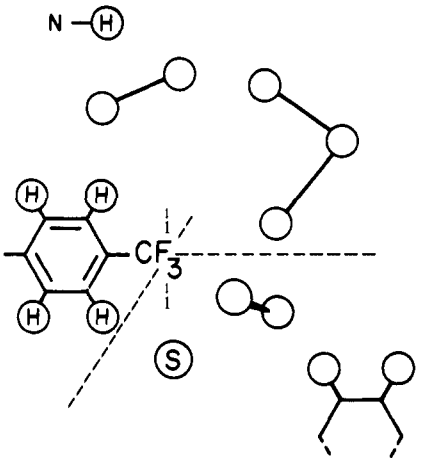

Figure 3. A sketch of the model for the local environment around the trifluoromethyl group of 4-trifluoromethylbenzenesulfonyl-α-chymotrypsin described in the text.

Spin–lattice relaxation rates calculated for protons in the model system are given in Table III. Plots of ln $(M_\infty - M_t)$ vs. time for these nuclei are predicted to be nonexponential by the model (M_∞ is the observed sample magnetization at equilibrium while M_t represents the magnetization at time t) and the entry in Table III is the computed *initial* slope of the relaxation curve. While these relaxation rates can hardly be expected to agree quantitatively with experiment (Table I), they are close to the experimental values. The entries in Table II show that the model correctly predicts the fluorine spin–lattice and spin–spin relaxation rates and the $^{19}F\{^1H\}$ nuclear Overhauser effects when all protons are simultaneously saturated in the derivatized enzyme; data for the deuterated variations of this system are also described well by the model. Finally, the frequency dependence of fluorine relaxation is accounted for by the model described, although there are disagreements between observed and calculated transverse relaxation rates in some cases. Until the nature of the concentration dependence of the R_2 parameters is elucidated and incorporated into the model, the significance of these discrepancies is not clear.

In making the calculations described here, we used the correlation times for overall tumbling (τ_c) and internal rotation (τ_i) of the CF_3 group that were indicated by our earlier analysis,[8a] namely, τ_c = 15 ns and τ_i = 0.019 ns. Attempts were made to compute the selective $^{19}F\{^1H\}$ NOE curves using larger values of τ_c, especially 30 ns, but we were unable to find any reasonable model system that simultaneously generated correct R_1 and R_2 relaxation rates and the saturation behavior of the NOE. These observations reinforce the earlier conclusions regarding the magnitudes of τ_c and τ_i in this protein system and indicate that the dominant species in these systems is the monomeric protein.

Discussion

Both ESR studies of spin-labeled chymotrypsin derivatives[21] and fluorescence polarization experiments[22] have indicated that the rotational correlation time (τ_c) for monomeric α-chymotrypsin is 11–16 ns. A pair of protons within the protein separated by 1.78 Å, as those in a methylene group, can be expected to have a R_1 relaxation rate of 1.8 s^{-1} at 100 MHz if the pair has no freedom of motion beyond the tumbling of the enzyme.[3d] Adjacent aromatic protons are about 2.5 Å apart and, in this case, the spin–lattice relaxation rate should be ~0.2 s^{-1}. The experimental R_1 values for various group resonances are of the same magnitude as these expectations and indicate that much of the protein structure can be regarded as rigid. However, our relaxation experiments measure only the conglomerate behavior of all nuclei which have a given chemical

shift and the presence of one nucleus with a larger or smaller relaxation rate than the norm or with nonexponential character to its relaxation would be difficult to detect, especially at our field strength.

Calculations by Kalk and Berendsen[3d] suggest that, for the proteins of up to mol wt 20 000, cross relaxation or spin diffusion effects are of, at most, moderate importance. α-Chymotrypsin (mol wt 25 000) is beyond this borderline and we must expect these effects to have a role in the relaxation of the fluorine and proton nuclei of our system. For spin diffusion to be effective, each proton in the molecule must be located within ~2.5 Å of another proton[3d] and protons within the side chain of a typical amino acid meet this requirement. Inspection of enzyme models shows that side chains of a polypeptide can interact with each other at this distance directly or by means of the amide proton of a peptide linkage. Bothner-By and Johner have indicated that the hydrogen atoms of a protein are not necessarily arranged so that spin diffusion effects are efficiently transmitted throughout the entire protein structure.[3h] Rather, protons in these structures may form "islands" within which spin diffusion is important but beyond which such effects are not transmitted. This may be expected especially in deuterium oxide solvent, as many of the amide NH protons would be replaced by deuterium and, thus, potentially introduce discontinuities in the paths which interconnect proton spins.

It can be shown that

$$f_F(S) = \left\{\frac{\sigma_{FS}}{R_F} - \sum_N \frac{\sigma_{FN} f_N(S)}{R_F} \frac{\gamma_H}{\gamma_F}\right\} \quad (2)$$

where $f_F(S)$ is the $^{19}F\{^1H\}$ NOE when proton S is saturated, the label N refers to all other protons in the system which interact with the fluorine nucleus but are not irradiated, and $f_N(S)$ represents the NOE on proton N when proton S is irradiated.[3b] We can distinguish two limiting cases. If the spin system is tumbling rapidly such that τ_c is small enough, the Overhauser effects, $f_N(S)$, experimentally will likely be small fractions[3b-d] and, as σ_{FN}/R_F is also less than one, the summation in eq 2 can be neglected. In this case, the observed $^{19}F\{^1H\}$ NOE will approximately be proportional to the fraction of the total fluorine spin–lattice relaxation (σ_{FS}/R_F) that is due to the interaction of proton S with the fluorine. The observed Overhauser effects will sum to about one-half under these conditions.

In the other limit, molecular motion and structure may be such that spin-diffusion effects are dominant and the values of $f_N(S)$ will all tend to be approximately -1. In this limit $-(\sigma_{FS} + \Sigma\sigma_{FN}) \approx R_F$ and the observed $^{19}F\{^1H\}$ NOE would be the same no matter what proton in the system is irradiated. Consideration of the extrapolated Overhauser effects (η_{max}) given in Table III as well as the near equivalence of the proton spin–lattice relaxation rates suggests that neither of these limiting situations applies to *p*-trifluoromethylbenzenesulfonylchymotrypsin, although the data indicate that the spin-diffusion limit is being approached. Thus, spin-diffusion effects must play a substantial role in the relaxation behavior of the protein-bound trifluoromethyl and of the nuclei which define its environment.

With the notion in mind that one need only consider a relatively small island of protons about the trifluoromethyl group in the inactivated enzyme, we sought a description of this collection of spins that would accord with the relaxation and NOE data available. The model system described above was the result of this search. The dynamical aspects of the system are necessarily approximate. If three protons were used to represent a methyl group or two protons taken for a methylene, one would have to introduce adjustable parameters to take into account internal motions of these sets of nuclei. Furthermore, means to calculate spectral densities for nuclei in one group that has a rotational degree of freedom (e.g., a methyl group) interacting with a second group (CF_3) which also has internal motion do not seem to be available. The nuclei in the model system are tightly interconnected in the sense that changing the properties (Cartesian coordinates, resonance frequency, or transverse relaxation rate) of one nucleus has an observable effect on the calculated effects from the remaining nuclei in the system. The saturation behaviors of the Overhauser effects are similarly closely linked. Thus, although the model is crude and cannot be guaranteed to be unique, it is not completely speculative and a number of features emerge from these calculational studies which we believe must be a prominent part of the nature of the proton matrix about the CF_3 group in the inactivated enzyme. These considerations include the following.

(1) On steric ground alone, only a limited number of proton-containing groups (amino acid side chains) can be juxtaposed to the trifluoromethyl. The chemical shifts of the groups near this function are likely ~0.9, ~2.8, and ~4.1 ppm. These groups of protons are close enough to the van der Waals contact distance (~2.6 Å) that it is unlikely that water molecules intervene between them and the fluorine nuclei. The chemical shifts mentioned could correspond to methyl groups of leucine, isoleucine, or valine, a methylene function, and a proton attached to an α carbon, respectively,[23] if it is assumed that there are no large ring-current or other effects present which would perturb "normal" chemical shifts.

(2) Solvent-exchangeable protons are nearly within van der Waals contact of the CF_3 group. However, it appears that at least one nonexchangeable N–H bond which resonates at $\delta \approx 8.5$ ppm is close enough to interact with the proton-containing groups at the active site. A structural feature of this nature is needed to reproduce the selective $^{19}F\{^1H\}$ NOE data in the 7–9-ppm range (Figure 2).

(3) A group of nuclei with the chemical shift of aromatic protons[24] may interact with the proton near the trifluoromethyl. Neither these protons nor the N–H proton(s) discussed above appear to be in direct contact with the fluorine nuclei, but rather exert their effects through spin diffusion.

We have considered a scale model of the structure of tosylchymotrypsin constructed using the coordinates of Birktoft and Blow,[6] seeking to find regions of the structure near the active site which are consistent with the features of the model system described above. If the structure of the tosylenzyme is modified by the replacement of the CH_3 group by CF_3 and all other atoms are held fixed, several protons are found to be very close to the fluorine nuclei. They include the C_α proton on cysteine-220 (~2.7 Å), the methylene group of serine-189 (~3.3 Å), and the C_α proton of cysteine-191 (~3.5 Å). The amide N–H protons of the peptide linkages to serine-217 and glycine-216 are also ~3.6 Å from a CF_3 group placed in the position of CH_3. While it is unlikely that the enzyme structure would tolerate the close contact with Cys-220 and probably would make some compensatory changes to minimize nonbonded interactions, it is clear that the groups expected to be in close contact with CF_3 should have the correct chemical shifts to account for $^{19}F\{^1H\}$ Overhauser effects generated by irradiation in the 2.5–4.5-ppm portion of the ^1H NMR spectrum. No methyl groups which could contact the trifluoromethyl function in this structure are apparent. However, a modest structural reorganization could bring a methyl of valine-17 into a closer orientation to the fluorine nuclei and yet retain most of the interactions noted above. Protons of the aromatic ring of tyrosine-146 are close enough to the α and β protons of Cys-220 for spin diffusion effects to be operative. Thus, one possible enzyme form which is consistent with our results is similar to, but not identical with, the structure of tosylchymotrypsin in the solid state.

Another collection of nuclei which potentially could meet

our specifications consists of valine-213, tyrosine-228, serine-190, and possibly glycine-226. This set of amino acid residues lies in a different region of the specificity pocket of α-chymotrypsin and, assuming that the basic integrity of the enzyme structure is retained in the derivative, could be reached by the CF_3 group of the fluorinated reporter group only if an orientation of the aromatic ring that is radically different from that observed in tosylchymotrypsin is taken up.

In δ-chymotrypsin the dipeptide linkage between tyrosine-146 and alanine-149 remains intact[24] so that the tyrosine residue likely has somewhat different conformational attributes in the δ form of the enzyme than in α-chymotrypsin. Fluorine–proton Overhauser effect studies of the *p*-trifluoromethylbenzenesulfonyl derivative of δ-chymotrypsin are underway (K. F. S. Luk, unpublished). While the NOE curves in the aliphatic and tertiary proton regions of the curves are similar to those observed with the α-chymotrypsin derivative, there appear to be appreciable differences in the aromatic proton section. These results suggest that tyrosine-146 is involved in the proton matrix about the CF_3 group in α-modified enzyme and we, therefore, tend to favor the first structural possibility discussed above.

Spin–spin relaxation rates (R_2) for the protons of the model system were regarded as adjustable parameters and the values of these needed to approximately match the bandwidths of the peaks observed in the NOE plots such as Figure 2 were three to four times larger than the experimental values (Table I). The R_2 value for a given spin in the model systems depends on the extent of intergroup interactions as well as interactions between equivalent spins within the group. In order to account for the magnitude of the R_2 values needed in the NOE calculations, it is necessary to postulate that internal rotation within the methylene or methyl groups near the CF_3 reporter function is slowed sufficiently that the correlation times, τ_i, describing these internal motions are less than τ_c, the correlation time for overall reorientation. Under these conditions, one calculates that a single proton–proton interaction at a distance of 1.78 Å (the interproton distance in a methylene or methyl group) would contribute about 120 s^{-1} to R_2 and, as indicated earlier, about 1.8 s^{-1} to R_1. Relaxation effects of this magnitude are consonant with the values of R_{xi}, used in the calculations as well. Our results therefore suggest a substantial decrease in the freedom of motion of the amino acid side chains near the trifluoromethylbenzenesulfonyl moiety of the derivatized enzyme. Attempts to substantiate this suggestion by other experiments are in progress.

The results described here give some encouragement toward hoping that three-dimensional structural information can be obtained from fluorine-labeled proteins by means of the Overhauser effect even when spin-diffusion effects become appreciable. An advantage of the $^{19}F\{^1H\}$ experiment is that proton irradiation frequencies throughout the entire proton spectrum, including the H_2O resonance, can be used. Further experiments of this nature are underway in our laboratory, and we trust that the utility and limitations of the technique will become better defined.

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Appendix

Spin–lattice relaxation in a multispin system is described, in the simplest approximation, by a system of differential equations of the form

$$dI_{z_i}/dt = -\rho_i(I_{z_i} - I_{0_i}) - \sum_j \sigma_{ij}(I_{z_j} - I_{0_j}) \qquad (A1)$$

where I_{z_i} is the longitudinal component of the magnetization of the ith spin and I_{0_i} is the value of this quantity at thermodynamic equilibrium.[25] The symbols I_{z_j} and I_{0_j} represent corresponding quantities for the remaining spin in the system. The dipole–dipole "cross-relaxation" interaction between nucleus **i** and nucleus **j** is represented by σ_{ij} while ρ_i includes the effects of dipole–dipole interactions between **i** and the remaining spins as well as the contributions of other mechanisms, such as chemical shift anisotropy, to the relaxation of spin **i**. The effect of an irradiating field at some frequency ω and intensity H_2 is represented by adding a "driving term" to each equation in the system defined by (A1) which has the form $-\gamma_i H_2 v_i$, where γ_i is the gyromagnetic ratio of nucleus **i** and v_i is the v-mode component of the magnetization of spin **i**. We assume that the transverse components of the magnetization for spin **i** in the rotating reference frame can be given by the Bloch equations

$$\frac{du_i}{dt} = (\omega_i - \omega)v_i - R_{2_i}u_i$$

$$\frac{dv_i}{dt} = -(\omega_i - \omega)u_i + \gamma_i H_2 I_{z_i} - R_{2_i}v_i \qquad (A2)$$

where R_{2_i} represents the transverse relaxation rate constant for nucleus **i** and ω_i is the Larmor frequency of the spin. For a steady-state experiment, the derivatives in eq A2 can be set equal to zero and thereby

$$\gamma_i H_2 v_i \equiv \frac{\gamma_i{}^2 H_2{}^2 T_{2_i} I_{z_i}}{1 + T_{2_i}{}^2(\omega_i - \omega)^2} \qquad (A3)$$

where $T_{2_i} = 1/R_{2_i}$. Setting the derivatives in eq A1 to zero, we can recast the equation into the matrix form

$$\bar{\mathbf{A}}\cdot\bar{\mathbf{I}}_{z_i} - \bar{\mathbf{A}}\cdot\bar{\mathbf{I}}_{0_i} = 0 \qquad (A4)$$

where $\bar{\mathbf{A}}$ represents the symmetric matrix of coefficients (ρ_i and σ_{ij}). $\bar{\mathbf{I}}_{z_i}$ is a column vector giving the values of the longitudinal magnetizations in the steady state, and $\bar{\mathbf{I}}_{z_0}$ represents the equilibrium values of these same quantities. Letting **D** be the diagonal matrix of driving terms, one has

$$(\bar{\mathbf{A}} + \bar{\mathbf{D}})\bar{\mathbf{I}}_{z_i} = \bar{\mathbf{A}}\cdot\bar{\mathbf{I}}_{0_i} \qquad (A5)$$

Solving for $\bar{\mathbf{I}}_{z_i}$ we obtain

$$\bar{\mathbf{I}}_{z_i} = (\bar{\mathbf{A}} + \bar{\mathbf{D}})^{-1}\cdot\bar{\mathbf{A}}\cdot\bar{\mathbf{I}}_{z_{0i}} \qquad (A6)$$

Equation A6 was used to generate the computed steady-state fluorine–proton Overhauser effect described in the text.

Elements of the matrix $\bar{\mathbf{A}}$ were obtained according to the methods described previously.[8a] All spins in the model system except the fluorine nuclei of the trifluoromethyl group were assumed to be attached to a rigid sphere undergoing rotational diffusion characterized by the correlation time, τ_c, of the enzyme. Internal rotation of the CF_3 group about its C_3 axis was explicitly included. The adjustable parameters in the calculation at the present level of approximation, in addition to τ_c and τ_i, the correlation time for CF_3 rotation, include the Cartesian coordinates, resonance frequency (ω_i), spin–spin relaxation rate (R_{2_i}), and the intragroup spin–lattice relaxation contribution R_{xi} of each proton. These parameters were varied until the experimental data such as those shown in Figure 2 were reasonably well reproduced and the calculated dependence of the NOE on the magnitude of H_2 at selected irra-

diation frequencies was in agreement with experiment. Because of the problems introduced by possible rotation of the aromatic ring holding the CF_3 group, the protons ortho and meta to trifluoromethyl were represented by single stationary protons. The position of the spin representing the ortho proton was adjusted so as to reproduce the contribution of these nuclei to fluorine relaxation that is revealed by selective deuteration experiments;[8a] the spin corresponding to the meta protons was placed 2.48 Å from the pseudo-ortho spin. Similarly, the position of a single spin was adjusted to take into account the relaxation effects of solvent or solvent-derived protons.

Initially the R_{xi} parameters were set to zero, but the need for nonzero values soon became apparent. The values for R_{xi} and R_{2i} given in Table IV could be varied ±10% without substantial effects on the computed $^{19}F\{^1H\}$ NOE curves.

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Crystal and Molecular Structures of 2,6-*cis*-Dimethylpiperidyl-*N*-phenylacetamidine and 2,6-*cis*-Dimethylpiperidyl-*N*-phenyl-2,2-dimethylpropionamidine. An X-ray Crystallographic Investigation of the C(sp^2)–N(piperidyl) Bond

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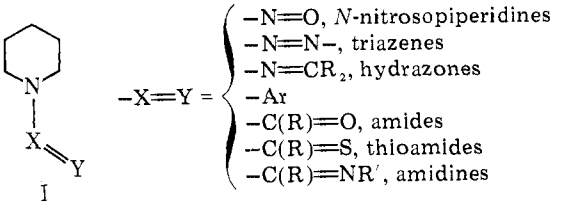
Abstract: The single crystal X-ray analyses of 2,6-*cis*-dimethylpiperidyl-*N*-phenylacetamidine (MA) and 2,6-*cis*-dimethylpiperidyl-*N*-phenyl-2,2-dimethylpropionamidine (TBA) are described. MA crystallizes in the space group $P2_1/c$ with four molecules in the unit cell of dimensions a = 10.238 (2), b = 10.189 (2), c = 12.875 (3) Å, and β = 95.82 (2)°. The structure was solved and refined from 1401 unique observed reflections collected on an automated four-circle diffractometer to final values of the discrepancy indices of R = 0.046 and R_w = 0.058. TBA crystallizes in the space group $P2_1/c$ with eight molecules in the unit cell of dimensions a = 8.470 (2), b = 16.095 (3), c = 24.900 (4) Å, and β = 96.29 (2)°. From 2633 unique observed reflections similarly collected the structure was solved and refined to final values of the discrepancy indices of R = 0.061 and R_w = 0.074. The structure analyses show, in agreement with ^{13}C NMR spectroscopic data, that the two molecules adopt different conformations around the C(sp^2)–N(piperidyl) bond, the amidinic group and the piperidyl ring being approximately coplanar in MA and orthogonal in TBA, respectively. The comparison of the present data with the data in the literature, supported by nonbonded intramolecular potential energy calculations and INDO calculations, allows clarification of the relationship among the torsion angle around the C–N bond, the bond distances in the amidinic group, the pyramidality of the N (piperidyl) atom and the conformation of the 2,6-*cis*-methyl groups in the piperidyl ring.

N-Substituted piperidines of type I have been the subject of several X-ray crystallographic investigations and most of them were found to adopt a nearly planar conformation (that is, with the N—X=Y group lying in the mean plane of the piperidyl ring). Such a conformation would not be preferred in terms of nonbonded interactions alone and is to be ascribed to restricted rotation around the N–X partial double bond, as



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