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Fluorine Nuclear Relaxation Studies of *p*-Trifluoromethylbenzenesulfonyl-α-chymotrypsin

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Abstract: Spin–lattice and transverse fluorine relaxation rates have been determined for the title enzyme derivative at pH 7. The data have been analyzed to provide an estimate of the rotational correlation time (τ_c) near the trifluoromethylbenzenesulfonyl group and the correlation time (τ_i) for internal rotation of trifluoromethyl. The major part of the fluorine relaxation is due to proton–fluorine dipole–dipole interactions. Specific deuteration experiments show that these interactions predominantly involve protons of the enzyme and solvent.

The use of spectroscopic reporter groups is an important aspect of modern protein biophysical chemistry and the past decade has seen an increasing utilization of reporter groups which are amenable to study by magnetic resonance techniques. These latter experiments are especially attractive because they often provide information about the dynamics of molecular motion near the reporter group and, by inference, of the protein itself.

Fluorine-substituted moieties can be introduced readily into protein structures, thereby providing materials which can be examined by fluorine magnetic resonance (^{19}F NMR) spectroscopy.[1] ^{19}F NMR in these systems offers the advantage of ease of signal detection as well as being characterized by chemical-shift effects and relaxation rates that are highly sensitive to the environment of the reporter (fluorine) nucleus.

Although covalently bound fluorine is similar in steric bulk to covalent hydrogen, fluorine is highly electronegative and potentially a hydrogen-bond acceptor. Fluorine substitution within the confines of a tightly structured protein may exert an effect on the structure of the protein, rendering data obtained by ^{19}F NMR spectroscopy irrelevant to the properties of the unmodified system. It is, therefore, necessary to have information on the possible structural consequences of fluorine substitution in a variety of biomolecular systems so that these perturbations may be recognized and possibly avoided.

The structure of the enzyme α-chymotrypsin has been investigated by a large number of experiments, including X-ray crystallography, and this work has provided a foundation for a detailed understanding of the mechanism of action of the protein.[2] An X-ray structure of tosylchymotrypsin, a derivative in which the serine-195 residue at the active site has been esterified, is also available.[3] We have previously reported the preparation and purification of α-chymotrypsin which had been inactivated by treatment with *p*-trifluoromethylbenzenesulfonyl fluoride.[4,5] This protein can be regarded as an analogue of tosylchymotrypsin in which a methyl group has been replaced by trifluoromethyl. In this and subsequent papers we report ^{19}F NMR studies of this protein in solution; the results bear on the question of the effects of fluorine substitution on protein structure and provide information about the dynamics of molecular motions at the protein active site.

Experimental Section

Materials. 4-Trifluoromethylbenzenesulfonyl fluoride was prepared as described previously.[4]

3,5-Dideuterio-4-trifluoromethylbenzenesulfonyl fluoride was synthesized according to the reactions in Scheme I. Dideuterated 4-nitrotoluene was obtained by heating 21 g (0.18 mol) of 4-nitrotoluene (Aldrich) in 70 g of deuterium sulfate (Stohler, 99% D) according to the procedure of Renaud et al.[6] After three exchanges, the ^{1}H NMR spectrum of the product showed a single resonance in the aromatic region of the spectrum at the chemical shift of the 2,6 protons. 3,5-Dideuterio-4-nitrobenzoic acid was obtained from this material by oxidation of 15 g (0.1 mol) with 40 g of potassium permanganate using the procedure of Bigelow.[7] The acid (9 g, 0.05 mol) was treated with sulfur tetrafluoride (Matheson, 14 g, 0.13 mol) in a stainless steel vessel at 135 °C for 20 h.[8] After this time, excess SF_4 was removed and the residual oil taken up in 100 mL of ether. The ether layer was dried over magnesium sulfate and the solvent removed in vacuo, leaving a crude product (mp 35–40 °C, lit. 41–43 °C[9]) which was used directly in the reduction step. 2,6-Dideuterio-4-aminobenzotrifluoride was obtained by reduction of crude 4-nitrobenzotriflu-

Scheme I. Preparation of 3,5-Dideuterio-4-trifluoromethylbenzenesulfonyl Fluoride

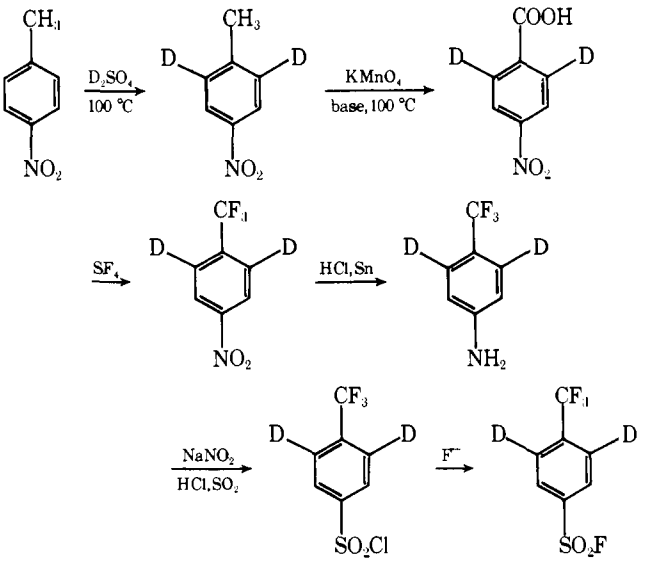

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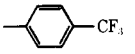
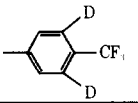
Table I. ^{19}F NMR Parameters for p-Trifluoromethylbenzenesulfonyl-α-chymotrypsin[a]

enzyme derivative				
solvent	H_2O/D_2O (80/20)	D_2O	H_2O/D_2O (96/4)	D_2O
R_1, s^{-1} [b]	5.1 (5.8)	3.3 (3.8)	4.2 (4.9)	2.7 (2.9)
R_1, s^{-1} [c]	4.4 (4.9)	3.6 (3.5)	4.3 (4.1)	2.7 (2.3)
R_2, s^{-1} [d]	~30. (35.)	~20. (29.)	~30. (32.)	~30. (26.)
$^{19}F\{^1H\}$ NOE	−0.81 (−0.82)	−0.78 (−0.81)	−0.80 (−0.84)	−0.81 (−0.76)
chemical shift, ppm[e]	−13.55	−13.67	−13.58	−13.70

[a] At pH 7 in 0.05 M KCl, 25 °C. Protein concentration was approximately 1 mM unless otherwise noted. Values calculated by the methods described in the text are given in parentheses. [b] Obtained with noise-modulated (full) proton decoupling at 100 MHz. [c] Estimated from a logarithmic plot of data obtained without proton decoupling (initial slope). [d] Estimated value at infinite dilution. [e] Parts per million from internal trifluoroacetate (0.5 mM); average of at least two determinations.

oride-*2,6-d*$_2$ (3.9 g, 0.02 mol) with 20 g of stannous chloride in 15 mL of concentrated hydrochloric acid as described by Jones.[10] 4-Trifluoromethylbenzenesulfonyl chloride-*3,5-d*$_2$ was prepared by diazotization of the amine in the presence of sulfur dioxide using the method described previously.[4] Conversion of the sulfonyl chloride to the desired sulfonyl fluoride was carried out with fluoride-loaded Bio-Rad AG1-X10 ion exchange resin according to the procedure of Borders et al.[11] 3,5-Dideuterio-4-trifluoromethylbenzenesulfonyl fluoride prepared by this scheme showed a single resonance in the ^1H NMR spectrum at 8.1 ppm ($CDCl_3$ solvent) downfield from tetramethylsilane and, after sublimation at 0.1 Torr, exhibited mp 70–71 °C (lit. 71 °C[4]). Absence of contaminating sulfonyl chloride was indicated by a negative silver nitrate test.

α-Chymotrypsin was a three times recrystallized Worthington product. Crude inactivated enzyme was prepared as described elsewhere.[4,5] Heterogeneity in the crude protein has been discussed.[5] In brief, up to four ^{19}F NMR signals can be observed with the crude modified enzyme preparation. Of these, one at about 13.6 ppm downfield from trifluoroacetate accounts for 70–80% of the total fluorine signal intensity. Chromatography of the protein on ion-exchange or affinity columns only partially purifies the material. For much of the work reported herein, modified protein which had been purified by chromatography on carboxymethylcellulose (Whatman CM-32) was used;[5] no differences beyond experimental errors between the results obtained with crude and purified protein were noted. ^{19}F NMR spectra of the inactivated enzymes showed no detectable changes over periods of 12 h at 25 °C and the inactive protein showed no activity toward the substrate *N*-glutaryl-L-phenylalanine *p*-nitroanilide.[12]

Procedures. Fluorine spectroscopy at 94.1 MHz employed a Varian Associates XL-100 spectrometer; deuterium in the solvent provided a field-frequency lock signal. A Nicolet Technology TT-100A Fourier transform accessory was used to accumulate and manipulate all spectra. Proton noise decoupling was carried out as described previously.[13] The sample temperature was controlled at 25 ± 1 °C using the Varian controller for all experiments. For most spectra a spectral width of 2000 Hz, represented by 4K data points, was used; typically 1K transients were collected for a given spectrum.

The protein concentration was 1 mM (25 mg/mL); samples were prepared gravimetrically and contained 0.05 or 0.1 M KCl and 0.5–0.7 mM sodium trifluoroacetate (K&K Labs). Sample pH was adjusted to pH 6–7 using microliter amounts of potassium hydroxide or hydrochloric acid solutions. All solvents were saturated with N_2 before use, but, given the large values of R_1 observed, no attempt to rigorously exclude oxygen was made.

R_1 values were obtained using the 180-τ-90 sequence.[14,15] At 94.1 MHz, the observed line width, less the instrumental line width represented by the width of the reference peak, was usually used to estimate R_2. However, in several cases, a Carr-Purcell-Meiboom-Gill experiment[14] was used to determine R_2. The results from either method agreed within experimental error. The estimated experimental errors for R_1 are 10%, based upon reproducibility of results for a number of enzyme preparations, while the estimated error in R_2 is ± 20%.

In acquiring the $^{19}F\{^1H\}$ NOE data, a waiting time of at least 2 s (~$10T_1$) between each accumulation was used.[16] The Overhauser effects are judged to be reliable to within ±10%.

Results

Crude preparations of *p*-trifluoromethylbenzenesulfonyl-α-chymotrypsin consist of a complex mixture of fluorine-containing and non-fluorine-containing proteins.[5] The results described herein are those afforded by the major fraction (~65%) in this mixture and this fraction is assumed to represent an enzyme form analogous to tosylchymotrypsin. Kinetic experiments have previously shown that regeneration of enzyme activity is slow at pH 6–7 in 0.05 M KCl, being about 1% per 24-h period,[5] and this is consistent with our observation that the ^{19}F NMR spectra were stable for at least 12 h at 25 °C. Small variations in pH from 6 to 7 appeared not to affect any of the observations reported herein.

The major trifluoromethyl-labeled enzyme form exhibits a broad spectral line that was well fit by a single Lorentzian function. Fluorine spin-lattice (R_1) and spin-spin (R_2) relaxation rates were determined for this signal as a function of protein concentration from 0.1 to 3.5 mM modified protein. There was no variation in the spin-lattice relaxation rates beyond experimental error over this concentration range. However, R_2 did change appreciably and, at the highest protein concentration, was at least triple the value observed at the lowest concentrations. Attempts to accurately extrapolate the R_2 values to infinite dilution were thwarted by the large experimental uncertainties in the data at very low enzyme concentrations, but, in all cases, it could be estimated that R_2 at infinite dilution was in the range 20–35 s^{-1}. The chemical shift of the fluorine resonance relative to trifluoroacetate (present in solution or in a separate capillary) was independent of concentration within the experimental error of ±0.02 ppm. Spectral parameters for the major fluorine resonance, obtained at a protein concentration of approximately 1 mM, are recorded in Table I. Spin-lattice relaxation rates were determined with and without irradiation of the protons of the enzyme and both sets of data are given in Table I.

The nuclear Overhauser effect on the fluorine signal intensity upon broad-band irradiation of the proton spectrum was obtained; the $^{19}F\{^1H\}$ Overhauser effect was found to be substantial and negative, implying that proton-fluorine dipole-dipole interactions play a significant role in relaxing the CF_3 group of the macromolecule.[17] The aromatic protons ortho to the CF_3 group on the inhibitor are reasonably close to the fluorine nuclei[18] and to assess the contribution of these hydrogens to fluorine relaxation the specifically deuterated inhibitor I was prepared. Preparation and purification of the inactivated, dideuterated enzyme followed the same procedures as used for the fully protonated system. Relaxation and Overhauser data for the deuterated system are also given in Table I.

Indole is a competitive inhibitor of the enzyme and it is known that certain labels covalently attached to the serine-195

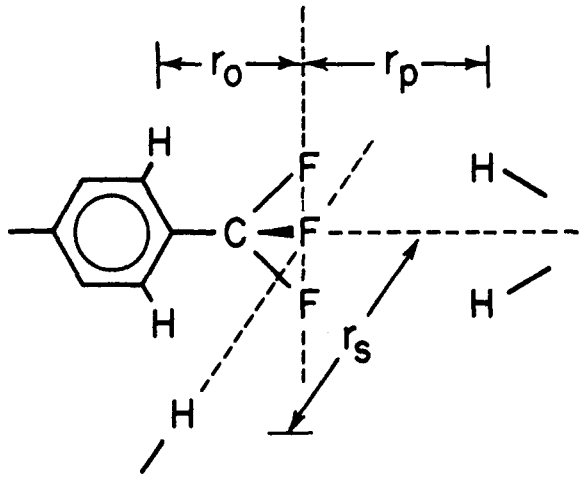
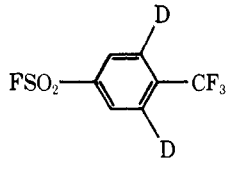


Figure 1. Model system used to analyze the fluorine relaxation data. For the dideuterated compound, the aromatic protons were omitted. The bond angles and bond length given in ref 18 were used to obtain the coordinates of the trifluoromethyl group.



I

residue of the protein can be displaced from the active site area with indole.[19,20] Using solutions saturated with indole (~15 mM), there was no detectable difference in the relaxation behavior of the ^{19}F NMR signal from the modified enzyme compared to data obtained in the absence of indole. This result suggests that the trifluoromethylbenzenesulfonyl group is not as easily displaced from the active site as are the covalently linked spin labels of Berliner and Wong.[19]

Data Analysis. Kalk and Berendsen have emphasized that complete relaxation expressions involving "cross relaxation" terms must be used to obtain reliable correlation times and internuclear distances from macromolecular relaxation data.[21] However, under conditions of complete proton decoupling the fluorine spin-lattice relaxation rates in *p*-trifluoromethylbenzenesulfonylchymotrypsin will not be affected by cross relaxation effects. Calculations show that the degree of multiexponential character introduced into the spin-lattice relaxation functions by cross-correlation effects in fluorine-fluorine dipole-dipole interactions should be small for this system.[22,23] We therefore expect, under conditions of complete proton decoupling, essentially single exponential relaxation for the fluorine signal that is characterized by a time constant which indeed reflects the molecular correlation time(s) and internuclear distances involved in the relaxation process(es). A major disadvantage in using spin-lattice relaxation data obtained under conditions of full proton decoupling is the negative Overhauser effect which makes highly accurate data difficult to collect in a reasonable time owing to the greatly diminished signal intensity.

The $^{19}F\{^1H\}$ Overhauser effect (η) for the present system is given by the equation

$$\eta = \frac{F_z - F_0}{F_0} = \frac{\gamma_H}{\gamma_F} \frac{\sum_i \sigma^i_{HF}}{\rho^*_{FF} + \rho_{csa} + \sum \rho^i_{HF}} \qquad (1)$$

where F_z is proportional to the observed fluorine signal intensity with irradiation while F_0 is the signal strength without proton decoupling.[24] The terms ρ^*_{FF}, ρ_{csa}, and ρ^i_{HF} represent the contributions of fluorine-fluorine interactions, fluorine chemical shift anisotropy, and proton-fluorine interactions, respectively, to fluorine relaxation while γ_H and γ_F are the gyromagnetic ratios for the nuclei involved. The terms σ^i_{HF} are the result of proton-fluorine cross-relaxation effects;[21] the term ρ^*_{FF} includes the effects of fluorine-fluorine cross relaxation. There is presumably a matrix of protons surrounding the CF_3 group in the modified protein and the summations are taken over all possible interactions between these protons and the fluorine nuclei.

The protein lattice which aids in relaxing the CF_3 group can be divided into three parts: (1) protons covalently attached to the enzyme, (2) the two protons ortho to the CF_3 function of the *p*-trifluoromethylbenzenesulfonyl group, and (3) protons of the solvent and protons on the enzyme which exchange rapidly with the solvent. To dissect these contributions to relaxation from our data, a specific model of the protein matrix must be proposed so that computations of the various relaxation parameters reported in Table I can be attempted. The model we chose is indicated in Figure 1; it includes two protons to represent the ortho hydrogens of the inactivator molecule, a pair of protons to represent the protons of the protein, and a proton normal to these four nuclei which represents a solvent or solvent-exchangeable proton. The distances r_o, r_p, and r_s are adjusted to control the contributions of each group to relaxation (ρ^i_{HF}). The entire assembly was assumed to tumble isotropically with a characteristic correlation time (τ_c) and the CF_3 group allowed to rotate independently of the structure at a rate defined by the correlation time τ_i. The choice of the number of spins used to represent the protons of the enzyme and the solvent is at this point arbitrary; we have shown that more elaborate models can be adjusted to give theoretical results equivalent to those described here.

In computing relaxation rates and Overhauser enhancements, each pairwise dipole-dipole interaction was treated according to the theory of Solomon.[25] Spectral densities were calculated using the equations of Woessner for cases where H-H distances remain fixed or for the F-F interactions where internal rotation must be considered.[26] For proton-fluorine interactions internal rotation makes the internuclear distances time dependent and, in these situations, the spectral densities of Rowan et al.[27] were used. The contribution of the chemical shift anisotropy mechanism depends on τ_c and τ_i and was estimated using the parameters of Hull and Sykes.[28] Fluorine-fluorine internuclear distances in the CF_3 group were fixed at 2.158 Å; the bond angles of this structure were those used by Scott et al.[18]

Data for fluorine relaxation in the dideuterated compound, obtained with D_2O as solvent under conditions of full proton decoupling, permit us to estimate the effects of fluorine-fluorine interactions and the chemical shift anisotropy effect ($\rho^*_{FF} + \rho_{csa}$) as well as the contribution of protons on the protein to trifluoromethyl relaxation. In the analysis, the correlation time τ_c was fixed at particular values between 10 and 60 ns and the internal rotation rate (τ_i) and the distance r_p were varied until the experimental R_1, R_2, and Overhauser effect at 94.1 MHz were reproduced as closely as possible. The best fit to the data occurred with τ_c = 12-15 ns, τ_i = 0.019 ns, and $r_p \simeq 2.72$ Å. The portion of fluorine spin-lattice relaxation due to fluorine-fluorine interactions and the csa effect was thereby estimated to be 0.6 s^{-1} while protons on the enzyme contributed through hydrogen-fluorine interactions (ρ^i_{HF}) 2.2 s^{-1} to the observed relaxation rate. Consideration of the remaining data in Table I shows that the ortho protons of the inhibitor generate a spin-lattice relaxation effect of 0.9 s^{-1} at the CF_3 group while the contribution of solvent and/or solvent exchangeable protons is substantial, about 1.9 s^{-1}. Values of r_o and r_s consonant with these relaxation effects were found

to be 2.96 and 3.34 Å, respectively. Although the uncertainties in these contributions to relaxation (about $\pm 0.4\ s^{-1}$) are high, it is seen in Table I that they lead to calculated values of R_1 with proton decoupling and the NOE at 94.1 MHz which are within experimental error of the observed quantities.

Without proton decoupling the fluorine spin-lattice relaxation process should be polyexponential[21,24,25] and the apparent R_1 observed can depend on the nature of the decay curve and the time at which the observation is made. Using the crude model described, the initial rates for fluorine spin-lattice relaxation were computed at 0.05 s after the start of the recovery curve. The experimental data seemed to be well fit by a single exponential function and the slopes if these curves agreed with the initial slopes computed with the model (Table I).

Spin-spin relaxation is expected to be a single exponential function in this fluorine-proton system if cross-correlation effects can be neglected.[22,23,25] All experimental observations of spin-spin relaxation in *p*-trifluoromethylbenzenesulfonylchymotrypsin were consistent with this expectation and R_2 values calculated using the model system were consonant with the experimental values estimated by extrapolation of R_2 data obtained as a function of concentration to zero protein concentration. Given the quality of the experimental data, it is not possible to distinguish reliably the (small) differences in R_2 anticipated for the various isotopically substituted systems.

Chemical Shifts. The chemical shift of the fluorine signal of *p*-trifluoromethylbenzenesulfonylchymotrypsin relative to the resonance of a trace (0.5 mM) of trifluoroacetate for each system is given in Table I. The reference signal was subject to a small positive Overhauser enhancement ($\eta \simeq 0.03$) under conditions of complete proton decoupling. If significant binding of the reference substance to the protein took place, a negative NOE would be expected[17] and we conclude that this interaction is weak if present at all. Small but significant isotope effects on the fluorine chemical shifts are apparent when the isotopic substitution is made either in the solvent or at the positions ortho to the CF_3 group.

Discussion

Since *p*-trifluoromethylbenzenesulfonylchymotrypsin is a mixture of proteins, we were concerned that the resonance exhibited by the major enzyme form, in fact, corresponds to a single protein species. The fluorine magnetization recovery curves in the R_1 determinations and the decay curves in the Carr-Purcell-Meiboom-Gill determinations of R_2 were examined to ascertain if the partially relaxed signals gave any evidence of asymmetry that would correspond to species with similar chemical shifts but distinctive relaxation times. There was no evidence for these effects. Also, the line shape for the fluorine resonance of the major modified enzyme form could be fit nicely to a single Lorentzian curve. Thus, there is no evidence from ^{19}F NMR data that the resonance observed represents more than a single protein form.

Chymotrypsin is known to associate with ease into various oligomeric forms.[29] There is evidence that tosylchymotrypsin associates less readily than does the native enzyme at low solution pH but near pH 6 the association behavior appears to be similar to the native enzyme.[30] The ultracentrifugation data of Neet et al. suggest that an appreciable variation in the extent of protein association should take place over the range of protein concentrations examined in the present work if the trifluoromethyl analogue aggregates in the same manner as the native or tosylated enzyme.[31] It is likely therefore that the variation in R_2 with protein concentration observed is the result of protein association. A detailed analysis of the relaxation rates in terms of the structure and dynamics of each of the oligomeric forms of the modified enzyme present will require much more data than we presently have available, but the fluorine R_2 relaxation rates extrapolated to zero protein concentration should be those of the monomeric protein.

Because of experimental errors in each relaxation rate, the estimated contributions of possible relaxation mechanisms to the relaxation of the CF_3 group in *p*-trifluoromethylbenzenesulfonylchymotrypsin have rather high uncertainties. However, the assumed model for the environment of the fluorine reporter group gives a reasonably good accounting of experimental spin-lattice and transverse relaxation rates and $^{19}\{^1H\}$ Overhauser effects under a variety of conditions. Estimates of τ_c for the monomeric form of the protein made by ESR spectroscopy of spin-labeled derivatives[32] or fluorescence polarization[33] give values in the range 11-16 ns and agree with τ_c calculated from the Stokes-Einstein relation.[34] The parameter τ_c of the model system basically reflects the tumbling time of the C_3 axis of the CF_3 reporter group and the agreement observed indicates that at low protein concentrations *p*-trifluoromethylbenzenesulfonylchymotrypsin exists as a monomeric protein with the benzenesulfonyl moiety held tightly enough to the surface of the enzyme that the symmetry axis which extends through the benzene ring has little freedom of motion beyond overall tumbling of the enzyme. However, until a detailed analysis of fluorine relaxation in concentrated protein samples is available, we cannot rule out the possibility that the modified enzyme at low concentrations is more highly associated than the monomer but that molecular motions in the vicinity of the reporter group take place on a time scale that is fortuitously close to the correlation time of the monomer.

In examining the effect of solvent or solvent-derived protons on the relaxation of the CF_3 group, no attempt was made to take into account the difference in τ_c expected because of the difference in the viscosity of water and deuterium oxide.[35] The question of how deuterium oxide affects protein structure at a local level complicates any such correction. In any event, τ_c would not be expected to be more than 11% larger in D_2O and a change of this magnitude would be difficult to detect reliably given our present experimental uncertainties.

With the exception of the positioning of the ortho hydrogen atoms, our model for the proton matrix around the CF_3 group in the enzyme derivative is arbitrary. The distances r_o, r_p, and r_s which correspond to the contributions of the various groups of protons to fluorine relaxation are 2.98, 2.76, and 3.35 Å. One notes that the sum of the van der Waals radii for hydrogen and fluorine is ~2.6 Å so that these distances are not unreasonable.[36] The estimated distances from the ortho protons to the atoms of the trifluoromethyl group are larger by about 0.7 Å than those expected from previous studies of the trifluorophenyl ring.[18] The distances demanded by our model could be generated by bond angle deformations which "close up" the CF_3 group or move the ortho hydrogen nuclei away from this function. At a greater cost in energy, C-H, C-C, or C-F bonds could be lengthened by some protein-CF_3 interaction. However, it should also be noted that the model system used to compute r_o may not be particularly appropriate for the present situation for it has been assumed that in the model the aromatic ring of the inhibitor is held rigidly enough to the enzyme that its motion, as described by τ_c, is essentially that of the protein. There is now ample evidence that aromatic rings in protein structures can rotate about their local symmetry axes at widely different rates[37] and one might expect, given the near-surface location of the *p*-trifluoromethylbenzenesulfonyl group, that this kind of rotational process would be rapid in the present case. To our knowledge, spectral density functions appropriate to this situation have not yet been reported but it is possible that certain rates of flipping the aromatic ring through 180° would make fluorine relaxation by the ortho protons less efficient and, in the present treatment, this would be reflected by an increase in the distance r_o.

The value for τ_i needed to fit the observed relaxation data corresponds to an internal rotation rate[38] ($1/2\tau_i$) of about 2.6 $\times 10^{10}$ s^{-1}, representing an activation barrier of ~3.2 kcal/mol. The rotational rate observed is certainly not unusual when compared to the rates of methyl (CH_3) rotation in various amino acid crystals or solid alkanes.[21] In solid benzotrifluoride the activation barrier to CF_3 rotation is 3.5 kcal/mol (estimated by NMR methods).[39] In the gas phase, thermochemical and vibrational spectroscopic data suggest that the rotational barrier is at most a few tenths of a kcal/mol.[18] Fluorine relaxation studies suggest that the rotational barrier is very low in solution.[40,41] It is hard to know which of these values to compare to the result obtained with the enzyme derivative or to know if the observed barrier is intrinsic to the trifluoromethylphenyl ring or is the result of interactions with the protein. Additional values of the rotational barrier obtained in studies of related systems that are now underway may illuminate this point.

Our dissection of the fluorine relaxation rates in *p*-trifluoromethylbenzenesulfonylchymotrypsin neglected any contribution that the spin-rotation mechanism might make to these rates. This mechanism is significant for fluorine relaxation in various benzotrifluorides[40-42] and it has been suggested that spin rotation can be involved even when the CF_3 group is part of a large molecule.[22] Spin-rotation coupling generates a fluorine spin–lattice relaxation effect of about 0.3 s^{-1} in benzotrifluoride and it is unlikely that this contribution will be any larger in macromolecular systems. Thus, relaxation by spin rotation cannot be more than 10% of the observed fluorine relaxation rate in any systems examined here and, given the apparently large barrier to internal rotation of the CF_3 group, likely is substantially less than this.

The contribution of the chemical shift anisotropy (csa) to R_1 and R_2 was included in the fitting of the relaxation data according to the prescription of Hull and Sykes.[28] Using their parameters for the CF_3 group we estimate that the contribution (ρ_{csa}) of csa relaxation to R_1 at 94.1 MHz is 0.06, about 2% of the total relaxation of the dideuterated enzyme derivative in deuterium oxide. The chemical shift tensor given by Hull and Sykes is for CF_3 next to a carbonyl group and this may not be appropriate for the present case.[43] However, the errors involved are probably less than 20% and thus will have a minor influence on the calculated fluorine–fluorine dipolar contribution ($\rho^*_{FF} = 0.54$); it is this latter quantity which largely determines the estimate of τ_i given above.

A major approximation in making the analysis of the fluorine relaxation data is the neglect of cross-correlation effects. Although this is a common assumption,[24] theoretical work shows that it can lead to errors when interpreting NOE data.[44] Because the various protons of the enzyme which relax the CF_3 group are likely in constant and uncorrelated motion relative to the trifluoromethyl, proton–fluorine cross-correlation terms can probably be neglected safely in our analysis. Werbelow[45] has shown that in the X{A} NOE experiment with an AX_3 system cross correlation of the motions of the X internuclear vectors always leads to an Overhauser effect which overestimates the importance of A–X dipolar contributions to the relaxation of X. Calculations using his equations indicate that cross-correlation effects at correlation times of *p*-trifluoromethylbenzenesulfonylchymotrypsin will change the $^{19}F\{^1H\}$ NOE by less than 10%. The experimental values for the various enhancements given above are uncertain by this amount at least and neglect of fluorine–fluorine cross correlation for this system seems justified until more accurate data are available.

Deuterium substitution for a proton three bonds away from a fluorine nucleus results in an appreciable upfield shift of the fluorine resonance signal.[46,47] A smaller (~0.02 ppm) shift upfield has been noted in the 4-trifluoromethylbenzenesulfonate ion when the protons ortho to the CF_3 group are replaced by deuterium (K. F. S. Luk, unpublished observation); in this case the deuterium nucleus is separated by four covalent bonds from the fluorine atoms. The trend to upfield chemical shift effects when H is replaced by D is not apparent in the modified enzymes used in the present work where small (~0.02 ppm) *downfield* shifts are observed. The origin of these isotope effects on fluorine chemical shifts is not apparent at present but may be related in some unappreciated way to the interactions between the trifluoromethylbenzenesulfonyl group and the enzyme surface.

Replacement of solvent protons by deuterons also generates a shift (~0.11 ppm to low field) of the fluorine signal from the enzyme derivative. Chemical shifts of fluorine nuclei in aqueous solution are generally shifted 0.2–0.3 ppm upfield when the solvent is replaced by deuterium oxide[48] and this effect has been used as a probe for the degree of exposure of fluorine-containing groups in proteins to the solvent.[49] If transfer from H_2O to D_2O does not produce a structural change in chymotrypsin,[50] our observations may indicate that the CF_3 reporter group is not highly available to solvent. It is clear, however, that solvent-derived protons are important in the relaxation of the trifluoromethyl attached to the enzyme and readily exchangeable amide or hydroxyl protons near the active site of the enzyme may interact sufficiently with the fluorine nuclei to play this role.

Summary

The *p*-trifluoromethylbenzenesulfonyl group of the modified enzyme appears to be tightly held to the enzyme surface, although rotation of the CF_3 group about its C_3 axis remains rapid in this situation. Proton–fluorine dipole–dipole interactions account for most of the observed R_1 and R_2 relaxation rates. Some characteristics of the proton matrix around the CF_3 group have been elucidated and it has been demonstrated that protons on the enzyme interact strongly with the reporter group. (Selective Overhauser experiments aimed at further describing these protons have been carried out and will be described in a subsequent paper.) Deuterium isotope effects on the chemical shift of the fluorine reporter are appreciable but have not, as yet, been rationalized.

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Insertion of Tetrafluoroethylene into the Fe–Fe Bond of $(\mu(SCH_3)Fe(CO)_3)_2$, Its Thermal Rearrangement to a Bridging Carbene Ligand, and the Transformation of the Carbene to a Perfluoromethylcarbyne Ligand. Structures of $\mu(SCH_3)_2\mu(C_2F_4)Fe_2(CO)_6$ and $\mu(SCH_3)_2\mu(FCCF_3)Fe_2(CO)_6$ at −162 °C

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Abstract: The insertion of tetrafluoroethylene into the Fe–Fe bond of the dinuclear complex $(\mu(SCH_3)Fe(CO)_3)_2$ is photochemically induced. When the temperature of the reaction is stabilized at 20 °C, the major product is the yellow dinuclear species $\mu(SCH_3)_2\mu(C_2F_4)Fe_2(CO)_6$ (**1**), where C_2F_4 bridges the Fe atoms with two σ(C–Fe) bonds, the C–C bond being parallel to the Fe–Fe axis. When the temperature is higher, i.e., 35 °C, the product is the red dinuclear species $\mu(SCH_3)_2\mu(FCCF_3)Fe_2(CO)_6$ (**2**), which contains a $>CF-CF_3$ carbene bridge. It is possible by heating **1** to obtain **2** and a mechanism for this reaction is proposed, based in part on a study of the action of BF_3 on **1**. The action of BF_3 on **2**, followed by the addition of trimethylphosphine, affords $[\mu(SCH_3)_2Fe_2(CO)_3(PCH_3)_3)_2(CCF_3)][BF_4]$ (**7**), which may be a perfluoromethylcarbyne complex. A proof for the two different kinds of insertion of C_2F_4 is presented in the form of crystal structure determinations of **1** and **2**. In **1** each iron atom is octahedrally coordinated to three carbonyl groups, two bridging S atoms, and one C atom of C_2F_4. The Fe–Fe separation is 3.311 (1) Å, the dihedral angle around the S atoms is 135.0°, and the average Fe–S–Fe angle is 91.6°. Compound **1** crystallizes in the orthorhombic space group D_{2h}^{15}-*Pbca* in a cell of a = 15.029 (8), b = 13.561 (5), c = 15.437 (8) Å. Compound **2** crystallizes with eight formula units in space group C_{2h}^5-$P2_1/c$ of the monoclinic system in a cell of dimensions a = 11.545 (3), b = 16.681 (5), c = 16.830 (6) Å with β = 97.86 (2)°. Based on 2471 and 4416 unique reflections for **1** and **2**, respectively, the structures were refined by full-matrix least-squares techniques to conventional agreement indices (on F) of R = 0.044 and R_w = 0.049 for **1** and R = 0.039 and R_w = 0.048 for **2**. In **2**, each iron atom is also octahedrally coordinated, being bound as in **1** to three carbonyl groups, two bridging S atoms, but here to the same bridging C atom of the $>CF-CF_3$ carbene group. The Fe–Fe separation averages 2.963 Å, the dihedral angle around the sulfur atoms is 107.2°, and the average Fe–S–Fe angle is 79.39°. The Fe_2S_2 unit is more compact in **2** than in **1** but less compact than in the starting material $(\mu(SCH_3)Fe(CO)_3)_2$. The flexibility of such molecules around the S–S axis, together with the reactivity of the Fe–Fe bond, is discussed.

The study of the reactivity of the metal–metal bond in dinuclear complexes toward alkynes, alkenes, or more generally small unsaturated molecules is an increasing field of interest. This is particularly true for dinuclear complexes with metal to metal multiple bonds.[2,3] However, insertion reactions of alkynes and alkenes into metal–metal single bonds in dinuclear

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