$$CH_3NO_2 \rightarrow CH_2NO_2^- + H^+ \quad \Delta H° = 357.0 \text{ kcal}^{36} \quad (11)$$

$$CH_3CN \rightarrow CH_2CN^- + H^+ \quad \Delta H° = 373.5 \text{ kcal}^{36} \quad (12)$$

dramatic attenuation of the gas-phase acidity difference is entirely normal, as the enthalpies of solution of small charged species are much more negative than those of neutrals. So transfer to aqueous solution will favor the dissociated states in reactions 11 and 12 where both the proton and anion will be strongly solvated. For reaction 10 the stabilization will be less as the enthalpy of solvation of the proton will be to some extent offset by that of the diazonium ion.

Rate of Protonation of Diazomethane by the Hydronium Ion. The rate of protonation of diazomethane by the hydronium ion of 4×10^8 M^{-1} s^{-1} is extremely high for a formally neutral carbon base. A number of factors are responsible for this surprising reactivity. First, the reaction is strongly exoenergetic, as judged by the difference in pK_a's of the acidic species involved. This in itself is not however a sufficient cause, as even more basic substrates, e.g., nitronate and enolate anions, are protonated much less rapidly.

The localization of negative charge on the site of protonation, minor in the above two cases, is of great importance.[4] Although a number of resonance forms involving charge separation may be written for diazomethane and the dipole moment indicates slightly greater charge density on N,[38] evidence from infrared[39] and ^1H NMR[40] spectroscopy indicates that considerable negative charge is localized on the carbon atom (^{13}C NMR evidence has been shown to be less reliable in this respect[41]). So little further energy expenditure is necessary to localize all the negative charge on carbon prior to proton transfer.[5,6] Furthermore indirect evidence exists that the diazomethane carbon is a good hydrogen bond acceptor.[42] Hence the energy necessary to desolvate the acid species may be partially compensated by hydrogen bond formation to diazomethane before proton transfer.[4]

Both these factors should diminish the W^r term in the Marcus formalism for the energetics of proton transfer—i.e., that part of the activation free energy which is independent of the thermodynamic driving force. Accordingly the observed rate constant is close to the diffusion-controlled limit.

Acknowledgment. We wish to thank the Swiss National Science Foundation for financial support and the Ciba-Geigy Fellowship Trust for a fellowship (to T.S.). We also gratefully acknowledge very helpful conversations with Professors C. A. Bunton and H. Dahn.

Supplementary Material Available: Figure S-1, diagram of the installation for continuous-flow pH measurement with the use of a microcapillary glass electrode (1 page). Ordering information is given on any current masthead page.

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Transient Fluorine–Proton Overhauser Effects in (4-(Trifluoromethyl)benzenesulfonyl)-α-chymotrypsin

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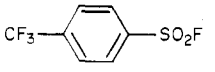
Abstract: Transient $^{19}F\{^1H\}$ nuclear Overhauser effects have been used to explore the protein environment of the trifluoromethyl group of the modified enzyme mentioned in the title. Computational results for a small model spin system which has dynamic properties appropriate for a protein the size of chymotrypsin are presented; these suggest that the NOE experiments should indicate the chemical shifts of those protons of the protein closest to the CF_3 group. It is shown that the data obtained are consistent with the structural model for the immediate environment of the CF_3 group that was proposed earlier (*J. Am. Chem. Soc.* **1979**, *101*, 7698).

The proteolytic enzyme α-chymotrypsin is inactivated by treatment with *p*-(trifluoromethyl)benzenesulfonyl fluoride (I).[1]



I

The reaction places a trifluoromethyl reporter group at the active center of the protein which can then be examined by fluorine magnetic resonance spectroscopy. Previous work has been aimed at defining the nature of the environment surrounding the CF_3 group in the protein derivative and has shown that there are interactions between protons of the enzyme structure and the fluorine nuclei, probably at internuclear distances defined by the van der Waals contact distances of hydrogen and fluorine.[2,3] Equilibrium $^{19}F\{^1H\}$ Overhauser effects, in which the modified protein was irradiated at a particular frequency in the proton spectrum until a steady state in the nuclear spin populations was reached, aided in the deduction of a crude model for the structure in the locale of the trifluoromethyl.[3]

In large molecules, cross-relaxation effects become important with the result that nuclear spins of the same type in such molecules rapidly acquire the same temperature.[4] An important

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ramification of facile equilibration of nuclear spin magnetizations is that perturbation of the magnetization of one spin, even if it is done in a selective manner, soon results in changes in the magnetizations of other spins. In the more graphic terms of Bothner-By,[5] making one spin "hot" by selective irradiation soon "warms" other spins. Thus, when a steady-state fluorine–proton NOE experiment with a large molecule is carried out, irradiation of *any* part of the proton spectrum may result in the *same* effect on the fluorine magnetization; selectivity is lost and no useful structural information, other than the fact that interactions with protons produce some fluorine relaxation, is obtained.[6] Calculations suggest that for spherical proteins much larger than ~20000 Daltons, cross-relaxation or spin diffusion effects remove specificity in the $^{19}F\{^1H\}$ NOEs, and experimental observations are generally in accord with these conclusions.[7] Although some fine structure was noted in equilibrium $^{19}F\{^1H\}$ NOE data obtained with (*p*-(trifluoromethyl)benzenesulfonyl)chymotrypsin, we were concerned that spin-diffusion effects might be largely negating reliable interpretation of these data.

A crucial variable in the spin-diffusion phenomenon is time. Several investigators have pointed out recently that, if observations can be made after a perturbation before all spins acquire a common spin temperature, selective Overhauser effects will be observed.[8-10] In the present work, the transient Overhauser experiment of Solomon[11] was applied to the CF_3-labeled enzyme. In this experiment selective perturbation of a particular proton magnetization over a time small relative to the proton spin–lattice relaxation time is attempted, after which time-dependent changes in the fluorine magnetization are followed until the system reaches equilibrium. We first describe the experimental observations to be expected under our conditions and then present the results of transient $^{19}F\{^1H\}$ NOE experiments with the trifluoro-methylated protein. The results confirm the conclusions reached earlier on the basis of the equilibrium NOE data.

Experimental Section

(4-(Trifluoromethyl)benzenesulfonyl)-α-chymotrypsin and (3,5-dideuterio-4-(trifluoromethyl)benzenesulfonyl)-α-chymotrypsin were prepared as described previously.[3] For most of the work described here the enzyme samples were ultrafiltered through an Amicon UM-10 membrane. Fluorine NMR spectra show that crude (4-(trifluoromethyl)-benzenesulfonyl)chymotrypsin is not homogeneous;[12] the ultrafiltration step reduced the component in the mixture giving the sharp resonance line described previously[12] to less than 5% of the total. The broad, major peak at -13.55 ppm from internal trifluoroacetate comprised >80% of the fluorine signal intensity observed, and it is the species represented by this resonance that is the subject of this paper. After 1 week at probe temperature there was a detectable increase in the intensity of the sharp signal due to slow hydrolysis or elimination of the reporter group.

Samples for fluorine NMR spectra were prepared as for the previous papers;[2,3] the protein concentration was approximately 25 mg/mL. All solutions were in D_2O (Stohler Isotope Chemicals) and were prepared by diluting concentrated H_2O solutions with deuterium oxide and ultrafiltering. This cycle was repeated three times to give solutions which were ~90% in solvent deuterium.

^{19}F NMR experiments were obtained at 94.13 MHz on a Varian Associates XL-100 by using the decoupling system indicated earlier.[3] For the transient NOE experiment a radio frequency pulse of known amplitude and duration at a particular proton frequency was applied to the sample. After a delay time τ, a 90° pulse at the fluorine frequency was used to sample the magnitude of the longitudinal component of the fluorine magnetization, F_z. A slight repatching of the Nicolet 293 panel provided computer-controlled gating of the ^1H radio frequency through a DAICO Industries radio frequency switch. Calibration of the ^1H field has been described earlier.[3] The sample temperature was controlled at 25 ± 1 °C by the Varian controller and checked with a Kontes 5-mm o.d. thermometer. Because of limited disk storage space and limited stability of the modified enzyme, much of the data collected used smaller data sets to represent free induction decays and fewer collected transients than we would have preferred. For scans through the proton chemical shift region, data at 100 proton frequencies were collected by using 2K (quadrature detection) data sets averaged over ~200 transients. We found that even with careful control of experimental variables that the reproducibility of the integrated signal intensities was not high. Setting the value for the integral of a fluorine resonance when no perturbation of the proton spins had taken place to 1.00, we typically found that the reproducibility of subsequent integrals was ±0.05. A waiting time of 3 s (about 10 times the proton T_1 values for this system) between successive accumulations was observed.

Computations were carried out on an Itel AS-6 computer, using the development described in the Appendix.

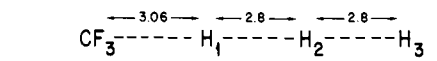

Figure 1. Model system used for calculation of the transient Overhauser effects presented in Figures 2 and 3. The correlation time for overall tumbling (τ_c) was 15 ns while internal rotation of the CF_3 group was characterized by τ_i = 0.02 ns.

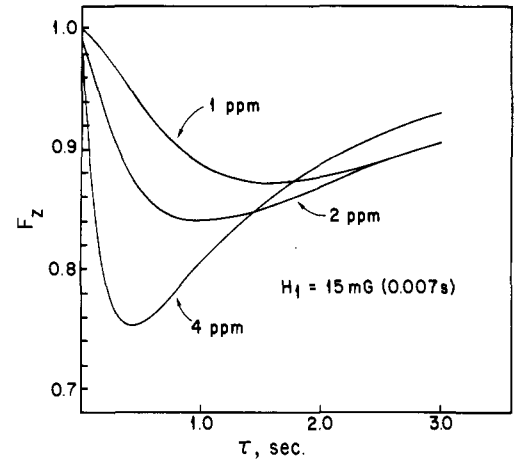

Figure 2. Calculated variations in the fluorine signal intensity for an experiment on the model system in which the proton spins are perturbed by a 15-mG pulse of duration 7.8 ms followed by an analyzing 90° pulse at the fluorine frequency. In a slowly relaxing system the proton pulse would produce a 180° inversion of magnetization when applied on resonance. For the results shown, the frequency of the ^1H radio frequency corresponded, *seriatim*, to the chemical shifts of spins 1, 2, and 3.

Results

A Model System. Previous authors have presented calculations for model systems which indicate the potential utility of the transient nuclear Overhauser effect (TNOE) in structural studies of large molecules.[8,9] These calculations have not taken into account adequately the specificity with which proton spins in a macromolecule can be selectively perturbed nor given an indication of how the TNOE data changes as the perturbing proton frequency is scanned. We therefore describe here computational results for a model system specifically parameterized to give fluorine and proton relaxation behavior in rough accord with the experimental data for (4-(trifluoromethyl)benzenesulfonyl)chymotrypsin.

This model is represented in Figure 1 and consisted of a trifluoromethyl group interacting with a linear array of proton spins. Proton 1, which was arbitrarily assigned a chemical shift of 4 ppm, was 0.31 nm from the average position of the fluorine nuclei while proton 2 (2 ppm) was located 0.28 nm from proton 1. Proton 3 with a shift of 1 ppm was set 0.28 nm from proton 2. The diagonal elements of the spin–lattice and spin–spin relaxation matrices (Appendix) were augmented so that the spin–lattice and spin–spin relaxation behavior of the spins of the model system agreed with those of the enzyme derivative. The correlation time for overall tumbling of the model system (τ_c) was set at 15 ns while internal rotation of the CF_3 group was characterized by τ_i = 0.02 ns. These

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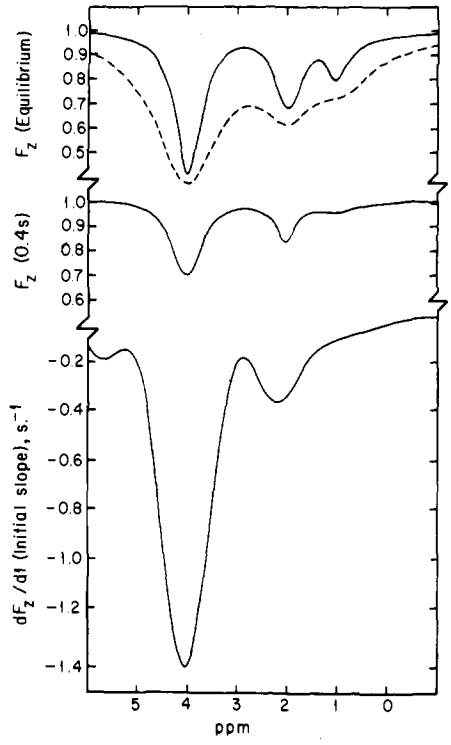

Figure 3. Overhauser effects in the model system when the ^1H frequency is scanned. In the top curve a steady radio frequency field of 1.5 mG is applied to the spin system and produces the fluorine signal intensity shown (steady-state Overhauser effect). In the second curve a continuously applied field of 4.0 mG is used. For the next curves, a pulse of radio frequency at the specified proton frequency is applied (15 mG for 7.8 ms). Curve 3 represents the fluorine intensity 0.4 s after the pulse while the bottom curve shows the initial rate of change of the fluorine intensity.

correlation times were found previously for (4-(trifluoromethyl)benzenesulfonyl)chyymotrypsin.[2,3]

If the magnetization of a proton interacting with the fluorine spins is perturbed, an initial decrease in the longitudinal component of fluorine magnetization is expected after the perturbation (Figure 2). A fluorine free induction decay excited by a 90° pulse at the fluorine frequency after the proton perturbation will thus show diminished signal intensity. As the time between the ^1H perturbation and accumulation of the fluorine fid increases, the fluorine signal intensity will pass through a minimum value and then recover back to equilibrium (Figure 2). The mathematical form of the curves in Figure 2 is complex, but one can empirically characterize and compare TNOE curves such as those shown by the value of the fluorine signal intensity at some arbitrary time, perhaps the time at the minimum signal. We see that the minimum appears sooner after the proton perturbation when the proton perturbed is physically close to the fluorine (spin 1). However, the minima are shallow and an accurate characterization in this way would appear to be difficult with noisy experimental data. More sensitive and revealing is the initial slope of the TNOE curves (Figure 2).

In Figure 3 the frequency of the proton radio frequency field is scanned across the chemical shifts of the protons of the model system. The computed initial slopes are largest when the spin adjacent to the CF_3 group is perturbed, while the distant spin 3 at 1 ppm has virtually no effect when it is excited. Plotting the fluorine intensity at 0.4 s after the ^1H pulse exhibits a smaller range of values. For comparison, Figure 3 also shows calculated $^{19}F\{^1H\}$ Overhauser effects when the spin system is irradiated continuously at a given proton frequency. The effects of spin diffusion are apparent; irradiation of spin 3 in this latter experiment produces a distinct effect on the fluorine intensity.

On the basis of these and related calculations we concluded that transient Overhauser effect data could, at least, qualitatively reveal the chemical shifts of nuclei which are close to the CF_3 group of (4-(trifluoromethyl)benzenesulfonyl)chymotrypsin, in a way not

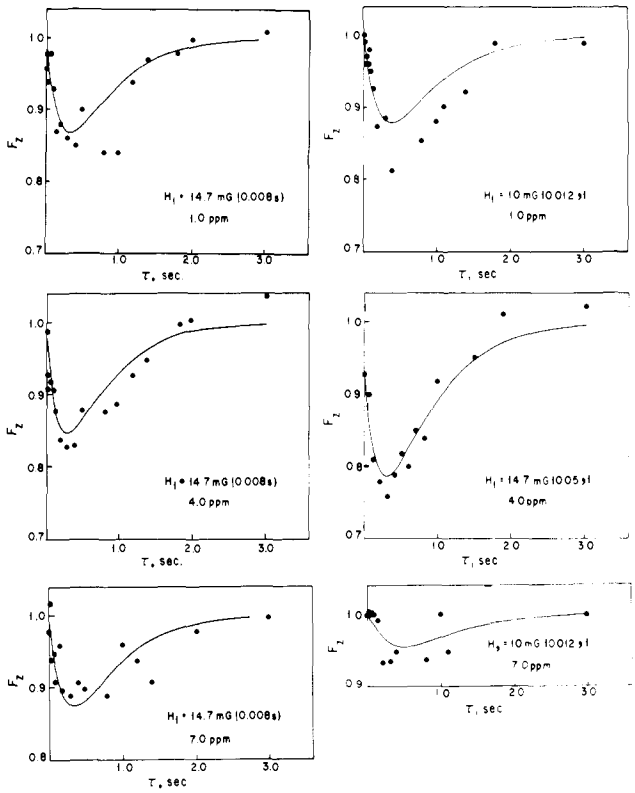

Figure 4. Transient $^{19}F\{^1H\}$ nuclear Overhauser effects in (4-(trifluoromethyyl)benzenesulfonyl-α-chymotrypsin (left column) and (3,5-dideuterio-4-(trifluoromethyl)benzenesulfonyl)-α-chymotrypsin (right column). A proton pulse was applied at the conditions indicated in each panel; the integrated fluorine signal intensity after the proton pulse is plotted in each case. The solid curves are calculated by using the model system given in Table I.

overly obscured by spin diffusion. The calculations also revealed that because of rapid proton transverse relaxation rates, it is unlikely that a selective full 180° inversion of a proton magnetization can be achieved in α-chymotrypsin at our magnetic field strength.

Modified Enzyme TNOEs. Selection of the magnitude of the field H_1 used to perturb proton magnetization in proteins in a selective manner must be made cautiously.[13] If H_1 is too large, specificity is lost, but if H_1 is too small, the time required for an appreciable perturbation becomes long relative to the T_1 and T_2 of the spin being irradiated. Calculations of the type described above suggested that 5 mG $\leq H_1 \leq$ 15 mG would be optimum for α-chymotrypsin. These conditions represent a compromise between the two extremes mentioned and, given the experimental values for the proton, relaxation times of the protein will not result in complete inversion of a given proton magnetization.

Transient Overhauser effects in the fluorine nuclei of (4-(trifluoromethyl)benzenesulfonyl)chymotrypsin following a radio frequency pulse at a particular proton frequency were determined at several frequencies and magnitudes of the radio frequency field. Some typical results are shown in Figure 4. The data are scattered, but it is unmistakeable that perturbation of proton spins which resonate at ~1, ~4, and ~7 ppm leads to appreciable decreases in fluorine signal intensity, followed by recovery of intensity, as predicted by the calculations described above. When the two protons ortho to the trifluoromethyl of the reporter group are replaced by deuterium, the magnitude of the effect observed in the ~7 ppm region is reduced, lending support to our earlier conclusion regarding the role of these nuclei in relaxing the fluorine spins.[2]

Given the large amount of time involved in constructing one of the plots shown in Figure 4, we did not obtain complete TNOE curves over the entire proton spectral region. Rather scans were

(13) Noggle, J. H. *J. Am. Chem. Soc.* **1980**, *102*, 2230–2232.

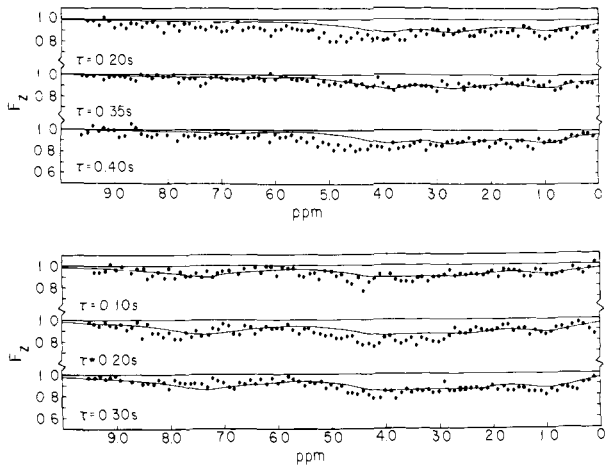

Figure 5. Transient NOE scans for (4-(trifluoromethyl)benzenesulfonyl)-α-chymotrypsin (bottom panel) and 3,5-dideuterio-4-(trifluoromethyl)benzenesulfonyl)-α-chymotrypsin (top panel). Each point represents an experiment in which the fluorine magnetization was sampled at a time τ after a proton pulse (14.7 mG of 0.8-ms duration) at the frequency indicated along the chemical shift axis. The solid lines are calculated by using the model system of Table I.

Table I. Properties of Model System

x	y	z	chem shift, ppm	$\bar{r}_F$,[a] Å	R_1^{ex} [b]	R_2^{ex} [c]	assignt
			Fluorine				
−0.623	1.079	0					
−0.623	−1.079	0					
1.246	0	0					
			Hydrogen				
0	2.4	−2.44	7.55	3.6	1.3	200	ortho H
0	2.4	−4.92	6.85	5.6	2.0	250	meta H
0	5.6	−2.44	3.60	6.2	2.5	170	CH
0	6.2	−0.1	4.00	6.3	2.5	170	CH_2
0	4.7	1.9	0.85	5.2	2.5	170	CH_3
0	3.1	3.9	2.10	5.1	2.5	170	CH
2.9	0.7	2.65	1.00	4.1	1.3	200	CH_3
−1.4	0.7	3.10	2.80	3.7	1.3	200	CH_2
0	−1.4	3.00	4.10	3.5	1.3	200	CH
0	−3.0	5.55	7.00	6.4	6.4	250	tyrosine
0	−3.0	8.03	7.20	8.6	2.0	250	tyrosine
−2.5	5.6	−2.44	8.50	6.7	2.0	250	N-H

[a] Average distance from the fluorine nuclei. [b] Spin-lattice relaxation contribution from intragroup nuclear interactions, as discussed in ref 3. [c] Spin-spin relaxation rate for the proton spin.

made in which the intensity of the fluorine signal at some particular time after the proton pulse was recorded as the proton frequency was scanned. Some typical results appear in Figure 5. Such scans of the enzyme system showed no fine structure although the intensity changes caused by irradiation in the aromatic proton part of the spectrum is less in the dideuterated material. Since the "line width" calculated for the TNOE scans of the model system is fairly narrow, the lack of sharply defined peaks in the enzyme data must mean either that there are present several peaks (minima) that overlap in such a way that fine structure is not obtained or that the transverse relaxation rates (R_2) for the protons that are being perturbed are substantially larger than those of the bulk proteins, or both.

Simulation of Enzyme TNOE Data. We have previously described a crude structural model of the immediate environment of the CF_3 group in the trifluoromethylated chymotrypsin which accounted semiquantitatively for the observed fluorine spin–lattice and spin–spin relaxation rates at several frequencies and the steady-state $^{19}F\{^1H\}$ NOE data available. With use of the method described in the Appendix, this model system was also used to simulate TNOE data of the type given in Figures 4 and 5. A few small adjustments in the Cartesian coordinates and other parameters of the model were made to improve somewhat the agreement between calculated and observed TNOE data; it was verified that the adjusted model still accounted for the relaxation and equilibrium NOE data as well as the original model. The solid line in Figures 4 and 5 are those calculated for the experiments reported in these figures. The agreement is within experimental uncertainty in most cases. Table I gives the properties of the revised model.

Discussion

Calculations using the model system shown in Figure 1 suggest that transient $^{19}F\{^1H\}$ nuclear Overhauser effects should be an effective means of minimizing the effects of spin diffusion when fluorine–proton multiple resonance experiments are used to characterize those protons near a CF_3 reporter group in a protein. Transient $^{19}F\{^1H\}$ NOEs are observed with (4-(trifluoromethyl)benzenesulfonyl)-α-chymotrypsin and, as comparisons of the data obtained for systems where protons or deuterons are present at the positions ortho to the CF_3 group of this enzyme indicate, can reveal the chemical shift of those hydrogen nuclei which interact strongly enough with the CF_3 group to cause relaxation. At the magnetic field strength used for the present work little fine structure was noted when the perturbing proton frequency was scanned through the range of proton chemical shifts. However, calculations show that all of the effects observed are consistent with the crude structural model of the local environment of the CF_3 group that was proposed earlier. Thus, groups of protons with chemical shifts near 1.0, 2.8, and 4.0 ppm relative to Me_4Si appear to be in close proximity to the fluorine nuclei.

One feature of the model that is particularly reenforced by the present experiments is the approximate value for the transverse relaxation rates of the protons near the CF_3 reporter. These relaxation rates appear to be approximately 200 s^{-1}, nearly three times larger than the proton R_2 values observed for the bulk protein. R_2 values of this magnitude permit the qualitative conclusion that nuclear motion is somewhat slower in this part of the protein (presumably the active site region) than is motion in the remainder of the structure, where $R_2 \approx 70$ s^{-1}.

A vexing aspect of these TNOE experiments is the inability to selectively invert the longitudinal magnetization for a particular spin of the protein. At the field strength used for these experiments such selectivity is difficult to obtain, especially in light of the apparently large resonance line widths observed for those protons near the CF_3 function. It will thus be of interest to carry out these experiments at higher field strengths where the proton chemical shift dispersion would be greater.

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM-25975) and the Cancer Research Coordinating Committee of the University of California. We thank these sponsors and also Professors W. E. Palke, S. Simons, and D. E. Mellichamp of the Departments of Chemistry, Mathematics, and Chemical Engineering, respectively, on this campus for their useful comments. Mr. M. Ando prepared many of the samples used.

Appendix

The transient $^{19}F\{^1H\}$ Overhauser effect experiment can be divided into two distinct phases. In the first, irradiation at a proton frequency is carried out with the goal of selectively perturbing the magnetization of a particular spin. During the second part of the experiment, the perturbed proton, all other protons and the fluorine nuclei recover their equilibrium magnetizations by spin–lattice relaxation. To simulate the recovery curves for the fluorine magnetization observed in the second phase requires calculations both of the effects of the proton perturbation and the recovery of the entire assembly of spins.

Since scalar coupling between protons and fluorine nuclei can be neglected in the CF_3-labeled protein, a generalized form of the Bloch equations was used to simulate the two phases of the experiment. For an n-spin system, in a reference frame rotating at the frequency ω

$$d\begin{vmatrix}M_x\\M_y\\M_z\end{vmatrix} = \begin{vmatrix}-R_2 & \Delta & 0\\ -\Delta & -R_2 & H\\ 0 & -H & -R_1\end{vmatrix}\begin{vmatrix}M_x\\M_y\\M_z\end{vmatrix} + \begin{vmatrix}0 & 0 & 0\\ 0 & 0 & 0\\ 0 & 0 & R_1\end{vmatrix}\begin{vmatrix}0\\0\\M_0\end{vmatrix} \quad (A-1)$$

where $\mathbf{M}_x$, $\mathbf{M}_y$, and $\mathbf{M}_z$ are each column vectors of length n representing the value for the two transverse and the longitudinal magnetizations of each spin, respectively. M_0 represents the equilibrium longitudinal component of each spin. The matrix Δ contains the offset parameters $(\omega_n - \omega)$ for each spin along its diagonal, where ω_n is the resonance frequency of spin n. $\mathbf{H}$ is a diagonal matrix of "driving terms" with the values $\gamma_n H_1$ along the diagonal, with γ_n being the gyromagnetic ratio of spin n and H_1 the magnitude of the perturbing radio frequency field. The spin-lattice relaxation matrix $\mathbf{R}_1$ is given by eq A-2. For the

$$\begin{vmatrix}\rho_1 & \sigma_{12} & \sigma_{13} & \sigma_{14} & \cdots & \sigma_{1n}\\ \sigma_{12} & \rho_2 & \sigma_{23} & \sigma_{24} & \cdots & \sigma_{2n}\\ \cdot & \cdot & \cdot & \cdot & & \cdot\\ \cdot & \cdot & \cdot & \cdot & & \cdot\\ \cdot & \cdot & \cdot & \cdot & & \cdot\end{vmatrix} \quad (A-2)$$

present work the F-F and H-F dipolar contributions to the ρ_i and σ_{ij}, as well as the chemical shift anisotropy contributions to the ρ for the fluorine nuclei, were computed as described in ref 2 and 3. The dipolar terms depend on the various internuclear distances and thus are a function of the Cartesian coordinates for each of the n nuclei. The matrix for spin-spin relaxation (R_2) was set up in a similar way, using the equations given by Solomon.[11] Solution of eq A-1 is straightforward[14] and in our implementation used the EISPAC routines.[15]

During the recovery phase of the experiment the driving field is absent, and the calculated behavior utilized only the equation along the bottom line of eq A-1. The initial values for each variable during the recovery phase were calculated by solution of the full equations parameterized according to our experimental conditions.

It will be recognized that the above description of relaxation and excitation in a complex spin system is extremely crude. Groups of spins with identical chemical shifts such as the three protons of a methyl group are treated as single, composite spins, spin coupling between proton spins is neglected, and all cross-correlation effects are neglected. More realistic theoretical approaches to describing intramolecular dipolar relaxation in multispin systems are available[16] but, given the current lack of *detailed* knowledge of protein dynamics in solution, we decided not to persue these more complicated descriptions at this time.

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Reduction of Flavins by Thiols. 1. Reaction Mechanism from the Kinetics of the Attack and Breakdown Steps[1]

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Abstract: In the reduction of 3-methylriboflavin (**3**) by dithiothreitol (DTT) and dithioerythritol (DTE) at constant pH, a change in rate-determining step was observed with increasing [buffer] from one which involves buffer catalysis to another which does not. The buffer catalyzed step followed the rate law $(k_{ac1}[HSS^-] + k_{ac2}[SS^{2-}])[HA][Fl]$, while the associated solvent terms obeyed $(k_{a0}[HSSH] + k_{a1}[HSS^-] + k_{a2}[SS^{2-}])[Fl]$. The Brønsted plot for k_{ac1} gave $\alpha = 0.58$, and all general acids including hydronium ion and water lay on a single straight line. The step which is not buffer catalyzed obeyed $k_{b1}[HSS^-][Fl]$. The corresponding reduction of **3** by mercaptoethanol (ME) obeyed $(k_{m0}[RS^-] + k_{m1}[RSH] + k_{mm}[RS^-][RSH])[Fl]$ and showed no buffer catalysis. The first-order terms were small compared to the second-order term, and their cause is unknown. The second-order term in the monothiol reaction and the results from the dithiol reaction establish that the redox reaction between flavin and thiols proceeds entirely or very largely by way of a covalent adduct which then breaks down to product disulfide and reduced flavin. It is argued that buffer catalysis must occur as general-acid catalysis at N(5) in a step other than breakdown. Mechanisms thus ruled out include hydride transfer, general-base-catalyzed thiol deprotonation in attack, general-acid catalysis at N(1), and attack by thiolate at C(1a), C(2), C(4), N(5), C(6), and C(8). Reduced **3** is oxidized by bis(2,2′-dithio-4,4′-dinitrobenzoic acid) (DTNB) and obeyed the rate law $(k_{r0}[FlH_2] + k_{r1}[FlH^-])[DTNB]$. The rate constant for reaction of hydrazine with DTNB was more than 10^4 times smaller than k_{r1} in spite of the greater basicity of hydrazine relative to N(1) of reduced flavin. Mechanisms involving adduct formation at N(1) are thus ruled out. Calculations show that mechanisms involving thiol and flavin radicals are too slow to account for the rate of the overall reaction. A mechanism fully consistent with the data is attack of thiolate at C(4a) with general-acid catalysis at N(5) followed by breakdown of the C(4a) adduct by displacement of reduced flavin anion upon attack of thiolate on sulfur.

Introduction

In this and the following two papers,[2,3] we consider the mechanism of reduction of flavins by thiols. The reaction is a model for the action of flavin-containing enzymes such as glutathione reductase[4] and lipoamide dehydrogenase,[5] which catalyze a redox exchange between thiols or dithiols and pyridine nucleotides.

Gascoigne and Radda[6] first studied the thiol-flavin reaction by using several flavins with lipoic acid and found the reaction to show buffer catalysis and to be first order in dithiol and flavin. Gibian and co-workers[7] later showed that the reaction of flavins

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(3) Part III: Loechler, E. L.; Hollocher, T. C., accompanying paper in this issue.
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