Examination of the Structure and Dynamics of Tosylchymotrypsin at pH 4 by Tritium NMR Spectroscopy

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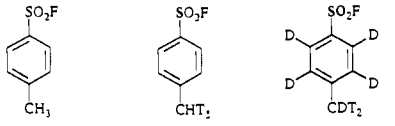
Abstract: Tritium NMR spectroscopy of specifically tritiated and tritiated/deuterated derivatives of tosylchymotrypsin has been used to examine the properties of the tosyl group in this protein. The presence of several tritiated isotopomers complicates analysis of experiments and extensive computer simulations of the composite relaxation behavior of the collection of tritiated species present were used in conjunction with models developed from crystallographic results to interpret the observations made. These analyses suggest that the tosyl group of tosylchymotrypsin at pH 4 is highly mobile in solution and, on average, only occupies the location in the protein that is observed in the solid state about 50% of the time.

An essential requirement for understanding protein structure and function is knowledge of three-dimensional structure. Historically, crystallographic methods have been used toward this end, but recently, these have been supplemented by NMR studies of proteins at high magnetic fields. In order for the methods used to define protein structures by NMR to be successful it is necessary to be able to resolve and assign virtually all of the proton resonances from the material of interest. However, this requirement becomes increasingly difficult to meet as the size of a protein increases since, with increasing molecular size, the number of proton signals that must be resolved increases at the same time spectral lines become broader due to a decreased rate of molecular tumbling. Introduction of three- and four-dimensional techniques, usually concomitantly with specific carbon-13 and nitrogen-15 isotope enrichment, provides powerful means to mitigate these considerations,[1] but even with these technologies in full force, it is probable that structure elucidation by NMR approaches will falter with molecular weights in excess of 50 kDal.[2]

Some of the unique structural and dynamical insights that NMR can provide about proteins can be obtained in systems where conditions are not favorable to multidimensional, multinuclear approaches by the introduction of extrinsic probes or "reporter" nuclei. Some desirable features of a good reporter nucleus include a spin number of $^1/_2$ so that lines are not broadened by quadrupolar relaxation, high sensitivity to detection, absence of background signals (either from the instrumentation used or spins intrinsic to the protein), ease of incorporation by synthesis into the system of interest, and absence of interactions between the reporter function and the protein which may perturb the structure or dynamics that are to be explored. The hydrogen isotope tritium has many of these properties: (1) tritium has the highest sensitivity to detection of any nucleus, (2) the natural abundance of tritium is virtually zero, (3) many decades of effort have provided a trove of synthetic methods for introduction of tritium selectively into structures, and (4) since tritium is after all hydrogen, there should be no structural consequences of its introduction into protein structures.[3] Balancing these desirable features of tritium as a reporter nucleus are some clear disadvantages, including the experimental inconvenience of dealing with the radioactivity (β, mean energy 0.0056 MeV) associated with tritium enrichment and the possibility of structural damage by radiolytic reactions. However, tritium NMR of specifically labeled materials has proven to be useful in addressing questions of mechanism and stereochemistry in small molecules[4] and is increasingly being applied to biochemical studies.[5]

The serine protease α-chymotrypsin reacts stoichiometrically with *p*-toluenesulfonyl fluoride (**Ia**) to give an inactive protein with a tosyl group attached to the critical Ser-195 residue of the active site. The native enzyme and its tosyl derivative have been



Ia Ib Ic

extensively studied by X-ray crystallography and it has been shown that in the solid state there is a cavity on the enzyme surface, often called the "tosyl pocket", into which the tosyl group fits.[6] The pocket is defined on one side by residues Cys-191 and Asp-194 and on the other by the stretch of residues between Val-213 and Ser-217. The tosyl group fits the pocket snugly and, if this structure is strictly maintained in solution, it would be anticipated that the tosyl group would be held to the protein surface with the aromatic ring unable to undergo rotation. In the present work the methyl protons of the tosyl group were replaced by tritons and various tritium NMR experiments with the isotopically

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labeled protein were carried out. Analysis of the results provides information about the dynamics of the tosyl group at the active site of the enzyme and indicates the degree to which the structure of this enzyme derivative in solution is similar to the structure observed in the crystalline state.

Experimental Section

Materials. Tritium gas was purchased from Oak Ridge National Laboratory and contained 97.9% T_2 with the major contaminant being 1.76% DT. Toluene-d_8 (99+ atom % D), deuterium oxide (99.9% atom % D), chlorine gas, *p*-toluenesulfonyl fluoride, and 10% Pd on carbon catalyst were purchased from Aldrich Chemical Co. α-Chymotrypsin was obtained as the 3X recrystallized, lyophylized product from Sigma Chemical Co. and was used without further purification.

Synthesis of methyl tritium- and deuterium/tritium-labeled tosyl fluoride used catalytic hydrogenolysis of the corresponding dichloromethylbenzenesulfonyl fluorides as indicated below.

4-Dichloromethylbenzenesulfonyl fluoride (Ib) was prepared by treating a solution of 4.0 g (230 mmol) of *p*-toluenesulfonyl fluoride and 0.04 g of benzoyl peroxide in 230 mL of CCl_4, contained in a 3-neck round-bottom flask, with Cl_2 gas at room temperature. The reaction was irradiated with a commercial sun lamp (200 W) 15 cm from the reaction flask for 3 h. The reaction was monitored by analyzing aliquots of the mixture with capillary VPC. It was observed that shorter reaction times lead to an increased proportion of monochlorinated product while longer times led to only trace amounts of trichlorinated products and predominant amounts of an insoluble side product which was not fully characterized. The desired product was purified by silica gel chromatography for an isolated yield of 23%, mp 77–80 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.07, 8.04, 7.84 and 7.82 (AA'BB', 4H, ring), 6.74 (s, 1H, $CHCl_2$); IR (KBr) ν_{max} (SO_2F) 1410 and 1211 cm^{-1}, C'–Cl 787 cm^{-1}; MS (*m/z*, relative intensity) 207, (M + –H^{37}Cl, 100.0), 209 (M + –H^{35}Cl, 37.1).

Catalytic tritiations of 4-(dichloromethyl)benzenesulfonyl fluoride to prepare tritium-labeled tosyl fluoride (**Ib**) were carried out inside a glovebox using a custom-built microhydrogenation apparatus. The reaction vessel was charged with a solution of 7.75 mg (32 μmol) of 4-(dichloromethyl)benzenesulfonyl fluoride and 10 μL of triethylamine in 3 mL of CH_3CN. The catalyst spoon of the apparatus was loaded with 7.8 mg of 10% Pd on carbon and the entire apparatus exhaustively degassed by the application of several freeze–pump–thaw cycles. Tritium gas was admitted via stainless steel vacuum lines to a pressure of 550 mmHg over the frozen solution and the solution was then allowed to thaw. The pressure in the vessel was adjusted to 760 mmHg and the catalyst added to the solution. The mixture was stirred at room temperature. The reaction was monitored by the uptake of T_2 gas and also by removing small aliquots of the reaction mixture with a syringe, followed by analysis using radio-TLC on precoated silica gel plates using hexane/ether (5:1) as the eluent. After 1 h the reaction mixture was frozen with liquid nitrogen, the system was flushed with nitrogen gas, and the solution was thawed. The catalyst was removed by filtration and the solvent stripped by evaporation with a stream of dry nitrogen gas. (Gentle conditions were used for solvent removal because of the high vapor pressure of the product and the attendant potential for product loss by sublimation). The residue was taken up in 1.7 mL of CD_3CN for analysis by NMR. Subsequent liquid scintillation counting showed that the product contained from 1.0 to 1.5 Ci. The scintillation results could be somewhat misleading since there could be some tritiated impurities left in the product from reduction of the solvent. The yield of product was not determined but was at least sufficient to give rapid and complete inactivation of the enzyme in the procedure described below.

4-Toluenesulfonyl chloride-d_7 was prepared from toluene-d_8 using a procedure based on published patents designed to optimize the yield of the para isomer.[7] To 1 mL (15 mmol) of chlorosulfonic acid in a 25-mL 3-neck round-bottom flask fitted with a reflux condenser, syringe adapter, and stopper was added 0.8 g (15 mmol) of ammonium chloride. The mixture was heated to 80 °C and 1 mL (8.6 mmol) of toluene-d_8 added over 0.5 h. The reaction was then cooled to 35 °C and another 1 mL of chlorosulfonic acid was added; the reaction mixture was stirred for an additional half hour. The mixture was poured onto 30 mL of ice and extracted with 3 × 10 mL of CH_2Cl_2. The organic layer was washed with 10% $NaHCO_3$ and water and then dried over $MgSO_4$ and filtered. The solvent was removed by rotary evaporation, yielding white crystals (54%). Capillary GC showed that the isolated material contained 92% of the para isomer and 8% of the ortho form and this material was used in the next step without any purification.

Conversion of the sulfonyl chloride to the sulfonyl fluoride was carried out as described by Sigler et al.[8] and preparation of the deuterated/tritiated inactivator **Ic** followed the same procedure as described for **Ib**.

Methods

General Procedures. Thin-layer chromatography was run using precoated silica gel plates and an elution system of 5:1 hexane–ether. The radio-TLC run used precoated silica gel plates which were analyzed using a Varian Aerograph Radio Scanner and Hewlett-Packard 3390A integrator. Liquid scintillation counting was done with a Packard 1500 LSC system using Packard Opti-Flor cocktail. Gas chromatography was run on a Hewlett-Packard 5890 with a 3392A integrator. An Ultra 11 column (5% phenylmethylsilane 25 M × 0.200 mm) was used isothermally at 150 °C with He carrier gas. Infrared spectra were obtained with a Bio-Rad/Diglab FTS-60 with the samples contained in KBr pellets. Carbon and proton NMR spectra were run on a General Electric GN500 with $CDCl_3$ as solvent and are referenced to tetramethylsilane.

The isotopic composition of the tritiation products had some variability from one reaction to the next and was determined by a combination of proton and tritium NMR spectroscopy at 300 and 320 Mhz, respectively, using an IBM/Bruker AF-300 spectrometer equipped with a ^{3}H/^{1}H dual probe. Spectra for determination of the relative amounts of each of the isotopomers were collected with delays between acquisitions of at least ten times the longest T_1 of the sample in order to avoid saturation effects.

For tritium-labeled tosyl fluoride the isotopic composition ratios as determined by NMR had an estimated error of about 20%, the large uncertainty arising primarily because the monotritiated and tritritiated products were present in low amounts. The synthesis of deuterated/tritiated tosyl fluoride was cleaner in that only two products resulted and the reproducibility was somewhat better. The estimated error for the ratio of the products is about 7% in this case.

Inhibition of Enzyme. One hundred twenty three milligrams (4.9 μmol) of α-chymotrypsin was dissolved in 15 mL of 0.05 M phosphate buffer adjusted to pH 4.0 ± 0.1. Tritiated inhibitor (ca. 30 μmol) prepared as described above was taken up in 1.5 mL CH_3CN and added to the enzyme solution. The resulting mixture was incubated at room temperature for several hours; loss of enzyme activity was monitored by hydrolysis of the substrate *N*-glutarylphenylalanine *p*-nitroanilide.[9] The inactivation reaction was halted when the activity had been reduced to less than 1% of its initial value.

Purification of Tritiated Tosylchymotrypsin. The solution of inactivated enzyme was loaded into an Amicon Centriprep-10 concentrator cell (10 kDal cutoff) for removal of excess inactivator and for solvent exchange with deuterated buffer. The solution was centrifuged through the filter to a volume of 3 mL and then diluted to 6 mL with 0.05 M KCl in D_2O and recentrifuged to 3 mL. This cycle was repeated 9 times with the final centrifugation going to a volume of 1.5 mL. The final sample had a calculated solvent deuterium level of greater than 99.8% and contained no free inhibitor detectable by NMR. Sample pH was adjusted to 4.0 ± 0.1 with microliter amounts of 1.0 M HCl in D_2O. The final protein concentration was 1.1 mM as determined spectrophotometrically at 282 nm using a molar extinction coefficient for the protein of 5 × 10^4 M^{-1} cm^{-1}.[10] The final sample radioactivities as determined by liquid scintillation counting were in the range of 60 to 80 mCi, with the average specific activity for the samples being 45 Ci/mmol.

Tritium NMR Spectroscopy. Enzyme samples (1.5 mL) were placed in Teflon tubes (Wilmad No. 6010) which were then placed inside a standard 10 mm glass NMR tube. Tritium NMR spectroscopy was carried out on the IBM/Bruker system mentioned earlier. All spectra were obtained with the sample spinning and with the sample temperature regulated at 297 K by the instrument controller. Data were collected on magnetic tape for transfer to a VAX computer and workup using the FTNMR program (Hare Research, Woodinville, WA).

Spin–lattice relaxation times (T_1) were determined by the inversion recovery method with a composite 180° pulse and were fit to a three-parameter function using a routine in the spectrometer software. The estimated error for the T_1 values obtained is ±10%. Transverse relaxation

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times (T_2) were estimated by fitting the observed ^3H line shapes of the inactivated enzyme with a composite of Lorentzian lines. The line widths so determined were converted to relaxation times by the relation $w_{1/2} = 1/\pi T_2$ where $w_{1/2}$ is the observed line width at half maximum intensity. Line widths were corrected for magnetic field inhomogeneity by subtracting the observed line width for the HOD signal in the proton spectrum and are believed to be reliable to ±20%. Equilibrium (steady state) $^3H\{^1H\}$ nuclear Overhauser effects (NOEs) were determined by comparison of the integrations of the spectra obtained with and without proton irradiation during the preacquisition period. A delay of at least 10X the tritium T_1 was observed between each accumulation.

Transient $^3H\{^1H\}$ NOEs were determined by application of a composite 180° proton pulse followed by a variable delay and a 90° tritium read pulse.[11] A spectrum taken with a zero delay between the two pulses was used to define the tritium signal intensity in the absence of any proton perturbation. The recycle delays used in both NOE experiments was at least 5 times the longest T_1 value for the sample.

Two-dimensional $^3H\{^1H\}$ heteronuclear NOE spectra[12] were obtained using the TPPI method to provide quadrature detection in the t_1 dimension.[13] Typically 512 transients were collected for each of 64 t_1 values with the delay between collections being greater than 5 times T_1 such that given the observations in the transient NOE experiments, the tritium magnetization would be fully recovered before the start of the next scan. About 60 h were required for each 2D NOE experiment. The 2D data sets spanned 3000 Hz (10 ppm) in the ^1H dimension, represented by 64 points initially. In processing, the ^1H dimension was zero filled to 1024 points. Assuming that only the first zero fill to 128 points gives an actual resolution enhancement,[14] the digital resolution of the spectrum is about 0.08 ppm/pt, but the situation is probably somewhat better. Proton chemical shifts were found using the peak picking facility in the FTNMR program; the chemical shifts reported are given to three significant figures, although only the first two are given with confidence. The ^3H dimension in the NOESY spectra was referenced to the chemical shift of the methyl signal of the free inactivator at 2.44 ppm (protonated) or 2.43 ppm (deuterated).

Results

Preparation of Tritium-Labeled Tosyl Fluoride. Although the starting dichlorides used as the starting material in the hydrogenolysis reactions were reasonably pure, the products of these reactions were mixtures containing species with a variable number of tritium atoms. Reduced efficiency of catalytic reduction with tritium gas is due to a number of factors and a single catalytic reduction step is only about 80% selective (H. Morimoto, private communication). In the present work two specific sites were targeted for tritium labeling by hydrogenolysis but a larger number of isotopomers including CT_3 were produced. That the isotopomer distribution is quite different for reduction of the $CHCl_2$ group compared to the $CDCl_2$ group in **Ib** and **Ic** indicates that the kinetics of the various processes operating during the hydrogenolysis are an important aspect in determining the product distribution observed.

Tritium NMR provides a convenient way to define the distribution of isotopic species formed in these reactions. Figure 1 shows spectra of **Ib**. An isotope shift of 0.025 ppm (8 Hz at 320 MHz) accompanies the introduction of each tritium into the methyl group and these effects in conjunction with the 16 Hz proton–tritium geminal coupling constant produce a proton-coupled spectrum that is misleadingly simple.

Consideration of the proton and tritium spectra of the sample of tritiated tosyl fluoride I used to modify the enzyme showed that the sample consisted of species containing 5%, 43%, 46%, and 7% of the CT_3, CHT_2, CH_2T, and CH_3 species, respectively. There was no detectable incorporation of tritium into the aromatic ring of the inactivator.

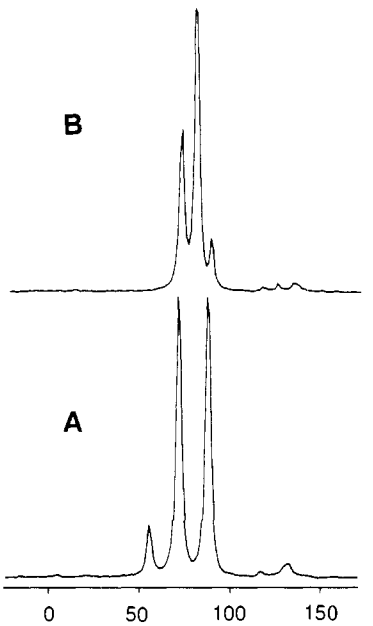

Figure 1. Tritium NMR spectra at 320 MHz of a preparation of **Ib**: (A) fully coupled, (B) proton decoupled. A tritiated impurity is evident at high field and possibly arises from catalytic exchange with triethylamine present during the tritiation reaction.

For the deuterated/tritiated tosyl fluoride **Ic** only two methyl-tritiated isotopomers were detected, with 54% of the tritiated molecules being the CD_2T species and 46% containing the CDHT methyl group. The presence of deuterium on the methyl group produces an upfield isotope shift of 0.0156 ppm per deuteron.[15] Proton, deuterium, and tritium spectra of the sample used for enzyme modification showed no detectable aromatic ring tritiation or any other species present other than the two components indicated.

Tritium Spectra of Tosylchymotrypsin. Tritium spectra of enzymes modified with the tritiated tosyl fluorides **Ib** and **Ic** exhibit broad lines due to the long correlation time of the protein and it was not possible to resolve signals for the various isotopic components. The line width for each species is of utility in helping to define the dynamics of the tosyl group, and to obtain an estimate of these we fit the observed line shape to a sum of Lorentzian functions. In this process it was assumed that the isotopic composition of the tosyl groups attached to the enzyme was the same as that observed for the corresponding tosyl fluoride and that the chemical shifts, including isotope shifts, and coupling constants that were obtained from analysis of the spectra of the inhibitor molecule are transferrable to the enzyme derivatives. In making the analyses it was assumed that the ^1H spectrum observed for the residue HOD in the solvent provided a reliable indicator of field inhomogeneity and the magnitude of spinning artifacts, both of which were taken into account.

For the enzyme modified with the protonated/tritiated I the tritium line shape was well-fit by assuming that the line widths for the Lorentzian functions for the CH_2T, CHT_2, and CT_3 groups were 13, 14, and 15 Hz, respectively. The ordering of line widths observed is that expected, since tritium is more effective in dipolar relaxation that protium, but the differences are within the reliability of the analysis and are not considered significant.

In the case of the deuterated/tritiated sample (**II**). it was found that the observed line shape could be fit by assuming that the line width of the CT_2D species was 15 Hz while that of the CTDH species was 14 Hz. Both proton and deuterium coupling was considered in the analysis; coupling to the geminal deuterium was not resolved in the tosyl fluoride spectrum and the magnitude of J_{DT} was computed to be 2.5 Hz by consideration of gyromagnetic

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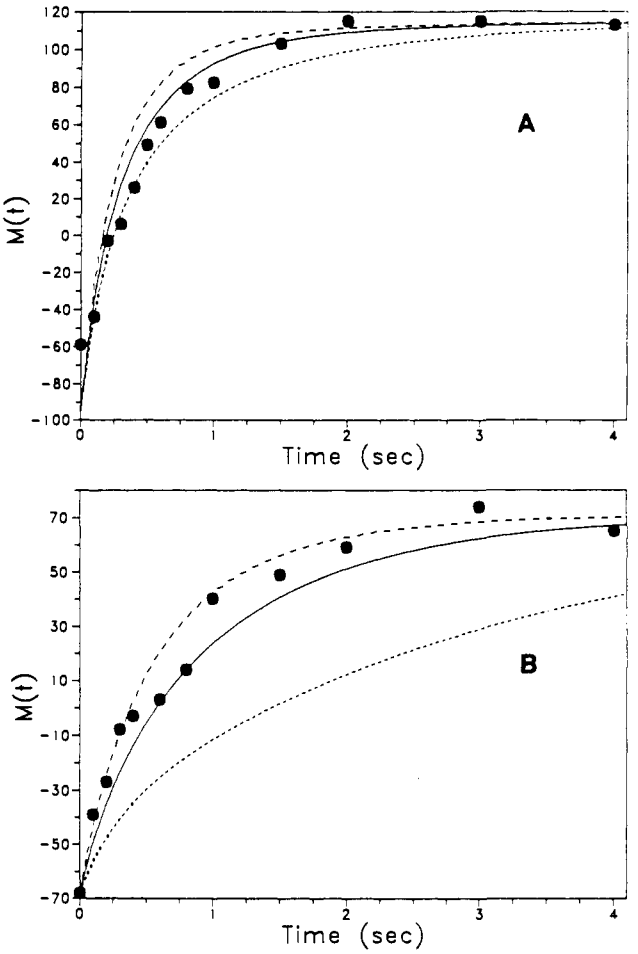

Figure 2. Representative inversion-recovery tritium T_1 experiments at 320 MHz with methyl tritium-labeled tosylchymotrypsin prepared from **Ib** (panel A) and tritium–deuterium-labeled enzyme prepared from **Ic** (panel B). The solid symbols represent experimental data while the coarsely dashed line shows computed behavior for the 100% "tosyl in" system, as described in the text. The finely dashed line is computed behavior for the 100% "tosyl out" conformation. The solid line arises from calculations based on a model in which equal amounts of the "tosyl in" and "tosyl out" structures are in rapid equilibrium. All calculations assumed $\tau_c \approx 15$ ns, $\tau_i = 0.02$ ns and used the 48 spin model described in the text. Calculations take into account the mixture of isotopomers present in each sample.

ratios of the value for J_{HT}.[16] Line shape fits were virtually identical with or without the inclusion of deuterium coupling, and it is likely that at the correlations times involved, the deuterium is effectively decoupled from the tritium.

Tritium Relaxation. The observed tritium spectrum of tosylchymotrypsin is a composite of the spectra of several species and it was not possible to follow the relaxation behavior of each component. The tritium line shapes observed at various τ values used in 180°–τ–90° inversion–recovery experiments were examined for asymmetry which would indicate differences in spin–lattice relaxation times, but no difference was detected and the spin–lattice relaxation behavior of the composite line was describable by a single exponential function (Figure 2). The apparent T_1 relaxation times obtained for enzyme modified with **Ib** and **Ic** are given in Table I. Replacement of protium in the tosyl methyl group with deuterium leads to an appreciable increase in the tritium T_1 since an important dipole–dipole relaxation interaction is reduced by this substitution.

Irradiation at the proton frequency of samples of the tritium labeled enzymes produces an $^3H\{^1H\}$ NOE which is substantial and negative (Table I), consistent with dipolar interactions with neighboring protons being an important component of tritium relaxation. The same experiment with enzyme containing the deuterated/tritiated tosyl group showed a $^3H\{^1H\}$ NOE of nearly the same magnitude and near-neighbor proton–tritium interactions must also be involved in tritium relaxation in this system as well. Since protons in the methyl group and on the aromatic ring of the tosyl moiety have been removed, the interactions must take place with protons attached to amino acids of the protein that are adjacent to the tosyl group.

Table I. Observed and Calculated Tritium Relaxation Behavior

	exp	calc[d] 100% in	calc[d] 50% in	calc[d] 100% out
Ring Protonated[a]				
T_1, s	0.53	0.36	0.50	0.61
line width, Hz	31	30	29	28
$^3H\{^1H\}$ NOE[b]	–0.79	–0.81	–0.80	–0.77
Ring Deuterated[c]				
T_1, s	0.81	0.63	0.96	2.
line width, Hz	30	25	24	23
$^3H\{^1H\}$ NOE[b]	–0.69	–0.79	–0.72	–0.44

[a] Enzyme inactivated with a sample of **1b** in which the ratio of the CH_2T, CHT_2, and CT_3 species present was 50:46:4. [b] Equilibrium NOE produced by constant irradiation of protons at high power. [c] Enzyme inactivated with a sample of **1c** in which the ratio of the CDHT and CDT_2 species present was 54:46. [d] Computed values obtained using the models and parameters described in the caption for Figure 2. The composite line widths include an additional 3 Hz broadening to account for apodization and instrumental line width effects.

The transient $^3H\{^1H\}$NOE which follows application of a 180° proton pulse to the system was also determined for each modified enzyme (Figure 3). These experiments help define the parameters appropriate for multidimensional NOE experiments and, again, gave similar results for the protonated/tritiated and deuterated/tritiated enzyme systems.

The nature of the tritium–protein proton interactions was explored further by means of two-dimensional proton–tritium Overhauser experiments. Under the conditions of our experiments only a single set of NOE crosspeaks were observed for the composite tritium signal and the information contained in a 2D NOE spectrum can be represented by a "skyline" projection plot, in which crosspeaks appear at positions along the ^1H axis corresponding to the chemical shifts of protons which contribute to tritium–proton dipolar relaxation pathways. Figure 4 presents typical skyline plots from two-dimensional NOE experiments with enzymes modified with **Ib** and **Ic**. The strong features that appear in these spectra near 2.2 and 7.0 ppm at short mixing times must arise from interactions between the tritium nuclei and protons on the methyl group and aromatic ring of the tosyl group, since these features in the 2D results are greatly attenuated when the protons on the tosyl group are replaced by deuterium (Figure 4).

Spin diffusion is expected to be rapid in proteins as large as chymotrypsin[17] and we therefore examined the buildup rates of the $^3H\{^1H\}$ NOE crosspeaks observed. Although accuracy is limited by unfavorable signal-to-noise ratios at smaller mixing times, it is clear from these experiments that other protons with shifts between 2.5 and 4.6 ppm, in addition to the protons of the tosyl group, appear in the 2D NOE spectra because of direct (nearest neighbor) interactions with the tritium nuclei of the tosyl methyl group. Using the signals from the protons of the aromatic ring as a standard, comparison of initial slopes of the buildup curves suggests that protein-bound protons with shifts of 2.9, 3.7, and 4.1 ppm are less than 0.3 nm away from the tritium nuclei.

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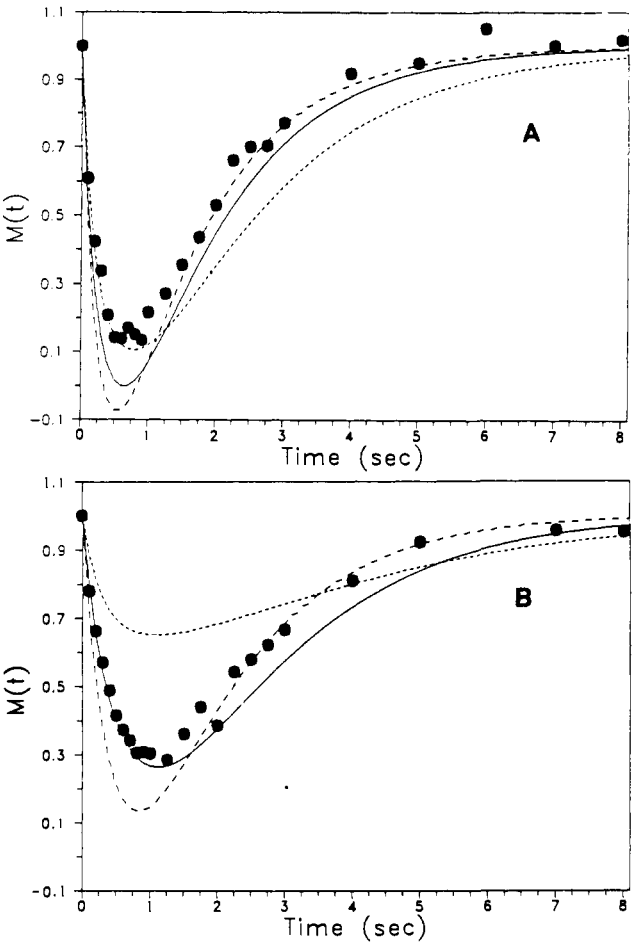

Figure 3. Representative transient $^3H\{^1H\}$ NOE experiments at 320 MHz with methyl tritium-labeled tosylchymotrypsin prepared from **Ib** (panel A) and tritium–deuterium-labeled enzyme prepared from **Ic** (panel B). The solid symbols represent experimental data while the significances of the computed lines shown are the same as described in the caption for Figure 2. For the computed curves an external T_1 relaxation contribution (ρ_{ex}) of 0.5 s was used for proton spins on the edges of the model system, as explained in the text.

A significant crosspeak was also observed in the 2D NOE experiments at 4.6 ppm. This feature likely includes the effects of interactions of the tritium spins with residual protons in the D_2O solvent or protons which are solvent-exchangeable since increasing the amount of protium in the solvent proportionately increased the intensity of this peak. However, the 2D NOE experiments were carried out with samples in which the solvent was highly deuterated and intensity in the NOE data at this chemical shift probably also represents an interaction with a carbon-bound proton of the enzyme.

Analysis of Data. Attempts to extract quantitative information from the tritium NMR spectroscopic observations made were complicated by the fact that these spectral responses are from a collection of isotopomers. To approach an analysis we used the crystal structure of tosylchymotrypsin as a starting point; hydrogens were placed on the heavy atoms whose positions are defined by the X-ray work[6] and bad contacts between these removed by empirical conformational energy minimization using the standard force field of Polygen's CHARMM/QUANTA package (Version 19). A collection of the hydrogens closest to the center of the tosyl methyl group were used to define a model system and, assuming that this collection tumbles isotropically with a correlation time τ_c while the tosyl methyl group rotates diffusively with a correlation time τ_j, the results of spin–lattice relaxation time, line width, and various $^3H\{^1H\}$ NOE determinations for the species containing one, two, and three tritiums in the tosyl methyl group were calculated. The program used is based on the Solomon equations and was largely derived from one described previously.[18] It assumes that dipole–dipole interactions are the only ones of significance for tritium relaxation. Standard formulations for the spectral density functions were used and rotation of protein methyl groups was included explicitly,[19] but cross-correlation effects were neglected in these calculations.[20] The correlation times for rotation of protein methyl groups were assumed to be the same as that of the tosyl methyl group. The computed behavior for each tritiated species was summed appropriately, according to the fraction of each species present in the mixture and the amount of tritium present in each species, to produce the computed composite behavior for the protonated/tritiated sample. In computing 2D NOE results chemical shifts corresponding to the random coil shifts of the protons of the enzyme were used.[21] The line width for these proton was taken to be 38 Hz, arbitrarily chosen to give computed 2D NOE cross sections that resembled those obtained experimentally. This choice of line width does not alter conclusions reached about relative peak intensities.

Tosylchymotrypsin is monomeric at pH 4.4 and is presumably monomeric under the conditions used in the present work;[22] the rotational correlation time τ_c for monomeric chymotrypsin is approximately 15 ns as deduced by several experimental methods.[23] It is probable that the rotation of the methyl groups is rapid enough that the calculations are insensitive to the value of τ_j; we chose to use 0.02 ns.[24,25]

Calculations were done with a model composed of the spins of the tosyl group plus the 39 non-exchangeable protons of the protein closest to the carbon of the tosyl methyl group and the spins of the three methyl groups closest to the tosyl methyl (methyls of Val–15, Met–188, and Val–109) since methyl groups may act as relaxation sinks in proteins.[19] Additional calculations were carried out with a model based on the 82 spins that are closest to the tosyl methyl carbon plus the 6 closest methyl groups. Comparison of these showed the tritium line width, spin–lattice relaxation behavior, and steady state $^3H\{^1H\}$ NOE are almost completely defined by the geometry and dynamics of spins directly adjacent to the tosyl methyl group while the time dependence of the NOE (transient $^3H\{^1H\}$ NOE and two-dimensional $^3H\{^1H\}$ NOE) are dependent on the nature of the entire proton lattice. The relaxation behavior of spins at the edges of the model is not correctly described in the model since these spins are missing critical interactions with spins which are nearby in the protein but are not included because they are beyond a cutoff distance. To account for these missing interactions an additional (external) spin–lattice relaxation contribution was added (ρ_{ex}) for the spins of the model that were on the periphery of the model system. This was regarded as an adjustable parameter that was varied until the recovery phases of the time-dependent $^3H\{^1H\}$ NOE simulations were in reasonable agreement with experimental observations.

Simulations of spectral results for the tritiated–deuterated form of the enzyme were made difficult by the incomplete deuteration at the aromatic ring positions of sulfonyl fluoride **Ic**. As a way to compensate for incomplete deuteration we assumed for our calculations a model in which both protons ortho to the tosyl methyl group are deuterium while a deuterium and a proton were assumed to be present at the meta position. In general, the

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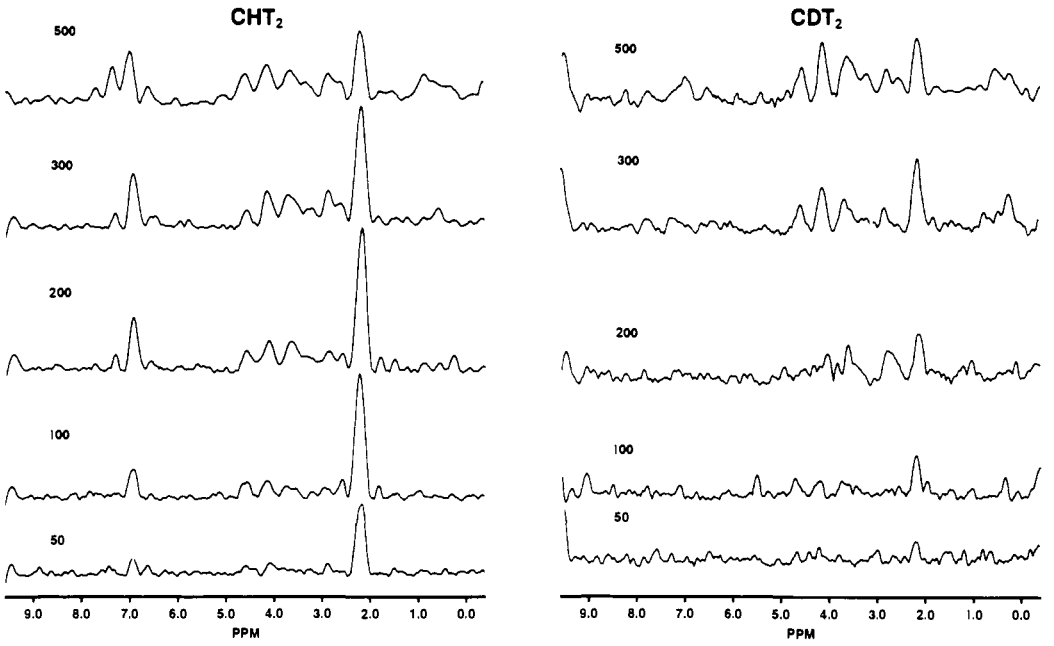

Figure 4. Skyline projections of two-dimensional $^3H\{^1H\}$ NOE experiments at 320 MHz with methyl tritium-labeled tosylchymotrypsin prepared from **Ib** and tritium–deuterium-labeled enzyme prepared from **Ic**. Mixing times (in ms) are indicated.

computed results for this system agree less well with experiment than those for the system derived from **Ib**, probably because of the crude way that incomplete ring deuteration is taken into account.

Calculations of T_1, line shape, and the steady state $^3H\{^1H\}$ NOE using the procedure outlined above gave results in good agreement with experiment (Table I), considering the approximations made and the neglect of conformational motions of the protein structure. However, as comparison of Figures 4 and 5 demonstrates, the predicted 2D NOE behavior as a function of mixing time was not in agreement with experiment. The intensities of the 2.2- and 7.0-ppm features of the 2D cross sections act as intensity/distance standards to be compared to intensities of the remaining features in the cross sections that arise from interactions of the tritium nuclei with protons of the protein. The predicted relative intensities for these are too large and we found that no reasonable adjustment of the parameters used in the calculation would improve these relative intensities while retaining the agreement between observed and calculated T_1, T_2, and steady state NOEs.

The disagreement between the experimental $^3H\{^1H\}$ 2D NOEs and those calculated on the basis of the structure of tosylchymotrypsin in the solid state must arise because the interaction distances between the protons within the tosyl binding site and the nuclei of the tosyl methyl group are not correct and the situation in solution must be such that the tosyl methyl group can move away from these contacts. A reasonable pathway for breaking these interactions would involve movement of the tosyl group out of the tosyl pocket. Computer graphics and adiabatic mapping studies of this possibility showed that a series of rotations about the single bonds along the side chain of Ser-195 and the toluenesulfonate group would provide a low-energy pathway for motion of the tosyl group away from the active site pocket and toward a more solvent-exposed position.[26] The results of a series of simulations of the NMR experiments done for a structure with $\tau_c = 15$ ns and $\tau_i = 0.02$ ns in which the tosyl group was maximally extended away from the protein are given in Table I and Figure 5. As expected, in this conformation of the tosyl group only intra-tosyl group interactions are apparent in the computed 2D NOE results and it is only at long mixing times where cross peak intensities for protein–tosyl interactions begin to appear through spin diffusion effects.

We suggest that the tosylchymotrypsin structure in which the tosyl group completely occupies the active site pocket in the manner found in the crystal structure ("tosyl in" structure) and the tosylenzyme structure in which the tosyl group has moved away from any contact with the enzyme ("tosyl out" structure) represent two extremes in a system that is conformationally mobile. Additional computer simulations in which the "tosyl in" and "tosyl out" structures are assumed to both be present, but in rapid dynamic equilibrium, were carried out. Calculations in which the amounts of these two structures were each 50% (±10%) gave predicted T_1, T_2, and $^3H\{^1H\}$ NOE behaviors that were in good agreement with experiment, including satisfactory agreement with 2D NOE cross peak intensities (Figure 5). There can, of course, be a larger number of conformations in equilibrium, and to be consistent with our experimental observations it is only required that the rate of interchange between conformations be rapid enough to average NOE-producing interactions and that the mix of conformations present permit the tosyl group to reside within the tosyl pocket about 50% of the time.

Discussion

Consideration of the crystal structure of tosylchymotrypsin indictes that the C_α protons of Gly-216 and Cys-220 are less than 0.3 nm from the methyl carbon of the tosyl group. Their random coil proton chemical shifts are 3.97 and 4.69 ppm, respectively. The amide proton of Ser-217 is also within this distance but can be assumed to be replaced by deuterium under the conditions of our experiments. At somewhat greater distances (less than 0.45 nm) are the C_α proton of Cys-191 (4.69 ppm), a C_β proton of Ser-217 (3.88 ppm), C_β protons of Cys-191 and Cys-220 (3.1 ppm), several N–H protons, and protons of two crystallographically detectable water molecules. There will be some alteration of these shifts by the native tertiary structure and possibly by the ring current of the tosyl group but the shifts of the enzyme protons which interact with the nuclei of the tosyl methyl group revealed by the 2D $^3H\{^1H\}$ NOE experiments are not inconsistent with the notion that a structure similar to the one found in the solid state is also present in solution.

The analysis of the relative cross peak intensities in the 2D NOE experiments indicates that in solution the tosyl group must

(26) We have been informed of an extensive computational study of motions of the tosyl group in tosylchymotrypsin carried out by Prof. M. E. Paulaitis, University of Delaware, that appears to be fully consistent with our more limited efforts. We thank Dr. Paulaitis for a preprint of this manuscript.

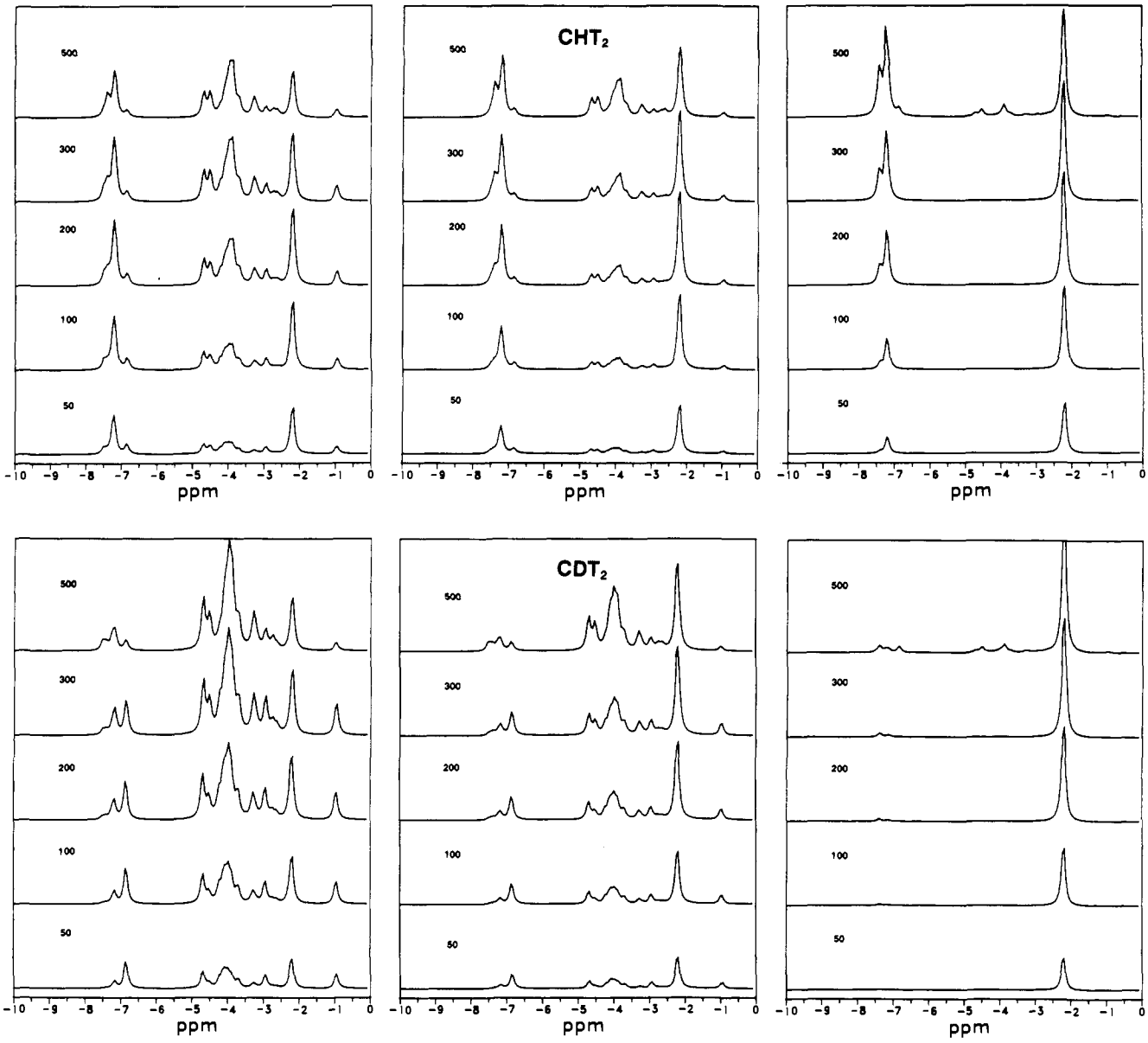

Figure 5. Computer simulations of two-dimensional $^3H\{^1H\}$ NOE experiments using the models used to prepare the theoretical curves shown in Figures 2 and 3 and described in their captions. Missing times (in ms) are indicated. The top series of calculations are for the methyl tritium-labeled tosylchymotrypsin prepared from **Ib** while the bottom series is for the tritium–deuterium-labeled system prepared from **Ic**. The left panel in each series presents computed skyline projections for proteins in which the tosyl group occupies the tosyl pocket 100% of the time, while the right panels give results for enzymes for the "tosyl out" conformation described in the text in which the tosyl group protrudes from the enzyme surface. The center panel shows the expected skyline projections when the tosyl group rapidly equilibrates between these two conformations, spending half of its time in each. Intensities as a function of mixing time for a given series are plotted on the same vertical scale but these scales vary from series to series.

break contact with the protons of the tosyl binding pocket for a significant fraction of the time. It is impossible to specify precisely the number of conformations that are available to the tosyl group that do not place the tosyl methyl group within 0.4–0.5 nm of protons of the enzyme; in our modeling efforts all of these are represented by a single structure ("tosyl out"). Thus, the quantitative aspects of the conclusion that the tosyl group is 50% in and 50% out of the tosyl pocket on average should not be taken too seriously. The qualitative conclusion that the tosyl group cannot exclusively occupy the position within the protein found in the crystal structure is well-supported by our observations, however.

The conclusion that the tosyl group experiences some solvent exposure in tosylchymotrypsin is consistent with the development of a $^3H\{^1H\}$ NOE between protons of the solvent and the nuclei of the tosyl methyl group. However, the tosyl methyl group is exposed to the protons of two (or more) water molecules within the tosyl binding pocket in addition to a number of N–H groups that presumably are solvent exchangeable, and there is ample opportunity for an NOE to develop at the water chemical shift even without the additional exposure attending the conformational change we suggest.[27]

The results and conclusions of the present study are consonant with NMR observations made with deuterium and carbon-13 labeled forms of tosylchymotrypsin. Consideration of line widths at pH 4 for the tosyl enzyme containing a CD_3 or $^{13}CH_3$ group led to the conclusion that the correlation time τ_c for the protein is 10–15 ns,[25] a result consistent with the analysis of tritium relaxation behavior discussed above. Consideration of the deuterium line widths for enzyme specifically deuterated at the aromatic ring positions indicated that the correlation time for the aromatic ring is only about 0.5 ns at pH 4, indicating that there is considerable mobility of the aromatic ring of the tosyl group beyond that provided by tumbling of the protein.[25] Rotation of

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the tosyl ring is surely slow when the ring occupies the tosyl pocket because of the close protein contacts involved and τ_c for the aromatic deuterons in this environment must be close to that for overall tumbling of the protein. By movement of the tosyl group out of this pocket, as suggested by the tritium NMR results described above, much freer rotation of the aromatic ring would be possible. A reduced local correlation time for the ring would arise by mixing of the slow motion characteristic of the "tosyl in" conformation and the rapid motions would be present in accessible "tosyl out" structures.

Side chain movement is an important aspect of protein structures[28] and conformational flexibility within enzymes is essential for the catalytic activity of these species.[29] The results of the present study join those of several others from our laboratory which indicate that some acyl groups or analogs of acyl groups attached to the Ser-195 residue of chymotrypsin may be quite mobile conformationally[30] while others become essentially immobilized when becoming bound to the enzyme.[31] A controlled variation of conformational mobility is likely essential through the catalytic cycle and it will be of interest to determine how conformational dynamics are regulated in this system.

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