A study of the relaxation parameters of a ^{13}C -enriched methylene carbon and a ^{13}C -enriched perdeuteromethylene carbon attached to chymotrypsin

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L-1-Chloro-4-phenyl-3-tosylamido[1- ^{13}C]butan-2-one (Tos-[1- ^{13}C]Phe-CH₂Cl) and Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl were prepared and used to alkylate δ -chymotrypsin. The relaxation parameters of the ^{13}C -n.m.r. signal resulting from the alkylation of histidine-57 in both enzyme-inhibitor complexes were determined at 1.88 T and 6.34 T as well as the spin-lattice relaxation times of the backbone α -carbon atoms of the unenriched Tos-Phe-CH₂- δ -chymotrypsin complex. It is concluded that the species examined do not have significant internal librational motions and that the rotational correlation time of the monomeric enzyme-inhibitor complex is 16.0 ± 3.2 ns. The signal from the ^{13}C -enriched atom of Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl is split into a quintet ($J_{\text{CD}} = 23$ Hz) whereas in the Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂- δ -chymotrypsin complex the signal from the ^{13}C -enriched inhibitor carbon atom is decoupled. This decoupled signal had linewidths of 16 ± 3 Hz and 52 ± 2 Hz at 1.88 T and 6.34 T respectively, whereas linewidths at 40 ± 2 Hz and 53 ± 4 Hz were obtained for the same signal in the Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin complex at 1.88 T and 6.34 T respectively. Therefore whereas deuteration produces a 2.5-fold reduction in linewidth at 1.88 T there is no significant decrease in the linewidth at 6.34 T. This result is explained by using the rigid rotor model, which predicts that the quadrupolar spin-lattice relaxation rate will be faster at low field strengths, resulting in more efficient deuterium decoupling by scalar relaxation of the second kind at lower field strengths. It is also predicted that deuterium decoupling by scalar relaxation will become less efficient as rotational correlation times increase. The consequences of these predictions for the detection of ^{13}C -enriched atomic probes of proteins are discussed. It is also shown that a spin-echo pulse sequence can be used to remove signals due to protonated carbon atoms without attenuating the signal due to deuterated carbon atoms.

INTRODUCTION

The specific ^{13}C enrichment of proteins and their ligands has the potential to provide a specific non-perturbing atomic probe that can be used to investigate the structure and function of proteins and their adducts. This potential has been realized for probes by using ^{13}C -enriched quaternary carbon atoms, as their signals are usually much narrower than those of the overlapping non-enriched protein carbon atoms (Malthouse, 1986). However, the full potential of using ^{13}C -enriched methine and methylene carbon atoms as atomic probes of proteins has not yet been fully achieved. This is because such ^{13}C -enriched carbon atoms will undergo efficient dipolar relaxation with directly bonded protons (Doddrell *et al.*, 1972; Oldfield *et al.*, 1975), which will result in broad signals that will increase in linewidth as the M_r increases (Malthouse, 1986). Therefore, as a ^{13}C -enriched methylene carbon atom attached to a protein with an M_r of approx. 25000 has a linewidth of approx. 50 Hz (Primrose *et al.*, 1985), it would be expected to have a linewidth in excess of 200 Hz when attached to proteins of $M_r > 100000$. Such broad signals will not be clearly resolved from those of the unenriched carbon atoms of the protein, and so difference spectroscopy will have to be used to identify the signals due to the ^{13}C -enriched carbon atoms. However, difference spectroscopy doubles the amount of protein required and it will decrease the signal-to-noise ratio. Consequently it will be difficult and sometimes not possible to identify and isolate the signals due to ^{13}C -enriched methine and methylene carbon atoms attached to large proteins.

According to the rigid rotor model for dipolar relaxation (Doddrell *et al.*, 1972; Oldfield *et al.*, 1975; Malthouse, 1986) the

replacement of directly bonded protons by deuterons will produce an 8–15.9-fold reduction in the linewidths of signals from ^{13}C nuclei at magnetic field strengths of 1.88–14.1 T provided that the rotational correlation time of the ^{13}C nucleus lies in the range 1–1160 ns (see Table 2 in the Results section). Several workers (Browne *et al.*, 1973; London, 1984; Malthouse, 1986) have pointed out that this reduction in linewidth on deuteration should greatly facilitate the resolution of signals due to deuterated ^{13}C -enriched carbon atoms attached to proteins. However, in the absence of deuterium decoupling, a perdeuteromethine carbon atom will appear as a triplet (peak intensities 1:1:1) and a perdeuteromethylene carbon will appear as a quintet (peak intensities 1:2:3:2:1). This would reduce the signal-to-noise ratio and make it difficult to resolve signals which were less than $2-4 \times J_{\text{CD}}$ apart. These problems could be overcome if a high-power deuterium decoupling field was applied. But this could lead to dielectric heating with aqueous biological solutions (Malthouse, 1986). Also, as most modern n.m.r. spectrometers are fitted with a deuterium lock channel, deuterium decoupling is difficult to implement. Consequently, deuteration of ^{13}C -enriched atomic probes has not been widely utilized in the study of proteins. However, an applied deuterium decoupling field will not be necessary if the deuterons undergo rapid quadrupolar relaxation, which decouples them from the directly bonded ^{13}C nucleus by scalar relaxation of the second kind (Pople, 1958; Abragam, 1961). It has recently been assumed (Jaffe & Markham, 1988) that this mechanism will lead to effective deuterium decoupling at high magnetic field strengths (7.05 T) with a large protein (M_r 280000). In the present study we show that a perdeuteromethylene carbon atom attached to δ -chymotrypsin is

Abbreviations used: Tos-Phe-CH₂Cl, L-1-chloro-4-phenyl-3-tosylamidobutan-2-one; Z-, benzyloxycarbonyl-.

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decoupled from the directly bonded deuterons by scalar relaxation of the second kind and that the efficiency of this deuterium decoupling decreases with increasing magnetic field strength.

These results are explained by the fact that the efficiency of deuterium decoupling is dependent on the efficiency of scalar relaxation of the second kind, which depends on the magnitude of the quadrupolar spin-lattice relaxation rate (Pople, 1958; Abragam, 1961). Therefore, using the rigid rotor model for quadrupolar relaxation (Abragam, 1961; McConnell, 1987), we calculate quadrupolar spin-lattice relaxation rates and show that for proteins the efficiency of deuterium decoupling will decrease with increasing rotational correlation times and magnetic field strength. We conclude that it should not be assumed that scalar relaxation will cause effective deuterium decoupling with large proteins at high magnetic field strengths. The implications of our results and predictions for the use of deuterated ^{13}C -enriched atomic probes in studying proteins of different M_r values at high and low magnetic field strengths are discussed.

MATERIALS

δ -Chymotrypsin (salt-free, from bovine pancreas), N -[^{13}C]methyl- N -nitrosotoluene- p -sulphonamide (99 atom %) and N -[^{13}C , $^2\text{H}_3$]methyl- N -nitrosotoluene- p -sulphonamide (99 atom % ^{13}C ; 99.5 atom % ^2H) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were as described by Finucane *et al.* (1989) or were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

METHODS

Synthesis of Tos-[1- ^{13}C]Phe-CH $_2$ Cl and Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl

N -[^{13}C] and N -[^{13}C , $^2\text{H}_3$]Methyl- N -nitrosotoluene- p -sulphonamide were used to generate the diazomethane required for the synthesis of Tos-[1- ^{13}C]Phe-CH $_2$ Cl and Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl respectively by the method of Schoellman & Shaw (1963). For the synthesis of Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl it is essential to ensure that all solvents and reagents with exchangeable protons are fully deuterated to prevent hydrogen/deuterium exchange at the ^{13}C -enriched atom during the synthesis. The ^{13}C -n.m.r. spectrum of Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl in [^2H]chloroform gave a quintet ($J_{\text{CD}} = 23$ Hz) centred at 46.5 p.p.m. (Fig. 1e). The deuterons of Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl were exchangeable with protons. Therefore it was stored in ethan[^2H]ol and its reaction with δ -chymotrypsin was carried out in 99.8 atom % $^2\text{H}_2\text{O}$. However, we did not detect any exchange of the protons of the ^{13}C -enriched methylene group in the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin complex at pH 3.3 in 99.8 atom % $^2\text{H}_2\text{O}$. The ^{13}C -n.m.r. spectrum of Tos-[1- ^{13}C]Phe-CH $_2$ Cl in [^2H]chloroform gave a triplet ($J_{\text{CH}} = 150$ Hz) centred at 47.1 p.p.m. in the absence of proton decoupling.

Assay of enzyme solutions

Enzyme solutions were assayed as described by Finucane *et al.* (1989).

Inhibition of δ -chymotrypsin by Tos-Phe-CH $_2$ Cl, Tos-[1- ^{13}C]Phe-CH $_2$ Cl and Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl

The inhibition of δ -chymotrypsin by Tos-Phe-CH $_2$ Cl and Tos-[1- ^{13}C]Phe-CH $_2$ Cl was carried out as described by Finucane *et al.*

(1989). This procedure was modified when δ -chymotrypsin was inhibited by Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl to prevent the exchange of the deuterons of the inhibitor. Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ Cl (98 mM) was dissolved in 1 ml of 99.5 atom % ethan[^2H]ol and added to 100 ml of 0.5 mM active δ -chymotrypsin in 99.8 atom % $^2\text{H}_2\text{O}$ containing 25 mM-potassium phosphate buffer, pH 7.0. When the catalytic activity of δ -chymotrypsin towards N -3-carboxypropionyl-L-phenylalanine p -nitroanilide (Finucane *et al.*, 1989) had been lowered to approx. 3 % of its original value, the solution was adjusted to pH 3.3 with 2 M- ^2HCl (99.8 atom %) and concentrated to a volume of 10 ml by ultrafiltration on an Amicon PM10 membrane. Excess inhibitor was removed by centrifuging at 15600 g (Finucane *et al.*, 1989). The ethan[^2H]ol was removed by freeze-drying. The Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex was dissolved in 99.8 atom % $^2\text{H}_2\text{O}$ and used for determining the relaxation parameters of the inhibitor complex at pH 3.3.

The samples of Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin used for pH titration studies were treated with phenylmethanesulphonyl fluoride to diminish the rate of autolysis at alkaline pH values. Typically, 0.05 ml of 0.1 M-phenylmethanesulphonyl fluoride in propan-2-ol was added to 30 ml of the Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex (0.5 mM) in 99.8 atom % $^2\text{H}_2\text{O}$ containing 3 mM-potassium phosphate buffer, pH 7.0. When the catalytic activity of δ -chymotrypsin had diminished to approx. 0.5 % of its original value, the solution was adjusted to pH 3.3 with 2 M- ^2HCl (99.8 atom %), centrifuged at 15600 g and then freeze-dried to remove the propan-2-ol. The Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex was dissolved in 99.8 atom % $^2\text{H}_2\text{O}$ for pH titration studies.

As the dialysis step was omitted when solutions of the Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex were prepared, all these samples contained 10–25 mM-potassium phosphate. Because of the low solubility of the inhibitor in fully aqueous solutions (Finucane *et al.*, 1989) signals due to the ^{13}C -enriched atoms of the free inhibitor were not observed. Dialysis of a 0.9 ml sample of the Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex against 1 mM- ^2HCl in 99.8 atom % $^2\text{H}_2\text{O}$ (4×5 ml) did not result in the loss of signals assigned (see the Results section) to the ^{13}C -enriched atoms of the Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin complex, confirming that these signals result from inhibitor covalently attached to δ -chymotrypsin.

Determination of pH and pH titrations

pH determination and pH titrations were carried out as described by Malthouse *et al.* (1985).

N.m.r. spectroscopy

N.m.r. spectra at 1.88 T were recorded with a Bruker WP80 wide-bore spectrometer operating at 20.115 MHz for ^{13}C nuclei. The pulse sequence of Belton *et al.* (1985) was used to minimize acoustic ringing. Spectral conditions were 2048 data points per spectrum and the spectral width was 220 p.p.m. Pulse widths of 11 μs and 22 μs were equivalent to 90° and 180° pulses. For linewidth measurements all spectra were zero-filled to give 8192 data points before being transformed using an exponential weighting factor of 10 Hz. With samples of Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin and unenriched control samples an acquisition time of 0.05 s was used with 1 W or 2 W broadband decoupling. $^2\text{H}_2\text{O}$ (20 %, v/v) was incorporated into all these samples in order to obtain a deuterium lock signal. For samples of Tos-[1- ^{13}C , $^2\text{H}_3$]Phe-CH $_2$ - δ -chymotrypsin and its unenriched control an acquisition time of 0.231 s was used with no broadband decoupling. Convolution difference spectra (Campbell *et al.*, 1973) were obtained by subtracting a spectrum transformed using a 200 Hz exponential weighting factor from one transformed using

a 10 Hz exponential weighting factor. The signals due to carbon atoms with directly bonded protons were suppressed using a J -modulated spin-echo pulse sequence with gated decoupling [see Benn & Gunther (1983) and references cited therein]. For these experiments, an acquisition time of 0.1 s was used with a relaxation delay of 0.1 s. A delay (the evolution time) of 0.004 s was used between the 90° and 180° pulses in this pulse sequence. The solvent for these samples was 99.8 atom % $^2\text{H}_2\text{O}$.

Spectra at 6.34 T were recorded with a Jeol JNM GX-270 spectrometer operating at 67.80 MHz for ^{13}C nuclei. Spectral conditions were 4096 time-domain data points, a spectral width of 260 p.p.m. and an acquisition time of 0.116 s. For the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin and its unenriched control a pulse width of 7 μs (14.5 μs = 90° pulse) was used with an inter-pulse delay of 0.084 s and composite pulse decoupling was implemented by using standard Jeol instrumentation. For Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH $_2$ - δ -chymotrypsin and its unenriched control, a pulse width of 14.5 μs was used with an inter-pulse delay of 5 s and no proton decoupling. Samples were the same as at 1.88 T and all spectra were zero-filled to 16384 data points before being transformed using an exponential weighting factor of 10 Hz. For Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH $_2$ Cl in 99.8 atom % [^2H]chloroform the conditions were a pulse width of 1 μs , 8192 time-domain data points, an acquisition time of 0.926 s and a relaxation delay of 2 s, and no broadband decoupling was used. All samples at 1.88 T and 6.34 T were at pH 3.3 and 29 $^\circ\text{C}$. N.m.r. signals were referenced as described by Finucane *et al.* (1989). Sample volumes at 1.88 T were 1.0–2.5 ml and 2.5 ml at 6.34 T. Protein concentrations were 2–4 mM.

Determination of spin-lattice relaxation times

Spin-lattice relaxation times were determined by the inversion recovery method (Vold *et al.*, 1968) with six and eight inter-pulse delay times at 1.88 T and 6.34 T respectively. In order to determine the spin-lattice relaxation times of the ^{13}C -enriched atoms of the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin and the Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH $_2$ - δ -chymotrypsin complexes, control spectra of the unenriched Tos-Phe-CH $_2$ - δ -chymotrypsin complex were obtained under identical experimental conditions and were subtracted from the spectra of the ^{13}C -enriched inhibitor complexes. Because of the low signal-to-noise ratio at 1.88 T and the fact that signals A and B were not resolved from each other in the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin complex (see the Results section), it was not possible to determine the spin-lattice relaxation time of signal A in the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin complex at 1.88 T.

Measurement of linewidths

Linewidths were measured at half the signal height. Signals A and B of the Tos-[1- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin complex are not resolved from each other at 1.88 T (see the Results section). Therefore, in this case, the linewidth of signal A was estimated by measuring the half width at half height on the high-field side of signal A and doubling this value. By comparing the linewidth of [1- ^{13}C]glycine in samples of 2–4 mM- δ -chymotrypsin over periods of time equalling the time taken to accumulate the spectra presented here, we estimated that sample viscosity and instrumental broadening contributed approx. 0.5 Hz and 1.0 Hz at 1.88 T and 6.34 T respectively. These values were subtracted from the observed linewidths.

Bond lengths and internuclear distances used for calculating rotational correlation times

A value of 0.110 nm was used for the α -carbon-proton bond length in amino acids and all other carbon-proton or carbon-

deuteron bond lengths were assumed to be 0.109 nm (see the Results section). The protons or deuterons directly bonded to sp^3 -hybridized carbon atoms were assumed to have tetrahedral geometry. The bond lengths and geometries of the inhibitor atoms in the Tos-Phe-CH $_2$ -chymotrypsin complexes were assumed to be the same as those obtained by Scott *et al.* (1986) using the 'weak-constraint' refinement procedure for the analysis of X-ray-crystallographic data on the Z-Lys-CH $_2$ -trypsin complex. All other bond lengths and angles used were standard values (Weiner *et al.*, 1984). It was calculated that the α -carbon atoms of the backbone amino acids were 0.2151 nm and 0.2124 nm from the amino acid β -methylene protons and peptide NH protons respectively. Likewise it was calculated that the ^{13}C -enriched carbon atom of Tos-[2- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin was 0.2137 nm, 0.2153 nm and 0.1932 nm from the protons directly bonded to C-1, C-3 and the C-2 oxygen atom of the tetrahedral adduct respectively.

Calculation of rotational correlation times

These were calculated from experimental linewidths and spin-lattice relaxation times by using the rigid rotor model (Doddrell *et al.*, 1972; Oldfield *et al.*, 1975) and the librational model (Howarth, 1978) for dipolar relaxation. According to the librational theory (Howarth, 1978) the carbon-proton vectors of a carbon with a directly bonded proton rotate stochastically at a fixed angle (θ) to a given axis and their random jumps between different directions are assumed to be governed by a single rotational correlation time (t_g). In this study the overall rotational correlation times were estimated by only considering directly bonded protons or deuterons, which were assigned values of 0.35 radians and 10^{-11} s for θ and t_g respectively, as suggested by Howarth (1978). When rotational correlation times were calculated by using the rigid rotor model for dipolar relaxation the contributions due to directly bonded protons or deuterons were allowed for, as well as those due to directly bonded nitrogen atoms and protons up to two bonds distant from the carbon nucleus being examined. All the amino acid residues with chemical shifts at 55 ± 3 p.p.m. have β -methylene protons (Jardetsky & Roberts, 1981). Therefore their contributions to the dipolar relaxation rates of α -carbon atoms were allowed for. It was assumed that the C-2 hydroxy group of the Tos-[2- ^{13}C]Phe-CH $_2$ - δ -chymotrypsin complex was fully protonated at pH 3.3 and that its proton was equilibrated with the protons or deuterons of the appropriate solvent used.

RESULTS AND DISCUSSION

When δ -chymotrypsin alkylated by Tos-[1- ^{13}C]Phe-CH $_2$ Cl is examined by ^{13}C n.m.r. at pH 3.3 and 1.88 T the signals (Fig. 1a) from the ^{13}C -enriched atoms are not clearly resolved from the natural-abundance signals of the protein (Fig. 1b). A difference spectrum (Fig. 1c) revealed the presence of two broad signals: the larger signal, peak A, was at 55.1 p.p.m. and the smaller signal, peak B, was at 58.1 p.p.m. (Fig. 1c). Signal A results from the alkylation of histidine-57 and signal B results from the alkylation of methionine-192 (Stevenson & Smillie 1968; Finucane *et al.*, 1989; M. D. Finucane & J. P. G. Malthouse, unpublished work).

Signal A had a linewidth of 40 ± 2 Hz at 1.88 T, which increased to 53 ± 4 Hz at 6.34 T (Fig. 2c). Doubling the decoupler power did not decrease the linewidths of these signals, confirming that they are fully decoupled. Also, halving the concentration (from 4 mM to 2 mM) of the inhibitor complex or including 0.4 M-KCl in samples did not decrease linewidths, which supports our earlier arguments (Finucane *et al.*, 1989) that the inhibitor complex is monomeric at pH 3.3. The linewidth of signal A

Fig. 1. ^{13}C -n.m.r. spectra of δ -chymotrypsin at 1.88 T after alkylation by Tos-Phe-CH₂Cl, Tos-[1- ^{13}C]Phe-CH₂Cl and Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl

Acquisition parameters were as described in the Methods section except that 1228 800, 1228 800, 262 144, 262 144, 368 640 and 4096 transients were recorded for spectra (a), (b), (d), (e), (h) and (i) respectively. Sample conditions were: (a) 2.9 mM-Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin in 20% (v/v) $^2\text{H}_2\text{O}$; (b) 2.9 mM-Tos-Phe-CH₂- δ -chymotrypsin in 20% (v/v) $^2\text{H}_2\text{O}$; (c) difference spectrum (a)–(b); (d) 4.0 mM-Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂- δ -chymotrypsin in 99.8 atom % $^2\text{H}_2\text{O}$; (e) 4.1 mM-Tos-Phe-CH₂- δ -chymotrypsin in 99.8 atom % $^2\text{H}_2\text{O}$; (f) difference spectrum (d)–(e); (g) convolution difference spectrum of spectrum (d); (h) same sample as (d) except that it was obtained by using a J -modulated spin-echo pulse sequence with gated decoupling; (i) 0.3 M-Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl in 99.8 atom % [^2H]chloroform. All samples had volumes in the range 1.0–2.5 ml and were at pH 3.3. The sample temperature was 29 °C.

(53 ± 4 Hz) at 6.34 T is very similar to the linewidth of 50 Hz obtained for the intact Z-[1- ^{13}C]Lys-CH₂-trypsin complex, where only histidine-57 is alkylated (Primrose *et al.*, 1985). This similarity is expected, as trypsin and chymotrypsin have similar M_r values (Malthouse, 1986).

When δ -chymotrypsin alkylated by Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl is examined by ^{13}C n.m.r., proton decoupling is not necessary as there are no directly bonded protons attached to the ^{13}C -enriched inhibitor carbon atom. As pointed out by Malthouse (1986), in the absence of proton decoupling the natural-abundance signals of the enzyme (Figs. 1e and 2e) are less well resolved than in the presence of proton decoupling (Figs. 1b and 2b). Therefore the signals due to the deuterated ^{13}C -enriched inhibitor carbon atom are better resolved from the natural-abundance signals of the enzymes in the absence of proton decoupling at 1.88 T and

Fig. 2. ^{13}C -n.m.r. spectra of δ -chymotrypsin at 6.34 T after alkylation by Tos-Phe-CH₂Cl, Tos-[1- ^{13}C]Phe-CH₂Cl and Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl

Acquisition parameters were as described in the Methods section except that 250 000, 250 000, 2000 and 2000 transients were recorded for spectra (a), (b), (d) and (e) respectively. Sample conditions were: (a) 2.9 mM-Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin in 20% (v/v) $^2\text{H}_2\text{O}$; (b) 2.9 mM-Tos-Phe-CH₂- δ -chymotrypsin in 20% (v/v) $^2\text{H}_2\text{O}$; (c) difference spectrum (a)–(b); (d) 2.6 mM-Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂- δ -chymotrypsin in 99.8 atom % $^2\text{H}_2\text{O}$; (e) 2.7 mM-Tos-Phe-CH₂- δ -chymotrypsin in 99.8 atom % $^2\text{H}_2\text{O}$; (f) difference spectrum (d)–(e). All samples had volumes of 2.5 ml and were at pH 3.3. The sample temperature was 29 °C.

6.34 T (Figs. 1d and 2d). Difference spectra at 1.88 T (Fig. 1f) and 6.34 T (Fig. 2f) confirmed that at pH 3.3 there were two signals due to the ^{13}C -enriched inhibitor carbon atom, one large signal (signal A) at 54.2 p.p.m. and a smaller signal (signal B) at 57.5 p.p.m. The deuterium isotope shift of signal B (0.6 p.p.m.) was identical with that of the free inhibitor, but the isotope shift of 0.9 p.p.m. for signal A was larger than that observed for the free inhibitor. On increasing the pH the chemical shift of signal A increased (Δ p.p.m. = 4.90 ± 0.01 p.p.m.) according to a single ionizing group of $\text{p}K_a$ 8.86 ± 0.01 , whereas peak B did not titrate. The pH-dependence of these signals is essentially the same as that for the non-deuterated Tos-[1- ^{13}C]Phe-CH₂- δ -chymotrypsin complex (M. D. Finucane & J. P. G. Malthouse, unpublished work), which confirms that signal A results from alkylation of histidine-57 and signal B results from alkylation of methionine-192 (Finucane *et al.*, 1989; M. D. Finucane & J. P. G. Malthouse, unpublished work).

Although deuterium couplings ($J_{\text{CD}} = 23$ Hz) are clearly visible for the free inhibitor (Fig. 1i), no such couplings are detected when Tos-[1- ^{13}C , $^2\text{H}_2$]Phe-CH₂Cl is bound covalently to δ -chymotrypsin (Figs. 1f and 2f). If the linewidth of the signals making up the quintet are $> J_{\text{CD}}$ then the individual signals of the quintet will not be resolved and the linewidth will be $> 3 \times J_{\text{CD}}$, i.e. > 69 Hz. However, signal A had a linewidth of 16 ± 3 Hz at 1.88 T and a linewidth of 52 ± 2 Hz at 6.34 T. Since these linewidths are less than $3 \times J_{\text{CD}}$ (69 Hz) then the failure to observe the quintet is not due to the linewidth being $> J_{\text{CD}}$. The

Table 1. Rotational correlation times of δ -chymotrypsin calculated from experimental relaxation parameters

Species observed	Magnetic field strength (T)	Experimental relaxation parameters		Rotational correlation times calculated from experimental relaxation parameters (ns)†		
		Spin-lattice relaxation time (s)	Linewidth (Hz)	Rigid rotor model		Librational model
				A	B	
δ -Chymotrypsin α CH*	1.88	0.045 ± 0.004		15.7 (13.4–17.8)	16.8 (14.5–18.9)	6.3§ (6.3–8.3)
δ -Chymotrypsin α CH*	6.34	0.40 ± 0.04		14.7 (13.1–16.2)	15.4 (13.8–17.0)	13.0 (11.2–14.9)
Inhibitor $-\text{CH}_2-\dagger$	1.88		40 ± 2	10.8 (9.9–11.6)	10.7 (9.8–11.6)	18.6 (17.4–19.8)
Inhibitor $-\text{CH}_2-\dagger$	6.34		53 ± 4	19.1 (17.6–20.6)	19.1 (17.6–20.6)	28.2 (26.0–29.8)
Inhibitor $-\text{CH}_2-\dagger$	6.34	0.26 ± 0.05		20.3 (16.3–24.4)	20.4 (16.4–24.4)	20.6 (16.3–27.3)
Inhibitor $-\text{C}^2\text{H}_2-\dagger$	1.88	0.13 ± 0.01		14.6 (10.8–17.8)	15.2 (11.5–18.3)	
Inhibitor $-\text{C}^2\text{H}_2-\dagger$	6.34	0.88 ± 0.18		14.1 (10.5–17.5)	14.3 (10.6–17.8)	9.3 (6.1–12.2)

* Tos-Phe-CH₂- δ -chymotrypsin α -carbon signals at 55.0 p.p.m.

† Signal A due to the ¹³C-enriched carbon of Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin or Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin as indicated.

‡ The rotational correlation times were calculated from the experimental relaxation parameters as described in the Methods section. The ranges of values expected based on the experimental errors are shown in parentheses. For the values calculated in columns A and C only interactions with directly bonded protons or deuterons were considered, whereas those calculated in column B allow for interactions with directly bonded protons, deuterons and the nitrogen atom, as well as non-bonded protons or deuterons up to two bonds distant from the carbon nuclei observed.

§ The librational model predicts a minimum spin-lattice relaxation time of 0.048 s, which occurs when the rotational correlation time is 6.3 ns.

|| The libration model predicts a minimum spin-lattice relaxation time of 0.168 ns. Therefore, as the experimental spin-lattice relaxation time is significantly less than this value, a rotational correlation time could not be calculated.

replacement of directly bonded protons by deuterons should result in a decrease in the efficiency of dipolar relaxation, and consequently on the basis of the linewidths of signal A at 1.88 T and 6.34 T in the Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin complex we would expect linewidths of approx. 3–6 Hz for signal A in the Tos[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complex (Malthouse, 1986). Therefore, if only dipolar relaxation occurred, the quintet structure of signal A should be resolved. The fact that it is not resolved confirms that 'scalar relaxation of the second kind' (see below) is decoupling the ¹³C nuclei from the directly bonded deuterons.

The linewidth of signal A in the Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complex is narrower (Fig. 1d) than that of the broad envelope of signals resulting from the natural-abundance spectrum of the protein in the absence of proton decoupling (Fig. 1e). Therefore a convolution difference spectrum (Campbell *et al.*, 1973) can be used to isolate signals A and B (Fig. 1g). This obviates the need for obtaining a separate control spectrum, but the resulting spectrum (Fig. 1g) had negative wings and cannot be used for determining the natural linewidth of the deuterated carbon atoms. An alternative approach is to use a *J*-modulated spin-echo experiment with gated decoupling [see Benn & Gunther (1983) and references cited therein]. If an evolution time (see the Methods section) of approx. $\frac{1}{2}J_{\text{CH}}$ is used this should null all the signals due to ¹³C nuclei with directly bonded protons. As shown in Fig. 1(h), a delay of 0.004 s (equivalent to $J_{\text{CH}} = 125$ Hz) gives good suppression of all the signals resulting from ¹³C nuclei with directly bonded protons. Therefore only the signals due to carbonyl carbon atoms and deuterated carbon atoms remain (Fig. 1h). Since a control spectrum is not required, this approach requires 50 % less enzyme and also reduces the experimental time by 50 %. It will also increase the signal-to-noise ratio as difference spectroscopy is avoided (compare Fig. 1f with Fig. 1h). Spin-lattice relaxation times (Table 1) were determined as described in the Methods section.

Determination of the rotational correlation time of the Tos-Phe-CH₂- δ -chymotrypsin complex

For *sp*³-hybridized ¹³C nuclei with directly bonded protons dipolar relaxation should predominate even at high magnetic

field strengths (Norton *et al.*, 1977). Therefore if it is assumed that the ¹³C nuclei are part of a rigid isotropically tumbling molecule then the rigid rotor model for dipolar relaxation can be used to calculate the rotational correlation time of the molecule provided that the distances of the interacting nuclei from the ¹³C nucleus are known (Doddrell *et al.*, 1972; Oldfield *et al.*, 1975). Since the magnitude of dipolar interactions is inversely proportional to the sixth power of these internuclear distances, then the dipolar relaxation rates are primarily determined by the directly bonded protons (Oldfield *et al.*, 1975). Dill & Allerhand (1979) have suggested that the widely used value of 0.109 nm for the α -carbon-proton bond length of amino acid residues in proteins may be an underestimate of this bond length. This suggestion is supported by neutron-diffraction data (Koetzle & Lehmann, 1976), which are consistent with a value of 0.110 ± 0.001 nm. However, Nemethy *et al.* (1983) have stated that the carbon-proton bond length is 0.109 nm for both the α -carbon and β -carbon atoms of amino acids. Using the data sources they quote, we find that the α -carbon-proton and β -carbon-proton bond lengths are 0.110 ± 0.001 nm and 0.109 ± 0.001 nm respectively. These values were therefore used in our calculations of rotational correlation times (Table 1). As the methylene carbon-proton and carbon-deuteron bond lengths of glycylglycine and perdeuteroglycylglycine are identical (Freeman *et al.*, 1970; Koetzle *et al.*, 1972), we have assumed that these distances are both 0.109 nm for the ¹³C-enriched methylene and perdeuteromethylene groups of the Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin and Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complexes respectively. Although scalar relaxation of the 2nd kind contributes to the linewidth of signal A in the Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complex it does not significantly contribute to the spin-lattice relaxation times of signal A (see below).

The linewidth of signal A from the Tos-[1-¹³C]Phe-CH₂- δ -chymotrypsin complex is approx. 2-fold smaller than that expected for a protein with an *M_r* of 25000 on the basis of earlier calculations made by using the rigid rotor model (Malthouse, 1986). It has been proposed (Howarth, 1978) that if there is a 'libration in the protein backbone' then the protein resonances will be narrower than those predicted by the rigid rotor model.

Table 2. Effect of magnetic field strength and rotational correlation time on the quadrupolar spin-lattice relaxation rate of deuterium and on the linewidths of methylene and perdeuteromethylene carbon atoms

Magnetic field strength (T) ...	1.88	6.34	14.1	1.88	6.34	14.1	1.88	6.34	14.1	1.88	6.34	14.1
Rotational correlation time (ns)	Linewidths due to dipolar relaxation (Hz)*						Quadrupolar spin-lattice relaxation rates of directly bonded deuterons (s ⁻¹)†			Linewidth of the perdeuteromethylene carbon when the quadrupolar spin-lattice relaxation rate is > 2·π·J _{CD} (Hz)‡		
	Methylene carbon		Perdeuteromethylene carbon									
0.1	1.4	1.3	1.2	0.1	0.1	0.1	42.8	42.7	42.3			
0.5	6.5	4.8	3.1	0.4	0.4	0.4	212.8	202.3	167.7	49.6	52.2	62.8
1.0	11.6	6.5	4.3	0.8	0.7	0.5	419.4	349.4	210.4§	25.8	30.7	50.3
2.0	17.5	9.1	6.6	1.6	1.2	0.7	792.2	462.9§	180.8	14.8	23.8	58.7
5.0	26.4	16.1	14.2	3.3	1.7	1.1	1445.0	378.6	95.2	10.5	29.3	
8.0	33.7	23.5	22.2	4.2	2.0	1.5	1580.3§	277.3	62.1	10.9	39.8	
10.0	38.3	28.7	27.6	4.7	2.3	1.8	1548.3	231.6	50.2	11.4	47.5	
15.0	49.8	41.9	41.2	5.4	3.0	2.7	1355.8	161.7	33.8	13.2	67.8	
20.0	61.8	55.4	54.8	6.0	3.8	3.5	1156.2	123.3	25.4	15.1		
50.0	139.9	137.0	136.8	10.0	8.7	8.6	551.9	50.3	10.2	29.0		
100.0	275.1	273.7	273.5	17.9	17.3	17.2	284.4	25.2	5.1	54.8		
145.0	397.7	396.7	396.6	25.4	25.0	24.9	197.2	17.4	3.5	78.5		
290.0	793.7	793.2	793.2	50.1	49.9	49.9	99.0	8.7	1.8			
580.0	1586.7	1586.4	1586.4	99.8	99.7	99.7	49.5	4.3	0.9			
870.0	2379.8	2379.6	2379.6	149.6	149.6	149.6	33.0	2.9	0.6			
1160.0	3172.9	3172.8	3172.8	199.5	199.4	199.4	24.8	2.2	0.4			

* Calculated by using the rigid rotor model for dipolar relaxation (see the Methods section), assuming that carbon-proton and carbon-deuteron bond lengths are 0.109 nm.

† Calculated by using the rigid rotor model for quadrupolar relaxation (see the Methods section), assuming a quadrupolar coupling constant of 170 kHz.

‡ Total linewidth due to dipolar relaxation and scalar relaxation of the second kind. Eqn. (4) was used to calculate the contribution of scalar relaxation to linewidths when $J_{CD} = 25$ Hz ($2 \cdot \pi \cdot J_{CD} = 157$ s⁻¹).

§ Maximum quadrupolar spin-lattice relaxation rate at the given magnetic field strength.

Therefore we have calculated rotational correlation times (see the Methods section) from the experimental relaxation parameters using both the rigid rotor model and the librational model (Table 1). The experimental value of the spin-lattice relaxation time for the Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complex at 1.88 T is outside the range of values predicted by the librational model (Table 1, column C). This, plus the fact that there is much better agreement between the rotational correlation times calculated by using the rigid rotor model compared with those calculated using the librational model (Table 1), leads us to conclude that the experimental data are best described by the rigid rotor model. When only directly bonded protons or deuterons are considered, then by using the rigid rotor model a mean rotational correlation time of 15.6 ± 3.2 ns is obtained (Table 1, column A). This mean value is increased to 16.0 ± 3.2 ns when dipolar interactions with directly bonded nitrogen atoms and hydrogen atoms up to two bonds distant are also considered (Table 1, column B). The same procedure was used to estimate the rotational correlation time of the Tos-[2-¹³C]Phe-CH₂- δ -chymotrypsin complex by using the spin-lattice relaxation time of 0.59 s (Finucane *et al.*, 1989) for the ¹³C-enriched *sp*³-hybridized quaternary carbon atom. The value of 15.7 ns is in good agreement with the values obtained for non-quaternary carbon atoms (Table 1, columns A and B) and therefore suggests that the corrections for dipolar interactions with directly bonded nitrogen atoms and non-directly bonded protons are valid. Assuming that the chemical-shift anisotropy tensor of an *sp*³-hybridized carbon atom is ≤ 40 p.p.m. (Vanderhart, 1976; Haberkorn *et al.*, 1981; Naito *et al.*, 1981, 1983; Janes *et al.*, 1983; Pines *et al.*, 1972; Stark *et al.*, 1983; Naito & McDowell,

1984) and that the rotational correlation time is 16 ns, then relaxation by chemical-shift anisotropy (Norton *et al.*, 1977) will contribute no more than 0.1 Hz to the linewidths and 1% to the spin-lattice relaxation times in Table 1. Consequently this relaxation mechanism can be neglected, and we conclude that the value 16.0 ± 3.2 ns is the best estimate for the rotational correlation time of the chymotrypsin-inhibitor complex. This value is in reasonable agreement with the values of 12 ± 2 ns (Shimshick & McConnell, 1972) and 16–22 ns (Haughland & Stryer, 1967; Vaz & Schoellman, 1976) obtained for monomeric chymotrypsin by using spin-labelling and fluorescence-depolarization measurements respectively. The value of 35 ns obtained by James *et al.* (1978) implies that these workers were observing dimeric chymotrypsin.

Relaxation parameters of the Tos-[1-¹³C, ²H₂]Phe-CH₂- δ -chymotrypsin complex at 1.88 T and 6.34 T

Scalar relaxation of the second kind is only significant when the quadrupolar relaxation rate $[(T_{1Q})^{-1}]$ is greater than or of a similar magnitude to the frequency ($2 \cdot \pi \cdot J_{CD}$) of the coupling constant (J_{CD}) resulting from the coupling of the quadrupolar nucleus (²H) to the nucleus (¹³C) of spin $\frac{1}{2}$ (Abragam, 1961). Coalescence of the multiplet occurs when $(T_{1Q})^{-1} \approx 2 \cdot \pi \cdot J_{CD}$, but only when $(T_{1Q})^{-1} \gg 2 \cdot \pi \cdot J_{CD}$ will the nucleus be fully decoupled from the ¹³C nucleus by scalar relaxation of the second kind (Pople, 1958; Abragam, 1961; Pyper, 1971). If it is assumed that J_{CD} is the same for both deuterons and that $(T_{1Q})^{-1} \gg 2 \cdot \pi \cdot J$, then eqns. (1) and (2) can be used to estimate the contribution of scalar relaxation of the second kind to the spin-lattice relaxation

time and linewidth of a ¹³C nucleus with two directly bonded deuterons (Abragam, 1961; Pyper, 1971):

$$\frac{1}{T_{1s}} = \frac{32}{3} \cdot \pi^2 \cdot J_{CD}^2 \cdot \left(\frac{T_{1Q}}{1 + (W_C - W_D)^2 \cdot (T_{1Q})^2} \right) \quad (1)$$

$$\text{Linewidth} = \frac{16}{3} \cdot \pi \cdot J_{CD}^2 \cdot T_{1Q} + \frac{1}{2 \cdot \pi \cdot T_{1s}} \quad (2)$$

$$\frac{1}{T_{1Q}} = \frac{3}{10} \cdot \pi^2 \cdot \left(\frac{e^2 \cdot q \cdot Q}{h} \right)^2 \cdot \left(\frac{T_R}{1 + W_D^2 \cdot T_R^2} + \frac{4T_R}{1 + 4 \cdot W_D^2 \cdot T_R^2} \right) \quad (3)$$

In eqns. (1), (2) and (3) T_{1s} and T_{1Q} are the scalar and quadrupolar spin-lattice relaxation times respectively, J_{CD} is the carbon-deuteron coupling constant, W_C and W_D are the resonance frequencies of ¹³C and ²H nuclei respectively at a given field strength, T_R is the rotational correlation time and $(e^2 \cdot q \cdot Q)/h$ is the quadrupolar coupling constant of the deuterons (Abragam, 1961; McConnell, 1987). By using a mean rotational correlation time of 16 ns (Table 1) and a value of 170 kHz for the quadrupolar coupling constant of an *sp*³-hybridized perdeuterated methylene group (Mantsch *et al.*, 1977) we calculate (by using eqn. 3) that, according to the rigid rotor model for quadrupolar relaxation (Abragam, 1961; McConnell, 1987), the quadrupolar spin-lattice relaxation times of the ²H nucleus will be 0.76 ms and 6.57 ms at 1.88 T and 6.34 T respectively. Therefore by using these values and $J_{CD} = 23$ Hz in eqn. (1) we calculate that the spin-lattice relaxation times for scalar relaxation at 1.88 T and 6.34 T will be 3.3×10^7 s and 3.3×10^8 s respectively. Consequently, the contribution of scalar relaxation of the second kind to the experimental spin-lattice relaxation times (Table 1) will be negligible and eqn. (2) reduces to eqn. (4):

$$\text{Linewidth} = \frac{16}{3} \cdot \pi \cdot J_{CD}^2 \cdot T_{1Q} \quad (4)$$

By using eqn. (4) we calculate that for a rotational correlation time of 16.0 ± 3.2 ns scalar relaxation of the second kind should contribute 6.1–7.5 Hz and 47.5–69.1 Hz to the linewidths at 1.88 T and 6.34 T respectively. Thus, allowing for dipolar relaxation by the directly bonded nitrogen atom and deuterons, we calculate that the experimental linewidths at 1.88 T and 6.34 T should lie in the ranges 11.4–13.5 Hz and 50.2–72.8 Hz respectively. The experimental linewidths of 16 ± 3 Hz at 1.88 T and 52 ± 2 Hz at 6.34 T are in good agreement with the predicted values. As we discussed above, relaxation by chemical-shift anisotropy should contribute no more than 0.1 Hz to the linewidths of the perdeuteromethylene carbon atom at 1.88 T or 6.34 T.

Therefore whereas perdeuteration of the ¹³C-enriched methylene carbon atom has resulted in a 2.5-fold decrease in its linewidth at 1.88 T there is no corresponding decrease in its linewidth when it is observed at 6.34 T (Table 1). This result is therefore explained by using the rigid rotor model for quadrupolar relaxation (Abragam, 1961; McConnell, 1987), which predicts (eqn. 3) a decrease in the quadrupolar spin-lattice relaxation rate with increasing magnetic field strength, resulting in a decrease in the efficiency of deuterium decoupling due to scalar relaxation.

Effects of rotational correlation time and magnetic field strength on the linewidths of methylene and perdeuteromethylene carbon atoms

The efficiency of deuterium decoupling due to scalar relaxation of the second kind depends upon the magnitude of the quadrupolar spin-lattice relaxation rate (Pople, 1958; Abragam,

1961), which depends on the magnetic field strength and the rotational correlation time (Abragam, 1961; McConnell, 1987). Therefore, by using the rigid rotor model for quadrupolar relaxation (Abragam, 1961; McConnell, 1987), we have calculated quadrupolar spin-lattice relaxation rates at 1.88 T, 6.34 T and 14.1 T for deuterium nuclei with rotational correlation times of 0.1–1160 ns (Table 2). When the quadrupolar spin-lattice relaxation rates are $\geq 2 \cdot \pi \cdot J$ a ¹³C nucleus will be decoupled from directly bonded deuterons (Pople, 1958; Abragam, 1961). In Table 2 we have used eqn. (4) to calculate the linewidth of a perdeuteromethylene carbon atom ($J_{CD} = 25$ Hz) when the quadrupolar spin-lattice relaxation rates are $> 2 \cdot \pi \cdot J$ (157 s⁻¹). At 1.88 T the linewidth of a perdeuteromethylene carbon atom will be 2.5–5-fold smaller than that of an equivalent non-deuterated methylene carbon atom provided that its rotational correlation time lies in the range 5–145 ns (Table 2). However, at 6.34 T and 14.1 T the deuterium decoupling due to scalar relaxation of the second kind is less efficient, and if the quadrupolar spin-lattice relaxation rate is > 157 s⁻¹ then the linewidths of methylene carbon atoms will be increased on perdeuteration (Table 2).

When the quadrupolar spin-lattice relaxation rate decreases the efficiency of deuterium decoupling due to scalar relaxation of the second kind decreases, and when it is $< \pi \cdot J_{CD}$ a coupled spectrum is obtained (Pople, 1958; Abragam, 1961). Therefore for proteins with rotational correlation times > 10 ns the quintet signal due to a perdeuteromethylene carbon atom should be resolved at 14.1 T (Table 2). However, for large proteins with rotational correlation times > 145 ns the linewidths of the individual quintet signals from a perdeuteromethylene carbon atom will be > 25 Hz owing to dipolar interactions with the directly bonded deuterons (Table 2). Consequently the individual quintet signals will not be resolved from each other. By computer simulation we have estimated that when the linewidths of the individual quintet signals are 25, 50, 100 and 200 Hz, then if $J_{CD} = 25$ Hz the experimentally observed linewidths of the unresolved quintet will be 73, 105, 138 and 222 Hz respectively. The linewidths of proton-decoupled methylene carbon atoms with the same rotational correlation times of 145, 290, 580 and 1160 ns will be 398, 794, 1587 and 3173 Hz respectively (Table 2). Therefore for large proteins with rotational correlation times of 145–1160 ns perdeuteration of methylene carbon atoms will decrease linewidths 5.5–14-fold in the absence of deuterium decoupling. Similar decreases in linewidth will occur when a methine carbon is deuterated.

Jaffe & Markham (1988) have reported that the perdeuteromethylene carbons of amino[5-²H,¹³C]laevulinate and [2-²H,¹³C,11-²H,¹³C]porphobilinogen have linewidths of 92 Hz and 75 Hz respectively when bound to porphobilinogen synthase (M_r 280000). Assuming that a protein with an M_r of 25000 has a rotational correlation time of 16 ns and that the rotational correlation times of proteins are proportional to their M_r values (Malthouse, 1986), we estimate that porphobilinogen synthase will have a rotational correlation time of 180 ns. By using this value for the rotational correlation time and assuming a normal value of 170 kHz for the quadrupolar coupling constant of an *sp*³-hybridized perdeuteromethylene group (Mantsch *et al.*, 1977), we calculate that the quadrupolar spin-lattice relaxation rate of the directly bonded deuterons will be 11.3 s⁻¹. Therefore, as the perdeuteromethylene carbon atoms have J_{CD} values of 20–22 Hz, the quadrupolar spin-lattice relaxation rate is $\ll 2 \cdot \pi \cdot J$ (126 – 138 s⁻¹) at 7.05 T and no deuterium decoupling will be observed. Thus the assumption by Jaffe & Markham (1988) that rapid deuterium relaxation will decouple ¹³C from ²H in such large molecules is incorrect. However, relaxation by chemical-shift anisotropy (assuming a value of 40 p.p.m. for the chemical-

shift anisotropy tensor) and dipolar interactions with the directly bonded deuterons should contribute 2 Hz and 32 Hz to the linewidth respectively. Therefore the quintet due to the perdeuteromethylene carbon atom ($J_{CD} = 20$ –22 Hz) will not be resolved. By computer simulation we estimate that the signals due to perdeuteromethylene carbon atoms in the enzyme-aminolaevulinate ($J_{CD} = 22$ Hz) and enzyme-porphobilinogen ($J_{CD} = 20$ Hz) complexes will have linewidths of 86 Hz and 80 Hz respectively. These values are in good agreement with the experimentally observed values.

General conclusions

Provided that the acquisition parameters are optimized (Malthouse, 1986) then the signal-to-noise ratio of a fully decoupled perdeuteromethylene or methine carbon atom with a rotational correlation time in the range 10–1160 ns will be 1–1.8-fold greater than its protonated equivalent depending on the magnetic field strength and rotational correlation time. In the absence of a deuterium decoupling field there will only be a 3-fold decrease in the signal-to-noise ratio when the individual multiplet signals have linewidths $< J_{CD}$. However, this decrease in the signal-to-noise ratio will diminish and it can become negligible when scalar relaxation causes deuterium decoupling or if the linewidths of individual multiplet signals exceed J_{CD} .

In the absence of proton decoupling the signals due to ^{13}C -enriched perdeuterated carbon atoms are clearly resolved from the broad natural-abundance signals of protein carbon atoms with directly bonded protons and these broad natural-abundance signals can be removed by using a J -modulated spin-echo pulse sequence. Therefore, although difference spectroscopy is usually required to identify and isolate signals due to ^{13}C -enriched carbon atoms with directly bonded protons, it is not required for ^{13}C -enriched carbon atoms with directly bonded deuterons. Consequently, as difference spectroscopy is avoided the signal-to-noise ratio will increase and there is a 50% decrease in the amount of time and protein needed to identify and isolate the signals due to ^{13}C -enriched perdeuterated carbon atoms in proteins. Therefore we conclude that even in the absence of an applied deuterium decoupling field the isolation and identification of signals due to ^{13}C -enriched methylene and methine carbon atoms in proteins will be facilitated by perdeuteration of the ^{13}C -enriched carbon atom.

The conclusions outlined above apply to ^{13}C -enriched perdeuteromethylene carbon atoms with non-exchangeable deuterons provided that the perdeuteromethylene group is rigidly attached to an isotropically tumbling macromolecule. With methylene and methine carbon atoms dipolar relaxation usually predominates and so their linewidths are directly proportional to their rotational correlation times and are independent of the magnetic field strength (Doddrell *et al.*, 1972; Oldfield *et al.*, 1975; Malthouse, 1986). However, when scalar relaxation of the second kind causes deuterium decoupling of a perdeuteromethylene carbon atom its linewidth will not be directly proportional to its rotational correlation time and it will not be independent of the magnetic field strength except when its rotational correlation time is < 0.5 ns (Table 2). Therefore, to confirm that a perdeuteromethylene carbon atom is rigidly attached to a macromolecule, its relaxation parameters should be determined at two magnetic field strengths or the linewidth of the non-deuterated carbon atom should be determined. Such studies are especially important with non-covalent ligands and with covalent ligands where the perdeuteromethylene group may not be rigidly bound to the macromolecule, e.g. the side chain of a lysine amino acid residue. Therefore the efficiency of deuterium decoupling due to scalar relaxation at different magnetic field strengths may be used to investigate the motional freedom of a

perdeuteromethine or perdeuteromethylene carbon atom. However, the main advantage of this approach is that it allows non-quaternary carbon atoms to be used as atomic probes of large macromolecules and macromolecular complexes. The chemical shifts of the ^{13}C -enriched carbon atoms of such atomic probes can be sensitive to changes in the hybridization state of the ^{13}C -enriched carbon atom, its substituent groups, the ionization state of adjacent atoms and its molecular environment (Malthouse, 1986; Jaffe & Markham, 1988).

We thank Badische Anilin und Soda Fabrik Aktiengesellschaft for a research award for M.D.F. and Dr. A. Rous for operating the Jeol GX-270 n.m.r. spectrometer.

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Received 28 March 1991/4 June 1991; accepted 12 June 1991