Effect of magnetic field strength on the linewidth and spin–lattice relaxation time of the thiocyanate carbon of cyanylated β -lactoglobulin B: optimization of the experimental parameters for observing thiocyanate carbons in proteins

J. Paul G. MALTHOUSE* and Paul PHELAN

Department of Biochemistry, University College Dublin, Dublin 5, Ireland

The linewidths and spin–lattice relaxation times of the ^{13}C -n.m.r. signal at 109.7 p.p.m. due to the thiocyanate carbon of intact [*cyanato- ^{13}C*]cyanylated- β -lactoglobulin-B have been determined at magnetic field strengths of 1.88, 6.34 and 11.74 T as well as the spin–lattice relaxation times of its backbone α -carbon atoms. The linewidths were directly proportional to the square of the magnetic field strength and we conclude that, at magnetic field strengths of 6.34 T or above, more than 70 % of the linewidth will be determined by chemical-shift anisotropy. We estimate that the spin–lattice relaxation time resulting from the chemical-shift anisotropy of the thiocyanate carbon is 1.52 ± 0.1 s and we conclude that for magnetic field strengths of 6.34 T and above

the observed spin–lattice relaxation time of the thiocyanate carbon will be essentially independent of magnetic field strength. Using the rigid-rotor model we obtain estimates of the rotational correlation time of [*cyanato- ^{13}C*]cyanylated- β -lactoglobulin-B and of the chemical-shift anisotropy shielding tensor of its thiocyanate carbon. We have calculated the linewidths and spin–lattice relaxation times of thiocyanate carbons at magnetic field strengths of 1.88–14.1 T in proteins with M_r values in the range 10000–400000. The effects of magnetic field strength on the resolution and signal-to-noise ratios of the signals due to thiocyanate carbons attached to proteins of M_r greater than 10000 are discussed.

INTRODUCTION

The cyanylation of a cysteine residue to produce a β -thiocyanatoalanine residue provides a simple and economical method of introducing a small ^{13}C -enriched reporter group into a protein (Malthouse, 1986; Doherty et al., 1992). By utilizing such reporter groups ^{13}C -n.m.r. can be used to investigate the structure and function of proteins. Proteins that have been examined in this way include glutamate aspartate aminotransferase (Boettcher and Martinez-Carrion, 1975), lactate dehydrogenase (Waldman et al., 1986), flavodoxin from *Megasphaera elsdenii* (Malthouse et al., 1989; Doherty et al., 1991; 1992), flavodoxin from *Clostridium pasteurianum* (Doherty et al., 1993) and β -lactoglobulins A and B (Phelan and Malthouse, 1994a,b).

Owing to the low sensitivity of ^{13}C nuclei, it is important that signal-to-noise ratio is optimized in n.m.r. experiments involving proteins. However, both the linewidth and spin–lattice relaxation time of a given ^{13}C nucleus must be known if we are to predict accurately what acquisition parameters will give optimal signal-to-noise ratios. ^{13}C -enriched carbon atoms will undergo efficient dipolar relaxation with directly bonded protons (Doddrell et al., 1972; Oldfield et al., 1975), and therefore we can predict (Malthouse, 1986) that, when such nuclei are rigidly attached to spherical proteins with M_r values greater than 10000, their linewidths will be independent of the magnetic field strength (H_0), and their spin–lattice relaxation times (T_1) will be directly proportional to the square of the field strength ($T_1 \propto H_0^2$). However, it has been predicted that for unsaturated quaternary carbons rigidly attached to proteins, relaxation by chemical-shift anisotropy (CSA) may be the dominant relaxation mechanism at

high magnetic field strengths (Norton et al., 1977). Therefore it is expected that, at high magnetic field strengths, CSA may make a significant contribution to the relaxation of the thiocyanate carbon of a cyanylated protein. Consequently both dipolar relaxation with non-directly bonded protons and relaxation by CSA may contribute to the relaxation parameters of the thiocyanate carbon. The aim of the present work is to determine the relative contribution of each of these relaxation mechanisms to the relaxation parameters of the thiocyanate carbon for a range of cyanylated proteins with M_r values of 10000 or higher at various magnetic field strengths. If relaxation by CSA predominates, then linewidths will be directly proportional to the square of the magnetic field strength whereas spin–lattice relaxation times will be independent of magnetic field strengths, provided that the ^{13}C nuclei are rigidly attached to a protein of M_r greater than 10000 (Norton et al., 1977). Therefore by determining the magnetic-field-dependence of the relaxation parameters of the thiocyanate carbons it should be possible to estimate the contributions from dipolar relaxation and CSA to its relaxation parameters.

In this paper we examine the linewidth and spin–lattice relaxation time of the thiocyanate carbon of cyanylated β -lactoglobulin B at magnetic field strengths of 1.88, 6.34 and 11.74 T. We show that dipolar relaxation is the predominant relaxation mechanism at a magnetic field strength of 1.88 T and that CSA is the predominant relaxation mechanism at magnetic field strengths of 6.34 T and above. We discuss how the magnetic field strength can affect the resolution and signal-to-noise ratio of signals due to thiocyanate carbons attached to proteins. Finally we present a table of spin–lattice relaxation times and linewidths for thiocyanate carbons attached to a range of globular proteins

* To whom correspondence and reprint requests should be addressed.
Abbreviation used: CSA, chemical shift anisotropy.

(M_r 10000–400000) at various magnetic field strengths. We hope that this will provide a rough guide to the optimum acquisition parameters that should be used when studying thiocyanate carbons attached to globular proteins.

MATERIALS AND METHODS

The material and methods for preparing and assaying [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B were as described previously (Phelan and Malthouse, 1994b).

N.m.r. samples

All protein samples were at pH 7.0 and $28 \pm 1^\circ\text{C}$. The sample size was 2.5 ml in 10 mm diameter sample tubes. The samples contained 3.4 mM subunits of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B, 25% (v/v) $^2\text{H}_2\text{O}$ and 7.5 mM potassium phosphate. [^{13}C]Glycine (50 mM) was incorporated into some samples to act as an internal reference for linewidth measurements.

N.m.r. spectral conditions used for determining linewidths

N.m.r. spectra at 1.88 T were obtained using a Bruker WP 80 wide-gap spectrometer operating at 20.115 MHz for ^{13}C nuclei. The spectral conditions used for determining the linewidth of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B were: 8192 time-domain data points; spectral width 240 p.p.m.; acquisition time 0.852 s; 32768 transients recorded per spectrum; 1 W broadband ^1H decoupling and a 28° pulse angle. The spectra were zero-filled to give 16384 data points before being transformed using an exponential weighting factor of 5 Hz.

N.m.r. spectra at 6.34 T were obtained using a JEOL JNM GX-270 spectrometer operating at 67.80 MHz for ^{13}C nuclei. The spectral conditions used for determining the linewidth of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B were: 8192 time-domain data points; spectral width 254 p.p.m.; acquisition time 0.238 s; an interpulse delay of 0.01 s; 20856 transients recorded per spectrum; composite pulse ^1H decoupling was implemented using standard JEOL instrumentation and a 25° pulse angle was used. The spectra were zero-filled to give 32768 data points before being transformed using an exponential weighting factor of 5 Hz or 15 Hz.

N.m.r. spectra at 11.74 T were obtained using a Varian Unity 500 spectrometer operating at 125.70 MHz for ^{13}C nuclei. The spectral conditions used for determining the linewidth of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B were: 16384 time-domain data points; spectral width 276 p.p.m.; acquisition time 0.238 s; an interpulse delay of 1.8 s; 2W ^1H decoupling power was used with a WALTZ decoupling sequence; 76416 transients were recorded per spectrum and a 16° pulse angle was used. For linewidth measurements, spectra were zero-filled to give 65536 data points before being transformed using an exponential weighting factor of 5 Hz or 30 Hz.

Measurement of linewidths

Linewidths were measured at half the signal height. By examining the linewidth of 50 mM [^{13}C]glycine in samples of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B we estimate that sample inhomogeneity and instrumental broadening contribute less than 2 Hz to the experimental linewidths.

Determination of spin-lattice relaxation times

Spin-lattice relaxation times were determined by the inversion recovery method (Vold et al., 1968).

Referencing of n.m.r. signals

All ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0 p.p.m.

RESULTS AND DISCUSSION

The relaxation rate ($R_{\text{obs.}}$) of a quaternary carbon in a non-paramagnetic macromolecule is expected to be determined by the sum of the relaxation rates due to CSA (R_{CSA}) and dipole-dipole relaxation (R_{DD}) (Doddrell et al., 1972; Norton et al., 1977). Therefore eqn. (1) should describe the relaxation rates of the signal at 109.7 p.p.m. due to the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B:

$$R_{\text{obs.}} = R_{\text{CSA}} + R_{\text{DD}} \quad (1)$$

For spin-spin relaxation, $R = 1/T_2$, where T_2 is the spin-spin relaxation time. But, as linewidth (LW) = $1/(\pi \cdot T_2)$, we can rewrite eqn. (1) as eqn. (2):

$$LW_{\text{obs.}} = LW_{\text{CSA}} + LW_{\text{DD}} \quad (2)$$

Linewidths due to CSA (LW_{CSA}) are directly proportional to the square of the magnetic field strength (Ho) whereas those due to dipolar relaxation are independent of magnetic field strength. Therefore eqn. (3) should describe the effect of magnetic field strength on the linewidth of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B.

$$LW_{\text{obs.}} = m \cdot Ho^2 + C \quad (3)$$

In eqn. (3), $m \cdot Ho^2 = LW_{\text{CSA}}$ and $C = LW_{\text{DD}}$ (where m is the shape and C is the intercept).

For spin-lattice relaxation, $R = 1/T_1$, where T_1 is the spin-lattice relaxation time. The T_1 values resulting from CSA are independent of magnetic field strength whereas those due to dipolar relaxation are directly proportional to Ho^2 provided that the rotational correlation time of the protein is more than 10^{-8} s or when the M_r is 10000 or higher (Malthouse, 1986). Therefore eqn. (4) should describe the effect of magnetic field strength on the spin-lattice relaxation time of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B (M_r 36400; Hambling et al., 1992).

$$1/T_{1,\text{obs.}} = m/Ho^2 + C \quad (4)$$

In eqn. (4) $m/Ho^2 = 1/T_{1,\text{DD}}$ and $C = 1/T_{1,\text{CSA}}$.

^{14}N like ^2H is a quadrupolar nucleus with a spin of 1. When the quadrupolar spin-lattice relaxation rates $[(T_{1Q})^{-1}]$ of these nuclei are much greater than $2\pi J$, they will be fully decoupled from the ^{13}C nucleus by scalar relaxation of the second kind. We have shown that, owing to decreases in the quadrupolar spin-lattice relaxation rate, the efficiency of scalar relaxation of the second kind is decreased as M_r values (provided that they are above 10000) and magnetic field strengths increase (Malthouse and Finucane, 1991). For ^{13}C nuclei directly bonded to ^2H , this leads to increased linewidths as M_r values and magnetic field strengths increase (Malthouse and Finucane, 1991). However, because of the larger quadrupolar coupling constant (Lehn and Kintzinger, 1973) and smaller gyromagnetic ratio of the ^{14}N nucleus, its quadrupolar spin-lattice relaxation rates are about 1600-fold larger than those of ^2H nuclei. Therefore as the ^{13}C - ^{14}N coupling constants are less than 10 Hz (Stothers, 1972; Mlynarik, 1986) we estimate that, in the present study, line-broadening of the thiocyanate carbon caused by scalar relaxation of the second kind will not make a significant contribution to linewidths (< 0.02 Hz, if $J_{^{13}\text{C}-^{14}\text{N}} = 10$ Hz) even at 11.74 T. Likewise we estimate that such line-broadening will be negligible (< 0.3 Hz, if $J_{^{13}\text{C}-^{14}\text{N}} = 10$ Hz) when we observe a thiocyanate carbon attached to a protein of M_r about 400000 at 14.1 T. Therefore we

Figure 1 ^{13}C -n.m.r. spectra of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B at 1.88, 6.34 and 11.74 T

All the spectra were transformed using a 5 Hz exponential weighting factor which was subtracted from the linewidth (measured at half signal height) to give the experimental linewidth (LW_{obs}). Acquisition parameters and sample conditions are given in the Materials and methods section. The magnetic field strength and experimental linewidth are indicated for each spectrum. When the spectra in Figure 1 were plotted, the signal at 109.7 p.p.m. was set to 0 Hz at each magnetic field strength.

Figure 2 Relationship between the linewidth of the signal at 109.7 p.p.m. due to the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B and the magnetic field strength (H_o)

Acquisition parameters and sample conditions are as described in the Materials and methods section. The data used are presented in Table 1. The continuous line was calculated by using the eqn. (3) and the fitted parameters $m = 0.289 \pm 0.022 \text{ Hz} \cdot \text{T}^{-2}$ and $C = 4.64 \pm 1.86 \text{ Hz}$.

conclude that line-broadening of the thiocyanate carbon by scalar relaxation of the second kind is not expected to make a significant contribution to the linewidths of the signals of thiocyanate carbons attached to proteins.

Table 1 Effect of magnetic field strength on the linewidth of the signal at 109.7 p.p.m. due to the thiocyanate carbon of intact cyanylated- β -lactoglobulin B

Best-fit linewidth was calculated using the equation $LW = 0.289 \cdot Ho^2 + 4.64$. Results for experimental linewidths are means $\pm$ S.E.M.

Magnetic field strength (T)	Linewidths (Hz)				Percentage of total fitted linewidth due to CSA
	Experimental	Best-fit	Due to CSA relaxation	Independent of magnetic field strength	
1.88	4.4 ± 0.2	5.66	1.02	4.64	18
6.34	18.0 ± 1.0	16.26	11.62	4.64	71
11.74	44.0 ± 2.0	44.47	39.83	4.64	90

Effect of magnetic field strength on the linewidth of the signal at 109.7 p.p.m. due to the thiocyanate carbon of intact cyanylated- β -lactoglobulin-B

The linewidth of the thiocyanate carbon of intact [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B increased as the magnetic field strength increased (Figure 1). This increase in linewidth was directly proportional to the square of the magnetic field strength (Figure 2). The experimental linewidths (LW_{obs}) at a given magnetic field strength were fitted to eqn. (3). The fitted values of m and C were $0.289 \pm 0.022 \text{ Hz} \cdot \text{T}^{-2}$ and $4.64 \pm 1.86 \text{ Hz}$ respectively (Figure 2). Using these fitted values of m and C in eqn. (3) we have calculated the proportions of the linewidths that are proportional to Ho^2 and the proportions that are independent of Ho^2 at 1.88, 6.34 and 11.74 T (Table 1). Therefore we conclude that at 1.88 T, 82% of the linewidth of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B will be determined by dipolar relaxation whereas at magnetic field strengths of 6.34 T or higher more than 70% of the linewidth will be determined by CSA (Table 1).

Effect of magnetic field strength on the spin-lattice relaxation times of the signal at 109.7 p.p.m. due to the thiocyanate carbon of intact cyanylated- β -lactoglobulin-B

The experimental spin-lattice relaxation times (Table 2) at 1.88, 6.34 and 11.74 T were fitted to eqn. (4). The fitted values of m and C were $1.00 \pm 0.26 \text{ T}^2 \cdot \text{s}^{-1}$ and $0.658 \pm 0.043 \text{ s}^{-1}$. Using these fitted values of m and C in eqn. (4) we have calculated that $T_{1\text{CSA}} = 1.52 \pm 0.1 \text{ s}$ at all magnetic field strengths. We have also calculated the values of $T_{1\text{DD}}$ at 1.88, 6.34 and 11.74 T (Table 2). As the experimental spin-lattice relaxation times at 1.88, 6.34 and 11.74 T (Table 2) have similar magnitudes they should give a reasonable estimate of $T_{1\text{CSA}}$ but only an approximate estimate of the contribution of $T_{1\text{DD}}$ to the observed spin-lattice relaxation rate. We conclude that at magnetic field strengths of 6.34 T and greater the spin-lattice relaxation time of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B will be essentially independent of magnetic field strength as the spin-lattice relaxation rate is largely determined by CSA (Table 2).

Determination of the rotational correlation time of intact cyanylated- β -lactoglobulin-B and estimation of the CSA shielding tensor of its thiocyanate carbon

Rotational correlation times were calculated from the spin-lattice relaxation times of the α -carbons of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B using the rigid-rotor model as described by

Table 2 Effect of magnetic field strength on the spin-lattice relaxation times of the signal at 109.7 p.p.m. due to the thiocyanate carbon of intact cyanylated- β -lactoglobulin B

The best-fit spin-lattice relaxation times were calculated using the equation $1/T_1 = 1/Ho^2 + 0.658$. Experimental results are means $\pm$ S.E.M.

Magnetic field strength (T)	Spin-lattice relaxation time(s)			
	Experimental	Best-fit	Due to CSA relaxation	Due to dipolar relaxation
1.88	1.06 $\pm$ 0.06	1.06	1.52	3.50
6.34	1.56 $\pm$ 0.09	1.46	1.52	40.20
11.74	1.42 $\pm$ 0.07	1.50	1.52	137.83

Malthouse and Finucane (1991). When only directly bonded protons are considered, a mean rotational correlation time of 23.0 ± 4.2 ns is obtained (Table 3, column A). This mean value is

Table 3 Rotational correlation times calculated from the spin-lattice relaxation times of the signals at 55.0 p.p.m. due to the α -carbons of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B

The rotational correlation times were calculated from the spin-lattice relaxation times of the α -carbons of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B using the rigid-rotor model as described by Malthouse and Finucane (1991). Experimental results are means $\pm$ S.E.M. The ranges of values expected based on the experimental errors are shown in parentheses. For the values calculated in column A, only interactions with directly bonded protons were considered, whereas those calculated in column B allow for interactions with a directly bonded proton and nitrogen atom as well as protons up to two bonds distant from the carbon nuclei observed.

Magnetic field strength (T)	Experimental spin-lattice relaxation times (s)	Rotational correlation times calculated from experimental spin-lattice relaxation times (ns)	
		A	B
1.88	0.07 $\pm$ 0.02	27.9 (18.5–36.5)	29.9 (28.9–30.8)
6.34	0.56 $\pm$ 0.07	20.8 (18.0–23.5)	22.1 (19.2–24.9)
11.74	1.87 $\pm$ 0.38	20.4 (16.0–24.5)	21.6 (17.2–26.1)

increased to 24.5 ± 4.6 ns if interactions with directly bonded nitrogen atoms and hydrogen atoms up to two bonds distant are also considered (Table 3, column B). A value of 16.0 ± 3.2 ns was determined (Malthouse and Finucane, 1991) for the rotational correlation time of chymotrypsin (M_r 25000). This value was calculated in the same way as the value of 24.5 ns for [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B. For globular proteins a linear relationship between rotational correlation times and M_r values is expected (Malthouse, 1986). Therefore we would predict that the [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B dimer (Phelan and Malthouse, 1994b) of M_r 36400 (Hambling et al., 1992) will have a rotational correlation time of about 23 ns. This value is in good agreement with the mean value of 24.5 ± 4.6 ns obtained in this study (Table 3).

Using the isotropic rigid-rotor model (Norton et al., 1977) with a rotational correlation time of 24.5 ns, we calculate that, on the basis of the LW_{CSA} (Table 1) at 11.74 T, the CSA shielding tensor of the thiocyanate carbon is 303 ± 12 p.p.m. This value is similar to the value of 307 ± 4 p.p.m. obtained by Kennedy and McFarlane (1975) for the CSA shielding tensor of the cyanide carbon of acetonitrile. A similar calculation using $T_{1\text{CSA}}$ (Table 2) yields a value of 348 ± 12 p.p.m. for the CSA shielding tensor of the thiocyanate carbon. This value is similar to the value of 354 ± 15 p.p.m. obtained by Wehrli (1978) for the CSA shielding tensor of the cyanide carbon of benzonitrile. We do not know the reason for the difference between the values of the CSA shielding tensors calculated from LW_{CSA} and $T_{1\text{CSA}}$. It may reflect the fact that the isotropic rigid-rotor model is only an approximate model for relaxation of the thiocyanate carbon of [cyanato- ^{13}C]cyanylated- β -lactoglobulin-B.

General discussion

By assuming that rotational correlation times are directly proportional to M_r values (Malthouse, 1986) and that eqns. (3) and (4) apply for thiocyanate carbons in all globular proteins at magnetic field strengths of 1.88–14.1 T, we have calculated the linewidths and spin-lattice relaxation times of thiocyanate carbons in a range of proteins with M_r values 10000–400000 (Table 4). From Table 4 we predict that for thiocyanate carbons attached to globular proteins of M_r greater than 10000, the

Table 4 Effect of magnetic field strength and rotational correlation time on the relaxation parameters of the thiocyanate carbon

M_r values were calculated assuming that rotational correlation times are directly proportional to M_r values (Malthouse, 1986) and that a globular protein of M_r 25000 will have a rotational correlation time of 16 ns (Malthouse and Finucane, 1991). Spin-lattice relaxation times (T_1) were calculated assuming that the equation $1/T_1 = 1/Ho^2 + 0.658$ applies when M_r 36400 and that spin-lattice relaxation times are directly proportional to M_r values. Linewidths (LW) were calculated by assuming that the equation $LW = 0.289 \cdot Ho^2 + 4.64$ applies when M_r 36400 and that linewidths are directly proportional to M_r values.

Magnetic field strength (T) ...	Rotational correlation time (ns)	1.88		4.70		6.34		11.74		14.10	
		T_1 (s)	LW (Hz)	T_1 (s)	LW (Hz)	T_1 (s)	LW (Hz)	T_1 (s)	LW (Hz)	T_1 (s)	LW (Hz)
M_r											
10000	6.4	0.3	1.5	0.4	3.0	0.4	4.5	0.4	12.2	0.4	17.1
15000	9.6	0.4	2.3	0.6	4.5	0.6	6.7	0.6	18.3	0.6	25.6
20000	12.8	0.6	3.1	0.8	6.1	0.8	8.9	0.8	24.4	0.8	34.1
25000	16.0	0.7	3.9	1.0	7.6	1.0	11.2	1.0	30.5	1.0	42.6
50000	32.0	1.5	7.8	2.0	15.1	2.0	22.3	2.0	61.1	2.0	85.3
100000	64.0	2.9	15.6	3.9	30.3	4.0	44.7	4.1	122.2	4.1	170.6
200000	128.0	5.8	31.1	7.8	60.6	8.0	89.3	8.2	244.3	8.3	341.2
400000	256.0	11.7	62.2	15.6	121.1	16.1	178.7	16.5	488.7	16.6	682.4

spin-lattice relaxation time of the thiocyanate carbon will be essentially independent of magnetic field strength provided that it is 4.7 T or more whereas its linewidth will increase as the magnetic field strength increases. The decrease in resolution resulting from this increase in linewidth will be largely offset by the increase in resolution due to the increased magnetic field strength. If we measure linewidths in Hz and p.p.m. for a thiocyanate carbon attached to a protein of M_r 100000, the linewidths will be 15.6 Hz (0.78 p.p.m.) at 1.88 T, 30.3 Hz (0.60 p.p.m.) at 4.7 T, 44.7 Hz (0.66 p.p.m.) at 6.34 T, 122.1 Hz (0.97 p.p.m.) at 11.74 T and 170.6 Hz (1.13 p.p.m.) at 14.1 T. Thus on going from 1.88 to 4.7 and 6.34 T, the linewidths in p.p.m. will decrease by about 23 and 15% respectively whereas on going from 1.88 to 11.74 and 14.1 T the linewidths will increase by about 24 and 45% respectively. Therefore, although on going from 1.88 to 14.1 T there is an approx. 11-fold increase in linewidth, there is only a 45% decrease in resolution of signals. In general, for optimal resolution of signals, magnetic field strengths of 6.34 T or less should be used. Any decrease in signal-to-noise ratio due to the increase in linewidth as the magnetic field strength increases should be offset by the increased sensitivity at higher magnetic field strengths provided that the acquisition parameters are optimized (Malthouse, 1986). Therefore different magnetic field strengths are not expected to produce large differences in the sensitivity and resolution of the signals due to the thiocyanate carbons of cyanylated proteins ($M_r > 10000$).

The thiocyanate carbons of intact cyanylated flavodoxin from *Megasphaera elsdenii* had signals at 109.6 and 115.5 p.p.m. (Malthouse et al., 1989; Doherty et al., 1992) which at 1.88 T had linewidths of 1.67 ± 0.25 Hz and 2.4 ± 0.14 Hz respectively (Malthouse et al., 1989) and spin-lattice relaxation times of 0.46 ± 0.08 s and 0.48 ± 0.02 s respectively (J. P. G. Malthouse, R. Motherway and S. G. Mayhew, unpublished work). These linewidths and spin-lattice relaxation times are in reasonable agreement with those predicted for thiocyanate carbons in a globular protein of M_r 15000 (Table 4). Therefore we hope that Table 4 can be used to provide reasonable estimates of the linewidths and spin-lattice relaxation times of thiocyanate carbons in globular proteins with M_r values of 10000–400000 at

magnetic field strengths of 1.88, 4.7, 6.34, 11.74 and 14.1 T. Such estimates should provide a guide to the optimal acquisition parameters (Malthouse, 1986) for observing thiocyanate carbons in most globular proteins (M_r 10000–400000) at different magnetic field strengths (1.88–14.1 T).

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