
Rossi-Fanelli, A., and Antonini, E. (1957), Biochimica 22, 335.

Determinant of Rotational Mobilities of Backbone and Side-Chain Carbons of Poly(γ-benzyl L-glutamate) in the Helical and Random-Coil States from Measurements of Carbon-13 Relaxation Times and Nuclear Overhauser Enhancements†

Adam Allerhand* and Eric Oldfield

ABSTRACT: We have determined the 13C spin-lattice relaxation times (T1) of the various types of carbons in helical and random-coil poly(γ-benzyl L-glutamate) ((BzlGlu)ₙ) samples of molecular weight 7,000, 17,000, and 46,000 in CDCl₃-CF₃-COOH mixtures, by means of proton-decoupled natural-abundance 13C partially relaxed Fourier transform nuclear magnetic resonance (nmr) spectra at 14.2 K. We have also measured the nuclear Overhauser enhancement (NOE) of the various carbons by means of integrated intensities in decoupled and undecoupled 13C spectra. The T1 values of Cα change by less than a factor of 2 when going from helical to random-coil (BzlGlu)ₙ. However, the NOE changes from about 1.1 (10% intensity enhancement) to more than 2 (100% intensity enhancement). The very low NOE for the α-carbons of helical (BzlGlu)ₙ is evidence for a rotational correlation time too slow to satisfy the extreme narrowing condition. The combination of T1 and NOE values yields effective rotational correlation times (τₑff) for Cα of 24–32 ns for the helix and only about 0.8 ns for the random coil. These results suggest that τₑff of Cα of the helical species is dominated by overall rotation with little or no contribution from segmental motion. When going to the random-coil species, τₑff of Cα decreases by a factor of about 30 as a result of the onset of rapid segmental motion. The α-carbon T1 values of helix and coil differ only slightly because of their particular positions on different sides of the minimum in the functional relation between the spin–lattice relaxation time and the correlation time. In both the helical and random-coil molecules, there is a progressive increase in T1 of the protonated carbons of the side chains when going away from the polymer backbone, as a result of fast internal rotations. In the helical molecules, there is a large increase in NOE when going from Cα to Cβ, because τₑff acquires contributions from internal motions with rates that satisfy the extreme narrowing condition. In contrast, in random-coil (BzlGlu)ₙ, τₑff of Cα is already in the extreme narrowing limit, and changes in NOE along the side chains are small.

Proton nuclear magnetic resonance (nmr) has been used extensively for studying the helix–coil transition of homopolymers (Bovey, 1972; Bradbury et al., 1973). Recently, 13C nmr has also been used for this purpose (Paoletti et al., 1972; Boccalon et al., 1972). The usual approach has been to observe chemical shift variations between the helical and random-coil species. However, theoretical calculations of differences in chemical shifts between helical and random-coil environments would be extremely difficult. Thus, the observed chemical shifts must first be empirically assigned to the helical and random-coil environments on the basis of other measurements.

We show here that measurements of 13C spin–lattice relaxation times (T1), spin–spin relaxation times (T2), and nuclear Overhauser enhancements (NOE) of α-carbons provide a probe for studying transitions from rigid to flexible forms of a polymer without a priori knowledge about conformations. The 13C relaxation and NOE measurements should distinguish the relatively immobile Cα–Hα groups of a helical polypeptide from the mobile Cα–Hα groups of a random-coil polymer undergoing fast segmental motion. We present 13C T1, T2, and NOE measurements for the α-carbons of poly(γ-benzyl L-glutamate) ((BzlGlu)ₙ Figure 1) of various molecular...
weights. In addition, we have measured $T_1$, $T_2$, and NOE values of various side-chain carbons. These results should be useful in analyzing the more complex $^{13}$C spectra of proteins (Allerhand et al., 1970, 1971a, 1973; Glushko et al., 1972; Moon and Richards, 1972).

Experimental Section

Samples of (BzlGlu)$_n$ were purchased from the Research Products Division of Miles Laboratories, Kankakee, Ill., and had the following reported molecular weights: 6,970 (code 71-110A, lot GL90), 17,300 (code 71-110A, lot GL101), and $\sim$46,000 (code 71-110B, lot GL103) as determined by ultracentrifugation (mol wt 6970 and 17,000) and viscosity (mol wt 46,000). Trifluoroacetic acid was obtained from Matheson, Coleman & Bell, Norwood, Ohio, and deuteriochloroform (99.8% $^2$H) was purchased from Mallinkrodt Chemical Works, St. Louis, Mo.

The nmr spectrometer has been described (Allerhand et al., 1971b, 1972, 1973). All spectra were recorded on samples with natural-abundance $^{13}$C content, at 15.18 MHz (14.2 kG) in spinning samples tubes of 20-mm o.d. (Allerhand et al., 1972). All spectra were obtained at 40 $\pm$ 2°. For studies of the helical species, samples were 0.8 M $\gamma$-benzyl glutamate residue, in a deuteriochloroform-trifluoroacetic acid solvent mixture (97:3, v/v). For studies of the random-coil form, samples were 0.6 M in residue, in a deuteriochloroform-trifluoroacetic acid mixture (71:29, v/v).

Carbon-13 spin-lattice relaxation times were measured by means of partially relaxed Fourier transform (PRFT) spectra (Vold et al., 1968), as described previously (Allerhand et al., 1971b), with an estimated accuracy of $\pm$10% for narrow resonances and about $\pm$15% for broad ones. Spin-spin relaxation times were determined from measured line widths, corrected for instrumental broadening. The estimated accuracy is $\pm$25% for $C^\delta$ and $C^\gamma$ in helical (BzlGlu)$_n$, of mol wt 46,000 (where non-Lorentzian lines overlap) and for $C^\alpha$ in helical (BzlGlu)$_n$, of mol wt 17,000 and 46,000 (where the line shape is asymmetric). All other line widths were measured with an estimated accuracy of $\pm$15%. Nuclear Overhauser enhancements were determined as follows. Undecoupled and fully proton-decoupled spectra were acquired using recycle times equal to or greater than five times the longest pertinent $T_1$. Integrated intensities were obtained digitally. Coupled spectra were recorded by offsetting the proton-decoupling frequency 50-kHz off-resonance and switching off the noise modulation (Schaefer and Natusch, 1972). In spectra where poor base lines could cause inaccuracies in measurements of integrals, base-line corrections and integrations were performed in parts. Peaks used for calculation of coupled single-carbon intensities are given in the footnotes to Table II. The estimated errors are $\pm$10–20%.

Theoretical Considerations

It has been shown (Allerhand et al., 1971b) that because of the very short C–H bond length, the $^{13}$C relaxation of protonated carbons in large molecules is overwhelmingly dominated by dipole-dipole interactions with directly bonded hydrogens. This fact greatly simplifies the interpretation of $^{13}$C relaxation times of protonated carbons in large molecules, because one does not have to be concerned with contributions to the relaxation from dipolar interactions with non-bonded hydrogens, and from nondipolar relaxation mechanisms (Lylera and Grant, 1972). Equations have been given elsewhere (Doddrell et al., 1972) that relate the measured $^{13}$C $T_1$ and $T_2$ values, under conditions of complete proton decoupling, with the rotational correlation times of the pertinent C–H vectors. In Figure 2 we show theoretical $^{13}$C $T_1$ and $T_2$ values (at 14.2 kG) of a carbon with $N$ directly-attached hydrogens, which is part of a rigid molecule rotating isotropically with a correlation time $\tau_R$. From the measured $^{13}$C $T_1$ and $T_2$ values one can, in principle, extract rotational correlation times. In practice, $T_2$ values are difficult to measure. Moreover, even though accurate $T_1$ values of resolved resonances can be obtained, the interpretation can be clouded by the fact that $T_1$ is not a monotonic function of the rate of rotation.

As an application of Figure 2, if a methine carbon has a $T_1$ of 50 msec, $\tau_R$ is either 1.1 or 37 msec. The corresponding $T_2$ values are 49 and 5.9 msec, respectively. In practice, it is desirable to obtain first the two solutions for $\tau_R$ from $T_1$ measurements, and then to choose between the two values on the basis of the usually less accurate $T_2$ and NOE values (see below). We follow this practice here.

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FIGURE 4: Natural-abundance $^{13}$C Fourier transform nmr spectra of poly($\gamma$-benzyl L-glutamate) of molecular weight 7,000 in CDCl$_3$–CF$_3$COOH solvent mixtures at 40°, recorded at 15.18 MHz with 4096 points in the time domain and 250-ppm spectral widths. Vertical presentations of spectra and their integrals vary arbitrarily. Integrated intensities were measured digitally and normalized for variations in number of accumulations per spectrum.

(A) Fully proton-decoupled spectrum of 0.8 M (in residue) (BzlGln), in 97% CDCl$_3$–3% CF$_3$COOH (v/v) after 2048 accumulations with a recycle time of 15.8 sec. Assignments are of Paolillo et al. (1972). Carbon designations are those of Figure 1. (B) Same as part A, but without proton-decoupling, after 4096 accumulations. (C) Fully proton-decoupled spectrum of 0.6 M (in residue) (BzlGln), in 71% CDCl$_3$–29% CF$_3$COOH (v/v) after 256 accumulations with a recycle time of 21.1 sec. (D) Same as part C, but without proton decoupling, after 2048 accumulations.

The nuclear Overhauser enhancement (NOE) may also be useful for determining rotational mobility of C-H groups. The NOE in proton-decoupled spectra is the increase in integrated intensity of the $^{13}$C resonance of a given carbon when going from the undecoupled spectrum to the proton-decoupled spectrum (Kuhlmann and Grant, 1968; Kuhlmann et al., 1970). We define the NOE as the ratio of the integrated intensities in the decoupled and undecoupled spectra (Kuhlmann et al., 1970). In Figure 3 we show theoretical NOE values as a function of $\tau_R$ for a carbon undergoing $^{13}$C–H dipolar relaxation at 14.2 kG. As in Figure 2, we assume here that the rotational motion is isotropic and that the pertinent C-H vector does not have internal motions. Unlike $T_1$ and $T_2$, the NOE is independent of the number of directly attached hydrogens (Kuhlmann et al., 1970). The results of Figure 3 apply even for a nonprotonated carbon, but only if it is undergoing purely $^{13}$C–H dipolar relaxation (Lyerla and Grant, 1972). This is the case for nonprotonated carbons in many large molecules (Allerhand et al., 1971b).

Figure 3 predicts the maximum NOE of 2.988 when $^{13}$C relaxation is purely dipolar and when the rotational motion is sufficiently fast to satisfy the “extreme narrowing” condition (Kuhlmann et al., 1970)

$$4\pi(v_1 + v_2)\tau_R^2 \ll 1$$

Here $v_1$ and $v_2$ are the resonance frequencies, in hertz, of $^1$H and $^{13}$C, respectively. Even when the relaxation is purely dipolar, less than the full NOE is expected when eq 1 is not satisfied. In the limit of very slow rotation, defined by

$$4\pi(v_1 + v_2)\tau_R^2 \gg 1$$

the NOE will only be 1.153 (Kuhlmann et al., 1970).

Experimental Results and Discussion

In Figure 4 we show proton-decoupled and undecoupled $^{13}$C spectra and the corresponding integrals for helical (in CDCl$_3$ with 3% CF$_3$COOH) and random coil (in CDCl$_3$ with 29% CF$_3$COOH) poly($\gamma$-benzyl L-glutamate) at 40°.

TABLE I: $^{13}$C Spin–Lattice Relaxation Times of Helical (in CDCl$_3$ with 3% CF$_3$COOH) and Random Coil (in CDCl$_3$ with 29% CF$_3$COOH) Poly($\gamma$-benzyl L-glutamate) at 40°.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Helix (msec)</th>
<th>Coi (msec)</th>
<th>Helix (msec)</th>
<th>Coi (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>35 5</td>
<td>52</td>
<td>39</td>
<td>70</td>
</tr>
<tr>
<td>$\beta$</td>
<td>65</td>
<td>85</td>
<td>71</td>
<td>107</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>108</td>
<td>139</td>
<td>127</td>
<td>159</td>
</tr>
<tr>
<td>Bzl</td>
<td>264</td>
<td>419</td>
<td>279</td>
<td>480</td>
</tr>
</tbody>
</table>

Carbon designations of Figure 1. $N$ is the number of directly attached hydrogens. Estimated accuracy of $T_1$ values is ±10 to ±15% (see Experimental Section). Molecular weight.
Table II: Nuclear Overhauser Enhancements of Protonated Carbons of Helical and Random-Coil Poly(γ-benzyl L-glutamate) at 40°.

<table>
<thead>
<tr>
<th>Carbona</th>
<th>Helixb</th>
<th>Coilc</th>
<th>Helixd</th>
<th>Coile</th>
<th>Helixf</th>
<th>Coilef</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>1.0</td>
<td>2.3</td>
<td>1.2</td>
<td>2.6</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td>β</td>
<td>1.6</td>
<td>2.4</td>
<td>2.0</td>
<td>2.8</td>
<td>1.9</td>
<td>2.7</td>
</tr>
<tr>
<td>γ</td>
<td>2.1</td>
<td>2.8</td>
<td>2.4</td>
<td>3.1</td>
<td>2.2</td>
<td>3.1</td>
</tr>
<tr>
<td>Bzl</td>
<td>2.0</td>
<td>2.7</td>
<td>1.9</td>
<td>2.5</td>
<td>1.9</td>
<td>2.6</td>
</tr>
<tr>
<td>2-6</td>
<td>2.6</td>
<td>2.9</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a Carbon designations of Figure 1. b Estimated accuracy ranges from ±10 to ±20%, depending on the extent of peak overlap (see Figures 4 and 5). c Molecular weight. d Average of all measurable integrated intensities in the undecoupled spectrum was used as the single-carbon undecoupled intensity. e Average of saturated-carbon integrated intensities in the undecoupled spectrum was used as the single-carbon undecoupled intensity. f Average of integrated intensities of Cβ, Cγ, and Cδ in the undecoupled spectrum was used as the single-carbon undecoupled intensity.

CDCl3 with 3% CF3COOH) and random-coil (in CDCl3 with 29% CF3COOH) poly(γ-benzyl L-glutamate) of mol wt 7000. Chloroform is a commonly used "helix-promoting" solvent for polypeptides. The addition of small amounts of an organic acid prevents aggregation (Doty et al., 1956). The presence of 3% CF3COOH is not sufficient to destroy the helical form of (BzlGlu)n (Marlborough et al., 1965). When the concentration of CF3COOH is raised to 29%, (BzlGlu)n exists in the random-coil form (Marlborough et al., 1965). The spectra of (BzlGlu)n of higher molecular weight have chemical shifts nearly identical with those of Figure 4. However, the resonances of Cγ, Cδ, and Cγ of the helical molecules get broader as the molecular weight is raised (Figure 5). In contrast, the line widths in the random-coil state are practically independent of molecular weight (Figure 5). A further discussion of these effects is given below.

In Table I we show 13C relaxation times of protonated carbons (excluding aromatic ones), measured by means of PRFT spectra (Vold et al., 1968; Allerhand et al., 1971b). In Figure 6 we show portions of a typical series of PRFT spectra.

α-Carbons. The T1 values of the α-carbons increase by only about 50% when going from helix to coil. The T1 curve of Figure 2 indicates that such a small change does not necessarily represent a small change in effective rotational correlation time. In fact, an examination of the α-carbon NOE values (Table II) and line widths (Tables III-IV) clearly shows that the effective correlation times of Cα in the helix and the random coil are on different sides of the T1 curve. It should be noted that Figures 2 and 3 are only approximately valid here. Because of their rod-like shape (Doty et al., 1956), helical (BzlGlu)n molecules may rotate anisotropically. Moreover, the equations used here can only be approximately valid for the τ_d of the segmental motions of the random-coil backbone.

We believe that our observation of a negligible NOE for the α-carbons of helical (BzlGlu)n is the first experimental verification of the theoretical prediction (Kühleman et al., 1965).
mental motions, the correlation times of some of which do not
in terms of I3C-’H dipolar relaxation dominated by seg-
polymers (Schaefer and Natusch, 1972). It has been explained
satisfy eq 1. However, our NOE values are not sufficiently
lish that the a-carbons of random-coil (BzlGlu), indeed have
not have the maximum NOE of 2.988 (Table
independent of molecular weight. All these observations are
only consistent with a drastic decrease in Teff, when going
segmental motion.

...linewidths decrease from about 28-48
significantly less than the maximum NOE of 2.988.
that are faster than the overall reorientation (see below).
Correlation times of 8-16 nsec have been reported for the
overall rotation of some aqueous proteins of mol wt 14,000-
15,000 (Irwin and Churchich, 1971; Dubin et al., 1971;
Rawitch, 1972; Norris and Crespi, 1972; Haugland and
Stryer, 1967; Shimshick and McConnell, 1972). If we assume
that similar overall rotational correlation times apply here,
our results suggest that τ_{eff} for C" of helical (BzlGlu),,
is determined by the overall rotational motion, with little or no
contribution from segmental motion. In contrast
τ_{eff} for C" of random-coil (BzlGlu), is dominated by segmental motions
that are faster than the overall reorientation (see below).

When going from the helical to the random-coil conforma-
tion, the change in T_1 of the a-carbons is small, while the
NOE increases from about 1.1 to about 2.5 (Table II), and
the linewidths decrease from about 28-48 Hz to less than 12 Hz
(Figure 5 and Tables III-IV). Moreover, τ_{eff} for the C'-H\_m groups
of random-coil (BzlGlu), is about 0.8 nsec (Table IV)
independent of molecular weight. All these observations are
only consistent with a drastic increase in τ_{eff}, when going
from helix to random coil, as a result of the onset of fast
segmental motion.

It appears that the a-carbons of random-coil (BzlGlu), do
not have the maximum NOE of 2.988 (Table II), even though
a τ_{eff} of 0.8 nsec satisfies the extreme narrowing condition
(eq 1). Such a result has been reported for other random-coil
polymers (Schaefer and Natusch, 1972). It has been explained
in terms of C'-H\_D dipolar relaxation dominated by seg-
mental motions, the correlation times of some of which do not
satisfy eq 1. However, our NOE values are not sufficiently
accurate (see Experimental Section) to unambiguously
establish that the a-carbons of random-coil (BzlGlu), indeed have
significantly less than the maximum NOE of 2.988.

It should be noted that measurements of a-carbon T_1, T_2,
and NOE can be used, as we have done above, to detect the
transition from a rigid to a flexible polymer backbone. These measurements do not yield information about the actual con-
formations.

**Carbons of the Side Chain.** In both the helical and random-
coil molecules, as one goes away from the polymer backbone,
there is a progressive increase in T_1 (Table I). In the helical
molecules, there is also a large increase in NOE when going
from C_\alpha to C_\beta, and additional increases when going to C_\gamma
and beyond (Table II). This is because in the helical species,
τ_{eff} of C_\alpha satisfies eq 2, but contributions from internal rota-
tions shorten τ_{eff} of side-chain carbons in the direction of the
extreme narrowing limit (eq 1). Peak overlaps in the spectra
of helical (BzlGlu), limited the accuracy of our NOE values
(see Experimental Section) and prevented the observation of
details of the change in NOE along the side chains. Never-
theless, there is an unambiguous change from practically no
NOE at C_\alpha to approximately the theoretical maximum at the
protonated aromatic carbons. In contrast, the change in NOE
along the side chains of the random-coil species is small
(Table II) because in this case τ_{eff} of C_\alpha is already close to the
extreme narrowing limit.

The resonances get progressively narrower, and the broad-
ening effect of increased molecular weight gets less significant,
as one goes further away from the polymer backbone of the
helical species (Figure 5). Note that C_\alpha, C_\beta, and C_\gamma are
methylene carbons while C_\alpha is a methylene carbon, and thus
the line widths of C_\alpha, C_\beta, and C_\gamma should be halved before com-
paring with the line width of C_\alpha (Allerhand et al., 1971b).
The resonances of random-coil (BzlGlu), are considerably
narrower than those of the helical species. The difference be-
comes progressively smaller as one goes away from the back-
bone. The resonances of the random-coil species are not
greatly broadened by increases in the molecular weight (Figure
5).

The above results can be used to reach the following con-
clusions. (1) For helical (BzlGlu),, the correlation time of
the a-carbons satisfies eq 2, while τ_{eff} of the aromatic carbons
satisfies eq 1. (2) Internal rotations of the C--H vectors of the
aliphatic side-chain carbons are sufficiently fast to contribute
to τ_{eff}, which gets progressively shorter as one goes away
from the backbone in both the helical and random-coil species.
(3) The effective correlation times of all carbons of
random-coil (BzlGlu), approach values that satisfy eq 1.

If we assume that overall rotation of helical (BzlGlu), is
isotropic (with τ_R given in Table III), then the T_1 value of the
\beta-carbons can be used to compute the correlation time for
internal rotation (τ_\alpha) about the C_\alpha-\beta bond. Details have
been given elsewhere (Allerhand et al., 1971b; Doddrill et
al., 1972). The resulting value of τ_\alpha is about 0.25 nsec, inde-
pendent of molecular weight. The model for internal rotation
and definition of τ_\alpha used here are those used by Doddrill
et al. (1972).

**Implications for 13^C\textit{NMR of Proteins.** The trends in T_1,
the line widths, and the NOE of helical (BzlGlu), that we have
presented here are helpful in making approximate estimates of
the corresponding values of similar carbons in native pro-
teins. One should bear in mind, however, that there are struc-
tural differences between the system we have studied and
naturally occurring residues of proteins in water. One
generalization that can be made with confidence is that when rec-
dording proton-decoupled 13^C spectra of proteins in their
native conformation, one should expect a negligible NOE for
a-carbons and other carbons which have a value of τ_{eff} that

<table>
<thead>
<tr>
<th>TABLE IV: Comparison of Experimental Line Widths and NOE Values of the α-Carbons of Random-Coil Poly(γ-benzyl L-glutamate) with the Theoretical Values Consistent with Measured Spin–Lattice Relaxation Times.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured T1 (msec)^b</td>
</tr>
<tr>
<td>Computed τ_R (nsec)^e</td>
</tr>
<tr>
<td>Calculated NOE^e</td>
</tr>
<tr>
<td>Experimental NOE/</td>
</tr>
<tr>
<td>Calculated W (Hz)^d</td>
</tr>
<tr>
<td>Experimental W (Hz)^d</td>
</tr>
<tr>
<td>7000^b</td>
</tr>
<tr>
<td>52</td>
</tr>
<tr>
<td>1.0 (39)</td>
</tr>
<tr>
<td>2.8 (1.2)</td>
</tr>
<tr>
<td>2.3</td>
</tr>
<tr>
<td>6.2 (56)</td>
</tr>
<tr>
<td>≤8</td>
</tr>
</tbody>
</table>

^a Calculated values inconsistent with experimental observations are given in parentheses. See text. ^b Molecular weight. ^c From Table I. ^d Computed from the measured T1 values using the theoretical T1 curve of Figure 2. Each T1 yields two possible τ_R values. ^e Computed from Figure 3 for each τ_R consistent with the experimental T1. ^f From Table II. ^g Computed line width, from the theoretical T1 curve of Figure 2 (W = 1/πT1), for each τ_R consistent with the experimental T1.

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satisfies eq 2. On the other hand, side-chain carbons with several degrees of fast internal rotation may have a significant NOE. It is important to consider these variations in the NOE when estimating the minimum concentration of protein required to observe single-carbon resonances in natural-abundance $^{13}$C spectra (Allerhand et al., 1973).

References


