Carbon-13 Nuclear Magnetic Relaxation in ^{13}C Uniformly Enriched Glycine and Aspartic Acid

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Abstract: ^{13}C longitudinal relaxation times (T_1) and nuclear Overhauser enhancement factors (η) have been measured in glycine and aspartic acid uniformly enriched with carbon-13. The enrichment (85%) allows simultaneous measurements on isotopomers containing a single carbon-13 and on those involving two or more adjacent carbons-13. From data relative to the carbon of the carboxylic groups, one would expect to extract the carbon–carbon dipolar spectral density (for carbons bearing protons, the carbon–carbon dipolar contribution is masked by the much larger carbon–proton interaction). It is observed, however, that although T_1 can be interpreted on the basis of Solomon equations, the latter yield η values in disagreement with experimental results. The problem is solved by including in Solomon equations an interference term (cross correlation spectral density) between chemical shift anisotropy and carbon–carbon dipolar interaction. The relevant theory is given where it is demonstrated that the interference term affects measurements performed only under proton broadband decoupling and hence contribute to the nuclear Overhauser effect. It is, however, demonstrated (theoretically and experimentally), by measurements carried out with decoupling gated on only during the free induction decay acquisitions, that this term does not need to be taken into account when considering the major part of the recovery curves (i.e., excluding its very beginning) resulting from the classical inversion recovery experiment. Finally, measurements performed on molecules uniformly enriched with carbon-13 and deuterium simultaneously further confirm the validity and the consistency of all the results. Dipolar spectral densities (carbon–proton and carbon–carbon) deduced from the whole set of experimental data provide effective correlation times which are discussed in terms of overall reorientation and internal rotations.

Introduction

Amino acids uniformly enriched with carbon-13[1] or both carbon-13 and nitrogen-15[2-4] have been hereto investigated mainly with the aim of determining the coupling constants in relation with conformation.[5-7] These important parameters have allowed a better understanding of the structural properties of peptides.[1,8]

Most of the dynamical information on these molecules has been obtained through carbon-13[9,10] and nitrogen-15[11,12] longitudinal relaxation times at the natural abundance level. Such measurements essentially probe the motion of the CH and NH vectors and consequently cannot provide a complete description of the molecular motion because they do not allow a separation between the overall and the internal contributions. In this respect, information about the reorientation around the C–C bonds could be helpful, and this in principle could be deduced from the carbon relaxation study of molecules uniformly enriched with carbon-13. Curiously, such measurements are scarce[13] though the enrichment could allow them to be performed at lower concentrations (10 times easily). As a matter of fact, the simplest amino acid, glycine, uniformly enriched with carbon-13, presents a serious interpretative problem since the carbonyl relaxation data (longitudinal relaxation time T_1 and nuclear Overhauser enhancements (NOE)), as compared with those of normal glycine, not only resist simple analysis performed with generalized Solomon equations, but cannot even be explained on the basis of a more general treatment involving dipolar cross-correlation terms. One of the purposes of this paper is to demonstrate that such a difficulty essentially concerns the nuclear Overhauser enhancements and can be solved by introducing an interference term arising from the dipolar and chemical shift anisotropy relaxation mechanisms. Interpretation of the experimental data will be carried out by means of extended Solomon equations including the latter effect. Examples of both glycine and aspartic acid will be considered with the purpose of delimiting the possibilities of the method in providing the spectral densities relative to carbon–carbon bonds. In addition, the results concerning carbon-13 enriched molecules, denoted in the following by [^{13}C], will be checked against molecules doubly enriched in carbon-13 and deuterium, denoted [^{13}C,^{2}H]. A study of [^{13}C,^{2}H]glycine has been previously published.[14] [^{13}C,^{2}H]Aspartic acid is investigated in the present paper.

Experimental Section

Uniformly enriched aspartic acid and glycine (85% ^{13}C) and aspartic acid (85% ^{13}C, 97% ^{2}H) were obtained by biosynthesis as described in a previous paper.[15] The 85% ^{13}C enrichment allows the simultaneous observation of isotopomers containing two or more carbon-13 atoms per molecule and those containing only one carbon-13 atom per molecule. Samples were prepared by dissolving aspartic acid or glycine in $^{2}H_2O$ (CEA), both treated according to previously described procedures which allow the elimination of paramagnetic impurities.[10-12,16] Oxygen was removed by bubbling argon into the solution contained in a 10-mm o.d. tube. The pH of the solution of aspartic acid (concentration 0.13 M) was

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Table I. Carbon-13 Longitudinal Relaxation Times (T_1, in seconds) and Nuclear Overhauser Enhancement Factors (η) of Normal (s: isotopomers containing a single carbon-13) and Enriched (d: isotopomers containing two carbons-13) Glycine in D_2O (concentration, 0.28 M; zwitterionic form) with the Labeling Indicated by $^-OOC_O-C_\alpha H_2-ND_3^+$

decoupling mode (T_1 measurements)	$^{13}C_O$	$^{13}C_\alpha$
normal	s $T_1 = 80.0 \pm 3.0$, $\eta = 0.87 \pm 0.08$	s $T_1 = 4.2 \pm 0.3$, $\eta = 1.61 \pm 0.40$
	d $T_1 = 61.0 \pm 2.0$, $\eta = 0.72 \pm 0.06$	d $T_1 = 4.5 \pm 0.3$, $\eta = 1.83 \pm 0.15$
gated	s $T_1 = 75.5 \pm 5.0$	
	d $T_1 = 60.0 \pm 3.0$	

about 1. Glycine in its zwitterionic form was studied at a concentration of 0.28 M.

Carbon-13 relaxation time measurements of the (^{13}C,^2H) doubly enriched aspartic acid molecule were performed at ambient temperature (ca. 25 °C) at two different frequencies: 25.2 MHz on a Varian XL-100 WG-12 spectrometer and 100.6 MHz on a Bruker WH-400 spectrometer. The classical inversion recovery pulse sequence [180°–τ–90°–acquisition–T]$_N$ was used taking the waiting time $T \simeq$ 8–10 times the longest T_1 since we are dealing with coupled systems and ca. 30 different τ values for each T_1 to be determined. A spectral window of 1000 Hz was used and the 90° pulse had a duration of 15 μs at 25.2 MHz and 22 μs at 100 MHz. Because of the complicated structure of the C^α and C^β multiplets, no reliable relaxation time could be obtained for these two carbon atoms in this molecule.

Carbon-13 relaxation measurements on the ^{13}C-enriched aspartic acid and glycine were performed at 22.6 MHz with the help of a homemade spectrometer.[17,18] A spectral window of 1200 Hz was used; therefore two separate experiments were carried out, one for the carbon atoms of the carboxyl groups and the other for the $C_\alpha H$ and/or the $C_\beta H_2$ carbons. The 90° pulse had duration of 14 μs. Inversion–recovery sequences were again adopted with the above-indicated conditions, and two types of longitudinal relaxation experiments were performed: one with continuous proton irradiation, the other with the decoupling gated on only during the free induction decay (FID) acquisition. The NOE factor determinations were carried out by the gated decoupling method with the following refinements: instead of gating off the decoupler, the irradiation frequency was shifted by an amount of 100 kHz and the decoupler simultaneously switched from the modulation to the CW mode. In addition to that, FID's corresponding to continuous proton irradiation and to gated proton irradiation respectively were alternatively acquired and stored in separate memory blocks so that instrumental drifts affect similarly the spectra obtained with and without NOE.

The experimental data are presented in Tables I and II. Within the experimental uncertainities, the observed recovery curves are all simple exponential. Contrary to the case of [^{13}C,^2H]glycine,[14] no biexponential character could be detected in the recovery curves of the magnetization of the two carbonyl carbons C_O and C_γ of [^{13}C,^2H]aspartic acid. Concerning the NOE factors of C_α in glycine and C_α, C_β in aspartic acid, they are in all cases close to their maximum value ($\eta = 1.98$). This maximum value will be assumed in the following. The slight decreases observed for C_α and C_β in aspartic acid isotopmers containing directly bonded carbon-13 is attributed to the effect of the small chemical shift difference between these two carbons.

Theory

We shall consider a spin system involving two carbons A and M, A denoting the carbon of the acidic function and M a carbon directly bonded to A and bearing one or two protons (denoted by X). In fact, this corresponds exactly to the [^{13}C]glycine molecule; regarding aspartic acid, a decomposition in two such systems can be considered as well be discussed below. We shall assume throughout extreme narrowing. Let us define R_1^A and R_1^M by:

$$R_1^A = R^A + 2\sigma^{AM} + 2\sum_X \sigma^{AX}$$
$$R_1^M = R^M + 2\sigma^{AM} + 2\sum_X \sigma^{MX} \quad (1)$$

Intramolecular dipolar contributions are given with the *notations* of the so-called cross-relaxation terms: σ^{AM}, σ^{AX}, and σ^{MX}, while R^A and R^M are associated with the other relaxation mechanisms treated as random fields acting at nuclei A and M, respectively. If proton transitions are strongly irradiated, the classical Solomon equations[19] can be written in the following form:

$$\frac{d}{dt}\langle I_z^A\rangle = -R_1^A(\langle I_z^A\rangle - 1) - \sigma^{AM}(\langle I_z^M\rangle - 1) - \frac{\gamma_H}{\gamma_C}\sum_X \sigma^{AX} \quad (2a)$$

$$\frac{d}{dt}\langle I_z^M\rangle = -R_1^M(\langle I_z^M\rangle - 1) - \sigma^{AM}(\langle I_z^A\rangle - 1) - \frac{\gamma_H}{\gamma_C}\sum_X \sigma^{MX} \quad (2b)$$

These equations represent the time evolution of longitudinal magnetizations $\langle I_z^A\rangle$ and $\langle I_z^M\rangle$ associated with the two carbons-13 which are normalized to unity at thermal equilibrium. Equations 2 are valid provided that (i) nuclei A and M are weakly coupled and (ii) all cross-correlation spectral densities are neglected. The first condition is verified here; the second one is not a priori valid since the importance of these cross-correlation terms has been demonstrated in numerous examples.[20,21] However, recent calculations including simulations of AM{X} and AM{X_2}[22,23] spin systems relevant to the actual situation of enriched amino acids have been performed on the basis of only two relaxation mechanisms: intramolecular dipolar and random interactions. They show that *dipolar* cross-correlation spectral densities do not play any significant role (i) on the main part of the recovery curves in the case of conventional inversion recovery experiments (it should be mentioned that this would not necessarily be true if other spin preparations, e.g., soft pulses, were employed) and (ii) on the NOE factor η. On the other hand, it proved impossible to analyze completely experimental data by means of eq 2 essentially because the NOE values were found to be inconsistent with T_1 values. Therefore, it is necessary to consider cross-correlation spectral densities other than dipolar. In order to determine those mechanisms which should have to be taken into account, we start with the more general relation yielding the time evolution of any observable $\langle \hat{G}\rangle$ in the extreme narrowing limit:[24]

$$\frac{d}{dt}\langle \hat{G}(t)\rangle = -\sum_{r,r'} J_{r,r'} \sum_{q=-2}^{q=+2} (-1)^q \,\mathrm{Tr}\{[\hat{A}_r^{-q},[\hat{A}_{r'}^{q},\hat{G}]](\hat{\sigma} - \hat{\sigma}^{eq})\} \quad (3)$$

In this relation, $\hat{\sigma}$ is the density operator, the subscripts r and r' refer to relaxation mechanisms giving rise through their spatial coordinate function to the spectral densities $J_{r,r'}$ while $\hat{A}_r^{-q}$ and $\hat{A}_{r'}^{q}$, are the relevant spin operators. Cross-correlation spectral densities ($r \neq r'$) generally refer to the same type of relaxation mechanism (e.g., intramolecular dipolar interactions). However, cross-correlation spectral densities involving two different types of relaxation mechanisms must be considered if the relevant space coordinate functions are expressed with spherical harmonics of the same order. This is especially the case for intramolecular dipolar interaction and chemical shift anisotropy; the importance of such interference terms has been recognized for some time[25,26] and has been recently outlined in a study of [^{13}C,^2H]glycine.[14] Now, the time evolution equation (3) is strictly applicable in the absence of any radiofrequency field. In fact, for systems such those studied here, it can be shown that it is still valid for quantites involving C-13 when proton transitions are strongly irradiated.[27] On the other hand, the density matrix, expressed in the eigenstate basis, is assumed to remain diagonal; this means that eventual

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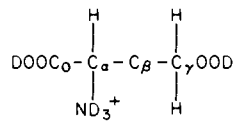
Table II. Carbon-13 Longitudinal Relaxation Times (T_1, in seconds) and Nuclear Overhauser Enhancement Factors (η) in Carbon-13 Enriched [^{13}C] and Carbon-13, Deuterium Doubly Enriched [^{13}C,^{2}H]Aspartic Acid in D_2O (concentration, 0.13 M; pH ≃ 1) with Labeling Shown in Table[a]

type of molecule and C-13 resonance frequency	decoupling mode (T_1 measurements)	$^{13}C_O$	$^{13}C_\gamma$	$^{13}C_\alpha$	$^{13}C_\beta$
[^{13}C]	normal	s $T_1 = 33.8 \pm 1.4$, $\eta = 1.13 \pm 0.15$	s $T_1 = 33.0 \pm 2.0$, $\eta = 1.18 \pm 0.09$	s $T_1 = 2.04 \pm 0.20$, $\eta = 1.85 \pm 0.15$	s $T_1 = 1.0 \pm 0.1$, $\eta = 1.95 \pm 0.15$
22.6 MHz		d $T_1 = 25.4 \pm 1.2$, $\eta = 0.85 \pm 0.05$	d $T_1 = 26.8 \pm 1.0$, $\eta = 1.02 \pm 0.06$	d $T_1 = 2.00 \pm 0.15$, $\eta = 1.64 \pm 0.10$	d $T_1 = 1.16 \pm 0.10$, $\eta = 1.68 \pm 0.08$
	gated	s $T_1 = 30.9 \pm 1.4$ d $T_1 = 23.5 \pm 1.3$	s $T_1 = 31.4 \pm 1.5$ d $T_1 = 23.1 \pm 0.9$		
[^{13}C,^{2}H]	nondecoupled	s $T_1 = 75 \pm 3$	s $T_1 = 79 \pm 3$		
25 MHz		d $T_1 = 48 \pm 2$	d $T_1 = 46 \pm 2$		
100 MHz		s $T_1 = 20 \pm 1$	s $T_1 = 24 \pm 1$		
		d $T_1 = 18 \pm 1$	d $T_1 = 21 \pm 1$		



[a] s indicates isotopomers containing carbon(s)-13 nondirectly bonded to other carbon(s)-13, and d indicates isotopomers containing two vicinal carbons-13.

transverse components created by proton irradiation are neglected. Equality will be further assumed between $\hat{\sigma}$ diagonal elements (level populations) which are associated with levels connected by irradiated proton transitions.

We shall retain three relaxation mechanisms: intramolecular dipolar interaction, random fields acting at each nucleus, and chemical shift anisotropy (CSA) of nucleus A (which will be here of the order of 200 ppm). All other mechanisms will be either neglected or supposed to behave as random fields. We shall apply (3) to $\langle I_z^A \rangle$ and $\langle I_z^M \rangle$. The relevant calculations lead to eq 2 plus terms arising from the CSA mechanism and all cross-correlation spectral densities. The CSA autocorrelation spectral density $J^{AA}_{(CSA)}$ appears only in R_1^A which becomes:

$$R_1^A = 2\sigma^{AM} + 2\sum_X \sigma^{AX} + R^A + 4J^{AA}_{(CSA)} \quad (4)$$

with

$$J^{AA}_{(CSA)} = \frac{1}{30}\gamma_A{}^2 B_0{}^2 (\Delta\tilde{\sigma}^A)^2 \tau^{(A)}_{(CSA)} \quad (5)$$

B_0 is the applied static magnetic field. The shielding anisotropy is expressed by:

$$(\Delta\tilde{\sigma}^A) = \tilde{\sigma}^A_{zz} - \frac{1}{2}(\tilde{\sigma}^A_{xx} + \tilde{\sigma}^A_{yy}) \quad (6)$$

x, y, and z denote here the shielding tensor principal axes. Their reorientation defines the effective correlation time $\tau^A_{(CSA)}$.

Referring to eq 3, σ^{AM}, σ^{AX}, σ^{MX}, R^A, and R^M can be expressed in terms of spectral densities; one has, for instance:

$$\sigma^{AM} = \frac{5}{3}J^{AMAM} = \frac{1}{2}\frac{\gamma_A{}^2\gamma_M{}^2\hbar^2}{\langle r_{AM}{}^6 \rangle}\tau^{AM}_{eff} \quad (7)$$

τ^{AM}_{eff} is the correlation time associated with the motion of the vector joining the two vectors A and M; R^A is twice the autocorrelation spectral density originating from the random fields acting on A. Among the cross-correlation spectral densities, we shall neglect in $d\langle I_z^A \rangle/dt$ and $d\langle I_z^M \rangle/dt$ all dipolar terms, following arguments given above. On the contrary, we shall take into account cross terms (interference terms) between intramolecular dipolar interactions and chemical shift anisotropy of nucleus A.

Evaluating all commutators implied by eq 3 leads to an impressive number of new average values of spin operators; fortunately, most of them are identically zero at thermal equilibrium and under strong proton irradiation by virtue of the above-indicated properties of the density matrix. A single average value remains: $\langle I_z^A I_z^M \rangle$; (2a) is therefore extended to:

$$\frac{d}{dt}\langle I_z^A \rangle = -R_1^A(\langle I_z^A \rangle - 1) - \sigma^{AM}(\langle I_z^M \rangle - 1) + \frac{\gamma_H}{\gamma_c}\sum_X \sigma^{AX} - 8J^{A,AM}\langle I_z^A I_z^M \rangle \quad (8)$$

whereas (2b) is unchanged. $J^{A,AM}$ is a cross-correlation spectral density between chemical shift anisotropy of nucleus A and the AM dipolar interaction:

$$J^{A,AM} = \frac{1}{10}\frac{\gamma_c{}^3\hbar B_0}{\langle r_{AM}{}^3 \rangle}(\Delta\tilde{\sigma}^A)\tau^{A,AM}_{CSA,dip} \quad (9)$$

$\tau^{A,AM}_{CSA,dip}$ is again an effective correlation time depending on the reorientation of the vector r_{AM} and of the principal axes of the C_A shielding tensor. By evaluating $\langle I_z^A I_z^M \rangle$with the help of the density matrix elements, it can be shown for AM{X} or AM{X_2} spin sysetms that (i) $\langle I_z^A I_z^M \rangle$ *is zero at thermal equilibrium* and (ii) $\langle I_z^A I_z^M \rangle$ *is nonzero under irradiation of the X transitions*. Therefore, this quantity does not contribute to the initial recovery rate of the considered carbons when proton transitions are not irradiated nor, of course, to the relaxation in molecules containing a single carbon-13 nucleus (the AM dipolar interaction is absent). It can, however, significantly contribute to the carbon relaxation and nuclear Overhauser effect in the enriched molecule under broadband decoupling. This latter feature is the key of the interpretation of the data presented here. The next step is to establish the time evolution equation of $\langle I_z^A I_z^M \rangle$. Applying relation 3, neglecting again all dipolar cross-correlation terms, and assuming proton broadband decoupling yield after tedious calculations:

$$\frac{d}{dt}\langle I_z^A I_z^M \rangle = -\left\{\sum_X \frac{6}{5}(\sigma^{AX} + \sigma^{MX}) + \frac{6}{5}\sigma^{AM} + R^A + R^M + 4J^{A,A}_{CSA}\right\}\langle I_z^A I_z^M \rangle - 2J^{A,AM}(\langle I_z^A \rangle - 1) \quad (10)$$

In view of calculating the NOE factor and the recovery of $\langle I_z^A \rangle$ by means of eq 8, we need the stationary value of $\langle I_z^A I_z^M \rangle$ under proton decoupling; this will be denoted $\langle I_z^A I_z^M \rangle^{DEC}$. It can be seen from (10) that, because σ^{MX} is much larger than $J^{A,AM}$, $\langle I_z^A I_z^M \rangle^{DEC}$ would represent a very weak fraction of $\langle I_z^A \rangle^{DEC}$ and therefore would not significantly contribute to eq 8. However, if the neglect of dipolar cross-correlation terms is fully justified in the evolution equation of $\langle I_z^A \rangle$, this is not necessaryily true with regards to $\langle I_z^A I_z^M \rangle$. In fact, for an AM{$X_2$} system, the most important dipolar cross-correlation terms is $J^{MXMX'}$, and it would contribute to $d\langle I_z^A I_z^M \rangle/dt$ by:

$$-4J^{MXMX'}[2\langle I_z^A I_z^M I_z^X I_z^{X'} \rangle + \langle I_z^A I_z^M (I_+^X I_-^{X'} + I_-^X I_+^{X'}) \rangle]$$

(with $I_\pm = I_x \pm iI_y$). These two average values are zero at thermal equilibrium but again *nonzero in the presence of irradiation of X transitions* and can possibly lead to a nonnegligible value for $\langle I_z^A I_z^M \rangle^{DEC}$.

In order to totally solve the problem, one would have to establish the time-evolution equations of all quantities involved by the consideration of dipolar cross-correlation spectral densities in $d\langle I_z^A I_z^M \rangle/dt$. This evidently would be a formidable task which cannot be performed by hand. In fact, in $d\langle I_z^A \rangle/dt$, $\langle I_z^A I_z^M \rangle$ will

be set equal to $\langle I_z^A I_z^M \rangle^{DEC}$ which will be treated as parameter, for the following reasons. (i) The evaluation of the NOE factor just requires $\langle I_z^A I_z^M \rangle^{DEC}$. (ii) $\langle I_z^A I_z^M \rangle$ remains unchanged by an inverting 180° pulse applied to A and M nuclei; the effect of such a pulse can be deduced from:

$$\exp[-i\pi(I_x^A + I_x^M)] I_z^A I_z^M \exp[i\pi(I_x^A + I_x^M)] = \exp[-i\pi I_x^A] I_z^A \exp[i\pi I_x^A]$$

$$\exp[-i\pi I_x^M] I_z^M \exp[i\pi I_x^M] = (-I_z^A)(-I_z^M)$$

Consequently, the initial recovery rate of $\langle I_z^A \rangle$ would strictly involve $\langle I_z^A I_z^M \rangle^{DEC}$. We can anticipate that, although $\langle I_z^A I_z^M \rangle$ and $\langle I_z^A \rangle$ evolve on the same time scale, $\langle I_z^A I_z^M \rangle$ will keep a value not too far from $\langle I_z^A I_z^M \rangle^{DEC}$. This is because $\langle I_z^A I_z^M(0) \rangle$ is identical with its stationary value (i.e., $\langle I_z^A I_{Mz} \rangle^{DEC}$) which $\langle I_z^A I_z^M \rangle$ will, of course, recover. We shall consequently assume that the term $-8J^{A,AM}\langle I_z^A I_z^M \rangle$ will not affect significantly the time evolution of $\langle I_z^A \rangle$. This is further supported by the monoexponential character of the recovery and by the results of measurements performed with decoupling gated on only during acquisition (vide infra).

For convenience, we shall deal with the following quantity:

$$K^A = -8J^{A,AM}\langle I_z^A I_z^M \rangle^{DEC} \tag{11}$$

which would have to be determined from experimental data and whose sign is a priori unknown.

Analysis of Experimental Results

We establish below expressions of the apparent longitudinal relaxation T_1^* and of the nuclear Overhauser effect factor η deduced from the above theoretical considerations. We start with the "extended" Solomon equations:

$$\frac{d}{dt}\langle I_z^A \rangle = -R_1^A(\langle I_z^A \rangle - 1) - \sigma^{AM}(\langle I_z^M \rangle - 1) + \frac{\gamma_H}{\gamma_c}\sum_X \sigma^{AX} + K^A \tag{12}$$

$$\frac{d}{dt}\langle I_z^M \rangle = -R_1^M(\langle I_z^M \rangle - 1) - \sigma^{AM}(\langle I_z^A \rangle - 1) + \frac{\gamma_H}{\gamma_c}\sum_X \sigma^{MX}$$

where, as explained before, K^A is nonzero only for molecules containing two carbons-13 at sites A and M under continuous proton decoupling.

As indicated above, the experimental recovery curves are all monoexponential, and it would be tempting to identify the apparent relaxation times with the inverse of their relaxation rates. In fact, simulations based on eq 12 show that this is perfectly valid as far as relaxation of nucleus M is concerned. However, the A magnetization recovery curve can be decomposed in three regions, each one being characterized by its own rate. Such behavior has already been noticed for AM{X} and AM{X_2} systems where intramolecular dipolar and random field interactions were the only mechanisms considered.[13,22] The importance of each region depends on the relative magnitude of A and M relaxation rates. It turns out that here, and in a more general way for peptidic fragments, the first region represents a tiny amount of the whole recovery (less than 10%), whereas the second region corresponds to its major part; the third region is experimentally unattainable. The above-mentioned simulations indicate that the relaxation rate pertinent to the second region is simply R_1^A; this can be easily understood by realizing that this second region occurs when carbon M has relaxed to a large extent, so that the evolution equation of $\langle I_z^A \rangle$ can be simply approximated by

$$\frac{d}{dt}\langle I_z^A \rangle = -R_1^A(\langle I_z^A \rangle - \langle I_z^A \rangle^{STAT})$$

As a consequence, the apparent relaxation time *does not depend on the dipolar chemical shift anisotropy interference term* but, as will be seen below, *the NOE factor η does*. It will be defined in the conventional way by:

$$\eta_i = \frac{\langle I_z^i \rangle^{DEC} - \langle I_z^i \rangle^{EQ}}{\langle I_z^i \rangle^{EQ}} = \langle I_z^i \rangle^{DEC} - 1 \tag{13}$$

The inverse of the apparent relaxation times will be denoted thereafter by $^k(1/T^i)^*$ where $k = 1$ indicates the isotopomer containing a single carbon-13 per molecule, and $k = 2$ indicates two (or more) linked carbon-13 atoms per molecule. The same conventions will be adopted for the NOE factors. One obtains:

$$^2(1/T_1^A)^* = R_1^A$$

$$^2(1/T_1^M)^* = R_1^M \tag{14}$$

and

$$^2\eta^A = \frac{R_1^M[(\gamma_H/\gamma_c)\sum_X \sigma^{AX} + K^A] - (\gamma_H/\gamma_c)\sigma^{AM}\sum_X \sigma^{MX}}{R_1^A R_1^M}$$

$$^2\eta^M = \frac{(\gamma_H/\gamma_c)\sum_X \sigma^{MX}}{R_1^M} \tag{15}$$

These equations and the following involve a number of approximations resulting from the weak value of σ^{AM} with respect to R_1^M.

Concerning the isotopomers containing only one carbon-13 atom per molecule, the classical expression hold. According to the definitions of R_1^A and R_1^M, they must be written:

$$^1(1/T_1^A)^* = R_1^A - 2\sigma^{AM} \qquad ^1(1/T_1^M)^* = R_1^M \tag{16}$$

$$^1\eta^A = \frac{(\gamma_H/\gamma_c)\sum_X \sigma^{AX}}{R_1^A - 2\sigma^{AM}} \qquad ^1\eta^M = \frac{(\gamma_H/\gamma_c)\sum_X \sigma^{MX}}{R_1^M} \tag{17}$$

For the deuterated molecules, eq 14 and 16 can be used provided that the necessary changes are made in the expressions for R_1^A and R_1^M (relations 1 and 4).

The above formulas show that the interpretation of the apparent relaxation times is straightforward and yield in the conventional way the spectral densities of interest:

$$J^{MXMX} = \frac{3}{5}\frac{\gamma_c}{\gamma_H}\eta^M(1/T_1^M)^* \tag{18}$$

(the superscript 1 or 2 has been omitted since it is postulated that identical results are obtained for all types of isotopmers; if carbon M is bound to two protons, the factor 3/5 has to be replaced by 3/10)

$$J^{AMAM} = \frac{3}{10}[^2(1/T_1^A)^* - ^1(1/T_1^A)^*] \tag{19}$$

The inconsistencies mentioned above, which occurred by application of the classical Solomon equations, concern only the NOE factor $^2\eta^A$. With our treatment it can be recalculated according to:

$$^2\eta^A = {}^1\eta^A\frac{^2(T_1^A)^*}{^1(T_1^A)^*} + K^A[^2(T_1^A)^*] - (^2\eta^M)(\tfrac{5}{3}J^{AMAM})^2(T_1^A)^* \tag{20}$$

It can be seen that the application of the classical Solomon equations would be equivalent to omitting the term involving K^A and thus the interference between dipolar interaction and chemical shift anisotropy. As will be shown in the next section, this would lead to $^2\eta^A$ values in disagreement with experimental results.

Results and Discussion.

The merging point of this study is the determination of the carbon–carbon dipolar spectral densities. For that, it was important first to check the validity of our theoretical treatment. J^{AMAM} obtained for glycine and aspartic acid are reported in Table III.

It should be mentioned that carbon–carbon dipolar spectral densities not involving a carbonyl are not obtainable because the carbon–carbon dipolar interaction yields too small a contribution to the relaxation of the involved carbons. For this reason, aspartic acid has been treated by assuming a separation into an AM{X} (C_OC_α{H}) and an AM{X_2} ($C_\gamma C_\beta${H_2}) spin systems.

Table III. Carbon-Carbon Dipolar Interaction Spectral Densities[a]

	$J^{O\alpha O\alpha}$	$J^{\beta\gamma\beta\gamma}$	$^2\eta^\alpha$	$^2\eta^\gamma$
glycine	(a) 0.00117 ± 0.0003		(f) 0.72 ± 0.06	
	(b) 0.00103 ± 0.0005			
	(c) 0.00094 ± 0.0002		(g) 0.46 ± 0.10	
aspartic acid	(a) 0.0029 ± 0.0009	(a) 0.0021 ± 0.0009	(f) 0.85 ± 0.05	(f) 1.02 ± 0.06
	(b) 0.0031 ± 0.0010	(b) 0.0034 ± 0.0006		
	(d) 0.0023 ± 0.0006	(d) 0.0027 ± 0.0004	(h) 0.61 ± 0.20	(i) 0.72 ± 0.18
	(e) 0.0017 ± 0.0019	(e) 0.0018 ± 0.0009		

[a] Determined from (a) continuous decoupling experiments, (b) gated decoupling experiments, (c) [^{13}C,^{2}H] molecule, from ref 24, (d) (d) [^{13}C,^{2}H] molecule at 25 MHz, (e) [^{13}C,^{2}H] molecule at 100 MHz, (f) experimental value. (g) Calculated according to the classical Solomon equations with $J^{O\alpha O\alpha} = 0.0010 \pm 0.00025$ and $^2\eta^\alpha \simeq 2$, (h) Calculated according to the classical Solomon equations with $J^{O\alpha O\alpha} = 0.0028 \pm 0.0005$ and $^2\eta^\alpha \simeq 2$,(i) Calculated according to the classical Solomon equations with $J^{\beta\gamma\beta\gamma} = 0.0027 \pm 0.0005$ and $^2\eta^\beta \simeq 2$.

Table IV. Dipolar Spectral Densities and Corresponding Effective Correlation Times of Glycine and Aspartic Acid[a]

type of molecule, concentration, ionic form	$J^{O\alpha O\alpha}$ (s^{-1}) $\tau^{eff}_{O\alpha}$ (ps)	$J^{\beta\gamma\beta\gamma}$ (s^{-1}) $\tau^{eff}_{\beta\gamma}$ (ps)	$J^{\alpha x\alpha x}$ (s^{-1}) $\tau^{eff}_{\alpha x}$ (ps)	$J^{\beta x\beta x}$ (s^{-1}) $\tau^{eff}_{\beta x}$ (ps)
glycine, 0.28 M, zwitterion	0.00104 ± 0.00025[b]		0.0348 ± 0.0036	
	20 ± 6		5.0 ± 0.5	
aspartic acid, 0.13 M, cation	0.0028 ± 0.0005[c]	0.0027 ± 0.0005[d]	0.136 ± 0.025	0.146 ± 0.030
	54 ± 10	36 ± 9	20 ± 4	21 ± 5

[a] $J^{O\alpha O\alpha}$ and $J^{\beta\gamma\beta\gamma}$ values represent the average of data (a), (b), (c), and (d) given in Table III. [b] Calculated with $C^OC^\alpha = 1.52$ Å. [c] Calculated with $C^OC^\alpha = 1.54$ Å. [d] Calculated with $C^\beta C^\gamma = 1.45$ Å.

Comparisons of results derived from the [^{13}C] molecule with normal and gated decoupling, on the one hand, and those derived from the [^{13}C,^{2}H] molecule are seen to agree fairly well. Those results deserve several comments. First, the apparent longitudinal relaxation times lead to consistent results and this justifies a posteriori the approximations involved in their analysis, especially the consideration of the second region and the fact that the initial portion of the recovery curves is effectively discarded. On the basis of previous simulations carried out on AM{X} systems,[22] the latter should depend on cross-correlation spectral densities and on the presence or absence of proton decoupling. Indeed, the classical behavior of the measurable part of the recovery (the second region) is in agreement with conclusions drawn by London et al.[13] and by ourselves.[22] This is further supported by the experiments carried out with decoupling gated on only during the acquistion. It has been noticed that the absence or presence of decoupling does not affect significantly this second region,[22] which consequently should occur when nonirradiated proton magnetization has essentially recovered. However, the introduction of the CSA dipolar interference term ($-8J^{A,AM}\langle I^A_z I^M_z\rangle$) in the Solomon equations could lead to different conclusions since $\langle I^A_z I^M_z\rangle$ is nonzero (and capable of evolution) when proton transitions are irradiated, whereas it is zero when decoupling is off. The fact that the two types of experiments (with decoupling on and off during the evolution period) yield essentially the same results justifies the assumption according to which $\langle I^A_z I^M_z\rangle$ should evolve quite slowly and has not to be taken into account in the analysis of conventional inversion recovery experiments.

This is, of course, no longer true for the NOE factors and is illustrated by the values of $^2\eta^A$ recalculated with classical Solomon equations (Table III), i.e., by using formula 20 without the term involving K^A. As expected from the considerations of the theoretical section, disagreement with experimental values concern mainly AM{X_2} (glycine and carbon γ of aspartic acid) rather than AM{X} (carbon α of aspartic acid) spin systems.

All autocorrelation spectral densities inferred from our experiments are gathered in Table IV. When several independent determinations are available, an average value is given. The effective correlation times are calculated by formula 7. Although it is out of the question to obtain a detailed picture of all motions occurring within these molecules, some of their trends can be discussed since we have in hand effective correlation times associated with carbon–carbon bond reorientation on the one hand, and CH reorientation on the other hand. As we stated in a previous report,[14] the effect produced by internal rotation in glycine is reflected by the value of $\tau^{eff}_{\alpha x}$ ($\simeq$5 ps) which is low as compared with the $\tau^{eff}_{O\alpha}$ value (20 ± 6 ps). All τ^{eff} values in aspartic acid do not exhibit as large differences as in glycine, an expected result indicating that internal rotation affects as well the carbon–carbon and carbon–hydrogen bond reorientation.

Finally, the measurements performed on [^{13}C,^{2}H]aspartic acid at two different frequencies allow one to have an idea of the C_O and C_γ chemical shift anisotropies from eq 5 by identifying τ^{CSA} with the mean values of correlations times given in Table IV. One obtains $\Delta\sigma^0 = (150 \pm 30)$ ppm and $\Delta\sigma^\gamma = (133 \pm 20)$ ppm, in agreement with the expected order of magnitude.[28]

Conclusion

It has been shown that ^{13}C relaxation data of amino acids enriched with carbon-13 can provide reliable information about the motion of carbon–carbon bonds involving a carbonyl group. Conventional T_1 measurements of the acidic function carbon can be interpreted in the usual way; the carbon–carbon dipolar contribution is simply deduced from the difference between relaxation rates measured in the enriched and normal molecules. On the contrary, NOE factors cannot be interpreted on the basis of simple Solomon equations. They are affected by a bias originating from an interference between carbon–carbon dipolar interaction and chemical shift anisotropy which manifest itself more significantly in AM{X_2} than in AM{X} spin systems.

The obtained results are in a agreement with those deduced from molecules enriched with both carbon-13 and deuterium. Whatever the procedure used, relatively large uncertainties reaching 30% are observed. In spite of the inherent origin of this uncertainty (the carbon–carbon dipolar interaction is in any case rather small with respect to the contribution of other relaxation mechanisms), we can anticipate interesting applications of this method in the study of backbone motions in small peptides.

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