Journal of Biomolecular NMR, 5 (1995) 362–366
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J-Bio NMR 252

The effect of ^{17}O on the relaxation of an amide proton within a hydrogen bond

Christopher J. Halkides* and Alfred G. Redfield

Department of Biochemistry, Brandeis University, 415 South Street, Waltham, MA 02254, U.S.A.

Received 12 October 1994
Accepted 11 November 1994

Keywords: Dipolar relaxation; ^{17}O; Hydrogen bond; Amide; p21; *Ras*

Summary

The relaxation rates of the multiple-quantum coherence for the amide hydrogen of Gly^{13} in *ras* p21•GDP were determined in the presence and absence of ^{17}O labeling in the β-phosphate of GDP. No significant difference could be observed between labeled and unlabeled samples, in spite of the fact that the hydrogen bond from the amide group of Gly^{13} to the β-phosphate is shorter than is typical, based on its chemical shift. For macromolecules in which an oxygen atom is the acceptor of a hydrogen bond, dipolar coupling between ^{17}O and hydrogen is unlikely to be observable, except for extremely short H-bonds.

Introduction

Hydrogen bonds are ubiquitous throughout biomolecules. NMR can detect the existence of hydrogen bonds, apart from complete structural determination, primarily from chemical shift changes of the donor atom and the hydrogen itself. It would be of interest to detect and identify the acceptor atom spectroscopically, and also to characterize the length of the hydrogen bond. As part of our continuing studies of the 21-kD products of the *ras* genes (p21), we are interested in the hydrogen bonds formed between the anionic oxygen atoms of tightly bound guanine nucleotides and the amide hydrogen atoms of the main chain. It is believed that the two most downfield amide hydrogen resonances in p21•Mg(II)GDP belong to Gly^{13} (Campbell-Burk et al., 1989) and Lys^{16} (Redfield and Papastavros, 1990; Campbell-Burk et al., 1992) and that these two residues form hydrogen bonds to the diphosphate portion of GDP (Tong et al., 1991). The resonance assigned to Gly^{13} moves upfield when GTP analogs replace GDP (Miller et al., 1993), an exchange which drives p21 into its active state (Marshall, 1993). An interpretation of this upfield change in chemical shift is that this residue forms shorter H-bonds to the nucleotide in the GDP than in the GTP form (Wagner et al., 1983), and the shift indicates that these signals are markers for the relatively small conformational change of this region of the protein accompanying nucleotide substitution (Pai et al., 1990; Tong et al., 1991).

We reasoned that the presence of ^{17}O bound to the β-phosphorus of GDP might lead to an observable increase in the relaxation rate of the amide proton of Gly^{13}, typically detected by $^{1}H/^{15}N$ correlation experiments. Two reports have appeared which bear on this question. In their study of (+)-CC-1065 bound to a specific DNA duplex, Hurley and collaborators observed a shortening of T_2 for protons bound to oxygen or nitrogen in the presence of ^{17}O in the phosphate backbone or as $H_2{}^{17}O$ (Lin et al., 1991). These workers used only 1D proton NMR. On the other hand, Fesik and co-workers found that $H_2{}^{17}O$ had essentially no effect on the ^{1}H relaxation parameters of the amide protons of the complex of (uniformly ^{15}N-labeled) FK506 binding protein with ascomycin (Yu et al., 1993). T_2 values are about 10% shorter for most of the amide groups in the ^{17}O sample, a fact which the authors suggest is the result of a difference in viscos-

*To whom correspondence should be addressed.

Abbreviations: p21, 21-kD protein product of the human H-*ras* gene; GMPPCP, guanylyl [β,γ-methylene]diphosphate; HPLC, high-performance liquid chromatography; CSA, chemical shift anisotropy; HMQC, heteronuclear multiple-quantum coherence.

ity between the two samples. These results cast some doubt on the changes in T_2 seen with (+)-CC-1065. Both of these studies examined the T_2 of ^1H nuclei.

For macromolecules, $1/T_2$ is dominated by the zero-frequency component of the spectral density function (Becker, 1980) and $1/T_2$ of the multiple-quantum coherence is not affected by dipolar coupling between the proton and the nitrogen, at least to a first-order approximation (Griffey and Redfield, 1987; Kay and Bax, 1990). The rate of (single-quantum) transverse relaxation of the amide proton, by contrast, is a function of the dipolar coupling to ^{15}N, and since this relaxation mechanism is in competition with dipolar broadening brought on by a more distant ^{17}O nucleus, we felt that the rate of multiple-quantum relaxation might be more sensitive to the presence of ^{17}O.

In this paper we report the relaxation rate of the $^1H/^{15}N$ multiple-quantum coherence of Gly^{13} in the presence of GDP compared with [^{17}O]GDP. Since GDP is anionic, it may form shorter H-bonds than H_2O and produce greater line broadening. We delineate the conditions under which observation of dipolar line broadening by ^{17}O is likely.

Materials and Methods

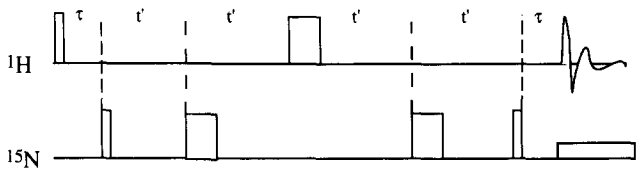
Synthesis of [^{17}O]GDP

[^{17}O]P_i was synthesized from 50% $H_2{}^{17}O$ (Cambridge Isotope Laboratories, Andover, MA) and PCl_5 (Hackney et al., 1980). After elution from a column of AG1-X8 with 30 mM HCl, the phosphoric acid was combined with distilled pyridine to give the pyridinium salt of phosphate.

[^{17}O]GDP was synthesized from guanosine 5'-monophosphate 4-morpholine-*N*,*N*'-dicyclohexylcarboxamidine (Sigma, St. Louis, MO) and [^{17}O]P_i (Moffatt, 1961). Trioctylamine was stirred with CaH_2 and distilled under reduced pressure. The GDP was purified on a DEAE Sephadex column (80 ml) with a 2 l gradient of 0.1–0.5 M triethylammonium bicarbonate at a flow rate of 1.5 ml/min (Reed and Leyh, 1980). The triethylamine had been stirred with CaH_2 and distilled. Isotopic enrichment of the [^{17}O]GDP was estimated to be 50% by integration of the ^{31}P signals of the α- and β-phosphates (Tsai, 1979).

Production of p21•[^{17}O]GDP

p21•GDP, labeled with [^{15}N]glycine, was expressed and purified as described previously (Halkides et al., 1994). A sample of p21 (14 mg) was treated with alkaline phosphatase and 1.5 equiv of GMPPCP (Boehringer Mannheim) as described previously (Halkides et al., 1994). Then 1 mM EDTA and 10 equiv of [^{17}O]GDP were added and the mixture was kept at room temperature for 1 h. Finally, $MgSO_4$ was added and the buffer was changed to 20 mM Tris•HCl (pH 7.5 at 23 °C)/50 mM NaCl/10 mM DTT/5 mM MgCl/0.02% sodium azide/1 μM GDP by means of size-exclusion chromatography using a PD-10 column (Pharmacia, Piscataway, NJ). Since the affinity of p21 for GDP is about 100-fold greater than for GMPPCP (Scherer et al., 1989), the labeled GDP was expected to replace the GMPPCP completely, and an HPLC assay (Halkides et al., 1994) confirmed that p21 was entirely in the GDP form.



Scheme 1. Pulse sequence of the modified difference-echo HMQC experiment.

NMR spectroscopy

All NMR experiments were performed on a Bruker AMX-500 spectrometer at 11 °C. We modified the difference-echo experiment by adding two 180° pulses on ^{15}N and four delays t' (Lowry, 1991). We used 15 or more values of t', ranging from 3 μs to 6 ms (see Scheme 1). In Scheme 1, all proton pulses are jump-return pulses with an interpulse delay of 95 μs for the 90° pulse, and nitrogen pulses are 25 and 50 μs for the 90° and 180° pulses, respectively. The spectral width was 7042 Hz and 2K data points were collected. GARP decoupling of ^{15}N was used during acquisition. FIDs were zero-filled twice and exponentially multiplied with a line broadening of 5 Hz.

Data analysis

The loss of phase memory can be expressed by:

$$M_x(t)/M_0 = \exp(-R_2 t) \quad (1)$$

where R_2 is the sum of the rate constants for all relaxation processes, except dipolar broadening from ^{17}O. When the correlation time is relatively long, the dominant dipolar relaxation from ^{17}O may be written as

$$R_{2(^{17}O)} = 1/20\,(35/3)\,(2\pi R)^2\,(4\tau_c) \quad (2)$$

where $R = (\mu_0/4\pi)r^{-3}\gamma_{^1H}\gamma_{^{17}O}(h/4\pi^2)$ (Harris, 1983). Higher frequency terms have not been included in this equation, because they make a small contribution to R_2 for macromolecules (Kay and Bax, 1990). The factor of 35/3 accounts for the fact that ^{17}O is a spin 5/2 nucleus and is the ratio of I(I+1) for a spin 5/2 to a spin 1/2 nucleus (Becker, 1980). τ_c is the correlation time. Evaluation of the constants shows that

$$R_{2(^{17}O)} = (2.444 \times 10^{-50})\,(r^{-6})\,\tau_c \quad (3)$$

where r is expressed in meters and τ_c in seconds.

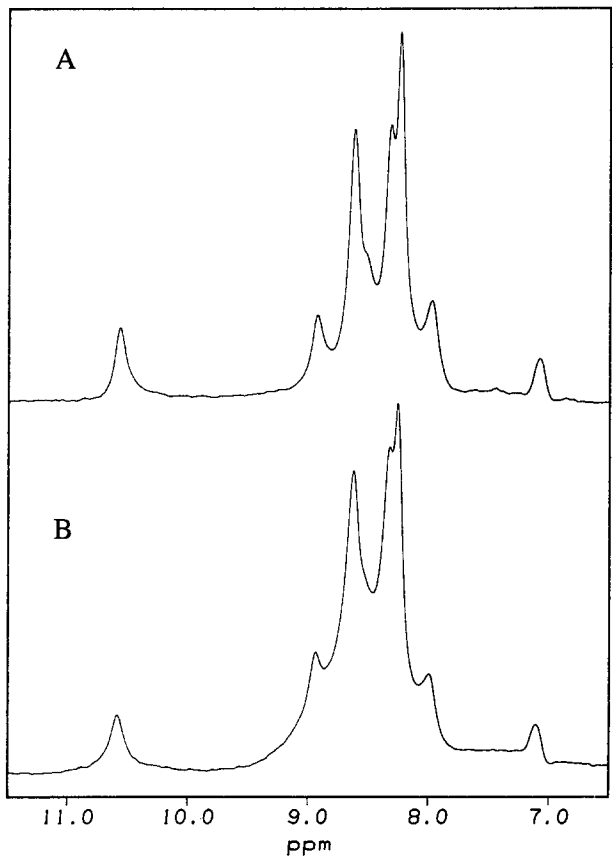


Fig. 1. (A) ^{1}H NMR spectrum of [^{15}N]Gly-labeled p21•GDP at 500 MHz. The resonance assigned to Gly^{13} has a chemical shift of ~10.5 ppm. The data were acquired with a modification of a difference-echo sequence (Scheme 1), with t'=3 μs. Exponential line broadening (5 Hz) was used as apodization. (B) ^{1}H NMR spectrum of [^{15}N]Gly-labeled p21•[^{17}O]GDP at 500 MHz. The conditions are identical to those described in (A).

Molecules bearing the ^{17}O label possess a relaxation rate

$$R_{2,tot} = R_2 + R_{2(^{17}O)} \quad (4)$$

Molecules without the label possess a relaxation rate R_2, which may be dominated by CSA broadening of either the ^{15}N or the proton spin (Kay and Bax, 1990).

We analyzed the relaxation data as follows. We used a value for the rotational correlation time of 20.9 ns at 11 °C (Hazlett et al., 1993). After fitting the relaxation rate for unlabeled GDP to obtain R_2, we fitted the data obtained with the ^{17}O-labeled sample to Eq. 5, treating R_2 as a constant to obtain $R_{2,tot}$. The fact that the ^{17}O labeling was incomplete means that there are two populations of NH protons, each relaxing at different rates, and that the expression for the relaxation rate of all observed magnetization is the sum of two exponentials:

$$M_x(t)/M_0 = (1-F)\exp(-R_2 t) + (F)\exp(-R_{2,tot} t) \quad (5)$$

where F is the fractional incorporation of ^{17}O at a single site on the β-phosphate of GDP.

We plotted Eq. 5 for different ratios of the two rate constants using the program TEMATH (Brooks/Cole Publishing, Pacific Grove, CA, 1991). When the natural log of the peak area is plotted versus the length of the delay, the degree of curvature in the graph is dependent on the difference in relaxation rates as well as on the isotopic enrichment (if $R_{2(^{17}O)}$ is small relative to R_2, then essentially a straight line is obtained). A twofold difference in rates produces noticeable curvature at 40–50% enrichment. We take this as the minimum difference that we can detect at the S/N level obtained with our experiment. Nonlinear least-squares fitting of the experimental data to Eq. 5 for both labeled (F=0.5) and unlabeled samples (F=0) was done using the program KaleidaGraph (Synergy Software, Reading, PA, 1992).

Results

Using the pulse sequence of Scheme 1, we recorded ^{1}H NMR spectra of p21•GDP with successively longer values of t'. Figure 1A shows the spectrum of p21 complexed with unlabeled GDP with t'=3 μs, and Fig. 1B depicts the spectrum of p21 complexed with [^{17}O]GDP under the same conditions. No obvious differences in intensity or line shape of the resonance at ~10.5 ppm (Gly^{13}) are apparent. We determined the relaxation rate of the multiple-quantum coherence of the amide group of Gly^{13} in the presence of unlabeled GDP using the sequence shown in Scheme 1. Since there are four delays in the sequence, we divided the slope of the best fit line by 4 to calculate the value of R_2 to be 0.117 ms^{-1} and T_2=8.5 ms. The value of R_2 agrees well with the ~40 Hz line width for this resonance in the ^{15}N dimension of HMQC spectra (data not shown).

We repeated the relaxation experiment twice in the presence of [^{17}O]GDP; the results are shown in Fig. 2. We fitted the ^{17}O data to Eq. 4, setting R_2=0.117 ms^{-1} and calculating the best value of $R_{2,tot}$. The first trial produced a value of 0.408/4=0.102 ms^{-1} and in the second trial a value of 0.507/4=0.127 ms^{-1} was found for $R_{2,tot}$. Because $R_{2,tot}$ is defined as the sum of R_2 and $R_{2(^{17}O)}$, the data of the second trial (in which $R_{2,tot} > R_2$) suggest that $R_{2(^{17}O)}$ is small. However, the data of the first trial (in which $R_2 > R_{2,tot}$) would yield a small, *negative* value of $R_{2(^{17}O)}$, which is absurd. Therefore, these data suggest that $R_{2(^{17}O)}$ is too small to be observed.

Discussion and Conclusions

We chose to use a modification of the 1D (difference-echo) version of the HMQC experiment (Scheme 1) on a sample of p21 that had been labeled with [^{15}N]glycine. It has been shown that the relaxation rate of the multiple-quantum coherence is (to a first-order approximation) unaffected by dipolar coupling between I and S (^{1}H and ^{15}N, in this case), whereas transverse relaxation of the single-

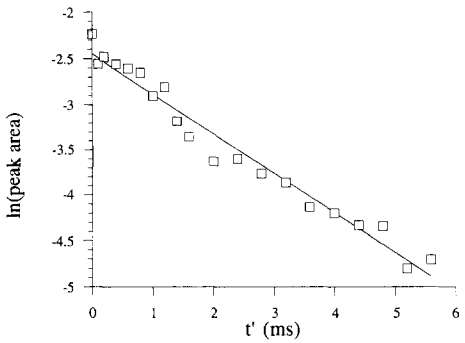


Fig. 2. The natural logarithm of peak area as a function of t'. The area of the resonance at ~10.5 ppm (Gly^{13}) is plotted. The solid curve is the best fit to Eq. 4. An increase in relaxation rate caused by the presence of ^{17}O would bring about curvature of this decay.

quantum coherence of the amide proton is affected by dipolar coupling to ^{15}N (Griffey and Redfield, 1987; Kay and Bax, 1990). This also implies that measurement of the decay rate of zero- and double-quantum coherence will be more sensitive to the presence of other nuclei, namely ^{17}O, which are dipolarly coupled to the proton, than a simple proton relaxation experiment. We focussed our attention on the signal at ~10.5 ppm, which has been assigned to the amide group of Gly^{13} (Campbell-Burk et al., 1989).

We consider three cases. The contribution to the relaxation rate from the presence of ^{17}O may be much faster than, about equal to, or much slower than the rate due to the sum of all other mechanisms. The first case would manifest itself as a decrease in intensity of the Gly^{13} resonance relative to all other peaks, even at short values of t'. Comparison of Figs. 1A and B shows that this is not observed. If the log of the intensity of this resonance is plotted versus the length of the delay, the second case would manifest itself either as curvature of this plot if the data were ideal, or possibly merely as an overall increase in relaxation rate. The third case would produce data which give insignificant curvature of such a plot.

The rate of ^{17}O-dependent relaxation increases with the inverse sixth power of the oxygen–hydrogen distance, and the expected distance between donor and acceptor in our system is uncertain. When both the phosphorus-containing species and the nitrogen-containing species are neutral, the length of the hydrogen bond from the NH group to the oxygen acceptor is 1.99 Å (Kostansek and Busing, 1972). On the other hand, a cationic nitrogen atom donating a hydrogen bond to an anionic oxygen atom within a phosphoryl group displays somewhat shorter distances. For anionic diesters of phosphate, forming hydrogen bonds to imidazolium counterions, the average distance between oxygen and hydrogen atoms is 1.83 Å as determined by X-ray crystallography (Holmes et al., 1992). However, X-ray crystallography is known to give significantly shorter (~0.1 Å) bonds between hydrogen and a more electronegative atom to which it is directly attached than does neutron diffraction. This is illustrated by comparing the N-H bond distances in the urea/phosphoric acid adduct studied by neutron diffraction (Kostansek and Busing, 1972) and by X-ray crystallography (Mootz and Albrand, 1972). We estimate that the distances between oxygen and hydrogen are about 0.1 Å less than 1.8 Å; therefore, we take 1.7 Å to be an approximate lower bound for the oxygen–hydrogen distance when both donor and acceptor atoms are charged.

The case of an *anionic* oxygen (from GDP) accepting a hydrogen bond from a *neutral* amide group (from Gly^{13}) would seem to be intermediate between these two cases. The relatively large downfield shift (~10.5 ppm) of this residue suggests that this group forms a shorter H-bond than most other amides in p21 (Wagner et al., 1983), many of which are involved in H-bonding to neutral carbonyl oxygen atoms of the peptide chain. The small-molecule models suggest the use of 2.0 Å as the upper bound and 1.7 Å as the lower bound for the oxygen–hydrogen bond distance between the amide of Gly^{13} and GDP.

From Eq. 2, we calculate that the distance between the hydrogen and oxygen atoms would have to be 1.27 Å to double the relaxation rate of the [^{17}O]GDP-containing species relative to the [^{16}O]GDP-containing species. The reason for the lack of any observed increase in relaxation rate is probably that the hydrogen bond is longer than this value. Even if the oxygen–hydrogen bond distance were 1.7 Å, the increase in relaxation rate is calculated to be about 17%. A second reason is that the effective correlation time (Lipari and Szabo, 1982) may be shorter than 20 ns, the rotational correlation time found from fluorescence studies (Hazlett et al., 1993). The ^{17}O relaxation pathway might still be observable for hydrogen bonds which are shorter and more constrained than the one between the amide proton of Gly^{13} and the β-oxygen atoms of GDP (Kostansek and Busing, 1972). Higher enrichment and, possibly, observation at a lower field where CSA broadening would be less, would facilitate observation of ^{17}O relaxation.

Acknowledgements

We thank David Lowry and Perry Frey for many stimulating discussions. This work was partially supported by U.S.P.H.S. Grant GM20168. C.J.H. was supported by N.I.H. postdoctoral fellowship CA08872.

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