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Measurement of ^{13}C Relaxation Times in Proteins by Two-Dimensional Heteronuclear ^{1}H-^{13}C Correlation Spectroscopy

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We have developed a heteronuclear 2D NMR experiment for measurements of ^{13}C T_1 values in proteins. This experiment was applied to determine spin-lattice relaxation times for the α-carbons of basic pancreatic trypsin inhibitor (BPTI). No isotope enrichment was used. The experiment consists of a 2D double DEPT sequence (Figure 1) that is related to 1D sequences proposed recently.[1,2] In a first step, polarization is transferred from protons to carbons by a DEPT sequence. This is followed by a carbon 90° pulse which converts S_x to $-S_z$. During the relaxation delay, unwanted transverse proton and carbon magnetization are destroyed by a long proton pulse and a homospoil pulse.[1] The initial DEPT sequence has the effect of saturating the magnetization of ^{13}C bound protons. This saturation is maintained by applying a train of proton 90° pulses for the remainder of the delay τ_2. This also eliminates magnetization of protons that are not coupled to ^{13}C. After the relaxation delay, the carbon z-magnetization is turned into the x-y plane, frequency labeled during t_1, and finally converted into proton magnetization for detection. The experiment was conducted on a 20 mM solution of BPTI in D_2O at 36 °C, pH 4.55. Measurements were performed on a General Electric GN-500 spectrometer. Sixty-four scans were collected for each of the 350 t_1 increments. The delay between scans was 3 s, corresponding to about twice the proton T_1's. Ten data sets were collected for τ_2 values of 8 ms, 53 ms, 93 ms, 128 ms, 178 ms, 228 ms, 278 ms, 353 ms, 2 s, and 5 s, respectively. The spectrum with τ_2 = 8 ms is shown in Figure 2. The assignments given in the figure have been obtained previously on a Bruker AM-360 spectrometer.[3] The remaining ambiguities of the α-carbon assignments were resolved due to the better resolution at the higher field strength. If the DEPT transfer were perfect, cross-peak intensities would relax from $-4 \times M_0$ at $\tau_2 = 0$ to $+1 \times M_0$ at $\tau_2 = \infty$, where M_0 corresponds to the carbon equilibrium polarization. In practice, the DEPT transfer reached only values of $-2.5 \times M_0$. Relaxation times were determined by measuring the cross-peak intensities, $I(\tau_2)$, as a function of the relaxation delay, and a linear least-squares fit of $\ln I = \ln [(I(\tau_2) - I(\infty))/(I(0) - I(\infty))]$ versus τ_2 was performed. The slope is equal to the inverse of the relaxation time T_1. To test the accuracy of the T_1 measurements obtained by this method, we used a sample of 20 mM α-^{13}C enriched alanine. The value of T_1 obtained by using a direct detection inversion recovery experiment was 4.4 s while the proposed pulse sequence gave a value of 3.8 s. For the β-carbon, the values were 2.3 and 2.1 s, respectively. The sequence of Figure 1 thus seems to yield values for T_1 that are consistently 10% to 15% shorter than those measured with direct detection methods. Being aware of this we have measured the T_1 values in BPTI for the α-carbons of all residues except for the glycines where the cross peaks are suppressed due to the DEPT sequence, and for a few other residues where the signals were not resolved or were broadened due to internal mobility (C14, Y23).[4] The whole relaxation time series took 10 days of instrument time. Figure 3 shows the experimental data points and the fitted curves for the residues Cys 51 and Ala 58. Figure 4 shows the distribution of T_1 values for the various residues in the protein. The average T_1 of the α-carbons for the whole protein is 358 ms with the longest T_1 being 477 ms for Ala 58 and the shortest being 305 ms for Arg 17. This may be compared with measurements at lower field strength (90.5 MHz) where an average value of 230 ms was estimated from the envelope of 1D spectra in a conventional inversion recovery experiment.[5] In this previous study it has been estimated that the overall rotational correlation time for BPTI is 4×10^{-9} s. This is consistent with our data which also confirm that we are in the regime of slow molecular motion ($\omega\tau_c > 1$). The variation in the T_1 values is smaller than the variation in the

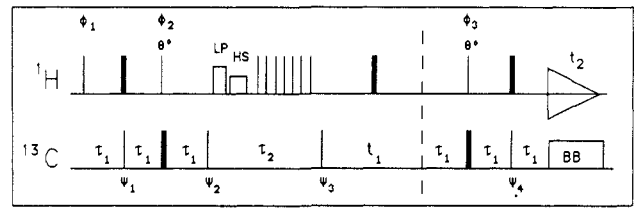

Figure 1. Double DEPT pulse sequence for measurements of ^{13}C relaxation times in 2D heteronuclear ^{13}C-^{1}H correlated spectra. The delays τ_1 are tuned to $1/2J_{CH}$. The length of the long pulse and the homospoil pulse were 1 ms each. The pulses in the train of 90° pulses were spaced 5 ms apart. We have used the following phase cycles: $\phi_1 = x, -x$; $\phi_2 = y, y, -y, -y$; $\phi_3 = x, x, x, x, -x, -x, -x, -x$; $\psi_1 = x$; $\psi_2 = y, -y, -y, y$; $\psi_3 = y, y, y, y, y, y, y, y, -y, -y, -y, -y, -y, -y, -y, -y$; $\psi_4 = x, x, -x, -x$; receiver = $x, x, -x, -x, -x, -x, x, x, -x, -x, x, x, x, x, -x, -x$. The phases of the 180° pulses were cycled independently. Quadrature detection in ω_1 was achieved using time-proportional phase incrementation of ψ_3. Decoupling of the ^{13}C spins during acquisition was done using the Waltz-16 decoupling sequence.

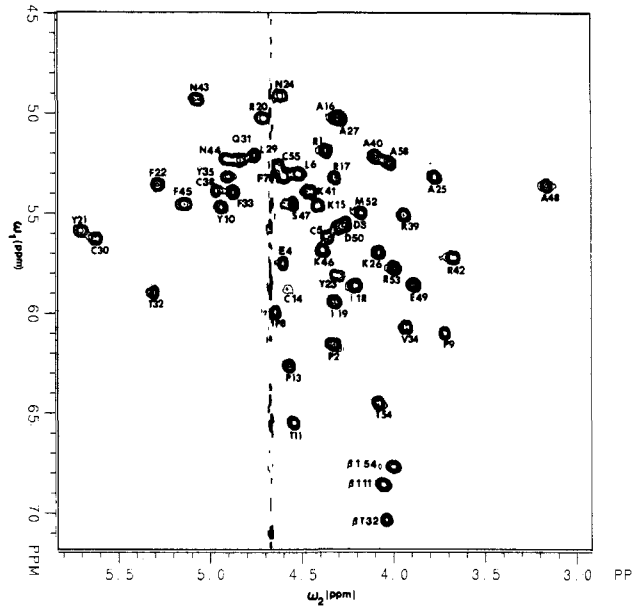

Figure 2. Region of the C$^\alpha$H cross peaks of a spectrum of a 20 mM solution of BPTI in D_2O recorded with the pulse sequence of Figure 1. The flip angle of the θ pulses was set to 90° to select for CH cross peaks. The value of τ_1 was set to 3.6 ms and τ_2 was 8 ms. Each FID was recorded with 4096 points and 350 FID's were collected. The data were Fourier transformed with use of a line broadening of 3 Hz and a 45° shifted sine bell window in ω_2 while a 60° shifted sine bell window was used in ω_1. The interferograms in t_1 were zero filled to 1024 points.

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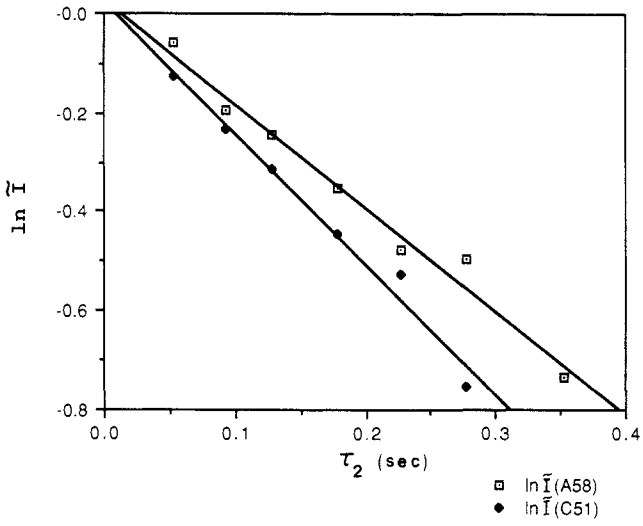

Figure 3. Linear least-squares fit of ln I versus τ_2 for the residues Cys 51 and Ala 58 where $I = [(I(\tau_2) - I(\infty))/(I(0) - I(\infty))]$. The value of T_1 obtained from the fit was 378 ± 6 ms for Cys 51 and 477 ± 30 ms for Ala 58. The average error in the measurement of T_1 was found to be 5.4%.

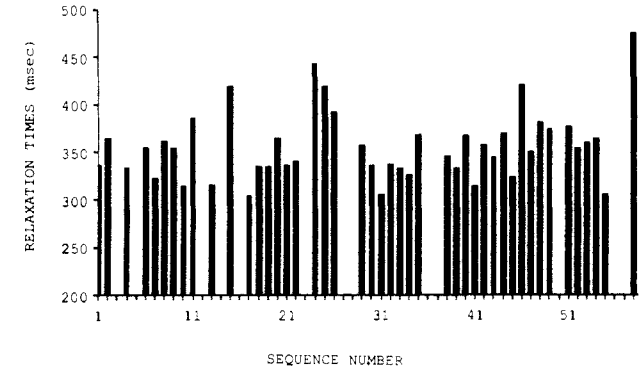

Figure 4. Relaxation times of the α-carbon signals of BPTI vs amino acid sequence. T_1 values of glycyl residues were not measured (see text). Asp 3, Asp 50, Ala 16, and Ala 27 could not be analyzed because of resonance overlap (Figure 2), and Cys 14 and Tyr 23 could not be analyzed due to strong line broadening at 36 °C.

B factors obtained from the crystal structure of BPTI.[6] This is reasonable since the main contribution to relaxation times originates from the overall rotational motions of the protein whereas *B* factors measure mainly internal mobility. It is more surprising, however, that the residues expected to have greater mobility (when comparing crystallographic *B* factors or when considering their location at the protein surface) have longer T_1 values. These residues are the C-terminal Ala 58, the residues at the β-turn (Asn 24 to Lys 26), or the residue in the reactive site (Lys 15). This observation is consistent with the results of Richarz et al.[5] where longer T_1's have been measured for the methyl groups in the same regions. This may indicate that for these surface regions of the molecule we have conditions of $\omega\tau_c < 1$, and there is a larger variation of effective correlation times in BPTI than indicated from the relatively small variations of T_1 values in Figure 4. On the other hand, the lack of dramatic variations of T_1 values assures us that measurements of nuclear Overhauser effects for determination of protein structures in solution are not significantly biased by internal motions, at least as far as the backbone is concerned.

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Preparation, Isolation, and Characterization of Transition-Metal η^2-Silene Complexes. X-ray Crystal Structure of $(\eta^5\text{-}C_5Me_5)[P(i\text{-}Pr)_3]Ru(H)(\eta^2\text{-}CH_2SiPh_2)$

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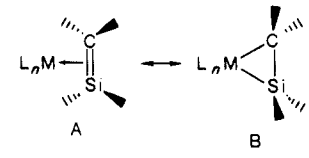
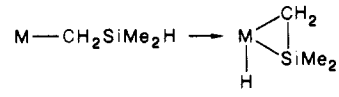
Silenes ($R_2C{=}SiR'_2$) are usually reactive organosilicon intermediates whose formation can be established indirectly with trapping reactions.[1,2] Recent interest in silenes has been stimulated by syntheses of isolable examples that are stabilized by steric protection of the Si=C double bond.[3,4] Given the well-known ability of transition metals to stabilize reactive species (e.g., carbenes, carbynes, cyclobutadienes, ketenes, and thiocarbonyl) by ligation, it seemed reasonable to assume that stable transition-metal silene complexes $L_nM(\eta^2\text{-}R_2CSiR'_2)$ (A,B) could be



isolated and studied. Silene complexes have been proposed as intermediates in a number of metal-mediated rearrangements of organosilicon ligands.[5] Intermediates generated by the β-hydrogen-transfer reaction shown in eq 1 were originally proposed by Pannell[5a] and have recently been observed spectroscopically



(1)

at low temperature by Wrighton.[6] Near-UV photolysis of $Cp^*(CO)_2FeCH_2SiMe_2H$ ($Cp^* = \eta^5\text{-}C_5Me_5$) in the presence of a ligand L (CO or PPh_3) was shown to result in formation of $Cp^*(CO)(L)FeSiMe_3$ via the intermediate $Cp^*(CO)Fe(H)(\eta^2\text{-}CH_2SiMe_2)$, which was stable to 225 K.[6b]

We have an interest in studying reactive organosilicon intermediates in the coordination sphere of transition metals[7] and have attempted syntheses of stable silene complexes that can be isolated and subjected to structural and chemical studies. The approach reported here involves use of the hydrogen-transfer process of eq 1 and stabilization of the formally more oxidized metal center with a noble metal (ruthenium) and electron-donating ligands (Cp* and trialkylphosphines). Additionally, since the hydrogen

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