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Study of protein dynamics in solution by measurement of $^{13}C^{\alpha}$-^{13}CO NOE and ^{13}CO longitudinal relaxation

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Summary

$^{13}C^{\alpha}$-^{13}CO homonuclear NOE and ^{13}CO T_1 relaxation were measured for a 20 kDa protein using triple-resonance pulse sequences. The experiments were sufficiently sensitive to obtain statistically significant differences in relaxation parameters over the molecule. The $^{13}C^{\alpha}$-^{13}CO cross-relaxation rate, obtained from these data, is directly proportional to an order parameter describing local motion and it is largely independent of the local correlation time. It is therefore a relatively straightforward observable for the identification of local dynamics.

We propose to study the backbone dynamics of doubly labeled proteins by measuring dipolar cross-relaxation processes between the $^{13}C^{\alpha}$ and ^{13}CO spins. The method consists of the measurement of steady-state homonuclear carbon–carbon nuclear Overhauser effects ($C^{\alpha}CO$ NOE) and ^{13}CO T_1 relaxation rates to obtain the cross-relaxation rates σ, according to the relation:

$$\sigma = [\mathrm{NOE} - 1] / T_1 \qquad (1)$$

The study of ^{13}CO relaxation complements well-documented ^{15}N-based relaxation measurements of the protein backbone (Nirmala and Wagner, 1988; Kay et al., 1989; Clore et al., 1990; Peng and Wagner, 1992) and $^{13}C^{\alpha}$ relaxation measurements (Yamazaki et al., 1994; Engelke and Rüterjans, 1995) because additional sites are probed, the internuclear vectors involved point in different directions, and other time scales can be investigated. Homonuclear carbon–carbon cross relaxation is interesting in particular for larger proteins at larger magnetic fields, as the effect is very sensitive to differences in apparent diffusional motion at the different sites. Figure 1A shows a theoretical correlation-time dependence of the steady-state NOE for the $^{13}C^{\alpha}$-^{13}CO system, given by (Solomon, 1955; Abragam, 1961):

$$\mathrm{NOE} \equiv \frac{I_{SAT}}{I_0} = 1 + \sigma/\rho = 1 + \left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_C^4 \hbar^2}{4} \left\langle r_{C^{\alpha}CO}^{-3} \right\rangle^2 \left[6J(\omega_{C^{\alpha}} + \omega_{CO}) - J(\omega_{C^{\alpha}} - \omega_{CO})\right] T_{1TOTAL}^{CO} \qquad (2)$$

where I_{SAT} is the steady-state ^{13}CO magnetization during $^{13}C^{\alpha}$ saturation, I_0 the equilibrium ^{13}CO magnetization, and σ and ρ the cross- and longitudinal relaxation rate, respectively; other symbols have their usual meanings. The effect varies from +1.5 to 0 for a two-spin system with dipolar coupling only and scales as indicated when all contributions to the ^{13}CO T_1 (see Eq. 3) are taken into account. It is evident that the rotational correlation times of larger macromolecules in the 20 kDa range ($\tau_R \geq 10$ ns) are in the NOE transition range and (apparent) local variations in these correlation times should be measurable with good precision. These variations can be due to non-isotropic overall tumbling, affecting directly the value of the effective correlation time τ_R for ^{13}CO-$^{13}C^{\alpha}$ vectors pointing in different directions with respect to the axes of the diffusion tensor (Woessner, 1962; Barbato et al., 1992; Hansen et al., 1994; Brüschweiler et al., 1995; Zheng et al., 1995), to the presence of (fast) local motions affecting the values of the apparent correlation times (Woessner,

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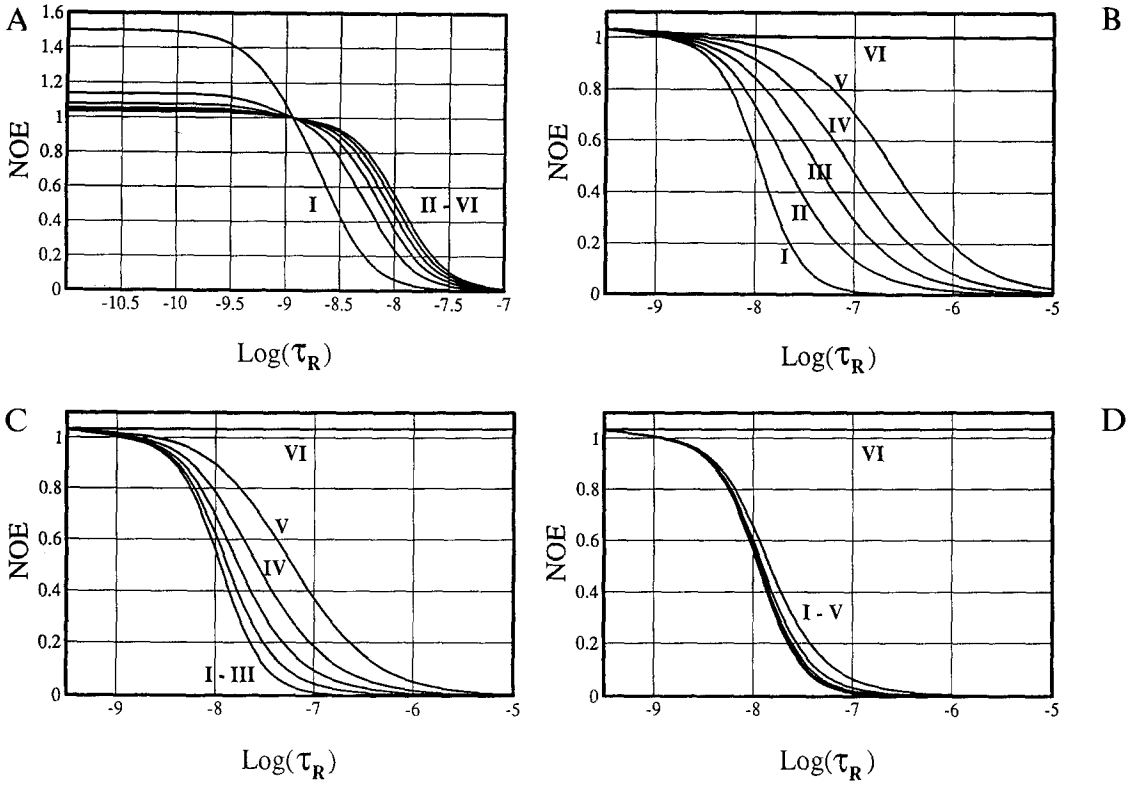

Fig. 1. Calculations of the steady-state nuclear Overhauser effect on ^{13}CO by saturation of the $^{13}C^{\alpha}$ spins, using Eqs. 2 and 3 for a 14.1 T magnetic field. (A) The effect for an isotropic rotor as a function of the overall rotational correlation time τ_R. Curve I shows a two-spin $^{13}C^{\alpha}$-^{13}CO system (without CSA relaxation); for curves II–VI, the dipolar relaxations with adjacent spins and the CSA relaxation ($\Delta\sigma = \sigma_{11} - (\sigma_{22} + \sigma_{33})/2 = 120$ ppm; Oas et al., 1987) are scaled in from 20–100%. (B–D) The effect of local disorder on the NOE. All terms contributing to T_1 were included, and a Lipari–Szabo model (Lipari and Szabo, 1982a,b) was used for all spectral density functions according to the relation: $J(\omega) = 2/5\{(S^2\tau_R)/(1+(\omega\tau_R)^2) + ((1-S^2)\tau_M)/(1+(\omega\tau_M)^2)\}$, where $1/\tau_M = 1/\tau_R + 1/\tau_L$. B: local correlation time $\tau_L = 10^{-9}$ s; C: $\tau_L = 10^{-10}$ s; D: $\tau_L = 10^{-11}$ s. Curves I–VI in B–D were drawn for order parameters ranging from 1 to 0 in decrements of 0.2.

1962; Lipari and Szabo, 1982a,b; Kay et al., 1989), or to a combination of both.

For doubly labeled proteins in H_2O, T_1 (= 1/ρ) in Eqs. 1 and 2 is given to a good approximation by:

$$\frac{1}{T_{1TOTAL}^{CO}} = \frac{c^2}{3} 6J(\omega_C) + \sum_{Q}^{C^{\alpha},H^{\alpha},N,HN} \frac{d_{CO,Q}^2}{4} \times \{3J(\omega_{CO}) + 6J(\omega_{CO} + \omega_Q) + J(\omega_{CO} - \omega_Q)\} \quad (3)$$

where the squares of the CSA and dipolar fields are indicated by c^2 and d^2, respectively (Goldman, 1988). The T_1 relaxation is thus complicated, even without relaxation interference terms (Werbelow and Grant, 1977; Vold and Vold, 1978; Goldman, 1984). Fast local motion superimposed on isotropic diffusional motion can be treated, as usual, with the model-free formalism (Lipari and Szabo, 1982a,b) and all individual spectral density functions J(ω) in Eqs. 2 and 3 are expressed by sums of Lorentzians, depending on the order parameters S^2 and fast local correlation times τ_L. In principle, for every individual ^{13}CO site, the internuclear vectors between that ^{13}CO nucleus and the neighboring $^{13}C^{\alpha}$, ^{15}N, $^{1}H^{\alpha}$ and ^{1}HN nuclei can have different order parameters and different local correlation times. T_1 may therefore be a difficult parameter to analyze quantitatively. In the following computations these complications are neglected and local motion is approximated by a single order parameter and local correlation time for all internuclear vectors (see the legend to Fig. 1). Accordingly, in Figs. 1B–D it can be seen that a reduction in the order parameter uniformly leads to larger steady-state NOEs at most global correlation times, confirming the intuition that the effect of local motion is somewhat equivalent to the effect of a shorter overall correlation time. However, this simple model breaks down when the correlation time of the local motion is very fast ($\tau_L \approx 10^{-11}$ s): the NOE becomes virtually independent of S^2, as is illustrated in Fig. 1D. Nevertheless, an increase (decrease) in the amplitude of local motion leads to an increase (decrease) in the steady-state $C^{\alpha}CO$ NOE for all relevant values of the local correlation time. Thus, the simple and sensitive $C^{\alpha}CO$ NOE experiment could be used as a monitor of motional changes in the protein backbone. For instance, effects of ligand binding, protein folding and mutagenesis on backbone dynamics (and on glutamine/asparagine side-chain dynamics) could be measured with this method without requiring extensive relaxation data analysis.

The effect of S^2 on the steady-state NOE (Figs. 1B–1D) is plotted directly in Fig. 2A for a global correlation time $\tau_R = 12$ ns, relevant for proteins in the 20 kDa size range. This shows again that the steady-state NOE by itself is not sufficient to quantitatively characterize protein local motion, since it is dependent on two parameters. A similar observation can be made for the ^{13}CO T_1 values (Eq. 3) plotted in Fig. 2B. However, the ratio of the two parameters, σ (Eq. 1), is independent of the local correlation time to a very good approximation, as shown in Fig. 2C. This is because the cross-relaxation rate σ is solidly in the slow motional limit for macromolecules with $\tau_R = 12$ ns at 14 T. We thus obtain that $\sigma \propto -S^2\tau_R$ to a very good approximation for most relevant values of τ_L and S^2. The measurement of the cross-relaxation rate σ thus gives direct access to the order parameter S^2, provided that the overall correlation time τ_R is known. We note that such a simple relationship does not hold for the ^1H-^{15}N cross relaxation.

The $^{13}C^\alpha$-^{13}CO dipolar cross-relaxation rate can be measured directly (as shown by Cordier et al. in their communication elsewhere in this issue) or, as we demonstrate here, by measuring the $C^\alpha CO$ NOEs and the ^{13}CO T_1 relaxation rates. An advantage of our approach is that, once the values for S^2 are obtained from the cross relaxation, local correlation times τ_L can be estimated from the already available T_1 relaxation curves. Figure 3A shows the pulse sequence of our steady-state $C^\alpha CO$ NOE experiment, which starts with the saturation of the $^{13}C^\alpha$ spins using a train of Gaussian pulses. The ^{13}CO magnetization is transferred to the HN resonance through a triple-resonance pathway (Ikura et al., 1990; Montelione and Wagner, 1990), with concurrent measurement of the ^{13}CO, ^{15}N and ^1HN frequencies in two or three dimensions, as indicated. The control experiment is carried out with the same sequence, but now with the saturating pulse train centered at the symmetrical off-resonance position. Two technical differences between this experiment and the ^1H-^{15}N steady-state NOE experiment stand out. First, as the magnetization is donated by carbon rather than nitrogen, the sensitivity of the $C^\alpha CO$ NOE is much better than that of the ^1H-^{15}N NOE experiment, and, second, the resonance intensities cannot be affected by solvent saturation transfer effects. The spectra have signal-to-noise ratios of 20:1 to 40:1 for 2 mM T4-lysozyme (19.6 kDa) and partial cross peak volumes (Rischel, 1995) were measured with a typical signal-to-noise ratio of 50:1, or with a precision of 2%. The $C^\alpha CO$ NOEs are obtained by taking the ratio of intensity/volume parameters of the two data sets. Assignments for the resonances were taken from Fischer et al. (1995) and the obtained NOEs are displayed in Fig. 4A and have a typical precision of 4%, as indicated.

Figure 3B shows the pulse sequence used for the determination of the ^{13}CO T_1 relaxation rates; the sequence is a member of a family of ^{13}CO relaxation measurements currently in development in our group. A more simple experiment, starting from inverted ^{13}CO magnetization, has a higher sensitivity per scan for T4-lysozyme; however, the sequence shown can be repeated much faster for higher overall sensitivity. The sequence as used did not contain the selective $^{13}C^\alpha$ pulses during the T_1 relaxation delay, which serve to suppress the cross correlation between the ^{13}CO CSA and $^{13}C^\alpha$-^{13}CO dipolar relaxation mechanisms (a small effect) and the cross relaxation with $^{13}C^\alpha$ (NOE). This latter effect causes biexponential relaxation curves and may cause up to 10% systematic error in the determination of the ^{13}CO T_1 relaxation rates for the system under consideration. However, since we obtain the ^{13}CO T_1 values with an average random error of 10% due to limitations in the signal-to-noise ratio (Fig. 4B), the presence/absence of the selective $^{13}C^\alpha$ pulses was not critical to the current data analysis. The calculated cross-relaxation rates, obtained from the NOE and T_1 experiments, thus have an average error of 14% and the data

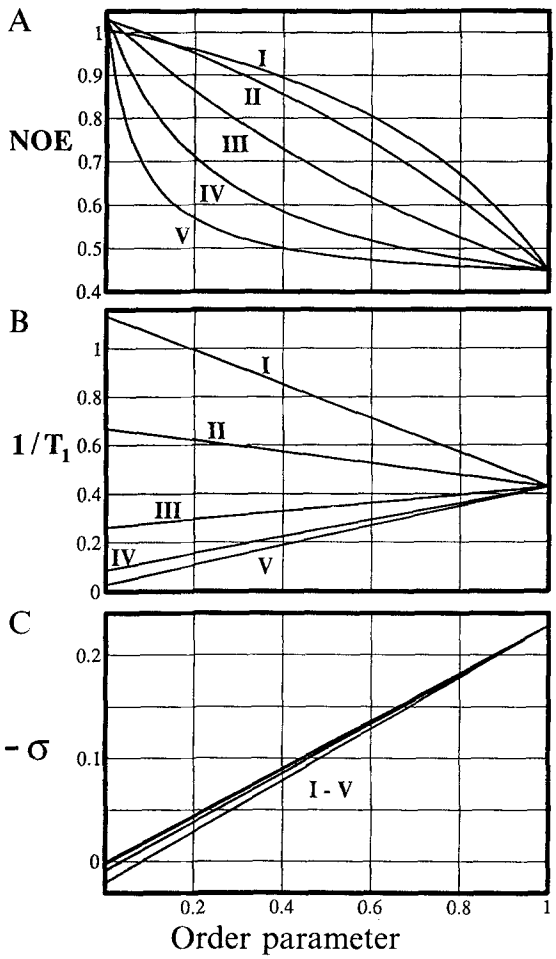

Fig. 2. Calculations of the dependence of ^{13}CO relaxation phenomena on order parameter and local correlation time for a global correlation time of 12 ns. (A) The steady-state nuclear Overhauser effect; (B) the ^{13}CO T_1 relaxation rate; and (C) the cross-relaxation rate σ. Curves I–V in A–C refer to local correlation times τ_L of 10^{-9}, 3×10^{-10}, 10^{-10}, 3×10^{-11} and 10^{-11} s, respectively.

take the shape as shown in Fig. 4C. It is evident that the cross-relaxation rates can be measured with sufficient precision to allow detection of interesting variations from site to site.

Full analysis of the cross-relaxation data for T4-lysozyme is complicated by the fact that T4-lysozyme is a nonglobular protein, for which the rotational correlation time differs from vector to vector. In the crystal, T4-lysozyme is a prolate ellipsoid, with aspect ratios of roughly 2:1:1 (Weaver and Matthews, 1987). We are currently in the process of determining the anisotropy of rotation of T4-lysozyme in solution from a detailed analysis of ^{15}N relaxation data (M. Fischer and E. Zuiderweg, unpublished results). For illustration purposes, we use the largest value (12 ns) of τ_R as obtained for T4-lysozyme from ^{15}N $T_1 / T_{1\rho}$ ratios to compute a theoretical cross-relaxation rate corresponding to an order parameter of 1 (horizontal line in Fig. 4C). The average ^{13}CO-$^{13}C^{\alpha}$ order parameter of 0.7 is seen to be less than commonly observed for ^{15}N-^{1}H order parameters. A general value for τ_L can be gauged from the observation that the site-to-site variation in steady-state NOE (Fig. 4A) is much less than the variation in T_1 relaxation data (Fig. 4B). According to Figs. 2A and B, this indicates that the local motions must be fast (10^{10}–10^{11} s^{-1}). The data also show that virtually all sites have order parameters less than 1, but that several sites appear to be much more ordered than other sites. Further analysis of relaxation data, for ^{13}CO, $^{13}C^{\alpha}$ and ^{15}N, is in progress in order to find explanations for these unexpected observations.

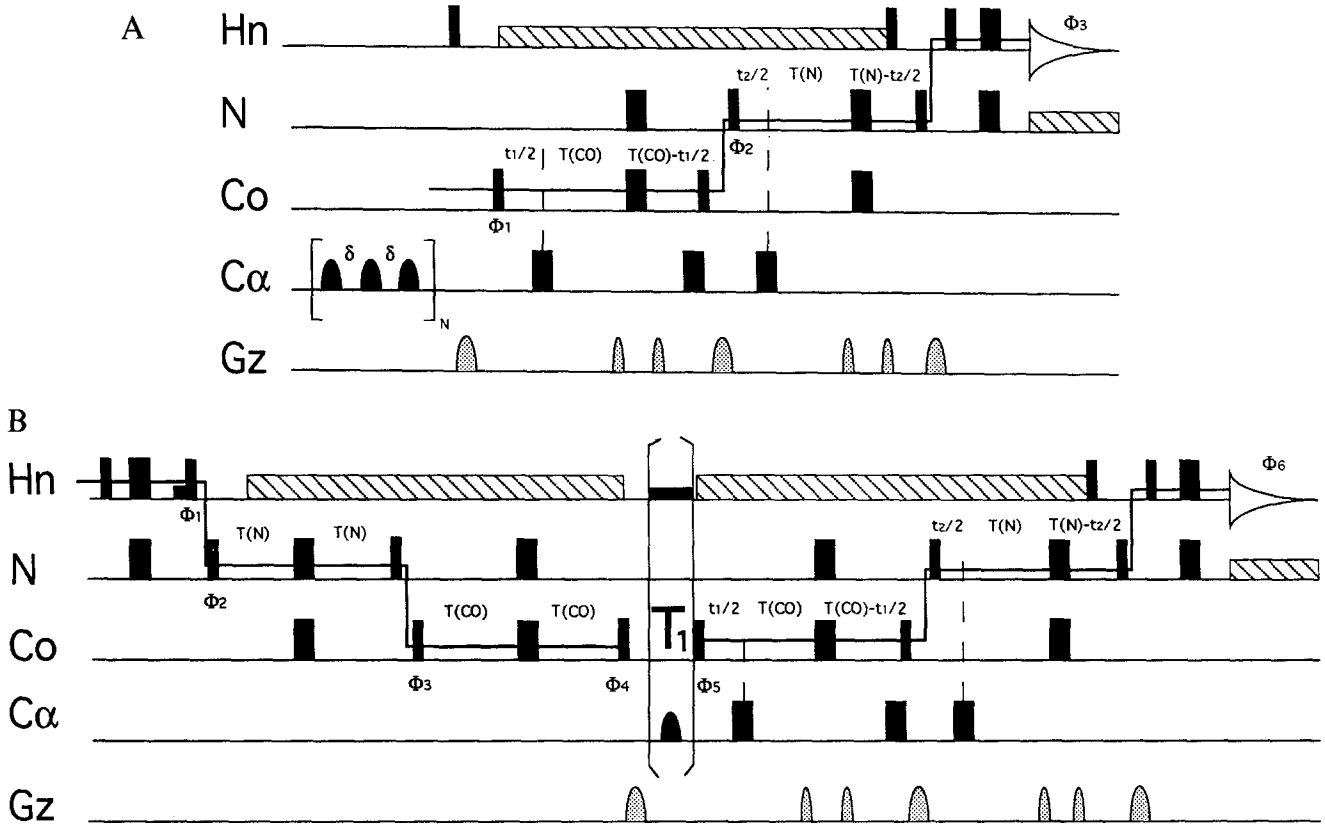

Fig. 3. Pulse sequences used for the determination of (A) the steady-state NOE between $^{13}C^{\alpha}$ and ^{13}CO and (B) the T_1 relaxation of ^{13}CO. Narrow and wide boxes indicate 90° and 180° rf pulses, respectively, with phase x, unless indicated otherwise. Parameters for the steady-state NOE experiment: phase cycles were: $\Phi_1 = y,-y$; $\Phi_2 = x,x,-x,-x$; $\Phi_3 = +,-,-,+$; T(CO) = 14 ms; T(N) = 13 ms. The saturation pulse train consisted of a train of Gaussian pulses of length 500 μs with a flip angle of approximately 120°. The delay δ was 20 ms and the cycle was repeated for a total time of 10.25 s, with no further recycle delay. This pulse train is adequate to fully saturate the $^{13}C^{\alpha}$ spectral envelope, as was verified by observing the ^{13}C spectrum directly after the pulse train. This sequence also leaves the ^{13}CO spectral envelope unperturbed, as was verified by centering the saturation pulse train at an equal separation to higher frequency (290 ppm) and observing the ^{13}CO spectrum. The NOE control experiment is carried out with the same sequence and identical parameters, but with the saturating pulse train centered at the symmetrical off-resonance position. Parameters for the T_1 experiment: phase cycles were: $\Phi_1 = y,y,-y,-y$; $\Phi_2 = 4(x),4(-x)$; $\Phi_3 = x,-x$; $\Phi_4 = y$; $\Phi_5 = 8(y),8(-y)$; $\Phi_6 = a,b,b,a$ with $a = +,-,-,+$ and $b = -a$. The recycle delay was 2 s; T(CO) = 14 ms; T(N) = 13 ms. Relaxation cross-correlation mechanisms with ^{15}N, $^{1}H^{\alpha}$ and ^{1}HN were not suppressed (Boyd et al., 1990; Palmer et al., 1991) during the T_1 delay for compatibility with the NOE experiment. The sequence as used did not contain the selective $^{13}C^{\alpha}$ pulses during the T_1 relaxation delay, which would serve to suppress the cross correlation between the ^{13}CO CSA and $^{13}C^{\alpha}$-^{13}CO dipolar relaxation mechanisms (which is suppressed in the NOE experiment) and the cross relaxation with $^{13}C^{\alpha}$. It is recommended to use the same selective pulses as described above, repeated at least every 50 ms. The only rf present during the T_1 delay in the performed experiments was a spin-lock on the H_2O resonance.

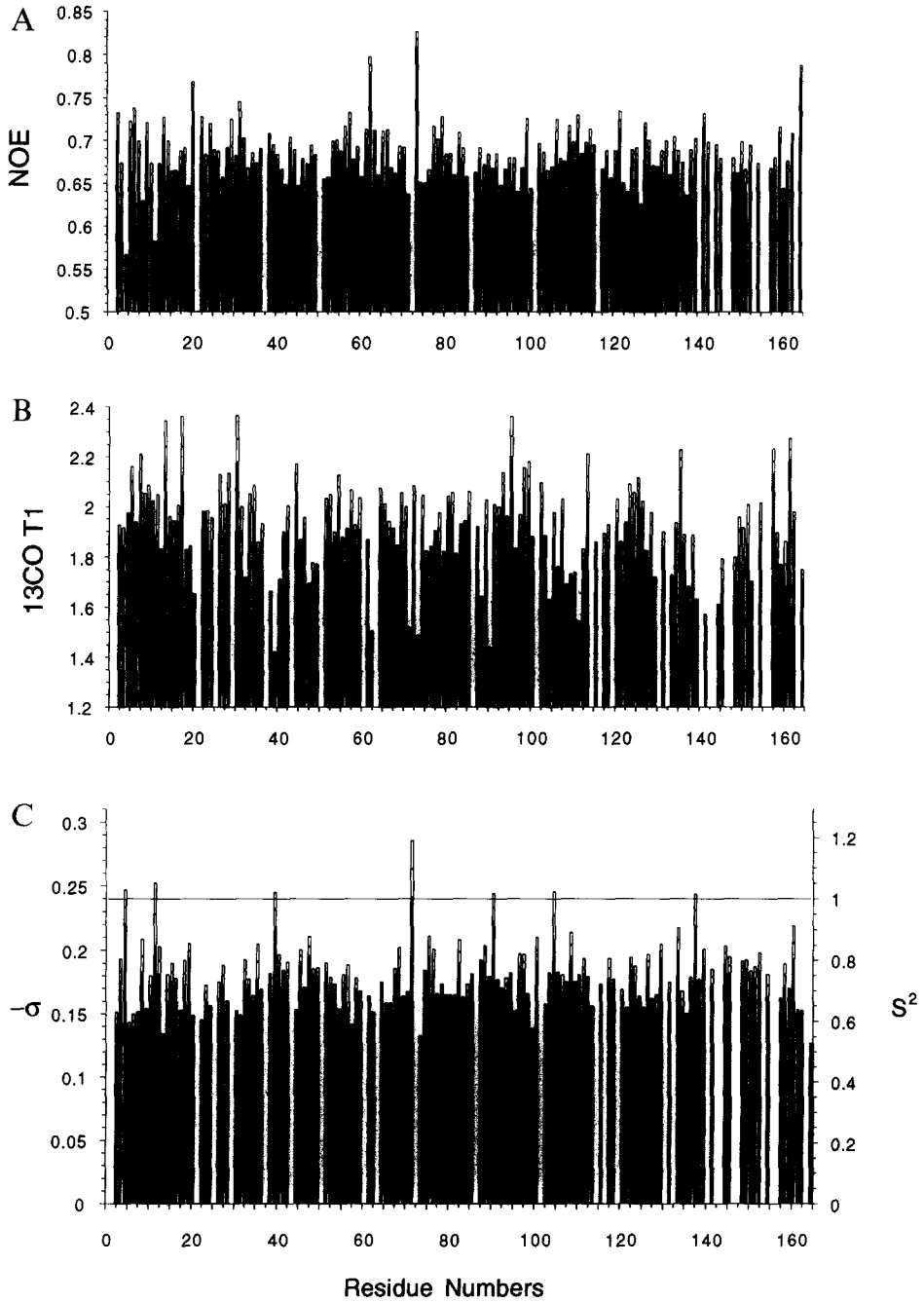

Fig. 4. Experimental ^{13}CO relaxation data for a 2 mM solution of doubly labeled T4-lysozyme (19.6 kDa) in 90% H_2O, pH 5.5, at 30 °C and 14.1 T. The experiments were acquired on a Bruker AMX 600 spectrometer, equipped with a 5 mm triple-resonance probe with a pulsed-field gradient coil. (A) The steady-state NOE. The experimental time was 70 h for the saturation experiment and the control experiment combined; both were acquired as 2D data sets (^{13}CO,^{1}HN). The error bars were obtained from an estimation of the enhancement of the one-dimensional signal-to-noise ratio by partial volume integration. (B) ^{13}CO T_1 relaxation parameters as obtained from three independent series of experiments using T_1 relaxation delays of 10, 150, 400, 1000 and 2000 ms. The total experimental time for all 15 experiments combined was approximately 120 h. The experiments were recorded as 2D data sets (^{13}CO,^{1}HN). The error bars were obtained using the Monte Carlo method as described by Nicholson et al. (1992). (C) The cross-relaxation rates σ for T4-lysozyme as obtained from (A) and (B). Error bars were obtained by adding the error percentages of (A) and (B). A cross-relaxation rate of $-0.24\ s^{-1}$ (horizontal line) is computed for a spherical molecule with a rotational correlation time of 12 ns (experimental value for T4-lysozyme) and an order parameter S^2 of 1.

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