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$^{13}C-^{13}C$ NOESY: An Attractive Alternative for Studying Large Macromolecules

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The set of NMR experiments commonly used for structure determination is largely based on direct ^1H detection. The constructive use of cross correlation phenomena (TROSY, CRINEPT)[1−3] has greatly contributed to solving the problems of fast proton relaxation that arise from increasing molecular mass of the systems.[4] This approach, however, works well only for NH and aromatic CH groups. ^{13}C direct detection provides a valuable alternative to ^1H detection to overcome fast relaxation due to the smaller magnetic moment of the ^{13}C nucleus. This is also the origin of the lower sensitivity of detection of ^{13}C, which, however, can be compensated for by the evolution of technology. Direct detection of ^{13}C has been successfully applied to paramagnetic proteins,[5−10] and it is promising as a general strategy for large systems.[11,12] For this purpose, we would like to exploit dipolar-based experiments for signal detection and assignment in large macromolecules, for which the scalar experiments become less effective.

It is well-known that transverse relaxation rates increase with increasing molecular mass, causing severe and unavoidable limitations for all experiments that rely on coherence transfer steps between in-plane magnetization. Longitudinal relaxation rates, however, often exhibit the opposite behavior: they decrease with increasing molecular mass.[13] Experiments in which magnetization is stored along the *z*-axis will thus take advantage of this property. When magnetization is along the *z*-axis, cross relaxation occurs at a rate σ that, for like spins, increases with molecular mass. Thus, for large systems, $^{13}C-^{13}C$ transfer through the nuclear Overhauser effect (e.g., NOESY-type experiments) will actually become competitive with transfer through scalar couplings (e.g., COSY-type experiments). We present here the potential of one of the simplest NMR experiments, the $^{13}C-^{13}C$ NOESY with direct ^{13}C detection, as a valuable tool for the investigation of large macromolecules. To overcome sensitivity problems, an NMR probe with an internal cryogenic ^{13}C coil, optimized for ^{13}C observation, has been used (DUAL $^{13}C\{^1H\}$ CryoProbe).

The 2D $^{13}C-^{13}C$ NOESY experiments were carried out on Cu,Zn superoxide dismutase (SOD), a dimeric enzyme of 32 kDa (τ_r = 26 ns, D||/D⊥ = 1.8),[14,15] and on a monomeric mutant of 16 kDa (τ_r = 9.3 ns, D||/D⊥ = 0.9)[14,15] on a 500 MHz spectrometer. Figure 1 shows the region containing C$\alpha-$Cβ connectivities of serines and threonines from two 2D $^{13}C-^{13}C$ NOESY experiments acquired with exactly the same parameters on monomeric (panel A) and dimeric (panel B) SOD. All the expected C$\alpha-$Cβ connectivities can be detected, and as theoretically predicted, the intensity of cross-peaks is much higher in the spectra of the dimer. Of course, a further increase in molecular mass is expected to be even more advantageous for this type of experiment.

The present results should be compared with those obtained using the experiment proposed by Zuiderweg, who replaced the COSY transfer step with a cross relaxation CC transfer step in HCCH

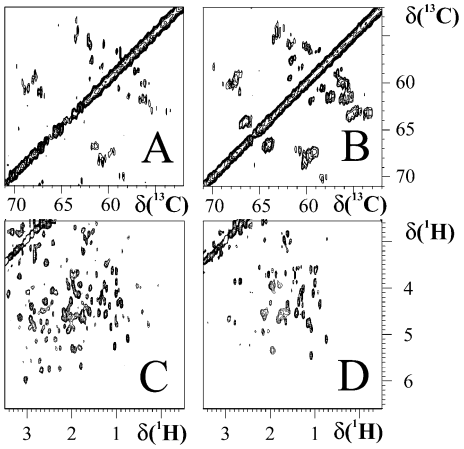

Figure 1. 500 MHz 2D $^{13}C-^{13}C$ NOESY (top) and $^1H-^1H$ 2D HCCH NOESY (bottom) experiments acquired at 298 K on ^{13}C, ^{15}N labeled monomeric (left) and dimeric (right) SOD with (B) and without (D) 70% ^2H labeling to be in the best conditions for the two experiments. Each experiment was acquired on the two samples with the same parameters. The $^{13}C-^{13}C$ NOESY experiments were acquired on a DUAL $^{13}C\{^1H\}$ CryoProbe with a recycle delay of 1.0 s, a mixing time of 300 ms, a spectral width of 200 ppm in both dimensions centered at 98 ppm, 4192 × 256 points, and with 128 scans. The $^1H-^1H$ 2D HCCH NOESY experiments were acquired on a TXI CryoProbe with 1024 × 256 data points, 128 scans, a mixing time of 320 ms, and a recycle delay of 1.0 s. All the other parameters were as described in the original paper.[16]

experiments when working with large systems.[16] Despite the improvement, ^1H detection is still a limitation. In Figure 1, the fingerprint region containing H$\alpha-$Hβ correlations of two HCCH NOESY spectra recorded on monomeric (panel C) and dimeric (panel D) SOD are reported. While nearly all expected correlations are detected in the monomeric SOD sample, only a fraction of them are detected in dimeric SOD, and a further decrease in the number of cross-peaks is observed when using the 70% ^2H labeled dimeric SOD sample (see Figure 1 in the Supporting Information). Therefore, this approach that can be useful for intermediate molecular mass proteins shows severe drawbacks with large molecular mass systems, in particular when fractional ^2H labeling becomes mandatory.

Another interesting consequence of direct ^{13}C detection is that we can monitor ^{13}C spins with no attached protons such as carbonyls, Cγ of aromatic residues, and quaternary carbon atoms. Through the 2D $^{13}C-^{13}C$ NOESY, for example, it is easy to connect the backbone of each aromatic residue of SOD with its side chain through the C$\gamma-$Cβ correlations as these fall in an uncrowded region of the spectrum (not shown).

The 2D $^{13}C-^{13}C$ NOESY experiment can also provide essential information for the assignment of large proteins and can be complementary to the set of TROSY triple resonance experiments. An interesting spectral region is the one containing correlations of carbonyls with Cα's. This region of the spectrum acquired with a

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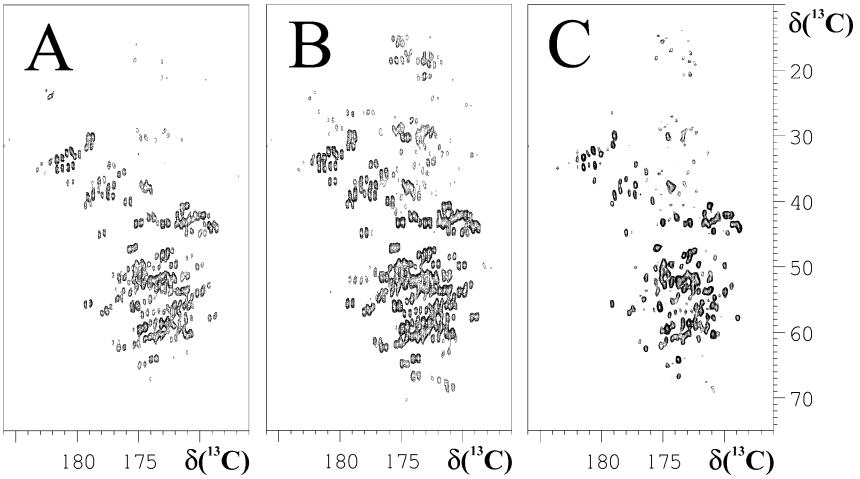

Figure 2. The part of 2D 500 MHz $^{13}C-^{13}C$ NOESY experiments (298 K) containing CO−Cα correlations obtained on dimeric SOD (^{13}C, ^{15}N, 70% ^{2}H labeled) with (A) 300 ms mixing time and (B) 800 ms mixing time. Panel C reports the experiment acquired with 800 ms mixing time but processed with the Rowland NMR Toolkit, kindly provided by Jeffrey C. Hoch, to remove the CO−Cα splitting through maximum entropy reconstruction. Experiments were acquired with the parameters reported in the caption to Figure 1, except for the mixing time, the relaxation delay, and the number of points in the indirect dimension of 800 ms, 1.2 s, and 512 for the experiment reported in panels B and C.

mixing time of 300 ms on dimeric SOD is reported in Figure 2A. It can be observed that all one-bond correlations that can be identified in a COCAMQ[10] experiment (not shown) are also present in the NOESY spectrum acquired with 300 ms. This means that CC dipole−dipole correlations at one bond distance can be detected. Additionally, some very weak peaks in the CO−Cβ region can be observed. By increasing the mixing time of the experiment to 800 ms, the CO−Cβ intraresidue correlations become more intense so that the majority of them can be observed (Figure 2B). The CO−Cα splitting in the acquisition dimension that complicates the spectra could be removed by using post-acquisition processing methods, such as deconvolution with maximum entropy reconstruction[11,17] (Figure 2C) or through bandselective homonuclear decoupling.[10] Therefore, this NOESY experiment provides information to correlate intraresidue CO, Cα, and Cβ spins. In other words, by knowing approximately the molecular mass of the molecule, it is possible to tailor $^{13}C-^{13}C$ NOESY experiments to detect mainly one bond or two bond distances, or eventually, even longer range correlations.

In summary, we have shown that $^{13}C-^{13}C$ NOESY experiments may be very useful for solving the problems arising from fast relaxation in large systems. The $^{13}C-^{13}C$ NOESY experiment could be included in the set of TROSY triple resonance experiments to study systems characterized by a large molecular mass.[4,18,19] As an example, it is useful in correlating the carbonyl with Cα and Cβ, which may become prohibitively insensitive with the traditional approaches as the molecular mass increases. The problem of resonance overlap could be overcome by developing software and hardware. However, even at the current stage, the $^{13}C-^{13}C$ NOESY experiment may be the preferred choice in situations where an increase in molecular mass is not accompanied by an increased number of signals, such as in multimeric proteins[20] or fairly small molecules tightly bound to large ones or membrane proteins solubilized in micelles.[21]

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Supporting Information Available: Figure 1 reports the HCCH NOESY experiments acquired, including the one acquired on the 70% ^{2}H labeled sample (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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