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^{13}C-^{13}C NOESY: A constructive use of ^{13}C-^{13}C spin-diffusion

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Abstract

^{13}C-^{13}C NOESY experiments were performed under long mixing time conditions on reduced human superoxide dismutase (32 kDa, ^{15}N, ^{13}C and 70% ^{2}H labeled). ^{13}C-^{13}C couplings were successfully eliminated through post-processing of in-phase-anti-phase (IPAP) data. It appears that at mixing time τ_m of 3.0 s the spin diffusion mechanism allows the detection of 96% of the two-bond correlations involving C′ and C$^{\beta}$. The interpretation was confirmed by simulations. This approach broadens the range of applicability of ^{13}C-^{13}C NOESY spectroscopy.

Introduction

Structural studies of proteins are critical for the understanding of biological processes at molecular level. Nuclear magnetic resonance spectroscopy (NMR) is a powerful technique for obtaining structural and dynamic information on small to medium size proteins. The development of novel NMR strategies for the investigation of high molecular mass biomolecules represents nowadays a major challenge for structural biology.

Direct detection of ^{13}C NMR has recently been applied to several paramagnetic proteins, where the contribution to line broadening coming from the paramagnetic center is so large that ^{1}H signals are broadened beyond detectable limits in a wide sphere around the metal ion (Kolczak et al., 1999; Bertini et al., 2001; Machonkin et al., 2002; Kostic et al., 2002; Amesano et al., 2003; Bermel et al., 2003). These applications are renewing the interest on low-γ NMR spectroscopy also in diamagnetic molecules, (Pervushin and Eletsky, 2003; Eletsky et al., 2003; Bertini et al., 2004) as heteronuclear-detected NMR avoids the detrimental fast ^{1}H transverse relaxation typical of large molecular mass proteins, that renders insensitive the usual triple resonance experiments used for signal assignment.

In this context we have shown that NOESY experiments based on dipole-dipole interaction with longitudinal magnetization transfer represent a valuable alternative to experiments based on scalar couplings to detect C-C one bond correlations in very fast relaxing systems, such as paramagnetic or large macromolecules (Bermel et al., 2003; Bertini et al., 2004). Indeed, magnetization transfer in NOESY experiments is affected by longitudinal relaxation while coherence transfer due to scalar coupling is affected by transverse relaxation, the latter being sizably faster than the former in these systems (Banci et al., 1991). Therefore NOESY type experiments become useful for signal detection and assignment. Moreover, we have already shown that, with a proper choice of the experimental parameters, ^{13}C-^{13}C NOESY experiments provide, in addition to one bond correlations, also some two bond correlations such as, for example, C′-C$^{\beta}$ ones (Bertini et al., 2004).

To fully exploit the potential of NOESY-based experiments, we investigated how efficient is multispin magnetization transfer in providing information useful for ^{13}C signal assignment in a perdeuterated protein, reduced human Cu,Zn superoxide dismutase (SOD). We will show that spin diffusion, usually considered

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detrimental for the determination of protein solution structures, can be precious for the assignment of intra-residue ^{13}C nuclei of a large molecular mass protein.

Experimental

Sample preparation

Dimeric human SOD (MW 32 kDa) was expressed in *Escherichia coli* TOPP1 strain (Stratagene). The ^{2}H (70%), ^{13}C, ^{15}N labeled protein was obtained by growing the cells in minimal medium (M9) as previously reported (Banci et al., 2000). The samples were isolated and purified according to previously published protocols (Getzoff et al., 1992). Reduction of the copper ion was achieved by addition of sodium isoascorbate to a total concentration of about 6 mM, in 20 mM phosphate buffer at pH 5.0 under anaerobic conditions. The NMR sample had a concentration of about 2 mM in dimeric protein and contained 10% D_2O for the lock signal.

NMR experiments

^{13}C direct detection experiments were acquired with a DUAL ^{13}C-{^{1}H} CryoProbe^{TM} optimized for ^{13}C sensitivity operating at 11.7 T (500.23 MHz for ^{1}H and 125.78 MHz for ^{13}C). Composite pulse decoupling on ^{1}H and ^{2}H was applied during the whole duration of the experiments with an RF field strength of 3.3 kHz for ^{1}H and of 1.0 kHz for ^{2}H (Waltz-16) (Shaka et al., 1983), respectively. The carrier frequencies for pulses applied on ^{1}H and ^{2}H were of 4.7 and 3.3 ppm, respectively, unless otherwise specified.

To check the quality of the sample an experiment to detect one bond C'-C^{α} multiple-quantum correlations, the CACO-MQ experiment (Bermel et al., 2003), was recorded with 1.0 s recycle delay, 128 dummy scans and 16 transients. The total acquisition time in the indirect dimension was $t_1^{max} = 8.7$ ms. Spectral windows of 40 and 60 ppm were acquired in the $^{13}C'$ and $^{13}C^{\alpha}$ dimensions, respectively, corresponding to a matrix of 1024 × 128 points. The carrier on the carbon channel is switched from 175 to 50 ppm before the first $\pi/2$ ^{13}C pulse and is switched back to 175 ppm after the second $\pi/2$ ^{13}C pulse.

Six ^{13}C-^{13}C NOESY experiments were acquired in total. Three were recorded with a spectral width of 200 ppm in the two dimensions, with 4096 × 256 points, with 81 ms acquisition time, 128 scans per increment (256 dummy scans), a relaxation delay between 1.0 and 1.2 s, ^{13}C carrier frequency of 98 ppm, unless otherwise specified. The mixing time used in the three cases were 300 ms, 800 ms, and 3.0 s, with 256, 400 and 256 increments in the indirect dimension, respectively. A fourth experiment was acquired with a mixing time of 1.0 s, a spectral width of 50.2 and 100.0 ppm in the direct and indirect dimensions respectively, 4096 × 128 points, 2048 scans per increment and 0.6 s of recycle delay. The first two $\pi/2$ pulses were semi-selective shaped pulses on the $C^{\alpha/\beta}$ region, while the third one was semi-selective on the C' region. Universal Gaussian cascades were used (Emsley and Bodenhausen, 1990a, 1992), 384 μs duration, Q5 shape for the $\pi/2$ $C^{\alpha/\beta}$ and C' pulses centered at 35 and 175 ppm respectively. The $\pi/2$ pulse with phase ϕ_2 (Figure 1A) was given with the time-reversed Q5 shape. The fifth experiment was run with a mixing time of 3.0 s, spectral widths of 30 and 197 ppm in the direct and indirect dimensions respectively, 2048 × 300 points, 64 scans per increment, and 1.0 s of recycle delay. The first two $\pi/2$ pulses were hard pulses (8 μs) centered at 98 ppm, while the last pulse was a semi-selective C' pulse (384 μs duration, Q5 shape) centered at 175 ppm. Finally, the latter experiment was repeated by including, in the last part of the pulse sequence, the IPAP building block (Ottiger et al., 1998; Andersson et al., 1998) to remove the C'-C^{α} coupling in the direct dimension (Duma et al., 2003a, 2003b). The block inserted is described in the following section. The π $C^{\alpha/\beta}$ pulses employed a Q3 shape with 308 μs duration. The experiment was acquired with 96 scans per transient and per component (one for the in-phase, one for the anti-phase) and in total 764 experiments were acquired.

Data were processed with the XWINNMR 3.1 Bruker software.

Description of the ^{13}C-^{13}C NOESY -IPAP experiment

The pulse scheme for the ^{13}C-^{13}C NOESY using semi-selective pulses (A) and its variations to implement the IPAP approach (B,C) are shown in Figure 1.

The variant of the pulse scheme reported in the figure is the one to detect dipolar correlations involving C' in the direct dimension and $C^{\alpha/\beta}$ in the indirect dimension. The first two $\pi/2$ NOESY pulses are centered on the aliphatic region, while the last one is centered on the carbonyl region; the π semi-selective pulses are necessary to refocus the C'-C^{α} coupling in

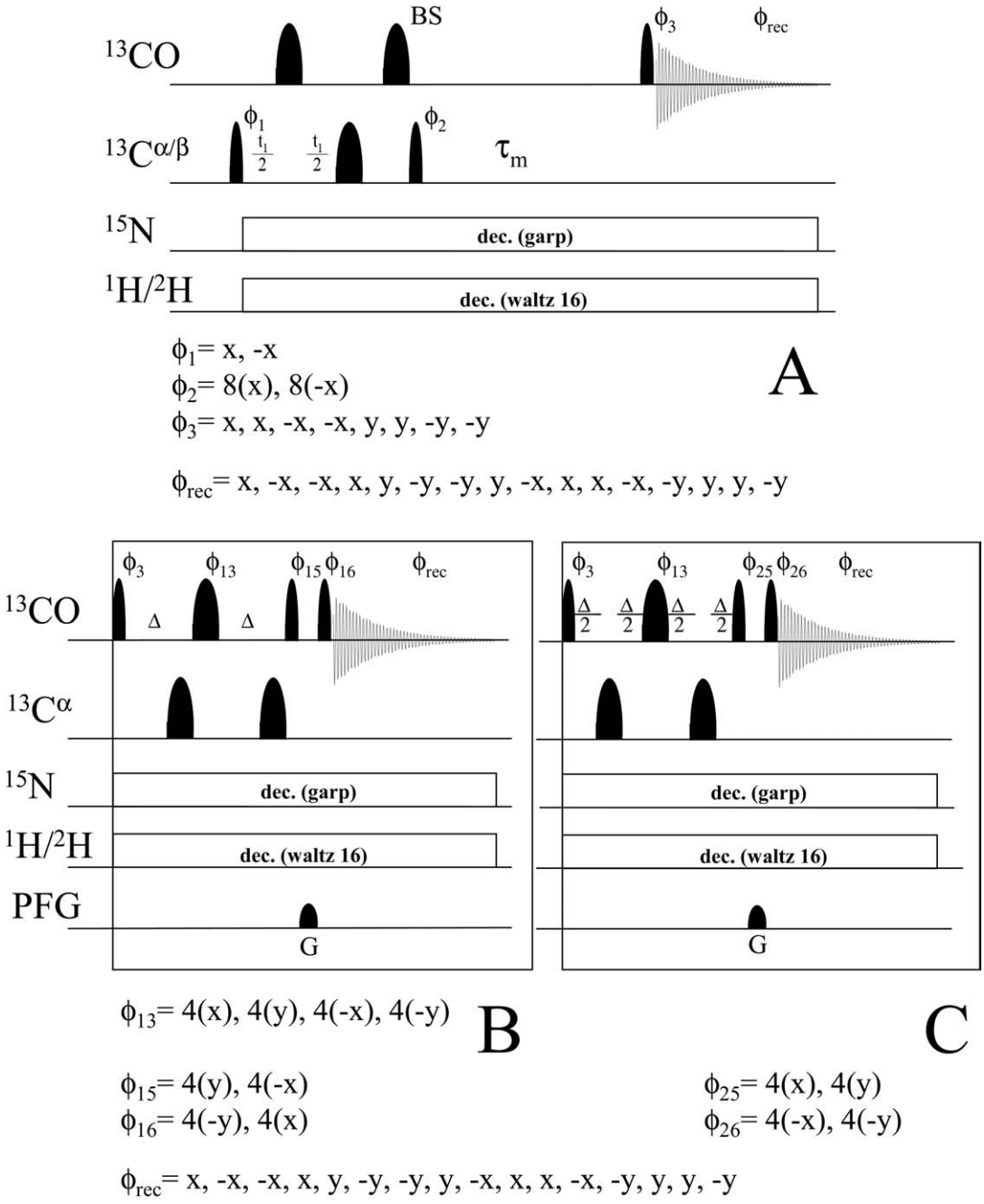

Figure 1. Pulse scheme for the ^{13}C-^{13}C NOESY using semi-se]ective pulses (A) and variations to implement the IPAP selection filter (B, C). The variant of the pulse scheme reported in the figure is the one to detect dipolar correlations between C′ in the direct dimension and $C^{\alpha/\beta}$ in the indirect dimension. Narrow and wide shaped pulses represent π/2 and π semi-selective pulses, respectively. The first two π/2 NOESY pulses are centered on aliphatic region (35 ppm); the π semi-selective pulses in between are necessary to refocus the C′-C^{α} coupling in the indirect dimension and to compensate tor Bloch–Siegert (BS) phase shifts. The last π/2 semi-selcctive pulse is centered on the carbonyl region (175 ppm). The IPAP approach to suppress the C′-C^{α} coupling in the direct acquisition dimension was implemented by replacing the last π/2 semi-selective C′ pulse with the building blocks illustrated in panels B (anti-phase component) and C (in-phase component). The πC^{α} pulses are centered at 50 ppm. The delay Δ is set to $1/4J_{C'C\alpha..}$. A *z*-filter is included before acquisition to improve separation of the in-phase and anti-phase components.

the indirect dimension and to compensate for Bloch–Siegert phase shifts (Bloch and Siegert, 1940; Emsley and Bodenhausen, 1990b).

The IPAP filter to separate the two C'-C^{α} multiplet components in the direct acquisition dimension (Ottiger et al., 1998; Andersson et al., 1998) was implemented by replacing the last $\pi/2$ semi-selective C' pulse (Figure 1A) with the building blocks illustrated in panels B (anti-phase component) and C (in-phase component). The C' in-phase magnetization present after the $\pi/2$ semi-selective pulse in each building block is converted during the delay 2Δ (with Δ equal to $1/4J_{C'C^{\alpha}}$) into anti-phase magnetization respect to C^{α} (Figure 1B) or is maintained in-phase (Figure 1C), keeping constant, in the two cases, the number of pulses and relaxation delays. The last two $\pi/2$ semi-selective C' pulses, separated by a gradient, improve the separation of in-phase and anti-phase components. Care should be taken to shift by $\pi/2$ the phase of the last two semi-selective $\pi/2$ pulses in panel B and C of Figure 1.

For each time increment in the indirect dimension two FIDs are stored separately, one for the anti-phase and one for the in-phase components. The two FIDs are then summed and subtracted to separate the two multiplet components. These are then shifted to the center of the original multiplet (by $J_{C'C^{\alpha}}/2$) and again summed to obtain a singlet. An automatic program to perform these manipulations directly in the time domain before transformation into the frequency domain, written for XWINNMR 3.1, is available upon request.

Simulations

Simulations were run with the Matlab software (The Mathworks Inc.). A simplified three spin system was considered (C', C^{α}, C^{β}). The elements of the ^{13}C relaxation matrix R ($R_1(C')$, $R_1(C^{\alpha})$, $R_1(C^{\beta})$, $\sigma_{C'C^{\alpha}}$, $\sigma_{C^{\alpha}C^{\beta}}$ and $\sigma_{C'C^{\beta}}$ were calculated including the following contributions: chemical shift anisotropy (Hiyama et al., 1988; Tessari et al., 1997; Veerman, 1984), one-bond dipole-dipole interactions (Ernst et al., 1987) and also a few short range interactions with ^{1}H for heteronuclei (i.e., C'-H^{N}). The evolution of the three spin system after an initial perturbation of C' was evaluated by numerical integration (Neuhaus and Williamson, 1989).

The same was repeated for a two spin system constituted only by C' and C^{β} to compare the data with a hypothetical direct two bonds transfer in the absence of spin diffusion.

Results and discussion

It has already been shown that the NOE between two carbons at one bond distance in macromolecules at high field is sufficient to yield measurable cross peaks in NOESY spectra, both through direct ^{13}C-^{13}C NOESY (Bertini et al., 2004) experiments as well as detecting it through ^{1}H (Fischer et al., 1996). The ^{13}C-^{13}C NOESY experiments acquired on dimeric SOD show, in addition to one bond correlations, also some specific sets of two bond correlations. This is potentially very interesting, as combinations of one-bond and two-bond correlations can be used for assignment purposes. However only two-bond ^{13}C-^{13}C correlations between two carbons that are separated by another carbon nucleus are detected with good sensitivity because this pathway is allowed by spin-diffusion (Fischer et al., 1996; Bertini et al., 2004). Particularly relevant is the C'-C^{α}-C^{β} correlation. Results of the simulation performed on a C'-C^{α}-C^{β} spin system for different values of the overall rotational correlation time (τ_r = 27, 50 and 100 ns) are shown in Figure 2. The curves report the decay of the diagonal peak ($\cdots$) and the buildup of the one bond direct (- - -), and the two bonds direct (—) processes, as well as the sum of the two bonds direct and spin-diffusion process (– $\cdots$ –). As expected the one-bond direct NOE and the two bond spin diffusion reach maximal intensities at different mixing times, the spin diffusion being at maximum at mixing times about two-four times longer than the direct effect. The figure clearly shows that the C-C spin-diffusion process that occurs through the one bond cross relaxation rates is very efficient, in particular at a suitably long mixing time. These expectations can be verified experimentally by increasing the mixing time of ^{13}C-^{13}C NOESY experiments, other conditions remaining virtually unaltered. As an example, the relative intensities of one bond and two bond correlations for Val 5 are shown in Figure 3. It can be noted that the relative intensity of the C'-C^{β} correlation increases as the mixing time increases with respect to the intensity of the C'-C^{α} correlation (to which the traces were normalized). The intensity of the C'-C^{β} correlation is definitely much larger than what expected on the basis of direct transfer, that is about 5% of the C'-C^{α} one-bond correlation (Figure 2).

Two interesting properties that emerge from Figure 2 are that i) the peak intensity of the spin diffusion increases and ii) as the overall correlation time increases, the maximum of both the one bond and two

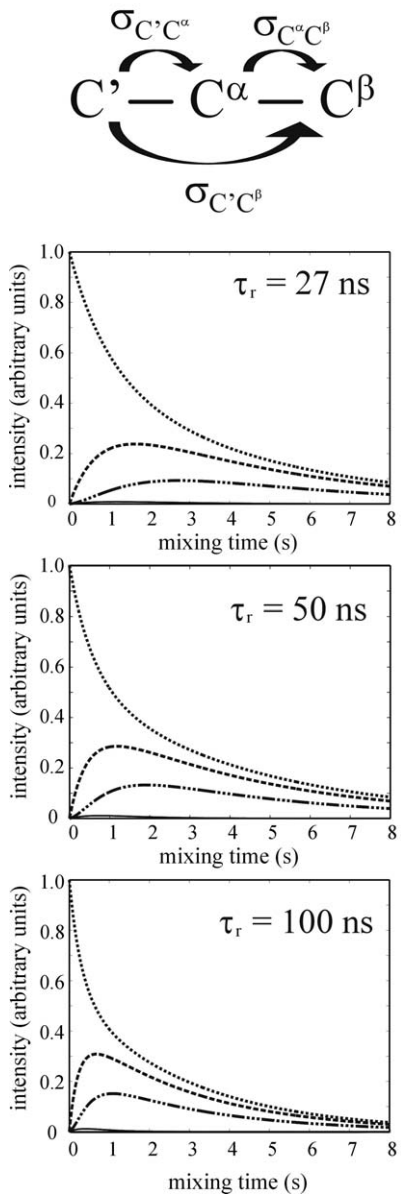

Figure 2. Simulations performed using a simplified three spin system including C^{β}, C^{α}, C' for three values of the overall correlation time (27, 50 and 100 ns). Relaxation rates ($R_1(C^{\beta})$, $R_1(C^{\alpha})$, $R_1(C')$, $\sigma_{C'C^{\alpha}}$, $\sigma_{C^{\alpha}C^{\beta}}$ and $\sigma_{C'C^{\beta}}$) as a function of the overall correlation time were calculated including the following contributions: chemical shift anisotropy (Hiyama et al., 1988; Tessari et al., 1997; Veerrnan, 1984), one-bond dipole-dipole interactions (Ernst et al., 1987) and also a few short range interactions for heteronuclei (i.e. C'-H^{N}, C'-H^{α}). The curves report the behavior of the three spin system after an initial perturbation of C'. The decay of the diagonal peak (C', · · · ·), the buildup of the one bond direct transfer (C'-C^{α}, – – –), as well as the sum of the two bonds direct and spin-diffusion process (C'-C^{β}, – · · · – · · · –), are reported as a function of the mixing time (Neuhaus and Williamson, 1989). For comparison, also the two bond direct transfer, obtained considering a simpJified two spin system is reported (C'-C^{β}, ——-).

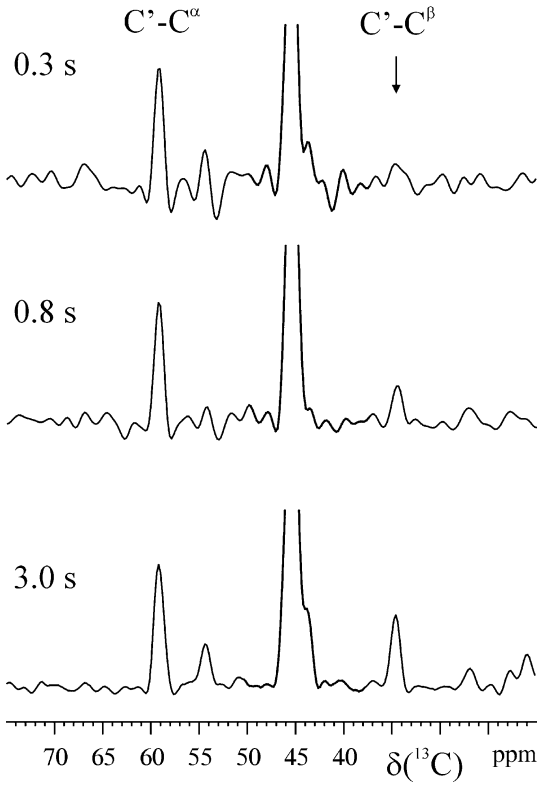

Figure 3. NMR traces along the indirect frequency dimension containing the C'-C^{β} and C'-C^{α} correlations of VAL 5. The traces were extracted from the 125.8 MHz ^{13}C-^{13}C NOESY spectra acquired with different mixing times (0.3 s, 0.8 s, 3.0 s) and are normalized to the intensity of the C^{α}-C' correlation.

bonds buildup curves is shifted to shorter values of the mixing time due to the increase in longitudinal relaxation rates. This means that shorter values of the mixing and recycle delays can be employed with increasing correlation time of the protein, i.e., with increasing molecular mass, thereby reducing the overall duration of the experiment.

The resolution of the experiment can be enhanced by the use of selective pulses (Brüschweiler et al., 1987). Indeed the ^{13}C chemical shift dispersion is much larger than the one on protons, so we can focus on a specific region of the spectrum (C', $C^{aromatic}$, C^{α}, C^{β}). Let us suppose to focus on the region containing C'-C^{β} correlations. Then the cross peaks will originate from the following transfer pathway:*

$$C_z^{\beta} \xrightarrow{\pi/2(C^{\beta})} C_x^{\beta} \xrightarrow{t_1} C_x^{\beta *} \xrightarrow{\pi/2(C^{\beta})} C_z^{\beta} \xrightarrow{\sigma_{CC}} (C_z^{\alpha}) \xrightarrow{\sigma_{CC}}$$
$$\underline{C_z'} \xrightarrow{\pi/2(C')} C_x' \longrightarrow t_2$$

*The evolution due to chemical shift is omitted from the notation for the sake of clarity.

Therefore, to detect the wanted cross-peaks the first two $\pi/2$ pulses, out of the three constituting a NOESY pulse scheme (Figure 1), should be centered in the C^{β} and C^{α} region, while the last $\pi/2$ pulse should cover the C' region. Obviously the spectrum is neither symmetric any longer nor it contains a diagonal, but the spectral width in the two dimensions can be measurably reduced. Furthermore, by using selective pulses an overall gain in signal intensity over experimental time can be obtained. Indeed, the intensity of an observed cross peak depends, among other factors, on the C^{α} and C^{β} longitudinal magnetization before the first pulse. Longitudinal magnetization is maximal when the relaxation delay between subsequent transients is long enough to allow for complete nuclear relaxation. A way for expanding the effective relaxation delay for C^{α} and C^{β} spins consists in avoiding to excite these spins with the last $\pi/2$ NOESY pulse, as it happens by using a semi-selective C' pulse. Therefore the effective relaxation delay for C^{α} and C^{β} spins becomes the relaxation delay plus the mixing time. Since the mixing time in these ^{13}C-^{13}C spin-diffusion experiments can be comparable, or even longer, than the relaxation delay, avoiding to excite the C^{α} and C^{β} spins with the last pulse should significantly influence the spectra (data not shown).

The resolution of the experiment in a specific region of the ^{13}C-^{13}C NOESY spectrum can also be improved through the use of the in-phase anti-phase (IPAP) method (Ottiger et al., 1998; Andersson et al., 1998) to separate the doublet components of the peaks. To increase also the sensitivity, we have exploited the uniformity of the $J_{C'C^{\alpha}}$ coupling constant value. Once the two separate matrices are obtained, a proper post-acquisition processing of the data lead to a C' singlet in the direct acquisition dimension. Details on how this was achieved are reported in the Experimental section. This approach can thus be used to yield an increase in sensitivity (in addition to improving the resolution) when the relaxation losses due to the inclusion of an additional IPAP building block are tolerable, as this is the case for dimeric SOD.

The portion of the 2D map containing C'-C^{β} as well as C'-C^{α} correlations is shown in Figure 4. For 96% of the residues, the expected C'-C^{α} and C'-C^{β} correlations could both be easily identified. Out of the six remaining residues, one residue (Val 81) could not be identified at all (the C' and C^{β} were not available from the previous assignment), while for the others (Lys 30, Pro 62, Arg 69, 79 and 143) the C'-C^{β} correlations could not be firmly identified. In two cases a tentative assignment was possible, but for the others the overlap with other signals prevent the assignment (in two cases the C^{β} was not available from the previous assignment), despite the presence of few unassigned peaks.

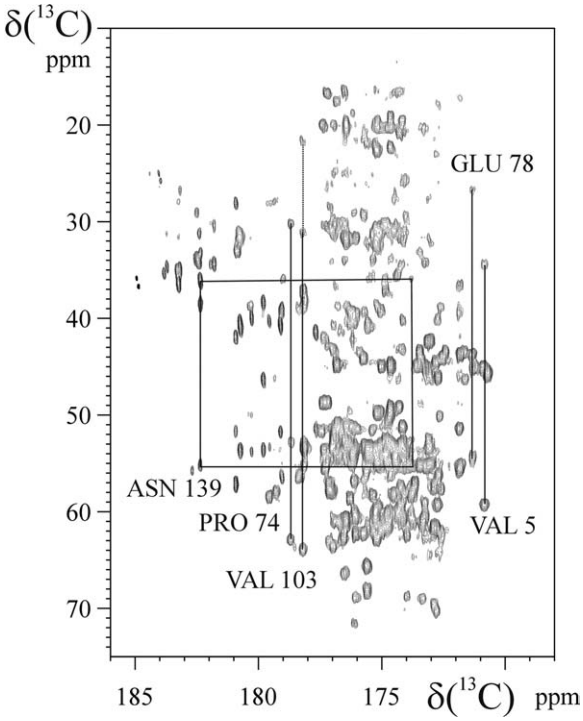

Figure 4. The 2D region containing C'-C^{β} correlations of the 125.8 MHz ^{13}C-^{13}C NOESY spectrum, with 3.0 s mixing time and with the IPAP pulse scheme to suppress the C^{α}-C' coupling.

Thus, the ^{13}C-^{13}C NOESY spectrum with long mixing time can be used with confidence to identify the C^{β} resonance starting on C' using a dipolar transfer, rather than a scalar coupling transfer. Furthermore, several C'-C^{γ} correlations could be seen. Among them, those involving methyl groups in VAL and ILE residues could be easily identified (Figure 4).

Other correlations that can be detected through this experiment are those involving side chain carbonyls and carboxylates (ASP, ASN, GLU, GLN) with the side chain carbons at one-bond and two-bond distances (e.g. C^{β}-C^{γ}-C^{δ} for GLU and GLN, or C^{α}-C^{β}-C^{γ} for ASP and ASN). In particular, for ASP and ASN, the C^{α} and C^{β} spins actually give correlations with both the backbone carbonyl and the side chain carbonyl or carboxylate, yielding a distinct square pattern (Figure 4) that allows to assign in a very straight-

forward way the side chain carbonyl or carboxylate resonance.

Concluding remarks

In summary, we have shown that multispin magnetization transfer contributes in making dipolar-based experiments a valuable alternative to scalar-based experiments to detect C-C correlations. These correlations are i) helpful in identifying the kind of aminoacid and ii) necessary to link the protein backbone to the corresponding side-chains resonances. This finding is particularly relevant in the investigation of large molecular mass systems, as coherence transfer mechanisms become less and less efficient as the linewidths of the signals approach the value of the scalar coupling constant used for the transfer itself, making it difficult to use the standard triple-resonance approach for signals assignment. On the other hand, dipolar-based experiments are expected to become more powerful with increasing molecular mass as the maximal spin diffusion effect i) becomes stronger and ii) occurs at shorter mixing times.

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