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Pseudo-4D triple resonance experiments to resolve HN overlap in the backbone assignment of unfolded proteins

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Abstract

The solution NMR resonance assignment of the protein backbone is most commonly carried out using triple resonance experiments that involve ^{15}N and ^1HN resonances. The assignment becomes problematic when there is resonance overlap of ^{15}N – ^1HN cross peaks. For such residues, one cannot unambiguously link the “left” side of the NH root to the “right” side, and the residues associated with such overlapping HN resonances remain often unassigned. Here we present a solution to this problem: a hybrid (4d,3d) reduced-dimensionality HN(CO)CA(CON)CA sequence. In this experiment, the Ca(*i*) resonance is modulated with the frequency of the Ca(*i*–1) resonance, which helps in resolving the ambiguity involved in connecting the Ca(*i*) and Ca(*i*–1) resonances for overlapping NH roots. The experiment has limited sensitivity, and is only suited for small or unfolded proteins. In a companion experiment, (4d,3d) reduced-dimensionality HNCO(N)CA, the Ca(*i*) resonance is modulated with the frequency of the CO(*i*–1) resonance, hence resolving the ambiguity existent in pairing up the Ca(*i*) and CO(*i*–1) resonances for overlapping NH roots.

Keywords

Assignment; Unfolded proteins; Triple resonance; Reduced-dimensionality

The resonance assignment of the protein backbone is most commonly carried out using solution triple resonance experiments that involve ^{15}N and ^1HN resonances (Cavanagh et al. 2007). The assignment becomes problematic when these are resonance overlap of ^{15}N – ^1HN cross peaks. Overlap occurs often in the spectra of unfolded proteins and in the spectra of flexible parts of larger proteins. If two ^{15}N – ^1HN cross peaks overlap, it is impossible to deduce which of the two H(*i*)N(*i*)Ca(*i*) cross peaks in the HNCA experiment belong to which of the two H(*i*)N(*i*)Ca(*i*–1) cross peaks in the HN(CO)CA experiment. Similarly, is

impossible to deduce which $H(i)N(i)CO(i-1)$ in HNCOC and/or which $H(i)N(i)CB(i-1)$ in HNCOCACB belongs to those $H(i)N(i)Ca(i)$ cross peaks, or, for that matter, belongs to which of the $H(i)N(i)CO(i)$ and $H(i)N(i)CB(i)$ peaks.

In the terminology of automatic assignments (Moseley et al. 2001; Crippen et al. 2010), for such residues that have overlapping HN resonances, one cannot link the “left” side of the Generalized Spin system (GS) to the “right” side. Therefore these residues become assigned only if they are the only ones left over after everything else is assigned, or, more commonly, remain unassigned.

Here we present a strategy to assign these overlapping HN resonances using a novel hybrid (4d,3d) reduced-dimensionality (Szyperski et al. 1993; Simorre et al. 1994)

HN(CO)CA(CON)CA pulse sequence, where the bold resonances are sampled conventionally, while the underlined resonance is sampled in pseudo 4D mode together with the bold CA. In the experiment, the coherence residing on $^{15}N(i)$ first goes to $Ca(i-1)$, then to $Ca(i)$, before returning to $N(i)$ and $H(i)$. In this manner, one can modulate the $Ca(i)$ resonance with the frequency of the $Ca(i-1)$ resonance, hence resolving the ambiguity associated with connecting the $Ca(i)$ and $Ca(i-1)$ resonances in GSs of the overlapping resonances. We also present a companion experiment, a hybrid (4d,3d) reduced-dimensionality **HNCOC(N)CA** sequence, resolving the ambiguity associated with connecting the $Ca(i)$ and $CO(i-1)$ resonances in GSs of the overlapping resonances.

Figure 1 shows the pulse sequences of the **HN(CO)-CA(CON)CA** and **HNCOC(N)CA**. The sequences are HNCOCA (Fig. 1a) or HNCOC (Fig. 1b) from point A to B.

For both experiments, the density operator at point B is given as:

$$\rho(B) \propto 4H_z C_z' N_y \cos(\Omega^{Ca(i-1)} \kappa t_1).$$

Between B and C, $J^{NC'}$, J^{NCai} and J^{NCai-1} are active, hence causing the density operator to evolve as:

$$\rho(C) \propto \cos(\Omega^{Ca(i-1)} \kappa t_1) \left\{ \begin{array}{l} 4H_z C_z' N_y \sin(\pi J^{NC'} \tau) \sin(\pi J^{NCai} \tau) \cos(\pi J^{NCai-1} \tau) \\ + 4H_z C_z^{ai-1} N_y \sin(\pi J^{NC'} \tau) \cos(\pi J^{NCai} \tau) \sin(\pi J^{NCai-1} \tau) + \text{other terms} \end{array} \right\}.$$

The transfer functions for these terms are plotted in Fig. 2.

Both $4H_z C_z' N_y$ terms are converted to $4H_z C_z' N_z$ at point D, after which a standard HNCA-TROSY or HNCA-HSQC sequence is executed. The $Ca(i-1)$ frequency of the HNCOCA part is jointly sampled with the Ca frequencies of the HNCA part in a pseudo-4d (GFT) manner (Shen et al. 2005; Atreya and Szyperski 2004). The given phase program ϕ_1 in Fig. 1 yields a cosine modulation of the t_1 period, giving rise to in-phase doublets with splittings corresponding to twice the frequency difference of the $Ca(i-1)$ resonance with the ^{13}C carrier (for $\kappa = 1$). A separate experiment is collected with phase ϕ_1 incremented by 90° , giving rise to an anti-phase doublet. Addition or subtraction of the sine and cosine experiments results in an HNCA-like spectrum, in which both $Ca(i)$ and $Ca(i-1)$ resonances are shifted by the signed difference frequency of the $Ca(i-1)$ resonance with the ^{13}C carrier position.

The **HN(CO)CA(CON)CA** sequence was used to complete the assignments for the 79-residue unfolded C-terminal region of human heme oxygenase-2 (Yi et al. 2009). Figure 3a

shows the results for several non-degenerate examples. Here we have overlaid the HNCA, HN(CO)CA and the sum of the cosine- and sine-modulated **HN(CO)-CA(CON)CA** experiments. As illustrated with the colored brackets, the HNCA peaks are shifted by the signed difference frequency of the HN(CO)CA peak with the ^{13}C carrier. As mentioned, both $\text{Ca}(i)$ and $\text{Ca}(i-1)$ cross peaks are shifted in the pseudo 4D experiment; however, the shifted $\text{Ca}(i-1)$ peak is in most cases too small to be observed. The dashed bracket in Fig. 3a points to a shifted $\text{Ca}(i-1)$ peak that did come through. Figure 3b shows the results for the resonances D29 and L68 of the protein for which the NH cross-peaks have identical frequencies, resulting in two HN(CO)CA peaks that have the H and N coordinates in common. The analysis shows how the ambiguity of $\text{Ca}(i)$ with $\text{Ca}(i-1)$ pairing is solved (dark blue: pairing for L68; light blue: pairing for D29). Several shifted $\text{Ca}(i-1)$ peaks are also showing, as indicated with the dashed brackets. For our project, this sufficed to obtain the connectivity and assignments for the overlapping HN peaks.

Although not used for initial assignments, the **HNC(O)N)CA** experiment was performed to confirm the connectivity between $\text{CO}(i-1)$ and $\text{Ca}(i)$ of residues with degenerate HN shifts. Figure 4a shows the results for the same non-degenerate examples as depicted in Fig. 3a. The left panel of the figure is an overlay of HNCA and the sum of the cosine- and sine-modulated **HNC(O)N)CA** spectra, whereas the right panel shows an HNCO spectrum synchronized in the H and N dimensions with respect to HNCA. Again, as illustrated with the colored brackets, the HNCA peaks are shifted by the (signed) difference frequency of the HNCO peak with the ^{13}C carrier. Figure 4b shows results for resonances D29 and L68 that have identical NH cross-peak frequencies and therefore the two corresponding $\text{Ca}(i)$ peaks fall on the same N, H coordinates. The analysis, again shows how the ambiguity of $\text{Ca}(i)$ with $\text{CO}(i-1)$ pairing is solved (dark blue: pairing for L68; light blue: pairing for D29).

The sensitivity of the **HN(CO)CA(CON)CA** and **HNC(O)N)CA** experiments is limited, since the extra transfer reduces S/N by 60% as compared to the HN(CO)CA or HNCO (see Fig. 2). The pseudo-4D cosine or sine modulations generate doublets, which when co-added further reduce sensitivity by a factor of $\sqrt{2}$. Finally, the $\text{Ca}(i)$ linewidth in **HN(CO)CA(CON)CA** is increased by $\kappa^*(\text{Ca}(i-1))$ linewidth, which is approximately a factor of 2 when $\kappa = 1$, assuming the $\text{Ca}(i)$ and $\text{Ca}(i-1)$ line widths are about the same. In total, the **HN(CO)CA(CON)CA** sensitivity is reduced by a factor of about 5 as compared to an HNCOCA. The **HNC(O)N)CA** sensitivity as compared to HNCO contains the same factors, but the effect of the pseudo-4D sampling of the composite $\text{CO}(i-1)$ - $\text{Ca}(i)$ linewidth as compared to the $\text{CO}(i-1)$ are more severe and also depends, because of the CO-CSA relaxation, on B_0 . We estimate that the total sensitivity of **HNC(O)N)CA** as compared to HNCO is down by a factor of 10. Hence the use of both experiments is restricted to unfolded proteins or unfolded protein areas with high intrinsic S/N. The TROSY version of the experiments may also be used for the folded areas of highly soluble larger proteins, if one is willing to invest a week of instrument time. Likely, the sensitivity of HNCA-SE-HSQC versions of the experiments (not shown) would be better for unfolded proteins when lower-field spectrometers are used.

The **HN(CO)CA(CON)CA** experiment could also be setup as a (5d,3d) **HNCOCA(CON)CA** in which the $\text{CO}(i-1)$ is co-sampled with the $\text{Ca}(i)$ or $\text{N}(i)$. This would unambiguously identify which $\text{CO}(i-1)$ belongs to which $\text{Ca}(i)$, which will also be of help in continuing assignments over two or more degenerate NH's. However, this additional (constant-time) evolution will cause further decrease in sensitivity. Better is to use the more sensitive (4d,3d) **HNC(O)N)CA** experiment to identify which $\text{CO}(i-1)$ belongs to which $\text{Ca}(i)$ (see Fig. 1b and 4). Extension of this approach can be visualized, e.g., a (4d,3d) **HNC(O)N)CACB** would identify which $\text{CO}(i-1)$ belongs to which $\text{Cb}(i)$. Obviously, the experiment's low sensitivity would severely limit its application.

Finally, the (4d,3d)HN(CO)CA(CON)CA andHNCO(N) CA experiments may be recorded with different values for κ , after which projection reconstructions methods could be applied to regenerate the full 4D spectrum (Kupce and Freeman 2004, 2006; Hiller et al. 2005). Whether such additional experiments and processing would be helpful for data interpretation depends on the severity of overlap in the NH spectrum.

Once the proper Ca(*i*) with Ca(*i*−1) and CO(*i*−1) pairings are accomplished, the remaining ambiguities can be solved with other (reduced dimensionality) experiments. For example, (4d,3d) GFT HNCOCA (Atreya and Szyperski 2004) or APSY6D HNCOCAHN (Fiorito et al. 2006) allows for the correct pairing of CO(*i*−1) with Ca (*i*−1)s; (4d,3d) GFT HNCACO (Atreya and Szyperski 2004) may be used to pair up CO(*i*)s with corresponding Ca(*i*)s. Similarly, (4d,3d) GFT HNCACB-CA (Atreya and Szyperski 2004) could provide for the correct pairing of Ca(*i*)s with Cb(*i*)s and possibly Ca(*i*−1)s with Cb(*i*−1)s, while APSY7D HNCOCACBHN (Hiller et al. 2007) will pair Ca(*i*−1) with Cb(*i*−1)'s.

Supplementary Material

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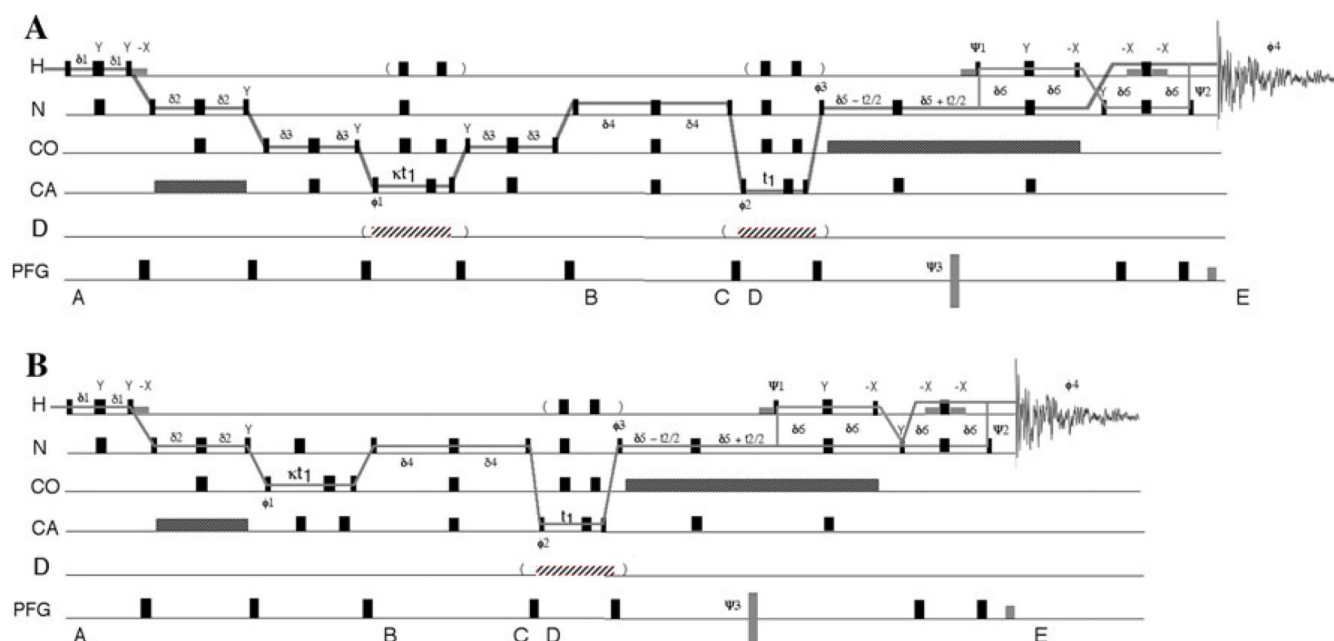
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**Fig. 1.**

a The (4d,3d) **HN(CO)CA(CON)CA** sequence. **b** The (4d,3d) **HNCO(N)CA** sequence. The sequences are HNCOCA (Fig. 1a) or HNCO(N)CA (Fig. 1b) from point A to B. From B to C is a transfer, and from D to E, the sequences are HNCA-TROSY, in an implementation combining publications of different groups (Rance et al. 1999; Schulte-Herbruggen and Sorensen 2000; Loria et al. 1999; Salzmänn et al. 1998). 90° pulses are narrow boxes, 180° pulses are wide boxes. The 180° CO/Ca pulses are selective by using a 41 μs pulse length (800 MHz). The gray pulses are selective for the H₂O resonance (≈ 800 ms). The ¹H 180° pulses in parentheses can be omitted if the protein is (perfectly) perdeuterated. The gray gradient pulses achieve ¹⁵N-¹H echo-anti-echo coherence selection. All pulses have x-phase, unless noted differently. $\psi_1 = -y$, $\psi_2 = -x$, $\psi_3 = \text{negative}$. Phase cycle: $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_3 = y$, $\phi_4 = +, -, -, +$. States-tppi quadrature in t_1 is achieved by incrementing ϕ_2 by 90° in odd t_1 -counts and both ϕ_2 and ϕ_4 with 180° in even t_1 -counts. Sensitivity-enhanced single-transition-to-single-transition echo-anti echo tppi in t_2 is achieved by incrementing ψ_1 , ψ_2 by 180° and the sign of the gradient ψ_3 in odd t_2 counts, and ϕ_3 and ϕ_4 with 180° in even t_2 counts. The delays are: δ_1 : optimum for $\sin(2\pi J^{\text{HN}} \delta_1) \exp(-2\delta_1/T_{2H}) \approx 2.4$ ms. δ_2 : optimum for $\sin(2\pi J^{\text{NC}} \delta_2) \exp(-2\delta_2/T_{2N}) \approx 11$ ms. δ_3 : optimum for $\sin(2\pi J^{\text{C}^{\text{Ca}}} \delta_3) \exp(-2\delta_3/T_{2C}) \approx 4.5$ ms. δ_4 : optimum for $\sin(2\pi J^{\text{NC}} \delta_4) \cos(2\pi J^{\text{NCa}(i-1)} \delta_4) \exp(-2\delta_4/T_{2N}) \approx 13$ ms. $\delta_5 + \delta_6$: optimum for $\sin(2\pi J^{\text{NCai}} (\delta_5 + \delta_6)) \cos(2\pi J^{\text{NCa}(i-1)} (\delta_5 + \delta_6)) \exp(-2(\delta_5 + \delta_6)/T_{2N}) \approx 12$ ms. δ_6 : $1/4 J_{\text{NH}} = 2.75$ ms. The pseudo 4D incrementation can be scaled with κ ; we used $\kappa = 1$. Cosine modulation is obtained with the given phase ϕ_1 ; sine modulation is obtained by using $\phi_1 = y, -y$ and applying a zero-order 90° phase correction in the ¹³C dimension during data processing

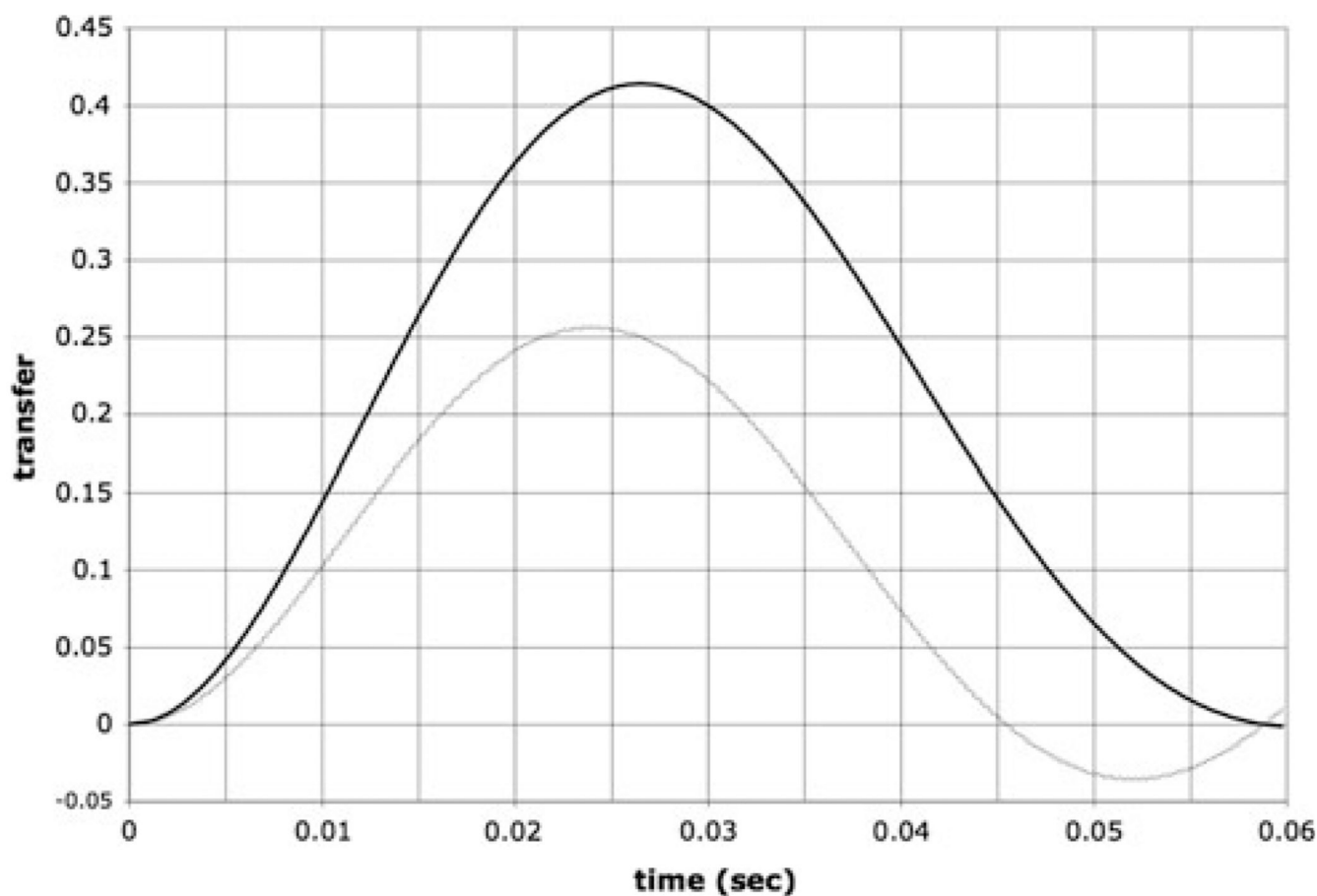


Fig. 2.

Transfer efficiencies for $\sin(\pi J^{NC'} t) \sin(\pi J^{NCai} t) \cos(\pi J^{NCa(i-1)} t) \exp(-R_{2N} t)$ (for $Ca(i)$ cross peaks in HNCA; solid) and $\sin(\pi J^{NC'} t) \cos(\pi J^{NCai} t) \sin(\pi J^{NCa(i-1)} t) \exp(-R_{2N} t)$ (for $Ca(i-1)$ cross peaks in HNCA; dashed) with $J^{NC'} = 17$ Hz, $J^{NCai} = 11$ Hz, $J^{NCa(i-1)} = 8$ Hz and $R_{2N} = 15 \text{ s}^{-1}$

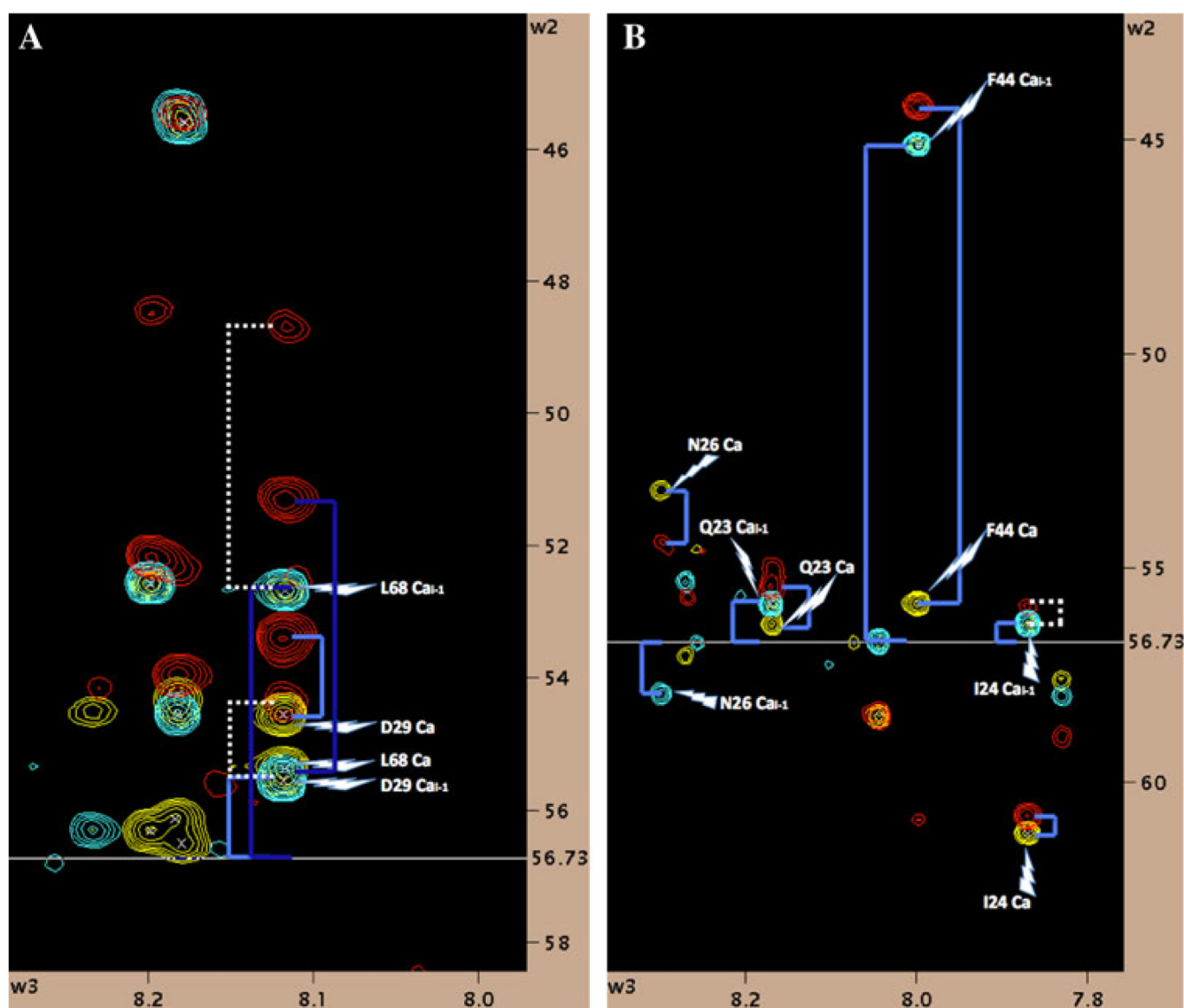


Fig. 3.

Overlays of standard 3d-HNCA-TROSY (yellow), standard 3d-HN(CO)CA-TROSY (cyan) and the sum of the cosine and sine modulated (4d,3d) HN(CO)CA(CON)CA experiments (red). The horizontal line at 56.73 ppm indicates the position of the ^{13}C carrier. **a** Blue “left-hand” brackets identify the $\text{Ca}(i-1)$ —carrier difference frequencies in the HNCOCA; the “right-handed” brackets show how the $\text{Ca}(i)$ in the pseudo 4D are shifted by these difference frequencies. The dashed bracket shows a shifted $\text{Ca}(i-1)$ peak. Resonance assignments obtained by SAGA (Crippen et al. 2010) are indicated. **b** Light and dark blue brackets show the pairing of $\text{Ca}(i)$ and $\text{Ca}(i-1)$ for two different spins systems, for which the NH frequencies are completely degenerate. The 79-residue disordered C-terminal region of human heme oxygenase-2 (HO2) was ^{13}C , ^{15}N labeled. Sample conditions: 1 mM HO2, 50 mM Tris, 100 mM KCl, pH 7.0, 18°C. The data was acquired on an Oxford 800 MHz magnet equipped with a Varian NMR SYSTEM console and a room temperature triple resonance gradient probe. The HNCA-TROSY, HN(CO)CA-TROSY, (4d,3d) HN(CO)CA(CON)CA cosine and (4d,3d) HN(CO)CA(CON)CA sine experiments were

collected with 75 and 45 complex points in t_1 and t_2 , respectively, and with 2 transients per increment in 9.5 h each

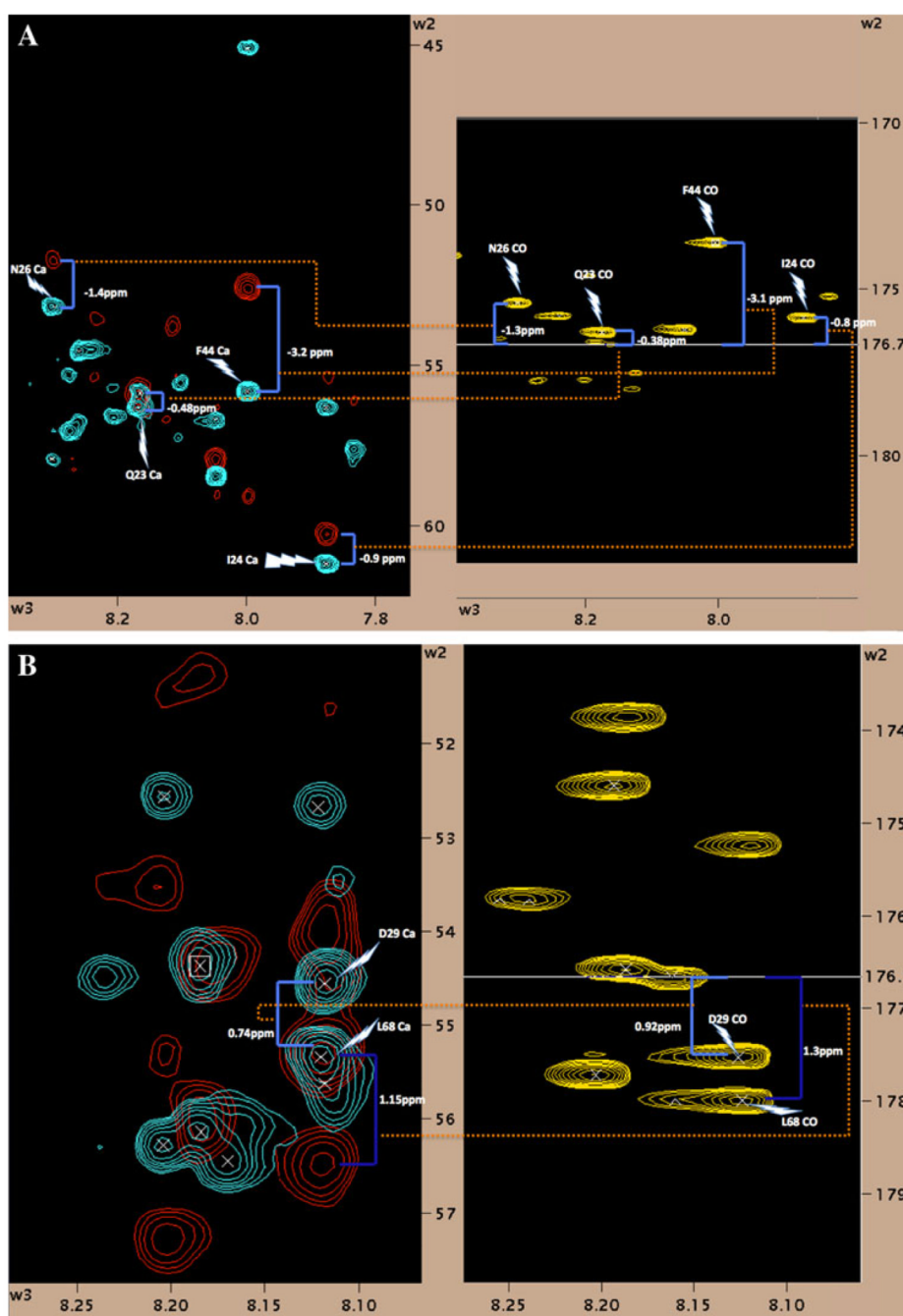


Fig. 4. Overlays of standard 3d-HNCA-TROSY (cyan) and the sum of the cosine and sine modulated (4d,3d) HNCO(N)CA spectra (red), at left. The standard 3D-HNCO-TROSY experiment, at right. The horizontal line at 176.7 ppm indicates the position of the ^{13}C carrier. The (4d,3d) HNCO(N)CA cosine and (4d,3d) HNCO(N)CA sine experiments were collected with 80 and 30 complex points in t_1 and t_2 , respectively, and with 4 transients per increment in 9.0 h each. Sample conditions were same as stated in Fig. 3. **a** Blue brackets in the right panel identify the $\text{CO}(i-1)$ —carrier difference frequencies in the HNCO; brackets in the left panel show how $\text{Ca}(i)$ in the pseudo 4D are shifted by these difference frequencies. Dashed orange connectors are used to point at the corresponding modulations

in the HNC0 and HNCA respectively. Resonance assignments obtained by SAGA (Crippen et al. 2010) are indicated. **b** *Light and dark blue brackets* show the pairing of CO($i-1$) and Ca(i) for two different spins systems, for which the NH frequencies are completely degenerate. The slight discrepancy in the chemical shift differences between the left and right spectra is accounted for by the fact that the HNC0 used here for comparison, was run earlier using a sample from a different preparation of HO2