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Minimisation of sensitivity losses due to the use of gradient pulses in triple-resonance NMR of proteins

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Summary

The use of pulsed field gradients in multiple-pulse NMR experiments has many advantages, including the possibility of obtaining excellent water suppression without the need for selective presaturation. In such gradient experiments the water magnetization is dephased deliberately; exchange between the saturated protons of the solvent water and the NH protons of a protein transfers this saturation to the protein. As the solvent is in large excess and relaxes relatively slowly, the result is a reduction in the sensitivity of the experiment due to the fact that the NH proton magnetization is only partially recovered. These effects can be avoided by ensuring that the water magnetization remains intact and is returned to the $+z$-axis at the start of data acquisition. General procedures for achieving this aim in any triple-resonance experiment are outlined and two specific examples are given. Experimental results confirm the sensitivity advantage of the modified sequences.

Introduction

Recently, it has been shown that the sensitivity of ^{15}N-^{1}H HSQC spectra of proteins may be compromised by the use of pulsed field gradients (Grzesiek and Bax, 1993; Li and Montelione, 1993; Stonehouse et al., 1994a). This effect arises due to a combination of circumstances. Firstly, in a typical gradient experiment the magnetization from solvent H_2O is dephased, resulting in saturation of the protons in the solvent. Secondly, exchange between these protons and the NH protons of the protein results in a transfer of saturation to the protein; as the solvent is in great excess (by a factor of 10^5) and as it relaxes much more slowly than the protein, the return of the NH magnetization back to equilibrium is retarded significantly. Thus, when the experiment is repeated at a rate typically used for protein NMR experiments, the NH protons do not have sufficient time to relax fully between scans and the signals observed from these protons are reduced in intensity. Furthermore, non-exchangeable protons may also become partially saturated due to spin diffusion between them and the NH protons (Smallcombe, 1993); the extent of saturation is less than for the exchanging protons, but it can be significant, especially for H^{α} protons. It has been shown that these saturation effects can be avoided by relatively straightforward modifications to the pulse sequence (Grzesiek and Bax, 1993; Stonehouse et al., 1994a). The improvement in sensitivity that is obtained by using these modified sequences depends on the exchange rate of the NH protons and we have shown that even for proteins at pH 6.5 these exchange rates are fast enough for a significant number of NH signals to be affected.

Many triple-resonance experiments frequently used in the assignment of protein spectra derive the observed signals from NH magnetization and, as a result, the use of gradients can lead to a loss of signal intensity in precisely the same way as has been described for the HSQC experiment. In this paper we describe, in general terms, how gradients can be used in these triple-resonance experiments in such a way as to avoid any sensitivity losses due to exchange. The application of these general principles to two particular experiments is described and

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experimental results are presented which demonstrate the sensitivity improvement which the modified experiments give when compared to their unmodified counterparts. Recently, Kay et al. (1994) have described modifications to the HNCO experiment based on considerations similar to those described here.

Theory

The sensitivity loss due to exchange with water can be minimised by ensuring that, at the start of data acquisition, as much of the magnetization from the water protons as is possible is returned to the $+z$-axis (Stonehouse et al., 1994a). This aim can usually be achieved by a careful choice of the phases of the proton pulses and, in cases where this alone cannot manipulate the magnetization into the desired position, by the inclusion of selective pulses to the water. The design of the experiment is aided by the fact that the signal from the water is generally placed on-resonance. Therefore, when viewed in the usual rotating frame, the magnetization does not precess and so its position at any point in the sequence is straightforward to determine. Of course, in selecting the phases of the proton pulses, due regard must be given to ensuring that the desired sequence of magnetization transfers between spins on the protein still takes place. If selective pulses to the water resonance are to be used, it must also be born in mind that the H^{α} protons are likely to be affected. Thus, such selective pulses can only be applied at points in the sequence during which the desired magnetization is neither on the H^{α} protons nor anti-phase with respect to a coupling to these protons.

At some point in the sequence a gradient may be applied in order to dephase and hence label a particular kind of magnetization (for example ^{15}N magnetization). After further coherence transfer steps, a second gradient rephases the required magnetization; often, the magnetization is present on the proton at this point and rephasing is followed immediately by measurement of the free induction decay. Alternatively, gradients may simply be used to dephase unwanted magnetization, such as that from the solvent.

In both types of experiment our aim is to return as much as possible of the water magnetization to the $+z$-axis before acquisition, and the best way of achieving this is to keep this magnetization intact throughout the sequence. Thus, when gradients are applied the magnetization from the water protons must either be along the z-axis, so that it does not experience the effect of the gradient, or if the magnetization is transverse it must be rephased immediately. This latter outcome is easily achieved by splitting the gradient into two parts, separated by a 180° pulse to a heteronucleus (S):

$$G_i - 180°(S) - \overline{G_i}$$

Here, the dephasing of the water magnetization caused by the first gradient, G_i, is immediately undone by the second gradient, $\overline{G_i}$, which is applied in the opposite sense. The magnetization on the heteronucleus S is, as a result of the refocusing pulse, dephased by both gradients. Such a 'split gradient' is a general method of dephasing and labelling magnetization on the heteronucleus whilst at the same time leaving the proton magnetization intact.

The large size of the magnetization from the water protons means that it is susceptible to radiation damping; as a result, the magnetization tends to rotate up to the $+z$-axis, even when no pulses are being applied. The rate at which radiation damping returns the magnetization towards $+z$ is proportional to the transverse component, and for magnetization starting in the transverse plane this process can be complete in only 20 to 30 ms. Radiation damping thus tends to alter the position of the magnetization from that expected on the basis of a simple consideration of the effect of pulses and delays. Since our aim is precise control of the position of this magnetization, it is clearly vital to consider the effects of radiation damping when redesigning the sequence.

The magnetization from the water must, of course, end up along $+z$ for all increments of the experiment. Since the water signal is on-resonance, the incrementation of indirect time variables (such as t_1) is not likely to be a problem. However, if there is an indirectly detected dimension which involves the evolution of proton magnetization, the phase of one or more proton pulses will have to be incremented as part of the TPPI (Marion and Wüthrich, 1983) or States et al. (1982) frequency discrimination procedures. If the phases of all the proton pulses in the sequence have been carefully chosen to ensure that magnetization from the water ends up along $+z$, incrementing the phase of one of these pulses is likely to result in some different outcome. Thus, if there is an indirectly detected proton dimension, we need to take further steps to ensure that the magnetization from the water ends up in the required position for all increments of the experiment.

This point requires some further amplification. Frequency discrimination in indirect dimensions hinges on the ability to alter the modulation with respect to the associated time variable from cosine to sine. Generally this can be achieved by incrementing the phase of a suitable pulse or pulses. Of necessity, if the pulse involved is applied to protons, the position of the water magnetization is affected. We cannot, however, make an arbitrary adjustment in the phase of subsequent pulses in order to compensate for this, since such changes would also alter the nature of the modulation. For example, starting with z magnetization the sequence $90(x) - t_1 - 90(-x)$ generates z magnetization which is cosine modulated with respect to t_1; magnetization from an on-resonance signal (i.e. the water) remains along the z-axis. Shifting the phase of the

first pulse to y results in sine modulation, but the magnetization from an on-resonance signal is left along the x-axis. This magnetization could be returned to z by changing the phase of the final pulse to $-y$, but this would of course result in cosine modulation with respect to t_1.

There are two ways in which this difficulty might be overcome. The first is to abandon the use of frequency discrimination in the indirect proton dimension. In some cases such a move might not be all that disadvantageous; for example, in the case of predominantly α-helical proteins the shifts of the H^{α} protons cover a small range which is already offset to one side of the 'natural' position for the transmitter, i.e., on-resonance with the water signal. For the same resolution, the resulting data sets with and without frequency discrimination would be identical in size.

The second way of controlling the position of the magnetization from water is by using selective pulses. However, to return the magnetization to a constant position, each increment of the TPPI or States procedure would require a *different* selective pulse to be introduced into the sequence. This difference might be one of phase or of flip angle. Whilst in principle this is not a conceptual problem, it does raise the practical problem of ensuring that the signals recorded in the different increments of TPPI have the same overall amplitude. Any imbalance in amplitude will result, in the final spectrum, in imperfect sign discrimination and the appearance of quadrature images.

In addition to being used for assignment, triple-resonance experiments are also used to identify proton–proton NOE connectivities. Typically, such experiments contain a NOESY-type sequence, e.g.:

$$...90°(\chi_1)... - t_1 - 90°(\chi_2) - \tau_m - ...$$

where the ellipsis ... indicates the possible presence of other elements in the sequence. We have described before the difficulties associated with controlling the position of the magnetization from water in such a sequence (Stonehouse et al., 1994b) and the effect of radiation damping on the line width of the water signal in two-dimensional NOESY and ROESY spectra has been discussed (Otting, 1994). In short, the main problem arises from the need to increment the phases χ_1 or χ_2 as part of a TPPI or States procedure. The effect of incrementing pulse phases, as part of a frequency discrimination procedure, on the orientation of the water magnetization has also been discussed by Bax et al. (1987) and Sklenář et al. (1987), with particular reference to water suppression. Assuming that the magnetization from water is along z at the start of this sequence, if χ_1 and χ_2 are both x this magnetization is placed along the $-z$-axis at the start of the mixing time; from this starting point radiation damping is relatively slow. On the other hand, if the phase χ_1 is shifted to $\pm y$ the magnetization is in the transverse plane at the start of the mixing time and radiation damping is much more rapid. Thus, the position of the magnetization at the end of the mixing time is both uncertain and dependent on the phases χ_1 and χ_2.

To make the design of the rest of the pulse sequence as simple as possible, it is desirable to ensure that the magnetization from the water is along $+z$ at the end of the mixing time τ_m. One simple way in which this can be achieved is to exploit the fact that radiation damping will rotate the magnetization back to $+z$ during the mixing time. To ensure that this damping proceeds reasonably quickly for all increments of TPPI we simply add a constant offset of 45° to χ_1 or χ_2 to give the sequence of phases needed for TPPI as 45°, 135°, 225° and 315°. With this choice of phases the magnetization from water is never rotated to $-z$ at the start of the mixing time and so we can be reasonably confident that the radiation damping will be fast enough to return the magnetization to the $+z$-axis within, typically, 30 to 50 ms.

Results

In this section we consider two triple-resonance experiments, namely HNCA and HNCAHA, which illustrate how the principles outlined above can be applied. Both experiments are used to make the critical connections in proteins between amide nitrogens and α-carbons and both identify these connections by transferring magnetization through the relatively small one- or two-bond ^{15}N-$^{13}C^{\alpha}$ coupling (7 to 11 Hz); as a result the experiments are rather insensitive. HNCA and HNCAHA both utilise the equilibrium magnetization of the NH protons, so we may expect significant sensitivity advantages to arise from appropriate control of the magnetization from water.

Constant-time HNCA

The HNCA experiment (Ikura et al., 1990; Kay et al., 1990; Farmer et al., 1992; Grzesiek and Bax, 1992) has no indirect proton dimension, and so the modifications needed to ensure that the magnetization from water ends up along the $+z$-axis are relatively straightforward. In the basic form of this experiment, magnetization from the NH proton is transferred successively to ^{15}N and then to $^{13}C^{\alpha}$. The magnetization is then allowed to evolve for a time t_1 before being transferred back to ^{15}N, where it evolves for time t_2, and finally back to the NH proton where it is detected. Once the magnetization has been transferred to ^{15}N and rephased with respect to the ^{15}N-^{1}H coupling, broadband proton decoupling is employed; strategically placed pulses to the carbonyl region of the ^{13}C spectrum effectively remove any modulation due to coupling to these nuclei. The incrementable time t_2 is placed within a constant-time period, T.

There are two ways in which gradients may be intro-

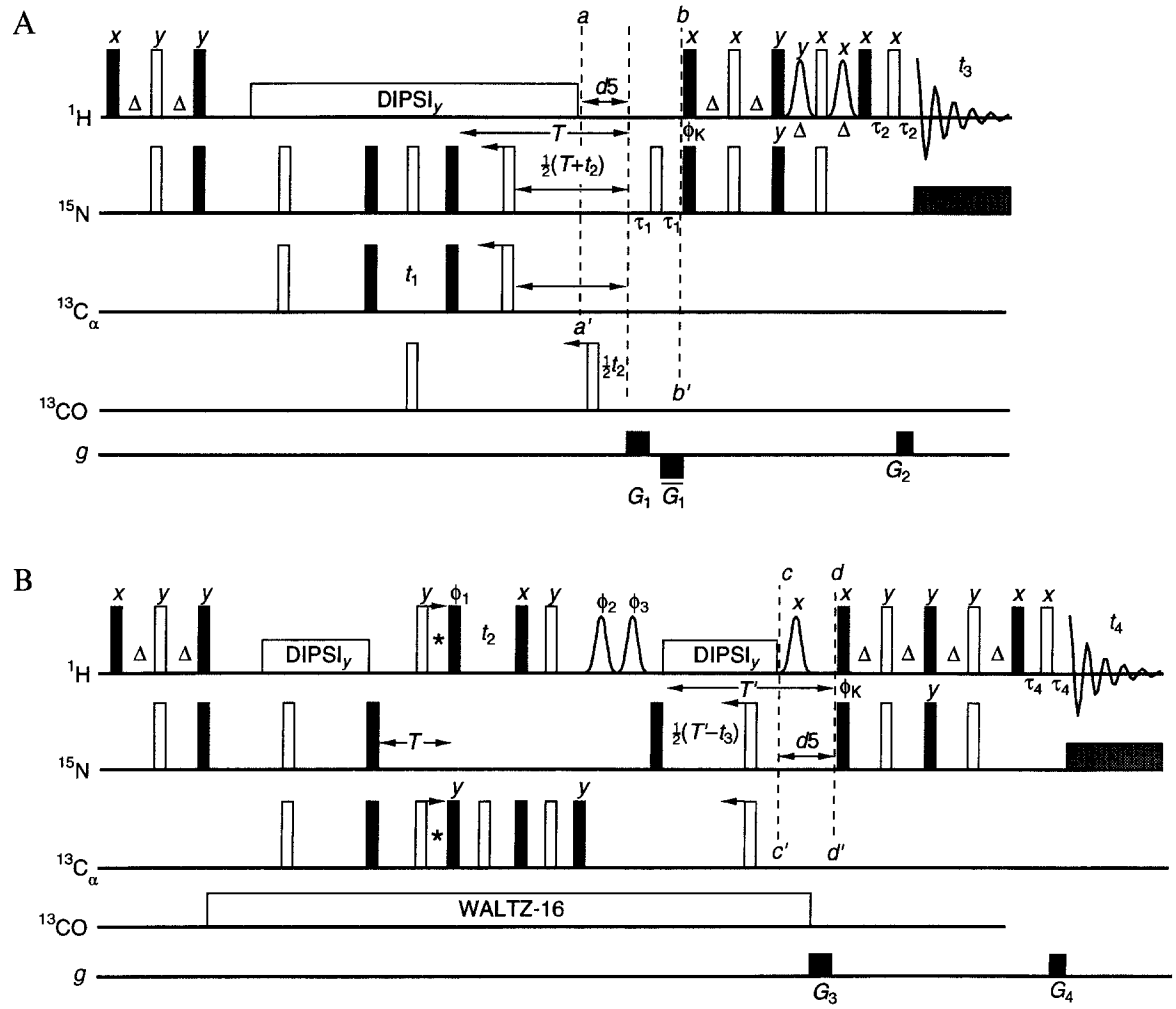

Fig. 1. Pulse sequences for recording (A) three-dimensional 'constant-time' HNCA spectra and (B) four-dimensional 'constant-time' HNCAHA spectra of globally ^{13}C,^{15}N-labelled proteins. These sequences have been designed so that, at the start of data acquisition, the magnetization from water is along the +z-axis. As explained in the text, use of these sequences minimises any signal losses due to exchange between water and the NH protons of the protein. The water magnetization is manipulated into the desired position by a combination of carefully chosen phases for the proton pulses and extra pulses to the water resonance. The two sequences illustrate alternative methods for incorporation of gradient pulses (see text). In the diagram 90° and 180° pulses are indicated by filled and open rectangles, respectively; selective 90° proton pulses, applied to the water resonance, are indicated by Gaussian-shaped profiles. The timing of pulses to protons, ^{13}C$^\alpha$, carbonyl carbons (^{13}CO) and ^{15}N are indicated separately; unless shown otherwise, all pulses have phase x. The timing of gradient pulses is indicated on the line marked g. In both sequences the final transfer step is achieved using the Kay sensitivity enhancement procedure (see text). The delay Δ is set to $1/(2J_{NH})$ and the delay marked * in sequence (B) has the value $\frac{1}{2}(T-t_1)$. The 180° pulses which move within constant-time periods are distinguished with an attached arrow which points in the direction in which the pulses move as the incremented time t_i increases. The full details of the timing of the pulses are not relevant here, and can be found in the original literature. All selective pulses were rectangular and of duration 2 ms. All field gradients were applied in the form of Gaussian-shaped pulses, truncated at the 5% level, with a peak amplitude of approximately 10 G cm^{-1}. The gradients G_1 and $\overline{G}_1$ were applied for 2 ms; G_3 for 4 ms; and G_2 and G_4 for 0.4 ms. As part of the Kay procedure, separate data sets were recorded with (i) the gradient pulses in the sense shown and the phase ϕ_K set to $-x$; and (ii) with the sense of the final gradient reversed and the phase ϕ_K set to $+x$. The delays τ_i are equal in length to the corresponding gradient pulses G_i.

duced into the HNCA experiment so as to keep the magnetization from the water intact and to return it ultimately to the +z-axis just before acquisition; the choice depends on the method used to achieve carbonyl decoupling. If such decoupling uses 180° pulses which are selectively applied to the carbonyl carbons, the pulse sequence of Fig. 1A illustrates how the gradients can be implemented. If decoupling is achieved with a WALTZ-16 sequence, the gradients may be introduced in a more elegant manner; this is shown for the HNCAHA experiment in Fig. 1B. At the end of the HNCA sequence the extra pulses needed to implement the procedure described by Kay et al. (1992a) and Muhandiram and Kay (1994) have been added; this technique is closely related to that described originally for non-gradient experiments by Cavanagh and Rance (1990) and Palmer et al. (1991). As has been described elsewhere, the procedure developed by Kay avoids, in principle, the sensitivity loss often associated with gradient selection (Kontaxis et al., 1994). However, for larger proteins, in which relaxation is more

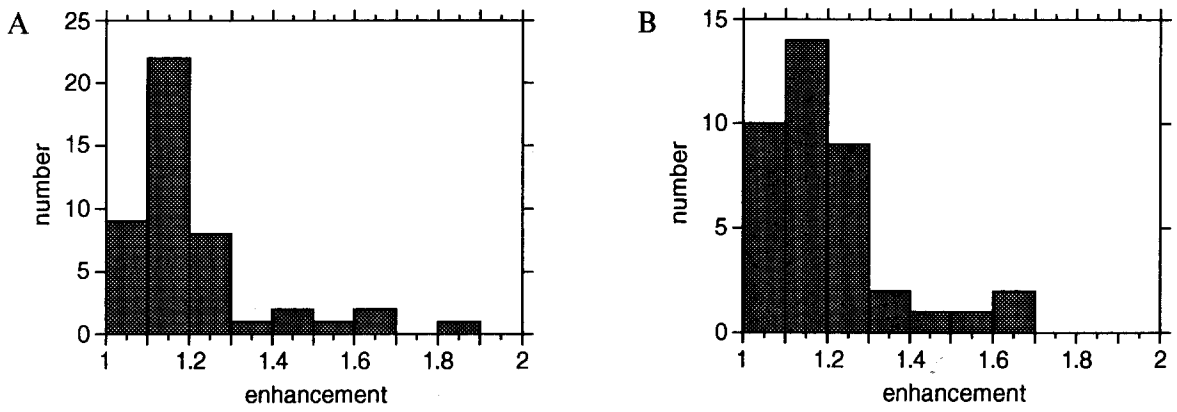

Fig. 2. Histograms showing the enhancement in signal intensity observed in (A) an HNCA spectrum and (B) an HNCAHA spectrum of the A domain of HMG1 (86 residues, pH 6.7, globally ^{15}N,^{13}C labelled, 293 K) obtained by using the sequences shown in Fig. 1. The enhancement is measured relative to equivalent spectra obtained using sequences in which the water magnetization is dephased at the start of data acquisition. The height of each bar gives the number of residues with the signal enhancement indicated on the horizontal axis; there is clearly an advantage to using the modified experiments of Fig. 1. These data were taken from two-dimensional versions of the sequences shown in Fig. 1; for the HNCA and HNCAHA sequences the indirect dimension contained ^{15}N and H^{α} shifts, respectively. All spectra were recorded at 600 MHz using a Bruker AMX-600 spectrometer equipped with a triple-resonance probehead incorporating a single shielded gradient coil.

rapid, experiments which use gradients simply to purge unwanted magnetization may give higher sensitivity than the sequences described here which use gradients for coherence selection (Otting and Wüthrich, 1988; Bax and Pochapsky, 1992; Kay et al., 1993; Vuister et al., 1993).

After the first three proton pulses, the water magnetization is along the $-y$-axis. In order to keep it in this position, a phase-alternating broadband decoupling sequence, in which the phases are set to $\pm y$, is chosen for proton decoupling; here we use the DIPSI sequence (Shaka et al., 1988). The water magnetization is effectively spin-locked, thereby preventing any radiation damping from turning it towards the $+z$-axis. The remaining delays in the sequence, during which proton decoupling is not applied, are sufficiently short to allow radiation damping to be ignored. The split gradient, $G_1 - 180°(^{15}N) - \overline{G_1}$, phase labels the ^{15}N magnetization of the protein and leaves the water magnetization intact. Finally, the ^{15}N magnetization is transferred back to the NH proton using a sequence of proton pulses whose phases are carefully chosen to ensure that the magnetization from the water ends up along the $+z$-axis at the start of acquisition; as described in our previous paper (Stonehouse et al., 1994a), this can be achieved by adding two selective pulses in the series of spin echoes needed for the Kay procedure.* The final gradient G_2, which rephases the magnetization which has been transferred from ^{15}N, also dephases any magnetization from water which, as a result of pulse imperfections or relaxation, has not been rotated onto z by the preceding proton pulses. As a result, the water suppression is excellent.

To assess the benefits of the modified sequence we have recorded spectra of the A domain of HMG1 (86 residues, pH 6.7) using (i) the sequence of Fig. 1A and (ii) an equivalent sequence in which the magnetization from the water is dephased by the final gradient. We have measured the peak heights, on a residue-by-residue basis, in the two spectra and the results are compared in Fig. 2A. In this histogram, the horizontal co-ordinate of each bar corresponds to a different range of signal enhancements, calculated as the ratio of the peak height in spectrum (i) to that in spectrum (ii), and the height of each bar gives the number of residues exhibiting that enhancement. As can be seen, for all residues spectrum (i) gives either the same or a higher intensity than spectrum (ii). The variations from residue to residue can be attributed to different NH-H_2O exchange rates.

Constant-time HNCAHA

Figure 1B shows the pulse sequence used to record HNCAHA spectra (Boucher et al., 1992; Clubb et al., 1992; Kay et al., 1992b); as with the HNCA experiment, the final stages have been modified to use the Kay sensitivity enhancement procedure. In this experiment magnetization is transferred from the NH proton to ^{15}N and then to $^{13}C^{\alpha}$. The carbon magnetization evolves for time t_1, is transferred to H^{α} where it evolves for time t_2 and is then transferred back to $^{13}C^{\alpha}$, and then to ^{15}N. The nitrogen magnetization evolves for time t_3, is labelled by the gradient G_3 and finally transferred back to NH using the Kay modification. The final gradient, G_4, refocuses the wanted magnetization.

Frequency discrimination in the H^{α} dimension (t_2/F_2) is achieved by incrementing the phase of the proton pulse marked ϕ_1. As was described above, for each increment a different sequence of pulses will be needed to restore the magnetization from the water to a fixed position. One

* Figure 1c of Stonehouse et al. (1994a) contains an error: the final ^{15}N 90° pulse shown in this figure is not required.

convenient way of achieving this is shown in Fig. 1B: two 90° pulses, of phases ϕ_2 and ϕ_3, are applied to the water resonance. The phases of these pulses depend on the phase ϕ_1 as follows:

$$\phi_1 = \{x,y,-x,-y\}$$
$$\phi_2 = \{x,-x,x,-x\}$$
$$\phi_3 = \{-x,y,x,-y\}$$

Using this set of phases, the two extra proton pulses, of phases ϕ_1 and ϕ_2, leave the water magnetization along the y-axis for all increments. Note that during these pulses the desired magnetization is present as zz order between ^{13}C and ^{15}N; there is thus no extra evolution of magnetization on the heteronuclei due to the inclusion of these pulses. Furthermore, losses due to relaxation are expected to be small as this magnetization relaxes relatively slowly. Although the results presented here have been acquired so that the extra proton pulses selectively affect the water magnetization, this need not be the case because the additional proton pulses will leave the desired magnetization unaffected. The experiment has been repeated with nonselective pulses and the results, not presented here, were virtually identical to those presented in this paper.

In the HNCAHA experiment there is a delay, *d5*, of duration $1/(2J_{NH})$ (approximately 5 ms), during which the ^{15}N magnetization dephases with respect to the NH coupling. During this delay a proton pulse is used to rotate the magnetization from water on to the z-axis; again, this pulse need not be selective as long as it is applied directly after the DIPSI sequence. The gradient G_3, used to label the ^{15}N magnetization, thus has no effect on the magnetization from the water and, in addition, the inclusion of the gradient in this position does not lengthen the sequence. Using this extra proton pulse to rotate the magnetization from water to the $+z$-axis also has the advantage that this magnetization can be returned to $+z$ at the start of acquisition simply by appropriate setting of the phases of the subsequent pulses (i.e. the pulses to the right of the line *dd'*); no additional selective pulses are needed. This is in contrast to the equivalent series of pulses in the HNCA sequence, i.e. those to the right of the line *bb'* in Fig. 1A. This difference arises because at the point indicated by the line *bb'* in Fig. 1A the magnetization from water is along y, whereas at the equivalent point *dd'* in Fig. 1B the magnetization is along z.

The way in which the gradients have been implemented in this experiment differs from the approach followed in the HNCA experiment in that the first gradient pulse is placed in the delay *d5*, and not afterwards. The method followed in the HNCAHA experiment has the advantage that it is not necessary to include an additional spin echo into which the gradient pulse is placed; the sequence is consequently not lengthened and involves one less 180° ^{15}N pulse.

If, in the sequence of Fig. 1B, a selective 180° carbonyl pulse, rather than WALTZ-16, is used for carbonyl decoupling, there would be a practical difficulty in this method of introducing the gradients. As t_3 is incremented, the 180° pulse to ^{13}CO would pass through the delay *d5* and hence through the selective 90° pulse to ^{1}H and the gradient pulse G_3; on our spectrometer such a sequence is very difficult to program. In order to circumvent this problem the sequence can be modified by replacing all the pulses to the right of the line *cc'* in Fig. 1B by those to the right of the line *aa'* in Fig. 1A; the phase of the final selective pulse should also be inverted.

The histogram of Fig. 2B illustrates the increase in peak heights found in an HNCAHA spectrum of HMG A, recorded using the modified sequence of Fig. 1B, compared to a spectrum recorded using an equivalent sequence in which the magnetization from water is dephased by the final gradient. Again, we see that for a significant number of residues the modified sequence offers an advantage.

Practical considerations

In practice, we have found that these modified experiments are straightforward to set up; some simple checks and precautions may be used to ensure that the experiment is working correctly. One is to verify that the water magnetization has indeed been returned to the $+z$-axis at the start of acquisition. This is done simply by adding a 90° proton pulse to the very end of the sequence; the observation of a suitably intense water signal from this modified sequence will confirm that the magnetization is indeed in the correct position. Typically, we find in this way that 70 to 80% of the equilibrium magnetization from water is returned to $+z$. The other point which needs some attention is to ensure that the DIPSI decoupling is indeed along the y-axis; if the power level is lowered for this decoupling, there is a possibility that a phase shift will also result. We find it convenient to check for this possibility simply by observing, in a separate experiment, the phase of the magnetization produced by a simple pulse at the two power levels. Any difference noted is compensated for in the pulse sequence by adjusting the phase of the DIPSI sequence. The same arguments apply to the phases of any selective pulses which may be present in the pulse sequence.

As was explained above, the choice of the phases of the proton pulses is crucial. For this reason, it is not appropriate to attempt any phase cycling of the *proton* pulses, as this would result in the magnetization from water not always ending up along the $+z$-axis, which is the whole aim of the modified experiment. Generally, with the use of field gradients, such cycling of the proton pulses is not required.

Finally, we have found that setting the phases of proton 180° pulses so that they coincide with the axis along

which the magnetization from water is aligned results in more of the magnetization remaining intact. This effect arises because, in such situations, the pulse has no effect and hence its calibration is unimportant. This is in contrast to the case where a pulse is applied about an axis perpendicular to that along which the magnetization is aligned; in such a case the pulse rotates the magnetization through a large angle and any imperfections result in incomplete refocusing of the magnetization.

Conclusions

We have described the general procedures which need to be adopted in order to minimise the sensitivity losses when recording triple-resonance spectra of proteins in H_2O using pulsed field gradients. The key to minimising this sensitivity loss is to ensure that most of the magnetization from water protons is returned to the $+z$-axis at the start of data acquisition. This can be achieved by careful choice of the phase of the proton pulses and, in some cases, the inclusion of extra proton pulses.

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References

Bax, A., Sklenář, V., Clore, G.M. and Gronenborn, A.M. (1987) *J. Am. Chem. Soc.*, **109**, 6511–6513.

Bax, A. and Pochapsky, S.S. (1992) *J. Magn. Reson.*, **99**, 638–643.

Boucher, W., Laue, E.D., Campbell-Burk, S.L. and Domaille, P.J. (1992) *J. Biomol. NMR*, **2**, 631–637.

Cavanagh, J. and Rance, M. (1990) *J. Magn. Reson.*, **88**, 72–85.

Clubb, R.T., Thanabal, V. and Wagner, G. (1992) *J. Biomol. NMR*, **2**, 203–210.

Farmer II, B.T., Venters, R.A., Spicer, L.D., Wittekind, M.G. and Müller, L. (1992) *J. Biomol. NMR*, **2**, 195–202.

Grzesiek, S. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 432–440.

Grzesiek, S. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 12593–12594.

Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.

Kay, L.E., Ikura, M. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.

Kay, L.E., Keifer, P. and Saarinen, T. (1992a) *J. Am. Chem. Soc.*, **114**, 10663–10665.

Kay, L.E., Wittekind, M., McCoy, M.A., Friedrichs, M.S. and Mueller, L. (1992b) *J. Magn. Reson.*, **98**, 443–450.

Kay, L.E., Xu, G., Singer, A.U., Muhandiram, D.R. and Forman-Kay, J.D. (1993) *J. Magn. Reson. Ser. B*, **101**, 333–337.

Kay, L.E., Yi Xu, G. and Yamazaki, T. (1994) *J. Magn. Reson. Ser. B*, **109**, 129–133.

Kontaxis, G., Stonehouse, J., Laue, E.D. and Keeler, J. (1994) *J. Magn. Reson. Ser. A*, **111**, 70–76.

Li, Y. and Montelione, G.T. (1993) *J. Magn. Reson. Ser. B*, **101**, 315–319.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

Muhandiram, D.R. and Kay, L.E. (1994) *J. Magn. Reson. Ser. B*, **103**, 203–216.

Otting, G. and Wüthrich, K. (1988) *J. Magn. Reson.*, **76**, 569–574.

Otting, G. (1994) *J. Magn. Reson. Ser. B*, **103**, 288–291.

Palmer III, A.G., Cavanagh, J., Wright, P.E. and Rance, M. (1991) *J. Magn. Reson.*, **93**, 151–170.

Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.

Sklenář, V., Tschudin, R. and Bax, A. (1987) *J. Magn. Reson.*, **75**, 352–357.

Smallcombe, S.H. (1993) *J. Am. Chem. Soc.*, **115**, 4776–4785.

States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982) *J. Magn. Reson.*, **48**, 286–292.

Stonehouse, J., Shaw, G.L., Keeler, J. and Laue, E.D. (1994a) *J. Magn. Reson. Ser. A*, **107**, 178–184.

Stonehouse, J., Shaw, G.L. and Keeler, J. (1994b) *J. Biomol. NMR*, **4**, 799–805.

Vuister, G.W., Clore, G.M., Gronenborn, A.M., Powers, R., Garrett, D.S., Tschudin, R. and Bax, A. (1993) *J. Magn. Reson. Ser. B*, **101**, 210–213.