Journal of Biomolecular NMR, 9 (1997) 317–322
ESCOM

J-Bio NMR 445

Identification of spin diffusion pathways in proteins by isotope-assisted NMR cross-relaxation network editing

Nenad Juranić, Zsolt Zolnai* and Slobodan Macura**

Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic and Foundation, Rochester, MN 55905, U.S.A.

Received 14 February 1997
Accepted 7 March 1997

Keywords: NOESY editing; Spin diffusion; Protein NMR

Summary

A new isotope-assisted cross-relaxation editing experiment, [^{1}H-^{13}C]DINE-NOESY[^{1}H-^{15}N]HSQC (DINE = Double INEPT Edited), is proposed. It is based on the selective inversion of CH/CH_3 or CH_2 protons in the middle of the mixing time. The experiment sorts out the spin diffusion paths according to the principal mediators, either the CH/CH_3 or the CH_2 protons. This is useful in the structure refinement process, as it enables proper alignment of the aliphatic protons in the vicinity of NH protons.

Homonuclear proton–proton cross-relaxation, the principal source of information in protein structure determination by NMR spectroscopy, is significantly improved by isotope labeling (Griffey and Redfield, 1987). Isotope editing and isotope filtering experiments (Marion et al., 1989; Ikura et al., 1990; Otting and Wüthrich, 1990; Clore and Gronenborn, 1991; Ikura and Bax, 1992) simplify cross-relaxation spectra by simultaneously improving their resolution and quantificability. Unfortunately, spin diffusion, a notorious problem that complicates the interpretation of macromolecular cross-relaxation spectra, also appears in isotope-assisted cross-relaxation spectra. It can be mediated even by filtered-out proton spins. A simple analysis shows that the protons eliminated by isotope-assisted filtering contribute to spin diffusion as if they are fully visible (Zolnai et al., 1995). Thus, most of the magnetization exchange network editing (MENE) methods are concerned with the elimination of spin diffusion (Massefski and Redfield, 1988; Fejzo et al., 1991,1992; Zwahlen et al., 1994; Zolnai et al., 1995; Vincent et al., 1996). However, spin diffusion, if properly identified, may contain valuable information about the cross-relaxation network. Since it involves more than one spin pair, it can help to identify the contributions to the cross-relaxation by filtered-out spins or spins that cannot be directly identified because of considerable overlap. In this communication, we present an isotope-assisted difference method that separates the spin diffusion pathways involving H^{CH}/H^{CH_3} and H^{CH_2}. In a novel isotope-assisted experiment, [^{1}H-^{13}C]-DINE-NOESY[^{1}H-^{15}N]HSQC (DINE = Double INEPT Edited), the double INEPT sequence (Bodenhausen and Ruben, 1980; Bax et al., 1990; Norwood et al., 1990) is used to selectively invert either H^{CH_2} or (H^{CH},H^{CH_3}) in doubly labeled proteins. The experiment achieves selectivity independently of the chemical shift. This is rather important, since the chemical shift ranges of CH, CH_2 and CH_3 groups overlap in both carbon and proton resonances. The experiment is designed for mapping the H^{N}-H^{N} spin diffusion mediated by aliphatic protons.

Any MENE experiment can be derived from the respective isotope-assisted NOESY sequence by inserting one or more evenly spaced selective inversions in the mixing period. Figure 1a shows the NOESY[^{1}H-^{15}N]-HSQC sequence (Norwood et al., 1990). The corresponding double INEPT edited NOESY sequence, [^{1}H-^{13}C]-DINE-NOESY[^{1}H-^{15}N]HSQC, is obtained by inserting the INEPT-reverse-INEPT (double INEPT) selective inversion in the middle of the mixing period, Fig. 1b. Depending on the phase φ (Fig. 1b), the double INEPT sequence selectively inverts either H^{CH_2} or (H^{CH},H^{CH_3}), see Fig. 1c.

The effect of selective inversion of a group of spins I with respect to an unperturbed group of spins P can be

*On leave from the Mathematical Institute, Knez Mihailova 35, 11000 Beograd, Yugoslavia.
**To whom correspondence should be addressed.

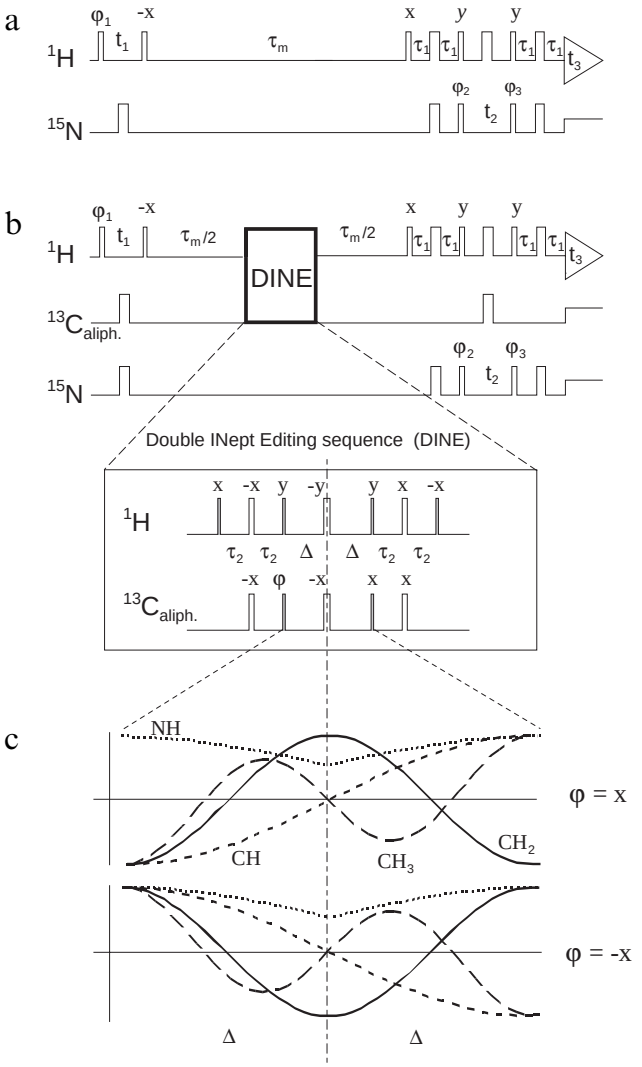

Fig. 1. (a) NOESY[^{1}H-^{15}N]HSQC sequence (Norwood et al., 1990). (b) The corresponding INEPT edited NOESY sequence [^{1}H-^{13}C]DINE-NOESY[^{1}H-^{15}N]HSQC, obtained by inserting the INEPT-reverse-INEPT (double INEPT) selective inversion pulse sequence in the middle of the mixing period. (c) Depending on the phase φ, the double INEPT sequence selectively inverts either H^{CH_2} or (H^{CH}, H^{CH_3}) for $\Delta = 1/2J_{HC}$. Note that the presented evolution of magnetization does not account for the relaxation and dephasing due to homonuclear couplings. Typically, ~10% of amide proton magnetization and ~30% of aliphatic proton magnetization is lost during DINE. Phase cycling: $\varphi_1 = 4x,4(-x)$; $\varphi_2 = x,-x$; $\varphi_3 = 2x,2(-x)$; receiver = x,2(–x),x,–x,2x,–x; delays: $\tau_1 = 1/(2J_{HN})$; $\tau_2 = 1/(4J_{HN})$. Spectra were recorded on a Bruker AMX-500 spectrometer equipped with a three-channel reverse detection gradient probe.

summarized by the following equations (Eq. A5 in the Appendix):

$$\begin{aligned} R_{ij}^{eff} &= 0 && i \in P,\ j \in I \text{ and } i \in I,\ j \in P \\ R_{ij}^{eff} &= R_{ij} && i,j \in P,\ i \neq j \text{ and } i,j \in I,\ i \neq j \\ R_{ii}^{eff} &= R_{ii} + \sum_j R_{ij} && i \in P,\ j \in I \text{ and } i \in I,\ j \in P \end{aligned} \quad (1)$$

The superscript eff indicates that, although the MENE experiment does not influence the original cross-relaxation rates R_{ij} and R_{ii}, it alters the magnetization transfer as though it is governed by the corresponding effective cross-relaxation rates defined in Eq. 1. The first relation indicates that MENE separates the cross-relaxation network into two noninteracting subnetworks (Macura et al., 1992; Zolnai et al., 1995). This results in the complete elimination of cross peaks between the inverted and noninverted groups of spins. The second relation shows that the cross-relaxation within the two groups of spins is not affected by MENE. Hence, the intensity of the cross peaks affected by spin diffusion through spins from the other group is reduced. The third relation demonstrates that spin inversion enhances the apparent autorelaxation rates of diagonal peaks. The increase in the relaxation rate is equal to the sum of the cross-relaxation rates over all excluded pathways.

In the [^{1}H-^{13}C]DINE-NOESY[^{1}H-^{15}N]HSQC experiment with H^{CH_2} inversion, the cross peaks between H^{CH_2} and H^N, H^{CH}, H^{CH_3} are removed and, additionally, the cross peaks among protons that have spin diffusion caused by H^{CH_2} are reduced by the exact amount contributed by this spin diffusion. Similarly, when (H^{CH}, H^{CH_3}) are inverted, the cross peaks between (H^{CH}, H^{CH_3}) and (H^N, H^{CH}) are eliminated, and those among H^N and H^{CH_2} are reduced. Focusing on the H^N-H^N interactions, in the first experiment the direct H^N-H^N cross peaks and H^N-H^N peaks with spin diffusion pathways over H^{CH}, H^{CH_3} and H^N protons remain intact, while the spin diffusion over H^{CH_2} is eliminated. Also, the H^N diagonal peaks will be attenuated roughly to the extent of their cross-relaxation with H^{CH_2}. Similarly, in the experiment with (H^{CH}, H^{CH_3}) inversion, cross peaks not related to the inverted protons remain intact, and the H^N-H^N cross peaks with spin diffusion mediated by the inverted protons are attenuated as well as the diagonals involved in their cross-relaxation.

Ideally, the attenuation of the H^N-H^N cross peaks in both DINE experiments reveals the dominant spin diffusion pathway. Peaks that are attenuated in the first experiment are influenced by spin diffusion over H^{CH_2} and in the second over (H^{CH}, H^{CH_3}). However, such a clear picture can only be observed when the inversions are perfect, the inversion rate is commensurate with the fastest magnetization exchange rate and the effective autorelaxation rates are similar. When the inversions are not perfect, or if $\sigma_{max}\,\tau_{in} \geq 1$ (σ_{max} is the maximal cross-relaxation rate that needs to be eliminated and τ_{in} is the interval between successive inversions; for a single inversion $\tau_{in} = \tau_m/2$), the magnetization transfer before inversion is not exactly compensated by transfer after inversion. Hence, the residual peaks among the inverted and noninverted spins may remain visible. Also, there is an attenuation of peaks due to the increased apparent autorelaxation rate in the edited experiments that makes the measurement of spin diffusion and the interpretation of MENE spectra ambiguous. To

alleviate this problem, we propose to use the difference between the spectra with inversion of H^{CH_2} and (H^{CH}, H^{CH_3}). To a first approximation, the imperfections in the two experiments have the same origin, hence their effects cancel, and the interpretation of the difference spectrum becomes straightforward.

Consider what happens with H^N-H^N cross peaks in the difference DINE-NOESY-HSQC spectrum with H^{CH_2} and with (H^{CH_3}, H^{CH}) inversions: Δ-DINE = DINE(H^{CH_2}) – DINE(H^{CH_3}, H^{CH}). Because the spin diffusion over H^{CH_2} is attenuated in the first experiment, and over (H^{CH}, H^{CH_3}) in the second, the H^N-H^N cross peaks with dominant H^{CH_2} pathways will be negative while those with dominant H^{CH} or H^{CH_3} pathways will be positive. Depending on whether the direct cross-relaxation is dominated by H^{CH}, H^{CH_3} or H^{CH_2}, the diagonal in the difference spectrum may be

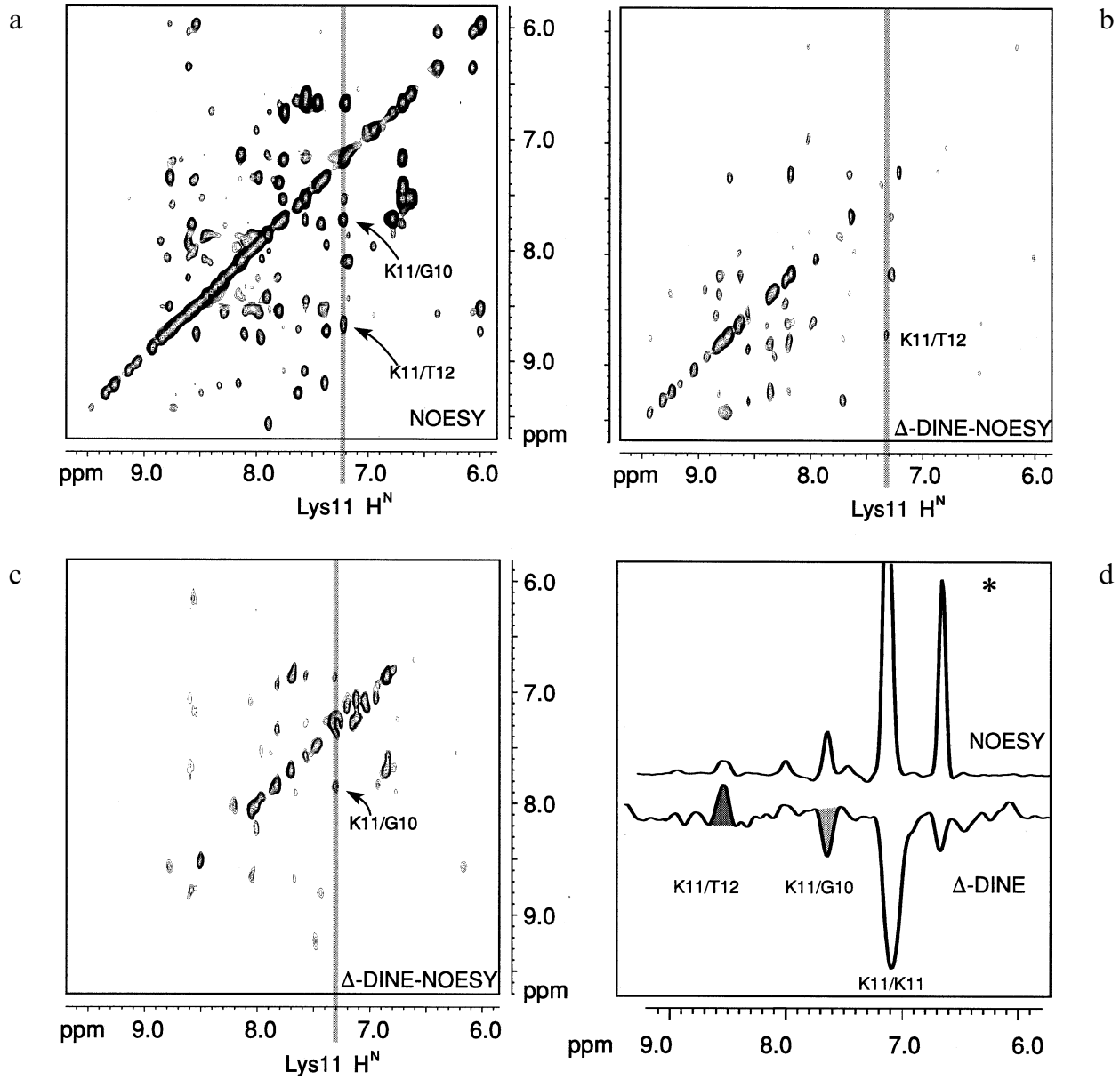

Fig. 2. (a) The amide region of a NOESY[^{1}H-^{15}N]HSQC spectrum of 2 mM doubly ^{13}C/^{15}N-labeled human ubiquitin (a small globular protein, M_w = 8565 Da) in 20 mM acetic acid-d_6, 90% H_2O/10% D_2O, pH 4.1, τ_m = 500 ms, τ_1 = 2.4 ms, 64 scans, 512 increments in t_1 and 512 t_2 data points; total acquisition time ~12 h. (b) Positive and (c) negative levels of the same region of a NOESY[^{1}H-^{13}C]Δ-DINE[^{1}H-^{15}N]HSQC spectrum (Δ-DINE = DINE(H^{CH_2}) – DINE(H^{CH}, H^{CH_3})). τ_m = 500 ms, τ_2 = 2.4 ms, Δ = 3.6 ms, 256 scans for each DINE experiment; total acquisition time ~4 days; other settings as in (a). In all experiments a 1 kHz rf field was used for WALTZ decoupling of ^{15}N during acquisition. The water signal was eliminated with the WATERGATE sequence. (d) Cross sections from a NOESY[^{1}H-^{15}N]HSQC spectrum (upper line, relative scaling 0.2) and from a NOESY[^{1}H-^{13}C]Δ-DINE[^{1}H-^{15}N]HSQC spectrum, taken along the proton resonance of Lys^{11} H^N. The positive cross peak Lys^{11}/Thr^{12} indicates that spin diffusion is mediated by (H^{CH}, H^{CH_3}) and the negative cross peak Lys^{11}/Gly^{10} (the peak labeled with an asterisk is a tail from an overlapping side-chain amide resonance) indicates that the principal mediator is H^{CH_2}.

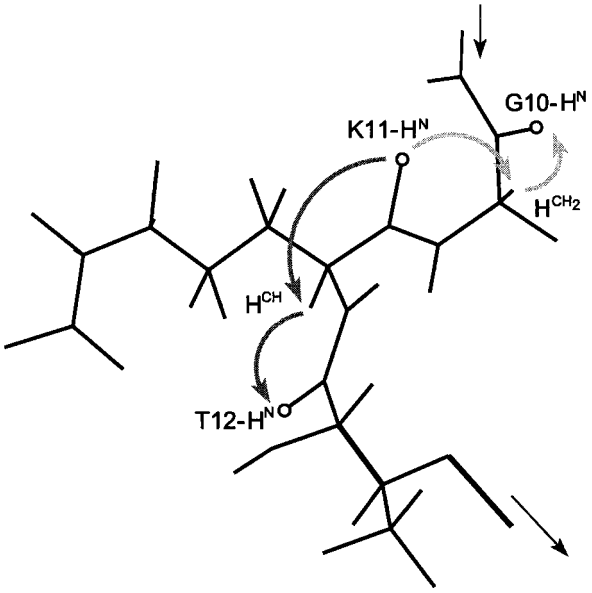

Fig. 3. Fragment 10–12 from the X-ray structure (Vijay-Kumar et al., 1987) of human ubiquitin with the spin diffusion pathways identified in Fig. 2.

either positive or negative. Consequently, in the difference spectrum the dominant pathway is determined by the sign of the cross peak rather than by the extent of attenuation as in a single DINE experiment. Up to the second order in mixing time τ_m, the volume of the Δ-DINE cross peak is given by (Eq. A11 in the Appendix):

$$\begin{aligned} A_{ij}^{\Delta\text{-DINE}}(\tau_m) &= A_{ij}^{CH_2}(\tau_m) - A_{ij}^{CH,CH_3}(\tau_m) \\ &= \frac{\tau_m^2}{2}\left[\left(R_{ik}R_{kj}\right)^{max}_{k\in CH,CH_3} - \left(R_{ik}R_{kj}\right)^{max}_{k\in CH_2} + R_{ij}\delta\right] \\ &\quad + o(\tau_m^2), \quad i,j \in NH \end{aligned} \qquad (2)$$

Equation 2 assumes that each group of pathways is dominated by a single, maximal term. It illustrates that the sign of a peak depends on the dominant pathway, and that the peak intensities are of second order, thus much weaker than the peaks in the original experiment. $R_{ij}\delta$ represents the contribution from differential enhancement of the apparent autorelaxation rates in the two MENE experiments. This complicates the interpretation of the Δ-DINE spectrum, as it can be either positive or negative and having the same order of magnitude as the first two terms. It is zero when both amide spins have the same sums of cross-relaxation rates to H^{CH,CH_3} and to H^{CH_2}. However, it may influence only the very strong peaks, and in a true spin diffusion case, when $R_{ij} \ll R_{ik}$, R_{jk}, can be safely neglected.

Figure 2 shows the amide section of an isotope-assisted two-dimensional NOESY spectrum of doubly labeled human ubiquitin, recorded using the sequences from Fig. 1. The amide part of the NOESY[^{1}H-^{15}N]HSQC spectrum recorded with $\tau_m = 500$ ms is shown in Fig. 2a. The [^{1}H-^{15}N]HSQC sequence helps to eliminate all but the H^N-originated NOEs, and allows an easy extension to the 3D HHN experiment needed for larger proteins. While the spin diffusion over carbon-bound protons can be identified from the heteronuclear BD-NOESY (Zolnai et al., 1995) or QUIET-BIRD-NOESY (Vincent et al., 1996), it would be beneficial if the dominant diffusion pathway could be identified. For example, the Lys^{11} H^N proton has cross peaks with Gly^{10} H^N and Thr^{12} H^N. The heteronuclear BD-NOESY experiment (Zolnai et al., 1995) (not shown) indicates that they are partially influenced by spin diffusion over H^{CH_n}, but the identification of proton type (H^{CH}, H^{CH_3} or H^{CH_2}) may provide additional information about their immediate environment. This information can be obtained from the NOESY[^{1}H-^{13}C]Δ-DINE[^{1}H-^{15}N]-HSQC spectrum ($\tau_m = 500$ ms) shown in Figs. 2b (positive contours) and 2c (negative contours). In the Δ-DINE spectrum, there is a positive cross peak between Lys^{11} H^N and Thr^{12} H^N and a negative cross peak between Lys^{11} H^N and Gly^{10} H^N, Fig. 2d. This indicates that the spin diffusion in the first pair is mediated by (H^{CH}, H^{CH_3}) and in the second by H^{CH_2}. Structurally, this means that either the H^{CH} or the H^{CH_3} proton is in close proximity to both Lys^{11} H^N and Thr^{12} H^N and that the H^{CH_2} is in the vicinity of both Lys^{11} H^N and Gly^{10} H^N. Indeed, inspection of the X-ray structure of human ubiquitin (Vijay-Kumar et al., 1987) reveals that Lys^{11} H^α is proximal to Lys^{11} H^N and Thr^{12} H^N and Gly^{10} $H^{\alpha 2,\alpha 3}$ to Lys^{11} H^N and Gly^{10} H^N, see Fig. 3.

In conclusion, we have proposed a new heteronuclear cross-relaxation edited experiment, [^{1}H-^{13}C]Δ-DINE-NOESY[^{1}H-^{15}N]HSQC, which enables the distinction between spin diffusion pathways over H^{CH_2} or (H^{CH}, H^{CH_3}). This information should be useful for (NMR-based) structural refinement when orienting side chains and backbone strands with nearby aliphatic protons that cannot be directly identified.

Acknowledgements

We thank Mr. Martin Moncrieffe for the critical reading of the manuscript.

References

Bax, A., Ikura, M., Kay, L.E., Torchia, D.A. and Tschudin, R. (1990) *J. Magn. Reson.*, **86**, 304–318.

Bodenhausen, G. and Ruben, D.J. (1980) *Chem. Phys. Lett.*, **69**, 185–190.

Clore, G.M. and Gronenborn, A.M. (1991) *Science*, **252**, 1390–1399.

Fejzo, J., Krezel, A.M., Westler, W.M., Macura, S. and Markley, J.L. (1991) *J. Magn. Reson.*, **92**, 651–657.

Fejzo, J., Westler, W.M., Markley, J.L. and Macura, S. (1992) *J. Am. Chem. Soc.*, **114**, 1523–1524.

Griffey, R.H. and Redfield, A.G. (1987) *Q. Rev. Biophys.*, **19**, 51–82.

Ikura, M., Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Am. Chem. Soc.*, **112**, 9020–9022.
Ikura, M. and Bax, A. (1992) *J. Am. Chem. Soc.*, **114**, 2433–2440.
Kalk, A. and Berendsen, H.J.C. (1976) *J. Magn. Reson.*, **24**, 343–366.
Macura, S. and Ernst, R.R. (1980) *Mol. Phys.*, **41**, 95–117.
Macura, S., Fejzo, J., Hoogstraten, C.G., Westler, W.M. and Markley, J.L. (1992) *Isr. J. Chem.*, **32**, 245–256.
Marion, D., Driscoll, P.C., Kay, L.E., Wingfield, P.T., Bax, A., Gronenborn, A.M. and Clore, G.M. (1989) *Biochemistry*, **28**, 6150–6156.
Massefski Jr., W. and Redfield, A.G. (1988) *J. Magn. Reson.*, **78**, 150–155.
Norwood, T., Boyd, J., Heritage, J.E., Soffe, N. and Campbell, I. (1990) *J. Magn. Reson.*, **87**, 488–501.
Otting, G. and Wüthrich, K. (1990) *Q. Rev. Biophys.*, **23**, 39–96.
Vijay-Kumar, S., Bugg, C. and Cook, W. (1987) *J. Mol. Biol.*, **194**, 531–544.
Vincent, S., Zwahlen, C., Bolton, P.H., Logan, T. and Bodenhausen, G. (1996) *J. Am. Chem. Soc.*, **118**, 3531–3532.
Zolnai, Z., Juranic, N., Markley, J.L. and Macura, S. (1995) *Chem. Phys.*, **200**, 161–179.
Zwahlen, C., Vincent, S., Di Bari, L., Levitt, M. and Bodenhausen, G. (1994) *J. Am. Chem. Soc.*, **116**, 362–368.

Appendix

Here we describe the DINE experiments in the framework of the theory of magnetization exchange network editing (MENE) that we have presented elsewhere previously (Zolnai et al., 1995). The relations between the cross-relaxation rates R_{ij} and the effective cross-relaxation rates in the MENE experiment, R_{ij}^{eff}, are obtainable from the scalar form of the master equation (Kalk and Berendsen, 1976):

$$\dot{m}_i = -\left(R_{ii} + \sum_{j=1,j\neq i}^{n} R_{ij}\right) m_i + \sum_{j=1,j\neq i}^{n} R_{ji}\langle m_j\rangle \qquad (A1)$$

Here, m_i is the observable magnetization at spin site i which is in exchange with spins at sites j, and $\langle m_j\rangle$ is the average magnetization at site j as seen from site i; in a standard experiment $\langle m_j\rangle = m_j$. R_{ij} and R_{ji} are the respective cross-relaxation rates. The leakage relaxation rate R_{ii} encompasses all sources of relaxation outside the cross-relaxation network. It includes contributions from all spins (like and unlike) that drain magnetization from the network, and describes the leakage of magnetization into the environment. For convenience, we split the network into two groups of spins, the positive P, which we observe but do not perturb, and the inverted I, which we manipulate in MENE. Then Eq. A1 becomes

$$\begin{aligned}\dot{m}_i = &-\left(R_{ii} + \sum_{j\in P,j\neq i} R_{ij} + \sum_{j\in I,j\neq i} R_{ij}\right) m_i \\ &+\left(\sum_{j\in P,j\neq i} R_{ji} + \sum_{j\in I,j\neq i} R_{ij}\right)\langle m_j\rangle,\quad i\in P\cup I\end{aligned} \qquad (A2)$$

In a standard experiment, there is no distinction between the P and the I spins. In MENE, the spins in group I are inverted during the mixing time. In an ideal case, with a high number of perfect inversions, the effective magnetization of inverted spins I, as seen from the spins P, is zero: $\langle m_j\rangle = 0$ for $j\in I$. For $j\in P$, analogous to the situation in a standard experiment, $\langle m_j\rangle = m_j$, and Eq. A2 becomes

$$\dot{m}_i = -\left(R_{ii} + \sum_{j\in P,j\neq i} R_{ij} + \sum_{j\in I} R_{ij}\right) m_i + \sum_{j\in P,j\neq i} R_{ji} m_j,\ i\in P \qquad (A3)$$

Equation A3 describes the magnetization evolution of spins P under selective inversion during the mixing time of the spin group I. Although the inversions do not affect the cross-relaxation rate constants R, they modify the magnetization transfer network. The character of modification becomes apparent if we put Eq. A3 into a form equivalent to the master equation (Eq. A1) (i.e., satisfying the principle of micro reversibility):

$$\dot{m}_i = -\left(R_{ii}^{eff} + \sum_{j=1,j\neq i}^{n} R_{ij}^{eff}\right) m_i + \sum_{j=1,j\neq i}^{n} R_{ji}^{eff} m_j \qquad (A4)$$

Comparing Eqs. A3 and A4, we find the relationships between the new, i.e., effective, relaxation rates and the original ones as:

$$\begin{aligned} R_{ij}^{eff} &= 0 && i\in P,\ j\in I \text{ and } i\in I,\ j\in P \\ R_{ij}^{eff} &= R_{ij} && i,j\in P,\ i\neq j \text{ and } i,j\in I,\ i\neq j \\ R_{ii}^{eff} &= R_{ii} + \sum_j R_{ij} && i\in P,\ j\in I \text{ and } i\in I,\ j\in P \end{aligned} \qquad (A5)$$

The volume of a cross-relaxation peak A_{ij} as a function of the mixing time τ_m can be expressed in a Taylor series as (Macura and Ernst, 1980):

$$\begin{aligned} A_{ij}(\tau_m) = &\\ &\delta_{ij} - R_{ij}\tau_m \\ &+\frac{\tau_m^2}{2}\left[\sum_{k\in P,k\neq i,j} R_{ik}R_{kj} + R_{ij}(R_{ii}+R_{jj}) + \sum_{k\in I,k\neq i,j} R_{ik}R_{kj}\right] \\ &+ o(\tau_m^2),\quad i,j\in P\cup I \end{aligned} \qquad (A6)$$

where, again, we have separated the summations over the P and I spins. In MENE, where the spins I are inverted, the cross-relaxation between the two groups is suppressed, which causes an increase in the leakage relaxation rates.

Substituting the effective relaxation rates from Eq. A5 into Eq. A6 for peaks ij in group P, we obtain:

$$A_{ij}^{I}(\tau_m) = \delta_{ij} - R_{ij}\tau_m + \frac{\tau_m^2}{2}\left[\sum_{k\in P} R_{ik}R_{kj} + R_{ij}\sum_{k\in I}\left(R_{ik}+R_{jk}\right)\right] + o(\tau_m^2), \quad i,j \in P \tag{A7}$$

Superscript I indicates that peak ij from group P is observed when peaks from I are inverted.

For labeled proteins, where N^H, H^{CH_2} and (H^{CH}, H^{CH_3}) can be selectively inverted, assuming that we observe H^N-H^N peaks only, we can rewrite Eq. A7 for (H^{CH}, H^{CH_3}) inversion as:

$$A_{ij}^{CH,CH_3}(\tau_m) = \delta_{ij} - R_{ij}\tau_m + \frac{\tau_m^2}{2}\left[\sum_{k\in NH} R_{ik}R_{kj} + \sum_{k\in CH_2} R_{ik}R_{kj} + R_{ij}\sum_{k\in CH,CH_3}\left(R_{ik}+R_{jk}\right)\right] + o(\tau_m^2), \quad i,j \in NH \tag{A8}$$

and for H_{CH_2} inversion

$$A_{ij}^{CH_2}(\tau_m) = \delta_{ij} - R_{ij}\tau_m + \frac{\tau_m^2}{2}\left[\sum_{k\in NH} R_{ik}R_{kj} + \sum_{k\in CH,CH_3} R_{ik}R_{kj} + R_{ij}\sum_{k\in CH_2}\left(R_{ik}+R_{jk}\right)\right] + o(\tau_m^2), \quad i,j \in NH \tag{A9}$$

Finally, taking the difference between the last two equations we obtain the cross-peak intensity in the Δ-DINE experiment:

$$A_{ij}^{\Delta\text{-DINE}}(\tau_m) = A_{ij}^{CH_2}(\tau_m) - A_{ij}^{CH,CH_3}(\tau_m) = \frac{\tau_m^2}{2}\left\{\sum_{k\in CH,CH_3} R_{ik}R_{kj} - \sum_{k\in CH_2} R_{ik}R_{kj} + R_{ij}\left[\sum_{k\in CH_2}\left(R_{ik}+R_{jk}\right) - \sum_{k\in CH,CH_3}\left(R_{ik}+R_{jk}\right)\right]\right\} + o(\tau_m^2), \quad i,j \in NH \tag{A10}$$

Thus, the size and intensity of Δ-DINE cross peaks depend on the number of the (CH, CH_3) or CH_2 pathways in a rather complex manner. In practice, because of the dependence of the cross-relaxation rates on the inverse sixth power of the interproton distance, usually one R_{ik} R_{kj} pair dominates in each sum, so that the peak sign and intensity qualitatively depend on the difference between the two dominant pathways:

$$A_{ij}^{\Delta\text{-DINE}}(\tau_m) = A_{ij}^{CH_2}(\tau_m) - A_{ij}^{CH,CH_3}(\tau_m) = \frac{\tau_m^2}{2}\left[\left(R_{ik}R_{kj}\right)_{k\in CH,CH_3}^{max} - \left(R_{ik}R_{kj}\right)_{k\in CH_2}^{max} + R_{ij}\delta\right] + o(\tau_m^2), \quad i,j \in NH \tag{A11}$$

$R_{ij}\delta$ represents the contribution from the differential enhancement of leakage relaxation rates in the two MENE experiments. It can be either positive or negative and can be of the same order of magnitude as the first two terms. Its presence complicates the interpretation of the Δ-DINE spectrum. However, it influences only very strong peaks and when spin diffusion is the dominant process, i.e., when $R_{ij} \ll R_{ik}, R_{jk}$, it can be safely neglected.