Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules

(NMR with biological macromolecules/cross relaxation-enhanced polarization transfer/heteronuclear correlation)

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ABSTRACT In common multidimensional NMR experiments for studies of biological macromolecules in solution, magnetization transfers via spin–spin couplings [insensitive nuclei enhanced by polarization transfer (INEPT)] are key elements of the pulse schemes. For molecular weights beyond 100,000, transverse relaxation during the transfer time may become a limiting factor. This paper presents a transfer technique for work with big molecules, cross relaxation-enhanced polarization transfer (CRINEPT), which largely reduces the size limitation of INEPT transfers with the use of cross-correlated relaxation-induced polarization transfer. The rate of polarization transfer by cross-correlated relaxation is proportional to the rotational correlation time, so that it becomes a highly efficient transfer mechanism for solution NMR with very high molecular weights. As a first implementation, \([^{15}\text{N},^{1}\text{H}]-\text{cross}-\text{relaxation}\)-correlation experiments were designed that make use of cross-correlation between dipole–dipole coupling and chemical shift anisotropy of the \(^{15}\text{N}–^{1}\text{H}\)-moieties for both CRINEPT and transverse relaxation-optimized spectroscopy (TROSY). When compared with INEPT-based \([^{15}\text{N},^{1}\text{H}]-\text{TROSY},\) these experiments yielded up to 3-fold signal enhancement for amide groups of a 110,000-Da protein in aqueous solution at 4°C. CRINEPT opens avenues for solution NMR with supramolecular structures such as membrane proteins solubilized in micelles or lipid vesicles, proteins attached to nucleic acid fragments, or oligomeric proteins.

Structure determination of proteins by NMR in solution (1) has so far been limited to the molecular weight range up to \(\approx 30\) kDa (2), and experiments yielding backbone assignments for \(^2\text{H}\)-labeled proteins up to \(\approx 60\) kDa have been reported (3). In larger molecular species, the standard experimental techniques (4, 5) lead to severe sensitivity loss because of transverse relaxation during the insensitive nuclei enhanced by polarization transfer (INSEPT) period \(T\), need to be considered (7). For isotropic rotational tumbling, \(J(0)\) is equal to \(\tauR/5\), where \(\tauR\) is the isotropic rotational correlation time of the molecule. The evolution of inphase coherence is coupled to the antiphase coherence via the cross-correlation relaxation rate \(R_{C}\).

Starting with inphase magnetization on spin I at the start of the magnetization transfer period \(T\), \(\langle I_{x},S_{y}\rangle(0)\), the buildup of antiphase coherence by CRIPIT during \(T\) can be described by ref. 14:

\[
\langle 2I_{x},S_{y}\rangle(T) = \sinh(R_{C}T) \exp(-R_{I}T)\langle I_{x}\rangle(0). \tag{1}
\]

with

\[
R_{I} = \frac{2}{5}\left[\frac{2}{9}(\gamma_{B}B_{0}\Delta\sigma)_{I}^{2} + \frac{1}{2}(\hbar \gamma_{S}/r_{1S})^{2}\right]\tauI + \frac{1}{2T_{1I}} + \frac{1}{T_{2I}} \tag{2}
\]

and

\[
R_{C} = \frac{4}{15}(\gamma_{B}B_{0}\Delta\sigma)_{I}(\hbar \gamma_{S}/r_{1S})\tauc. \tag{3}
\]

Abbreviations: CSA, chemical shift anisotropy; CRIPIT, cross relaxation-induced polarization transfer; CRINEPT, cross relaxation-enhanced polarization transfer; HMQC, heteronuclear multiple-quantum correlation; INEPT, insensitive nuclei enhanced by polarization transfer; TROSY, transverse relaxation-optimized spectroscopy.
where $r_{ij}$ is the distance between the two nuclei involved, $\Delta \sigma_i$ is the CSA of nucleus I, $B_0$ is the static magnetic field, and $\gamma_I$ and $\gamma_S$ are the gyromagnetic ratios of I and S. $T_{2i}$ and $T_{1S}$ account for the transverse relaxation of spin I and the longitudinal relaxation of spin S (7, 11, 16).

The relative efficiencies of polarization transfer with CRIPRT (Eqs. 1-3) or with INEPT (10) at variable rotational correlation times $\tau_c$ are compared in Fig. 1a. The buildup of antiphase magnetization in INEPT is described by

$$\langle 2I_zS_y(T) \rangle = \sin(\pi J_{IS}(T))\exp(-R_I T)I_z(0).$$

[Equation 4]

Because, in Eq. 1, the transfer time $T$ appears always in a product with $\tau_c$, the optimal transfer period for CRIPRT is inversely proportional to $\tau_c$. Therefore, with proper adjustment of $T$, the maximal amount of magnetization that can be transferred by CRIPRT is independent of the molecular size (Fig. 1a), where one has to consider, however, that the optimal T for $\tau_c$ values shorter than ~20 ns would be unreasonably long.

In contrast, the efficiency of INEPT falls off rapidly with increasing size (Eq. 4, Fig. 1a). The magnetic field dependence of CRIPRT for a $^{15}$N-$^1$H-moiety located in a $\beta$-sheet of a fully $^{15}$N,$^2$H-labeled protein (Fig. 1b) shows that the maximum theoretical magnetization transfer with CRIPRT is about half of the maximum transfer by INEPT and that maximal CRIPRT transfer for a $^{15}$N-$^1$H moiety is obtained at ~1 GHz (7-9). Fig. 1 further shows that CRIPRT becomes more efficient than INEPT for molecules with $\tau_c \approx 100$ ns, but that INEPT contributes significantly to the polarization transfer up to $\tau_c \approx 300$ ns.

Based on the observations on CRIPRT and INEPT in Fig. 1, and considering that systems with $\tau_c$ values in the range 50–300 ns will be of special practical interest, we combined the two polarization transfer mechanisms in CRINEPT, where proton antiphase coherence is generated during a delay $T$ devoid of radio-frequency pulses, which results in the terms in Eqs. 5 and 6 for the CRINEPT transfer (obtained from the differential Eq. 32 in ref. 11):

$$\langle 2I_zS_y(T) \rangle = A_{1I}(I_y(0)) = \cos(\pi J_{IS} T) \sinh(R_C T) \exp(-R_I T)I_z(0)$$

[Equation 5]

$$\langle 2I_zS_y(T) \rangle = A_{2I}(I_y(0)) = \sin(\pi J_{IS} T) \cosh(R_C T) \exp(-R_I T)I_z(0)$$

[Equation 6]

Eqs. 5 and 6 are the $x$ and $y$ components of the resulting antiphase magnetization, respectively. The relative orientation of the resulting total magnetization therefore depends on $\tau_c$ and the mixing time $T$, and the transfer efficiency of CRINEPT represented by the signal amplitude $A_I$ (Fig. 1) is proportional to the absolute value of the total antiphase magnetization:

$$A_I = \sqrt{A_{1I}^2 + A_{2I}^2} = \sqrt{\sinh(R_C T)^2 + \sin(\pi J_{IS} T)^2}\exp(-R_I T)$$

[Equation 7]

With Eq. 7, the relative contributions of INEPT and CRIPRT to the total polarization transfer can readily be evaluated whereas Eqs. 5 and 6 contain a mix of polarization transfer via scalar coupling (trigonometric functions) and CRIPRT (hyperbolic functions) in both terms. For short $\tau_c$, the rate $R_C$ is negligibly small and only INEPT contributes to CRINEPT whereas, for long $\tau_c$, $R_C$ becomes large and CRIPRT is the dominant polarization transfer mechanism (Eq. 3; Fig. 1a). In principle, CRINEPT is always superior to INEPT or CRIPRT (Fig. 1a). However, free proton chemical shift evolution during CRINEPT transfers (Figs. 2e and 3) has to be handled by additional pulse sequence elements, which may reduce the overall sensitivity (see below).

**Pulse Schemes for Comparative Studies of Magnetization Transfer by CRIPRT, INEPT, and CRINEPT.** The three experimental schemes in Fig. 2 were used for measurements of the efficiency of a single transfer from in-phase magnetization on $^1$H to antiphase magnetization on $^{15}$N (arrows in Fig. 2b) by the three transfer types considered in the preceding section. In each of the experiments, the $^{15}$N antiphase coherence is frequency labeled during $t_1$ and is transferred identically to $^1$H to antiphase magnetization with the two 90° pulses on I and S. In all experiments, the water magnetization is kept along $+z$ during the whole sequence by using water-selective pulses (17).

In the scheme used for CRIPRT (Fig. 2a), the inphase $^1$H magnetization generated by the first 90° pulse is transferred to antiphase magnetization by cross-correlated relaxation, according to Eq. 1. The proton chemical shift evolution is refocused by a 180° pulse, which also decouples the protons from $^{15}$N. At the end of the period $T$, 90° pulses on I and S generate the antiphase coherence $2I_zS_y$. The magnetization flow can be described in short notation as $I_z \rightarrow 2I_zS_y \rightarrow 2I_zS_y$ (Fig. 2a). In the INEPT scheme (Fig. 2b), the flow of coherence is $I_z \rightarrow 2I_zS_y \rightarrow 2I_zS_y$.

In the CRINEPT transfer measurement (Fig. 2c), the absence of 180° radio frequency pulses during $T$ results in magnetization transfer by cross-correlated relaxation as well as by scalar coupling. In addition, $^1$H chemical shift evolution occurs during $T$. The resulting antiphase coherence at time $a$ can be represented by the density matrix.

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**Fig. 1.** (a) Plot of the relative magnetization transfer efficiencies using optimal transfer delays $T$ (see Eqs. 1–7) for CRIPRT, INEPT, and CRINEPT at 750-MHz proton frequency versus molecular size represented by the isotropic rotational correlation time $\tau_c$. The CRIPRT graph is shown with a broken line for small $\tau_c$ values, to indicate that the optimal transfer time $T$ would be unreasonably long. (b) Plot of the maximal polarization transfer obtainable with CRIPRT versus the static magnetic field $B_0$, represented by the corresponding $^1$H frequency. The curves were calculated by using Eqs. 1-7 for a $^{15}$N-$^1$H-moiety located in a $\beta$-sheet of a fully $^{15}$N,$^2$H-labeled protein. The following parameters were used (8): $\tau_{1N} = 1.04 \mu$s, $\Delta \sigma_N = 15$ ppm, and $\theta_{1N} = 10^\circ$. Remote protons considered are $^1$H($i-1$), $^1$H($i+1$), and $^1$H($i$) at distances of 4.3, 4.3, and 3.3 Å, respectively. These are typical values for a $\beta$-sheet in a $^{15}$N,$^2$H-labeled protein, where $i$ is the observed residue, ($i-1$) and ($i+1$) are the sequential neighbors, and $j$ indicates a long-range contact across the $\beta$-sheet (1).
where $A_1I$ and $A_2I$ are given by Eqs. 5 and 6. The CRINEPT transfer efficiency can be measured with two experiments that use, respectively, $x$ and $y$ phases for the second 90° proton pulse (Fig. 2c).

\[
\sigma(a) = -2I_sS_x(\cos(\omega_1T)A_{21} + \sin(\omega_1T)A_{111}) + I_yS_y(-\sin(\omega_1T)A_{21} + \cos(\omega_1T)A_{111}), \quad [8]
\]

where $A_{11}$ and $A_{21}$ are given by Eqs. 5 and 6. The CRINEPT transfer efficiency can be measured with two experiments that use, respectively, $x$ and $y$ phases for the second 90° proton pulse (Fig. 2c).

\[
\sigma(b) = 2I_sS_x(\cos(\Gamma + \omega_1T)A_{21} + \sin(\Gamma + \omega_1T)A_{111}) + 2I_yS_y(-\sin(\Gamma + \omega_1T)A_{21} + \cos(\Gamma + \omega_1T)A_{111}), \quad [9]
\]

The dephasing along the $z$ axis because of the gradient is indicated by $\Gamma = G_1\gamma I_t \tau z$, where $\tau$ is the length of the pulsed field gradient, $G_1$ is its strength, and $z$ describes the position of the observed spins along the $z$ axis. Direct use of the CRINEPT transfer element as shown in Fig. 2c would result in reduced sensitivity because only half of the components of Eq. 9 can be recovered. In addition, a refocusing element has to be introduced in the pulse sequence to get a phase-sensitive $^{15N,1H}$-correlation experiment, as is demonstrated in the $^{15N,1H}$-CRINEPT-TROSY experiment of Fig. 3a. Alternatively, when omitting the second 90° proton pulse (Fig. 2c), zero- and double-quantum coherences are generated, and all of the terms of Eq. 9 can be transferred and refocused as demonstrated in the $^{15N,1H}$-CRINEPT–heteronuclear multiple-quantum correlation (HMQC) experiment of Fig. 3b.

The scheme of Fig. 3a represents a fully relaxation-compensated CRINEPT-correlation experiment. The following description retains only the magnetization components that lead to a detectable signal during the acquisition period. At time point $a$ of the pulse scheme, after the first time period $T$, the magnetization is described by Eq. 9. Because of the subsequent pulses, only the first term of Eq. 9 is transferred to transverse magnetization on $^{15N}$, which is subsequently frequency-labeled during the time $t_1$ yielding the following terms at time $b$:

\[
\sigma(b) = (2I_sS_x(\cos(\omega_1T)A_{21} + S_x\cos(\omega_1T)\sin(\pi J_1^{15N}) + A_{21}\cos(\Gamma + \omega_1T) + A_{11}\sin(\Gamma + \omega_1T) \quad [10]
\]

The CRINEPT-based sequence elements between time points $b$ and $d$ refocus the precession of the proton chemical shift during the first CRINEPT element as well as the effect of the first gradient and immediately before the last 90° pulse on $^{15N}$ the inphase term of Eq. 10 has evolved into the following coherences:

\[
\sigma(c) = 2I_sS_x\cos(\omega_1T)\sin(\pi J_1^{15N})(A_{21} + A_{11})A_{25}A_{18}S_xA_{18}, \quad [11]
\]

\[
2I_yS_y\cos(\omega_1T)\sin(\pi J_1^{15N})(A_{21} + A_{11})A_{13}A_{18}S_y, \quad [11]
\]
where $A_{IS}$ accounts for the relaxation of $I_{S1}S_2$ and $A_{I1}$ are calculated with Eqs. 5 and 6 after exchange of the indices I and S by using the relaxation rates $R_1$ and $R_C$ (Eqs. 2 and 3).

Finally, applying the last 90° pulse on $^{15}$N with the phase $\Psi_2 = x + \arctan(A_{2S}/A_{IS})$ the following proton antiphase coherence is generated ($A_{IS}$ is calculated with Eq. 7 by replacement of the indices I with S):

$$\sigma(d) = 2I_{S1}S_2 \cos(\omega_c t_1) \sin(\pi I_{S1}I_{S1}) (A_{I1} + A_{I2}) A_s A_{IS}$$

[10]

The antiphase term of Eq. 10 is transformed to inphase:

$$\sigma(d) = I_2 \cos(\omega_c t_1) \cos(\pi I_{S1}I_{S1}) (A_{I1} + A_{I2}) A_s A_{IS}$$

[13]

where $A_{IS}$ accounts for the reduction of the signal amplitude by relaxation of the $I_{S1}S_2$ state during the first period T within the refocusing element of Fig. 3a. The inphase and antiphase components in Eqs. 12 and 13 are separated by recording two free induction decays with inverted phase $\Psi_2$ (Fig. 3a).

A modified $[^{15}$N, $^1$H]-HMQC experiment (18), $[^{15}$N, $^1$H]-CRINEPT-HMQC-$[^{1}$H$]$-TROSY (Fig. 3b), is included here as an example of a scheme in which the complete CRINEPT-transferred polarization can contribute to the observed signal. Both transfer elements in this scheme are based on CRINEPT, and the different phases and chemical shift modulations obtained with the transfer according to Eq. 9 are optimally refocused by the 180° pulse on protons and the second CRINEPT element. The experiment does not include TROSY compensation during the $^{15}$N evolution period and therefore yields broader lines along the $^{15}$N frequency axis than the scheme of Fig. 3a.

Experimental. The NMR experiments were recorded with 7,8-dihydroneopterin aldolase from Staphylococcus aureus. This protein is a homooctamer with subunits of 121 amino acid residues. For the experiments in this paper, it was isotope-labeled uniformly with $^{15}$N and in the extent of 75% with $^1$H, and it was studied at 4°C in 95% H$_2$O/5% D$_2$O by using a protein concentration of 0.4 mM. Based on measurements of the $^{15}$N T$_1$ and T$_2$ relaxation times (19) at 20°C, a preliminary estimate for a lower limit on the rotational correlation time $\tau_\rho$ under the conditions of the present experiments was established at 70 ns. All NMR spectra were measured on a Bruker (Billerica, MA) DRX-750 spectrometer equipped with four radio-frequency channels and a shielded pulsed-field gradient along the z direction.

RESULTS

Magnetization Transfer by CRIP, INEPT, and CRINEPT. To provide a foundation for the use of CRINEPT, we evaluated the optimal transfer times T for each of the three transfer mechanisms of Fig. 2 in a macromolecular system with an effective rotational correlation time of $\sim 70$ ns by using the $^{15}$N-$^1$H-labeled S. aureus aldolase in H$_2$O solution at 4°C. The transfer efficiencies were measured from serial experiments by using the pulse sequences in Fig. 2 with variable transfer times T. The buildup curve for CRIP from 0 to 15 ms shows a fast increase followed by a plateau and an exponential decay, which corresponds to Eq. 1 as demonstrated by the close fit obtained with the simulation (Fig. 4). The optimal transfer delay is $\sim 6$ ms, with an observed range of 4–13 ms for different $^{15}$N-$^1$H groups in the aldolase.

For INEPT transfer with the same $^{15}$N-$^1$H-moiety as shown for CRIP, the optimal delay is $\sim 3$ ms (Fig. 4), and only $\sim 50%$ of the maximal transfer that would be obtainable without relaxation is achieved (Figs. 1a and 4). Nonetheless, for the presently studied system with $\tau_\rho = 70$ ns, the observed INEPT transfer maximum exceeds the maximal CRIP transfer by $\sim 1.5$-fold (Fig. 4).

![Fig. 4. Buildup curves of magnetization transfer for CRIP, INEPT, and CRINEPT for the well resolved $^{15}$N-$^1$H cross peak of [u-$^{15}$N, 75%H]-labeled S. aureus aldolase (32) at $\omega_1$($^{15}$N) = 121.0 ppm, $\omega_1$(H) = 11.1 ppm measured at 750 MHz (protein concentration 0.4 mM, solvent 95% H$_2$O/5%D$_2$O, T = 4°C). The buildup curves were obtained from serial measurements with variable T values by using the pulse sequences of Fig. 2. For CRINEPT, two free induction decays with phases x and y for the second $^1$H radio-frequency pulse, respectively, were measured for each value of T (Fig. 2c), which doubled the measuring time when compared with a or b. The two subspectra thus obtained are cosine- or sine-modulated with regard to the proton chemical shift during the time period T and were used to calculate the absolute value intensity of the individual cross peaks, which represents the total CRINEPT-transferred polarization (Eq. 7). CRIP, INEPT, and CRINEPT buildup curves were fitted by using Eqs. 1, 4, and 7, respectively, which resulted in $R_1 = 226 \pm 10$ s$^{-1}$ and $R_C = 172 \pm 13$ s$^{-1}$ for CRIP, $R_1 = 254 \pm 18$ s$^{-1}$ for INEPT, and $R_1 = 238 \pm 13$ s$^{-1}$ and $R_C = 153 \pm 13$ s$^{-1}$ for CRINEPT.

The experiments with CRINEPT (Fig. 4) and the fitting of Eq. 7 to the measured signal buildup confirm the theoretical prediction (Fig. 1a) that CRIP is in principle superior to INEPT and CRIP for all correlation times. Depending on the experimental used, however, some coherence terms of Eq. 9 may not contribute to the detectable signal. In the system of Fig. 4, the optimal transfer delay for CRINEPT is $\sim 4$ ms and thus lies between the optimal T values for the two basic experiments. The relative maximal transfers for CRINEPT, INEPT, and CRIP are $\sim 7.6$;5;3.4 (Fig. 4), which coincides well with the theoretical predictions for a protein with $\tau_\rho = 70$ ms (Fig. 1a).

$[^{15}$N, $^1$H]-Correlation Experiments with CRINEPT Transfers. The $[^{15}$N, $^1$H]-CRINEPT-TROSY experiment (Fig. 3a) includes transverse relaxation-optimization during the transfer delays as well as the evolution periods. For the peak shown in Fig. 5, comparison with $[^{15}$N, $^1$H]-TROSY demonstrates that the new scheme preserves the narrow line shape and yields a 2-fold signal increase. For other fast relaxing aldolase signals, sensitivity gains between 1.5 and 3 were obtained. The experimental scheme of Fig. 3a has been designed to select the two multiplet components of the $^{15}$N-$^1$H signal indicated by the filled circles in the top row of Fig. 5 (Eqs. 12 and 13). In the spectra obtained with the aldolase, the more rapidly relaxing one of these two components is in most signals broadened beyond detection (Fig. 5, contour plot on the left). Both components could be observed only for one highly flexible backbone $^{15}$N-$^1$H group and for some of the arginine side chains.

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DISCUSSION

Currently, nearly all heteronuclear multidimensional NMR experiments use INEPT to transfer magnetization between different spin species (5), but the efficiency of INEPT deteriorates with increasing rotational correlation time $\tau_c$ (Fig. 1a). In contrast, transfer of polarization by CRINEPT is independent of $\tau_c$. Further, in amide groups, the efficiency of CRINEPT increases with the strength of the external magnetic field up to $\approx 1$ GHz proton frequency whereas the sensitivity of the INEPT transfer deteriorates further because of increased CSA relaxation. The present experiments with the 110-kDa $S$. aureus aldolase validate the theoretical considerations of Fig. 1, which predict that, for molecules with rotational correlation times from $\approx 50$ to 300 ns, both INEPT and CRINEPT promote substantial polarization transfer between $^1$H and $^{15}$N in amide groups. This observation is exploited in CRINEPT.

The $^{15}$N,$^1$H-HMQC experiment (18) contains inherently two CRINEPT-type transfers. Based on the present treatment of polarization transfer, the delays $T$ in the HMQC transfer (Fig. 3b) can now be optimized for work with large molecules. However, because TROSY compensation of transverse relaxation can only be applied for $^1$H (Fig. 3b) and the rate of transverse $^{15}$N relaxation will therefore increase with increasing molecular size, the performance of this scheme, when compared with $^{15}$N,$^1$H-CRINEPT-TROSY, is predicted to deteriorate for systems with significantly longer $\tau_c$ values than 70 ns (Fig. 5).

For systems with $\tau_c \geq 300$ ns, CRIPRT is predicted to be nearly as sensitive as CRINEPT (Fig. 1a). CRIPRT then also could be used as a “filter” to eliminate resonances originating from smaller molecules because the optimal transfer time $T$ is inversely proportional to $\tau_c$ (Eq. 1). CRINEPT-based $^{15}$N,$^1$H-correlation experiments may be used as building blocks for more complex NMR experiments with large molecules, which may include simple two-dimensional experiments (1, 20, 21), triple-resonance experiments for sequential and intrarresidual backbone assignments (4, 22, 23), experiments for side-chain assignments (24–26), and experiments for studies of molecular dynamics (19, 27–29). Although this paper is focused on optimizing magnetization transfer by cross-correlated relaxation between dipole–dipole coupling and CSA, more general use may cover other types of cross-correlated relaxation. Overall, CRINEPT combined with TROSY opens avenues for solution NMR studies with particle sizes of several hundred kilodaltons, such as membrane proteins solubilized in micelles or lipid vesicles, proteins attached to large nucleic acid fragments, or oligomeric proteins.

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