Abstract: Interference between dipolar interactions in covalently linked $^{13}$C--$^1$H and nonlinked $^1$H--$^1$H pairs can be used to generate antiphase magnetization between noncoupled spins. The buildup rate of such antiphase terms is highly sensitive to local geometry, in particular the interproton distance and the $^{13}$C--$^1$H internuclear angle. These rates have been measured for opposing C$_2$H$_3$ pairs in antiparallel β-sheets in the third IgG-binding domain of protein G (GB3) and in HIV protease, complexed with the inhibitor DMP323. For GB3, good agreement with the 1.1-Å crystal structure is found. However, this agreement rapidly deteriorates with decreasing resolution of the corresponding X-ray structure. For HIV protease, two separate crystal structures that differ by less than 0.2 Å from one another exhibit lower agreement in their predicted cross-correlated relaxation rates relative to one another than is found between experimental rates and the average of the rates predicted for the two structures. These data indicate that quantitative measurement of these cross-correlated relaxation rates can provide highly accurate structural information in macromolecules.

Although it has been well recognized that, at least in principle, measurement of cross-correlated relaxation is applicable to any two pairs of interactions in a macromolecule, irrespective of their relative distance, in practice most work has focused on pairs of spins connected by a short J-couplings network that permits efficient generation of multipin coherences. Measurement of cross-correlated double-quantum and zero-quantum relaxation rates has been used to measure torsion angles in proteins and in nucleic acids and recently also has been used to obtain structural information on spins connected by J coupling through hydrogen bonds.

A different set of experiments is based on cross-correlated relaxation between a local interaction and the dipolar interaction with the magnetic-field-dependent net magnetization of a fast relaxing electron of a paramagnetic site in the protein. Owing to the fast electron relaxation, only one electron magnetization component is affected, and this electron magnetization component can be induced using the antiphase dipolar term. The electron magnetization transfer is a spin-echo process and therefore can be straightforwardly measured using standard NMR pulse sequences.

NMR has become a well-established method for determining the three-dimensional structure of proteins and nucleic acids. Most such structural studies have been based primarily on interproton distances, determined from $^1$H--$^1$H NOEs, and torsion angles, derived from J couplings. More recently, a host of other structural parameters have been introduced, including the measurement of internuclear vector orientations from dipolar couplings in partly oriented systems, and relative internuclear vector orientations from cross-correlated relaxation.

Cross-correlated relaxation relies on the interference between two separate relaxation mechanisms, which leads to nonexponential relaxation in a manner that is a function of the relative orientations of the magnetic interactions underlying these relaxation mechanisms. Besides providing structural information, in cases of known geometry, interference effects between covalently linked spins also can yield information on the CSA tensor, which is related to local conformation and hydrogen bonding.

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to the large dipole moment of such Curie spin magnetization, relaxation interference effects can be observed up to substantial distances away from the paramagnetic site, in addition to exploiting the direct paramagnetic effects such as pseudo-contact shifts and relaxation enhancement for structural purposes.

The present study relies on a different type of cross-correlated relaxation measurement in proteins: the transfer of magnetization between hydrogens that are not connected by a J-coupling network. The possibility of transferring net magnetization from one spin to another through relaxation interference has long been recognized, and several applications to magnetization transfer have been described. For example, Wimperis and Bodenhausen demonstrated that, in a nearly linear arrangement of four protons, A, B, C, and D, magnetization transfer between B and C could be generated in the absence of B–C scalar coupling. Here, we focus on relaxation interference between the $C_\text{II} - H_\text{II}$, $H_\text{III} - H_\text{II}$, and $H_\text{III} - C_\text{III}$ dipolar interactions in opposing $13C_\text{II} - H_\text{II}$ groups in $\beta$-sheets. We demonstrate that net magnetization transfer is readily possible and contains information that is very sensitive to the relative position of such groups. Relaxation interference effects depend primarily on zero-frequency spectral density terms, causing cross-correlated relaxation rates to increase with molecular size. Application of these new techniques is therefore not restricted to small systems.

### Materials and Methods

All NMR experiments were carried out on Bruker DRX spectrometers, operating at 600-MHz $^1$H frequency. For all experiments, the $^1$H carrier was positioned on the HOD resonance and the $^{13}$C carrier at 43 ppm. All data were processed and analyzed with NMRPipe. Data apodization utilized a 90°-shifted squared sine-bell function in the directly detected dimension and a sine-bell function shifted by 90° in the indirect dimension. Data were zero-filled by at least a factor of 2 in all dimensions prior to Fourier transformation.

Two different samples were used in the present study. The first sample contained 1.8 mM $^{13}$C$\text{II}$,$^{15}$N-labeled third lig-binding domain from streptococcal protein G (further referred to as GB3) dissolved in D$_2$O, pH 5.6, 20 mM sodium phosphate. For unidirectional transfer schemes, data were recorded at 15 °C on a spectrometer equipped with a triple-resonance, three-axes pulsed field gradient probehead, optimized for $^1$H detection. For the “out-and-back” experiments, the sample temperature was set to 6.5 °C, and the spectrometer used was equipped with a cryogenic, triple-resonance probehead equipped with a z-axis pulsed field gradient. A second sample contained 0.4 mM (dimer) $^{13}$C$\text{II}$,$^{15}$N-labeled HIV-1 protease complexed with unlabeled DMP123 inhibitor, dissolved in D$_2$O, pH 5.6, 25 mM sodium phosphate. Data were acquired at 27 °C using a cryogenic probehead with a triple-resonance, z-axis pulsed field gradient probehead, optimized for $^1$H detection.

### Theoretical Basis

Here, we consider the case of an isolated system of three spins, $A$, $M$, and $X$, each with negligible CSA. The master equation describing the spin dynamics has a block-diagonal matrix representation, where the evolution of the four $M$-spin single-quantum coherences is described by

$$\frac{d}{dt} \begin{pmatrix} M_{AA}^\text{X} \\ 2M_{AX}^\text{X} \\ 2M_{AX}^\text{X} \\ 4M_{AX}^\text{X} \end{pmatrix} = -(L + iK) \begin{pmatrix} M_{AA}^\text{X} \\ 2M_{AX}^\text{X} \\ 2M_{AX}^\text{X} \\ 4M_{AX}^\text{X} \end{pmatrix}$$

with

$$L = \begin{pmatrix} R(M_A^\text{X}) & 0 & 0 & 0 \\ 0 & R(2M_A^\text{X}) & \Gamma_{DD,DD} & 0 \\ 0 & \Gamma_{DD,DD} & R(2M_A^\text{X}) & 0 \\ 0 & 0 & 0 & R(4M_A^\text{X}) \end{pmatrix}$$

and

$$K = \begin{pmatrix} \omega_M & \pi J_M & \pi J_M & 0 \\ \pi J_M & \omega_M & 0 & \pi J_M \\ \pi J_M & 0 & \omega_M & \pi J_M \\ 0 & \pi J_M & \pi J_M & \omega_M \end{pmatrix}$$

where $\omega_M$ is the Larmor frequency of spin $M$, $J_{IS}$ is the scalar coupling between spins $I$ and $S$, $R(I)$ is the autorelaxation rate of coherence $I$, and $\Gamma_{DD,DD}^\text{MA,MAX}$ represents the dipole–dipole cross-correlation rates between spin vectors $MA$ and $MX$. Equation 1 describes how $M$-spin in-phase magnetization can be transferred to two-spin antiphase coherence, either by scalar coupling or by cross-correlated relaxation. Neglecting internal dynamics, the relaxation rates for a spherical macromolecule in the slow tumbling limit, are given by

$$R(2M_A^\text{X}) \approx R(2M_A^\text{X}) \approx R(4M_A^\text{X}) \approx R(M_A^\text{X}) \approx R_2$$

where $\theta$ is the angle between vectors $MA$ and $MX$, $\xi_{DD}^\text{MA,MAX} = -(\mu_0 \gamma_I \gamma_S)/(4\pi r_{IS}^2)$, and $r_{IS}$ is the distance between nuclei $I$ and $S$. In the case of anisotropic rotational diffusion, the correlation time $\tau_c$ needs to be replaced by the corresponding spectral density functions. Due to its geometrical dependence, the dipole–dipole cross-correlation rate is maximum for a linear alignment of the three spins, with $M$ occupying the central position. For the $13C_\text{II} - H_\text{II} - H_\text{III}$ spin system considered below, $J_{AX} = 0$. For such an isolated three-spin system, the resulting violation of the secular approximation introduces only spectral density terms at $J(\omega_M)$, which are negligible in the slow tumbling
Results and Discussion

The feasibility of cross-correlated relaxation for generating net magnetization transfer between uncoupled spins in a protein will be demonstrated for \{^{13}C,^{1}H,^{1}H\} spin systems, as found in antiparallel \(\beta\)-sheets. However, a range of other applications can also be envisioned. The relevant geometric parameters are illustrated in Figure 2. The close proximity typically found between opposing \(H_2\) spins in \(\beta\)-sheets and the relative orientation of the \(^{13}C-^{1}H-^{1}H\) vectors result in substantial cross-correlated relaxation rates, involving dipolar interaction of the \(C_\alpha-^{1}H\) \((C_\alpha-^{1}H\) \(H_2 \) pair and the dipolar interaction between the nonbonded \(H_2-^{1}H\) pair. Below, these dipole–dipole cross-correlated relaxation rates will be denoted \(\Gamma_1 = \Gamma_{\text{DD,DD}}\) and \(\Gamma_2 = \Gamma_{\text{C13CH2,1H2}}\).

As dipole–dipole cross-correlated transfer of magnetization involves up to four spins, the most general implementation of the method would be a four-dimensional scheme with each frequency dimension corresponding to one of the nuclei involved. In practice, it is more convenient to implement the experiments as 2D or 3D versions of such a 4D scheme, such that adequate digital resolution can be obtained within a limited total measuring time. As is the case for the regular triple-resonance NMR experiments, different transfer modes can be employed: unidirectional and “out-and-back”. In the unidirectional experiments, magnetization originating on the first proton is transferred by the dipole–dipole cross-correlation mechanism (originally named RACT, for relaxation-allowed coherence transfer)\(^{32}\) to antiphase magnetization on the second proton, which subsequently is rephased by a second RACT process, prior to detection of the second proton. The net transfer then depends on two separate RACT processes, each with its own geometric dependence. In the “out-and-back” mode, magnetization on the first proton is transferred by RACT to antiphase magnetization on the second proton, where it evolves for a variable amount of time, prior to transfer back to the first proton by the same RACT process. Quantitative analysis of the spectral intensities observed in the “out-and-back” version is therefore simpler as, besides the \(H_2-^{1}H\) distance, it depends only on the angle \(\theta_2\) in Figure 2, whereas intensity in the unidirectional version is a function of both \(\theta_1\) and \(\theta_2\). Both modes of implementation are discussed below.

Unidirectional Transfer Schemes. Figure 3 shows 2D and 3D implementations of the unidirectional transfer. The 2D RACT pulse scheme employed here is very similar to a standard inverse heteronuclear two-dimensional HSQC experiment,\(^{44}\) but with the last INEPT transfer replaced by a two-step RACT
by the $^1$H 90° pulse. This last term evolves again under influence of cross-correlated relaxation between points $c$ and $d$, converting $2^1H_1^2$ into $2^2C_2^2$. However, the value of $\Delta_2$ is tuned to $(n + 0.5) \gamma / J_{1C}$, such that this term is converted into in-phase $H_2^2$ magnetization at time point $d$:

$$\sigma_d = \sinh(-\Gamma_1 \Delta_2) [4^1H_1^2 C_2^2 \cosh(-\Gamma_2 \Delta_3) + 2^1H_1^2 \sinh(-\Gamma_2 \Delta_3)] \exp[-R_2 (\Delta_1 + \Delta_2)]$$  \hspace{1cm} (7)

$^{13}$C decoupling during $t_2$ data acquisition destroys the three-spin term in eq 7, and only the in-phase $H_2^2$ signal will be detected. Simultaneously to the $C_1 \rightarrow H_2$ transfer described above, a similar $C_2 \rightarrow H_1$ transfer results from $\Gamma_2$ evolution during $\Delta_1$ and $\Gamma_1$ during $\Delta_2$. Therefore, if the $\beta$-sheet geometry corresponds to sufficiently large dipole–dipole cross-correlation rates, two in-phase cross-peaks at frequencies $(\omega_{CH}, \omega_{H2})$ and $(\omega_{CH}, \omega_{H1})$ will be observed for each $H_n$ pair.

For the 3D analogue of the unidirectional RACT scheme, it is advantageous to insert the cross-correlation steps at the start of the pulse scheme (Figure 3B). This permits a gradient-enhanced reverse INEPT back to $^1$H to be used for transfer of both the $x$ and $y$ components of evolved $^{13}$C magnetization, thereby optimizing sensitivity. The $\Delta_1$ cross-correlation delay is tuned to $(n + 0.5) \gamma / J_{1C}$, such that $H_1^2$ magnetization at time point $a$, which is a result of cross-correlation evolves into $4^1H_1^2 C_1^2$, rephases under the influence of $J_{CH}$ into $2^1H_1^2$. Delay $\Delta_1$ simultaneously serves as a constant-time evolution period for $^1$H, and therefore does not require any additional delay duration relative to the 2D scheme. The $90^\circ \phi_2$ $^1$H pulse subsequently transfers $2^1H_1^2$ into $2^2C_1^2$, which as a result of cross-correlation during the subsequent delay $\Delta_2$ (adjusted to $m \gamma / J_{1C}$) evolves into $2^2C_2^2$. At the end of the two-step RACT, the observable detected signal has been transferred from $H_1^2$ (present at time point $a$) to the two-spin term $2^1H_1^2 C_2^2$ at time point $b$. The magnitude of this term is given by

$$\sigma_b = 2^1H_1^2 C_2^2 [\sinh(-\Gamma_1 \Delta_1) \sinh(-\Gamma_2 \Delta_2) \times \exp[-R_2 (\Delta_1 + \Delta_2)]]$$  \hspace{1cm} (8)

To minimize relaxation losses during the subsequent $^{13}$C $t_2$ evolution period, the term is converted to $2^1H_1^2 C_2^2$ two-spin coherence, prior to conversion back to single-quantum $^{13}$C coherence, phase-encoding by the $G_n$ gradients, and a Rance–Kay transfer back to $^1$H. The net result is that, for each pair of opposing $H_n$ atoms in an antiparallel $\beta$-sheet, two in-phase coherence cross-peaks at frequencies $(\omega_{CH}, \omega_{C2}, \omega_{H2})$, and $(\omega_{CH}, \omega_{C1}, \omega_{H1})$ will be observed.

**Out-and-Back Transfer Scheme.** As discussed above, intensities observed in the unidirectional transfer scheme depend on both the $\theta_1$ and $\theta_2$ angles, complicating quantitative analysis. However, an out-and-back RACT experiment can be used which depends on only a single angle, thereby facilitating the evaluation of the dependence of dipole–dipole cross-correlation rates on local geometry. This scheme (Figure 4) is quite similar to that of Figure 3B, but magnetization originating on proton $H_1$, and converted into $2^1H_1^2$ (time point $b$) as a result of $\Gamma_1$ cross-correlation and $J_{CH}$ rephasing during $\Delta_1$, is again transferred

$$\sigma_b = 2^1H_1^2 C_2^2 [\sinh(-\Gamma_1 \Delta_1) \sinh(-\Gamma_2 \Delta_2) \times \exp[-R_2 (\Delta_1 + \Delta_2)]]$$  \hspace{1cm} (8)


magnetization transfer accomplished through 2H into 2H. Figure 5. Contour plot representation of the geometrical factor ($P_2(\cos \theta)\sin(\theta)$) versus the distance $r_{HH}$ (nm) and the angle $\theta$ in degree. In the plot region shown, the geometrical factor ranges from 0 to 15,625 nm$^{-2}$.

Figure 4. Pulse scheme for the 3D "out-and-back" RACT experiment, used for quantitative analysis. The open 13C-180° pulses are applied at the indicated positions in the "out-and-back" RACT experiment; in the corresponding reference experiment the first open 13C pulse is omitted, and the second one is shifted to 1.74 ms prior to the 1H 90° pulse. Phase cycling: $\phi_1 = 4(\times), 4(-\times); \phi_2 = 2(\times), 2(-\times); \phi_3 = 8(\times), 8(-\times); \phi_4 = \pi; \phi_5 = x, -x; \phi_6 = -x; \phi_7 = y, -y; \phi_8 = -y$. Quadrature detection in the 1H dimension ($t_1$) is achieved by simultaneous incrementation of $\phi_3$ and $\phi_4$. All other parameters are as marked in the legend to Figure 3B.

Ratio of the two intensities may be written as

$$I_T = I_0 \sin(-\Gamma_1 \Delta_1) \sin(-\Gamma_2 \Delta_2) \exp[-R_1(\Delta_1 + \Delta_2)] \quad (9)$$

Of course, magnetization originating on H gives rise to a similar cross-peak, but with its intensity governed by $\sin(-\Gamma_2 \Delta_2) \sinh(-\Gamma_2 \Delta_2)$. To determine the quantitative magnitude of this cross-correlation term, a reference experiment is designed of the same total duration, but with essentially complete $1H \rightarrow 1C \rightarrow 1H$ magnetization transfer accomplished through $J_{CH}$ de- and rephasing (Figure 4), yielding an intensity $I_{Ref}$. As $\Gamma \Delta \ll 1$, the ratios of the two intensities may be written as

$$\frac{I_T}{I_{Ref}} = \sin(-\Gamma_{CHHH} \Delta_1) \sin(-\Gamma_{CHHH} \Delta_2)$$

$$\approx 4\Delta_1 \Delta_2 \left(\frac{\mu_0}{4\pi} g_H^2 \frac{D_{CH}}{J_{CH}} \right)^2 \left(\frac{P_2(\cos \theta)}{r_{HH}^3}\right)^2 \quad \left(10\right)$$

The theoretical dependence of the $I_T/I_{Ref}$ ratio on the geometry is presented in Figure 5. For a local geometry close to regular antiparallel $\beta$-sheets ($r_{HH} = 2.4 \text{ Å, } \theta = 25^\circ$), the experimental ratio will be very sensitive to small variations of the conformation: an independent modification of the angle by $4^\circ$ or of the interproton distance by 0.1 Å results in a variation of the $I_T/I_{Ref}$ ratio by more than 25%.

Equation 10 applies to the integrated intensities of the reference peaks and cross-peaks. However, for maximum $t_1$ delays that are shorter than the difference in the inverse relaxation rates of 2H and 1H, the difference in cross-peak and reference line shapes is negligible, and peak heights may be used instead.

Application to Protein G. The feasibility of magnetization transfer via dipole–dipole cross-correlated relaxation in proteins is demonstrated for two systems: GB3 and HIV protease. We first discuss application to GB3, a domain for which a 1.1 Å X-ray structure is available. This atomic resolution structure also allows for quantitative validation of eq 10. GB3 contains two sets of antiparallel $\beta$-strands. A region of the 2D RACT correlation spectrum, recorded with the pulse sequence of Figure 4A, is shown in Figure 6A. It shows eight cross-peaks, corresponding to five different H pairs (the two remaining cross-peaks fall outside of the region shown). As expected, they all involve correlations between proximate H nuclei on
antiparallel-paired β-strands. For reference, the corresponding HSQC spectral region is shown in Figure 6B. Correlations marked by asterisks in Figure 6A correspond to direct, one-bond correlations which are incompletely suppressed as a result of the heterogeneity of $J_{CH}$ values, which range from 135 to 153 Hz in this domain (B. Ramirez, personal communication). To minimize these residual signals for β-sheet residues, $\Delta_1$ and $\Delta_2$ were optimized for $J_{CH} = 143.4$ Hz, which corresponds to the middle of the range of values expected in β-strands.47 These residual peaks are particularly strong for small proteins such as GB3, for which the optimal $\Delta_1$ and $\Delta_2$ durations are rather long (ca. 20−25 ms at 15 °C). For larger proteins, such one-bond correlations are much less intense, owing to the shorter optimal transfer delays (Figure 1). Nevertheless, they frequently remain observable for α-helical residues, which typically have larger $J_{CH}$ values.

One-bond and RACT correlations can easily be distinguished by the 3D version of the RACT experiment (Figure 3B). For example, consider the correlation between residues 43 and 54 of protein GB3 in Figure 6A. Because the chemical shifts of the two $^{13}$C nuclei are very similar, it is unclear if the correlation observed in spectrum 6A corresponds to a residual diagonal peak, caused by J-mismatching, or a real cross-peak due to RACT. Analysis of the 3D spectrum shows a well-resolved peak at $(\omega_{H53},\omega_{C53},\omega_{H43})$ frequencies (Supporting Information), confirming that the peak results from cross-correlation.

Correlation with Structure. Inspection of the β-sheet topology of GB3 suggests that there are six pairs of H$_{13}$ nuclei for which RACT correlations are expected. However, no correlations are observed for the pair involving residues 8 and 13; all others are observed (Figure 6A and Supporting Information Figure 1). A more quantitative analysis of the 3D structure of GB3 (PDB code 2IGD) reveals a noncanonical geometry for the 8−13 pair. The angle between vectors $C_{\alpha}^8-H_{\alpha}^8$ and $H_{\alpha}^8-H_{\alpha}^{13}$ ($\theta_1$ in Figure 2) when substituting $C_{\alpha}^8 = C_{\alpha}^{13}$, $H_{\alpha}^8 = H_{\alpha}^{13}$, and $H_{\beta}^8 = H_{\beta}^{13}$) equals 53.9°, which is very close to the condition where the angular part of $\epsilon$ 3 equals zero (magic angle). This therefore precludes the observation of unidirectional transfer between residues 8 and 13 (cf. eqs 7 and 8). In contrast, in spectra recorded with the out-and-back transfer scheme (Figure 4), an additional correlation at frequencies $(\omega_{H11},\omega_{C13},\omega_{H13})$ is observed (data not shown). In this out-and-back experiment, the observed intensity does not depend on the $C_{\alpha}^8-H_{\alpha}^8-H_{\alpha}^{13}$ angle, which is responsible for the near-zero intensity in the unidirectional experiment.

The good qualitative agreement between the observed cross-peaks and the GB3 structure suggests that cross-correlated relaxation rates can yield quantitative information for structure determination. However, the structural dependence of eq 10 applies for an isolated three-spin system. In a protein, the presence of other spins complicates the situation. In particular, the $^1$H−$^1$H dipolar interactions with additional neighboring protons in the protein need to be considered. The cross-correlated relaxation rate itself, $\Gamma_{DD}^{DD}$, remains unaffected by the presence of additional $^1$H spins. For the autorelaxation rates, to a very good approximation, only the $^1$H−$^1$H dipolar interactions need to be considered. Dipolar interactions between $^1$H and additional neighboring spins affect the autocorrelated relaxation rate $R(H_1^1)$, $R(2H_1^1,H_1^1)$, $R(2H_1^1,3H_1^1)$, and $R(4H_1^1,3H_1^1)$ equally, and therefore to first order do not affect the intensity ratio of eq 10. Dipolar interactions between $^2$H and neighboring protons affect only the $^2$H longitudinal relaxation induced by additional neighboring protons. In the slow tumbling limit, and neglecting internal motion, the increase in effective relaxation rates falls in the 3−9% range if the additional neighboring protons are approximated by a single “pseudo-proton” placed at a distance, $r = (\sum(r_i)^{-6})^{-1/6}$, in the 2.3−1.9 Å range from $^2$H.

The implication of the violation of the secular approximation in eq 2 when applied to a protein was determined using eq 40 of ref 33, extended to take into account neighboring protons. For the GB3 data, the $I_{H/Ref}$ ratio (eq 10) decreases by a factor 0.97 ± 0.01 when again accounting for the additional protons by a single “pseudo-proton” placed at a distance of 2.1 ± 0.2 Å from $^2$H. This 3% systematic underestimate in the $I_{H/Ref}$ ratio is comparable to the measurement error and therefore may be safely ignored.

Figure 7 plots the $I_{H/Ref}$ ratio measured for the 11 H$_{13}$ protons in GB3 as a function of the geometric factor ($P_2(cos \theta)/\gamma_H^2$)‡, calculated from its 1.1-Å resolution X-ray structure. The slope of the solid line in this figure corresponds to a $\tau_z$ value of 6.3 ns, which is within 10% from previous NMR relaxation results.48−50 corrected for differences in temperature and solvent viscosity (D$_2$O vs H$_2$O).51 Considering the extremely steep

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dependence of the ratio on local geometry, with a Pearson’s correlation coefficient $R_p = 0.89$, the observed correlation is remarkably good. The largest outlier in Figure 7 is observed for Gln$^2$-H$_a$, but this residue differs by a T2Q substitution from the amino acid sequence used for the X-ray crystallographic study. The root-mean-square (rms) deviation between experimental and predicted data is 504 nm$^{-1}$. Assuming an average $\beta$-sheet geometry ($r_{\text{HH}} = 2.4$ Å and $\theta_1 = 25^\circ$; see Figure 2 for notation), this deviation corresponds either to a variation of 0.07 Å for the interproton distance or to a 3.5$^\circ$ change in $\theta$, which is comparable to the errors in these distances and angles on the basis of the atomic coordinate uncertainties in the 1.1-Å X-ray structure. For comparison, even with the most careful analysis of NOE data using relaxation-matrix-based refinement, distance restraints generally carry an uncertainty of at least 0.2 Å.

Comparison of the geometric factor ($P_2(\cos \theta)/r^3_{\text{HH}})^2$ for GB3 (PDB code 2IGD)$^{46}$ and the highly homologous crystal structure of the first Igg-binding domain of protein G (PDB code 1PBG)$^{33}$ shows a lower correlation ($R_p = 0.64$) than comparison of our experimental, NMR-derived geometric factors to 1PBG ($R_p = 0.77$). It is also interesting to note that correlation of the NMR-derived geometric factors with the 1.1-Å crystal structure of GB3, refined with anisotropic $B$-factors (PDB code 1IGD),$^{46}$ yields a lower $R_p (0.79)$ than the correlation with the GB3 X-ray structure refined with anisotropic $B$-factors ($R_p = 0.89$). These results therefore underscore the high precision of the structural restraints that can be extracted from the cross-correlation data.

**Application to a 22-kDa Protein.** As discussed in Theoretical Basis section, one important feature of RACT transfer is that its efficiency does not decrease with increasing size of the molecule (Figure 1). Therefore, the RACT experiment is well suited for the study of larger $\beta$-sheet-rich proteins too, provided that high-quality $^1\text{H}-^1\text{C}$ one-bond correlation spectra are obtainable. To demonstrate the utility for larger proteins, 3D unidirectional and out-and-back spectra have been recorded for a sample of the 22-kDa homodimeric HIV-1 protease, complexed with inhibitor DMP323.$^{54}$

Examples of unidirectional correlations observed in the HIV-1 protease are shown for the $H_a$ protons of residues Gly$^{48}$, Glu$^{65}$, and Ile$^{72}$ (Figure 8). On average, the same correlations in the out-and-back version of the experiment are about 15% weaker, owing to signal decay during the additional (non-constant-time) $^1\text{H}$ evolution period (Supporting Information Figure 2). Despite the low concentration of this sample (0.4 mM), the spectrum shows a total of 23 correlations involving $H_a$ protons, with a signal-to-noise ratio ranging from 5 to 26. All the observed correlations correspond to 12 distinct pairs of $H_a$ protons on adjacent $\beta$-strands (Figure 8B). Comparison with the secondary structure of the protein$^{35}$ shows that only pairs Ile$^{13}$–Ile$^{66}$, Ile$^{15}$–Ile$^{64}$, and Thr$^{31}$–Ile$^{35}$ do not give rise to any observable correlations. A more detailed analysis of different crystal structures (PDB codes 1MET and 1MES) reveals noncanonical structure for the two last pairs, with interproton distances larger than 3 Å, which correspond to RACT rates that are too low to observe such transfers. From the remaining 13 pairs, one involves an intermonomer contact between the H$_a$ protons of Leu$^{97}$, yielding only a single correlation. From the remaining

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25 expected cross-peaks, only the correlations between $H_{13}$ protons of Ile$^{13}$ and Ile$^{66}$ are not observed. This interaction involves an unfavorable angle between vectors $C_{\alpha}^{13}-H_{\alpha}^{13}$ and $H_{\alpha}^{13}-H_{\alpha}^{66}$ ($\theta = 41^\circ$, making the corresponding cross-correlated relaxation rate the smallest predicted one).

Quantitative comparison of $I/I_{Ref}$ ratios with the HIV protease structure is limited by the resolution of the available X-ray structures. Two structures of highly homologous HIV-1 proteases in complex with the DMP323 inhibitor have been solved by X-ray crystallography at a resolution of 1.9 Å. The two crystal structures differ from the NMR sample used by six-point mutations (residues 7, 33, 63, 67, 95, 82 for PDB entry 1MES, and 7, 33, 63, 67, 95, 82 for PDB entry 1MET), and by two-point mutations from one another. Despite the fact that the structures have very similar backbone coordinates, with pairwise backbone rmsd’s of less than 0.2 Å over the secondary structure regions of the protein, comparison of the relevant interproton distances and angles between the two structures results in a standard deviation of 0.13 Å and 5.8°. As expected, therefore, a poor correlation ($R_P = 0.46$) is obtained when comparing the geometrical factors, $[P_2(\cos \theta)I_{\text{Ref}}^{13}]^2$, for the two 1.9-Å resolution X-ray structures (Supporting Information Figure 3).

When structural differences are small, the averages of the corresponding geometric factors are very similar to the geometrical factors of the average structure. This averaging procedure therefore reduces the effect of small, largely random coordinate errors in the X-ray structures. As a result, a slightly better degree of correlation ($R_P = 0.62$) is obtained when comparing the experimentally measured geometrical factors with the average of the geometric factors for the two crystal structures (Supporting Information Figure 4). However, considering that the scatter between the geometrical factors derived from the different crystal structures is even larger, it is likely that the spread in the correlation between the NMR data and the averaged X-ray derived geometric factors remains dominated by the uncertainty in the X-ray coordinates. This indicates that it will be beneficial to use the experimental RACT rates as additional parameters in structure calculation and refinement.

**Conclusions**

Our study demonstrates that magnetization transfer between uncoupled nuclei by means of dipole–dipole cross-correlated relaxation is feasible in proteins. In the slow tumbling limit, the efficiency of such transfer is insensitive to the rotational correlation time, and therefore the RACT experiments are particularly promising for the study of larger proteins. In β-sheets, RACT between $H_{\alpha}$ protons on adjacent strands is exquisitely sensitive to the local geometric parameters. This suggests that RACT rates will be particularly useful for refining the local structure of β-sheets in proteins.

Quantitative measurement of RACT rates is relatively straightforward. A wide range of different types of interactions in proteins and nucleic acids, involving atoms in separate, non-covalently linked segments, can be envisioned to yield structurally meaningful information. Structure refinement to improve agreement with the experimental RACT rates simultaneously involves angular and distance terms and is relatively similar to refinement of protein structures against $^1\text{H}$$\leftrightarrow^1\text{H}$ dipolar couplings. Together with the use of dipolar couplings, RACT rates are expected to make it possible to determine the structure of biological macromolecules with unprecedented accuracy.

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**Supporting Information Available:** Four figures showing (1) selected regions from the unidirectional 3D RACT spectrum of GB3, (2) selected regions from the “out-and-back” 3D RACT spectrum of HIV-1 protease, (3) correlation of the geometrical factors for opposing $C_{\alpha}H_{\alpha}$ pairs in antiparallel β-sheets in HIV protease, derived from two crystal structures, and (4) plot of experimental $I/I_{Ref}$ ratio against the averaged geometrical factors in antiparallel β-sheets in HIV protease, derived from two crystal structures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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