Transferred Cross-Correlated Relaxation: Application to the Determination of Sugar Pucker in an Aminoacylated tRNA-Mimetic Weakly Bound to EF-Tu

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Transfer-NOE[1] has proven to be a valuable tool to study the conformation of small molecular weight ligands when bound to macromolecules. If the bound and free forms of the ligand are in fast exchange on the NMR time scale, the measured NOE between two spins *i* and *j* results from the average of the cross-relaxation rates σ_{ij} in the bound and in the free form; due to the dependence of σ_{ij} on the correlation time, the contribution of the bound form NOE to the observable peak is much larger than that of the free form, even in excess of free ligand. This allows adjustment of the concentration of the macromolecule, to minimize the line-broadening while still observing clear NOE contributions from the bound form. Unfortunately, the same concept cannot be applied to coupling constants, since they do not depend on the correlation time of the molecule; in this case the major contribution to the averaged coupling comes from the species present with the highest molar fraction, namely, the free form of the ligand, so that no information is available on the bound form. Traditionally, coupling constants were considered to be the only direct source of structural angular information. Recently it has been demonstrated that dipolar cross-correlated relaxation can be used to obtain projection angles between two internuclear vectors.[2] Since cross-correlated relaxation rates linearly depend on the correlation time, they can be used, as NOEs, in transfer-type experiments. In this article, we show that transfer cross-correlated relaxation can provide projection restraints of the bound conformation of a ligand when it is in fast exchange with the free form. This constitutes unique information, since it fills in the gap left by coupling constants in giving direct angular structural information on small ligands in large molecular weight complexes.

The binding of a ligand *L* to a macromolecule *M* can be described by the equilibrium reaction

$$M + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ML$$

When the exchange between the bound (*ML*) and free conformation (*L*) of the ligand is fast on the NMR time scale, one resonance will be observed with the following transverse relaxation rate:

$$\Gamma^{av} = p_{ML}\Gamma_{ML} + p_L\Gamma_L + p_L p_{ML}\tau(\Delta\Omega)^2 \qquad (1)$$

where p_{ML} and p_L are the molar fractions of the bound and free conformation, Γ_{ML} and Γ_L are the corresponding transverse relaxation rates, $\Delta\Omega$ is the difference in precession frequencies, and τ is the characteristic lifetime of the process defined as $\tau^{-1} = \tau_{ML}{}^{-1} + \tau_L{}^{-1} = k_{+1} + k_{-1}$, which needs to be smaller than $\Delta\Omega$ for the fast exchange condition to be fulfilled (see Figure 1).

A similar equation can be written for all NMR parameters with respect to which the bound and free forms of the ligand are in fast exchange. For a cross-correlated relaxation rate $\Gamma^{c,av}_{\mathbf{VW}}$ between two magnetic interactions **V** and **W**, eq 1 yields in fast exchange

$$\Gamma^{c,av}_{\mathbf{VW}} = p_{ML}\Gamma^{c}_{\mathbf{VW},ML} + p_L\Gamma^{c}_{\mathbf{VW},L} \qquad (2)$$

where $\Gamma^{c}_{\mathbf{VW},ML}$ is the cross-correlated relaxation rate of the bound conformation and $\Gamma^{c}_{\mathbf{VW},L}$ that of the free one. No chemical shift related term, like the third one in eq 1 (exchange term), is present for cross-correlated rates, since they are extracted from differential line broadening of multiplet components, which are equally affected by exchange, so that the corresponding term cancels out.

We focus now on the cross-correlated relaxation rate due to the dipolar coupling of two different C−H pairs, C_i−H_i (**V**) and C_j−H_j (**W**) Then the cross-correlated relaxation rate is given by

$$\Gamma^{c}_{C_iH_i,C_jH_j} = \frac{2}{5}\frac{\gamma^2{}_C\gamma^2{}_H\mu_0{}^2\hbar^2}{(4\pi)^2 r^3_{C_iH_i} r^3_{C_jH_j}} S^2_{ij}\frac{(3\cos^2\theta_{ij} - 1)}{2}\tau_c \qquad (3)$$

where γ_C and γ_H are the gyromagnetic ratios of carbon and proton respectively, $r_{C_iH_i}$ and $r_{C_jH_j}$ are the C−H interatomic distances of C_i−H_i and C_j−H_j, τ_c is the correlation time of the molecule, θ_{ij} is the projection angle between the two C−H vectors, and S^2_{ij} is the order parameter which takes into account possible internal motion. Due to the linear dependence of the

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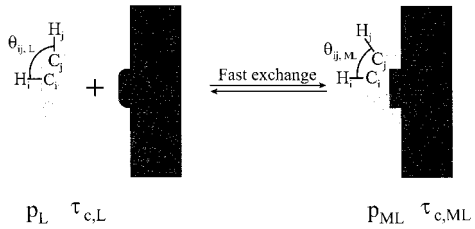

Figure 1. Schematic representation of the binding of a small ligand to a macromolecule. p_L, $\tau_{c,L}$, and θ_{ijL} are the molar fraction, the correlation time, and the projection angle between the C_i-H_i and C_j-H_j vectors in the free ligand. Analogously p_{ML}, $\tau_{c,ML}$, and θ_{ijML} represent the corresponding parameters for the complex.

cross-correlated relaxation rate on the correlation time τ_c, we can rewrite eq 3

$$\Gamma^{c,av}_{C_iH_i,C_jH_j} = \frac{2}{5}\frac{\gamma^2_C\gamma^2_H\mu_0{}^2\hbar^2}{(4\pi)^2 r^3_{C_iH_i} r^3_{C_jH_j}} \times \left[S^2_{ij,L}\, p_L \frac{3\cos^2\theta_{ij,L}-1}{2}\tau_{c,L} + S^2_{ij,ML}\, p_{ML}\frac{3\,cos^2\,\theta_{ij,ML}-1}{2}\tau_{c,ML}\right] \quad (4)$$

Obviously, when the condition $p_{ML}\tau_{c,ML} > p_L\tau_{c,L}$ is fulfilled, the observed cross-correlated relaxation rate will depend strongly on $\theta_{ij,ML}$. In this case it is possible to gain knowledge on the relative orientation of the two C_i-H_i and C_j-H_j vectors in the ligand when bound to the macromolecule by measuring the value of the average cross correlated rate $\Gamma^{c,av}_{C_iH_i,C_jH_j}$. The condition $p_{ML}\tau_{c,ML} > p_L\tau_{c,L}$ is satisfied when $\tau_{c,ML} \gg \tau_{c,L}$, which is often the case for very large macromolecules that bind small ligands, even though the population of the bound conformation p_{ML} may be much lower than that of the free conformation.

By contrast, the averaging of coupling constants is described by eq 5 and does not show any dependence on the correlation time

$$J^{av} = p_L J_L + p_{ML} J_{ML} \quad (5)$$

In this case when the population of the bound form p_{ML} is much lower than that of the free form p_L, the average coupling constant J^{av} is dominated by the first term $p_L\ J_L$, and no information is available relative to the bound form.

The cross-correlated relaxation rate between two vicinal C_i-H_i and C_j-H_j vectors can be used to determine the torsional angle χ_{ij} about the connecting C_i-C_j bond. In fact the projection angle θ_{ij} between the C_i-H_i vector and the C_j-H_j vector, which can be extracted according to eq 4 for the bound form from the value of $\Gamma^{c,av}_{C_iH_i,C_jH_j}$, is connected to the dihedral angle χ_{ij} by

$$\cos\theta_{ij} = -\cos(\gamma_{H_iC_iC_j})\cos(\gamma_{H_jC_iC_j}) + \sin(\gamma_{H_iC_iC_j})\sin(\gamma_{H_jC_iC_j})\cos(\chi_{ij}) \quad (6)$$

where $\gamma_{H_iC_iC_j}$ and $\gamma_{H_jC_iC_j}$ are the $H_i-C_i-C_j$ and the $H_j-C_i-C_j$ bond angles, respectively. Thus, cross-correlated relaxation $\Gamma^{c,av}_{C_iH_i,C_jH_j}$ uniquely yields the angular information that the $^3J(H_i,H_j)$ coupling cannot provide in the fast exchange regime due to the unfavorable averaging of coupling constants (eq 5).

The method is applied to the mixture of 2′ and 3′ esters of anthranilic acid with ribosyl ^{13}C-labeled adenosine, 2′-Ant-Ado and 3′-Ant-Ado (Figure 2), that are mimetics of amino-acid loaded tRNA and both weakly bind to the bacterial elongation

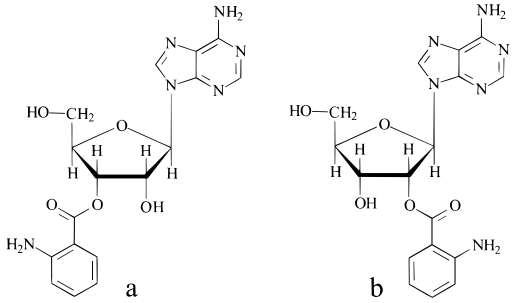

Figure 2. Constitution of the two isomers of the ester of anthranilic acid with adenosine: (a) 3′-Ant-Ado; (b) 2′-Ant-Ado.

factor Tu−GDP complex (EF-Tu·GDP).[3] Adenosine that is labeled with ^{13}C in the ribosyl ring is obtained by converting uniformly ^{13}C-labeled glucose into 3′,5′-benzoyl-1′,2′-acetyl-ribose, followed by glycosylation with adenine to give adenosine after deprotection.[4] Anthranoylation of ^{13}C adenosine, as previously described,[3d] leads to the final compounds 2′- and 3′-Ant-Ado. 3′-Ant-Ado binds stronger to EF-Tu·GDP, as can be derived from the degree of line broadening upon addition of protein.[3a] The conformation of the ribose ring has been determined for the free Ant-Ado from $^3J_{H1'H2'}$ and $^3J_{H3'H4'}$ coupling constants.[3b] For both 2′-Ant-Ado and 3′-Ant-Ado the C2′-endo conformation is preferred. From transfer-NOE studies on the complex between the 3′-Ant-Ado and the protein EF-Tu·GDP, it has been inferred that the ligand retains the C2′-endo conformation[3c] when bound to EF-Tu·GDP. However, spectroscopic evidence for the pucker of the sugar ring is expected to be stronger from the measurement of torsional angles than from that of interproton distances.

The CH−CH dipole−dipole cross-correlated relaxation rates can distinguish between the C2′-endo and the C3′-endo conformations of the ribose ring with high sensitivity.[5] As can be derived from Figure 3, the $\Gamma^c_{C1'H1',C2'H2'}$ and the $\Gamma^c_{C3'H3',C4'H4'}$ cross-correlated relaxation rates show typical values for the C2′-endo and the C3′-endo conformations, namely, in the C2′-endo (south) conformation $\Gamma^c_{C1'H1',C2'H2'}$ is positive [$(3\ cos^2\ \theta_{1'2'} - 1)/2 = 0.9$], and $\Gamma^c_{C3'H3',C4'H4'}$ is negative [$(3\ cos^2\ \theta_{3'4'} - 1)/2 = -0.4$]. Also the absolute value of $\Gamma^c_{C3'H3',C4'H4'}$ is larger than that of $\Gamma^c_{C3'H3',C4'H4'}$. In the C3′-endo (north) conformation exactly the reverse situation applies. Little difference is expected for the $\Gamma^c_{C2'H2',C3'H3'}$ rate in the two conformations. The ratio between the $\Gamma^c_{C1'H1',C2'H2'}$ and the $\Gamma^c_{C3'H3',C4'H4'}$ rates, together with their signs, represent sufficient information to determine the conformation of the sugar ring unequivocally. NOEs show much smaller differences upon changing of the conformation of the sugar and are plagued by the problem of spin diffusion. For these reasons a much stronger evidence of the sugar pucker can be obtained in an easier and quicker way by cross-correlated relaxation measurements rather than by NOEs.

The $\Gamma^c_{C1'H1',C2'H2'}$ and the $\Gamma^c_{C3'H3',C4'H4'}$ rates have been measured on a 1 mM solution of Ant-Ado in D_2O/d_4-methanol, 7:1 at 281 °K, with 0, 20, and 35 μM concentration of EF-Tu·GDP, respectively. The recently developed *quantitative*-Γ-HCCH

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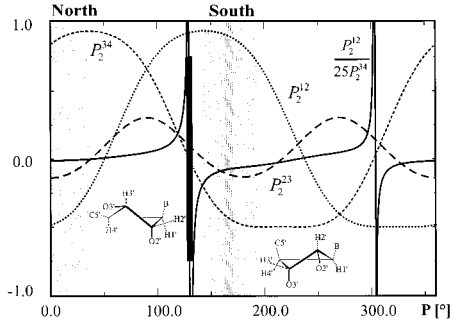

Figure 3. Dependence of the $P_2(\cos\theta)$ on the conformation in a ribose ring [$P_2(x) = (3x^2 - 1)/2$]. θ_{ij} is the projection angle between the C_i-H_i vector and the C_j-H_j one. The dotted line (···) represents the $P_2(\cos\theta_{1'2'})$ for the projection angle between the *C*1′*H*1′ and the *C*2′*H*2′ vectors, P_2^{12}; the dashed light line (- - -) the $P_2(\cos\theta_{3'4'})$ for the θ between the *C*3′*H*3′ and the *C*4′*H*4′ vectors, P_2^{34}; the dashed dark line (- - -) the $P_2(\cos\theta_{2'3'})$ for the θ between the *C*2′*H*2′ and the *C*3′*H*3′ vectors, P_2^{23}. The full line (—) shows the ratio, divided by 25, between the $P_2(\cos\theta)$ for $\theta_{1'2'}$ and $\theta_{3'4'}$ [$P_2^{12}/(25P_2^{34})$], which is equal to the ratio between the two corresponding cross-correlated rates assuming equal internal motion for both pairs of vectors. *P* is the pseudorotation phase of the puckering of the ribose ring; the pseudorotation amplitude χ^{max} has been fixed to the common value of 40°. The allowed values for typical C2′-endo (south) and C3′-endo (north) conformations are shown by the light gray bands. The dark gray box indicates the allowed conformational region for the 2′-Ant-Ado bound to EF-Tu·GDP. This corresponds to $\Gamma^c_{C1'H1',C2'H2'}/\Gamma^c_{C3'H3',C4'H4'} > 20$ and $\Gamma^c_{C1'H1',C2'H2'} > 0$.

sequence[5] was used. The sequence consists of a HCCH correlation: the magnetization starts on the H_i spin, is transferred to the spin C_i, whose chemical shift is recorded in t_1, while the coupling $J(C_i,C_j)$ is let evolve to obtain the operator $4H_{i,z}C_{i,x}C_{j,y}$. Two different experiments are recorded, yielding a *cross* and a *reference* spectrum. The operator $4H_{i,z}C_{i,x}C_{j,y}$ is transferred to the operator $4C_{i,y}C_{j,x}H_{j,z}$ in the cross experiment via the cross-correlated relaxation rate $\Gamma^c_{C_iH_i,C_jH_j}$, during a constant time *T*, and in the reference experiment via the two coupling constants $^1J(H_i,C_i)$ and $^1J(H_j,C_j)$, evolving during $\Delta' = {}^1/_2{}^1J_{HC}$. The coherence is then transferred back to the proton, and the chemical shift of the spin H_j is recorded in t_2. The value of the rate is obtained, as explained in ref 5, from the ratio of the volumes of the two corresponding peaks (C_i,H_j) in the *cross* and *reference* experiment. The intensity of the (C_i,H_j) peak in the cross experiment is in fact proportional to $\sinh(\Gamma^c_{C_iH_i,C_jH_j}\tau_M)$, whereas in the reference experiment it is proportional to $\cosh(\Gamma^c_{C_iH_i,C_jH_j}\tau_M)\sin(\pi^1J_{C_iH_i}\Delta')\sin(\pi^1J_{C_jH_j}\Delta')$. From this one obtains

$$I_{cross}/I_{reference} = \sinh(\Gamma^c_{C_iH_i,C_jH_j}\tau_M)/\{\sinh(\Gamma^c_{C_iH_i,C_jH_j}\tau_M)\cos(\pi^1J_{C_iH_i}\Delta')\cos(\pi^1J_{C_jH_j}\Delta') + \cosh(\Gamma^c_{C_iH_i,C_jH_j}\tau_M)\sin(\pi^1J_{C_iH_i}\Delta')\sin(\pi^1J_{C_jH_j}\Delta')\} \quad (7)$$

which allows the extraction of $\Gamma^c_{C_iH_i,C_jH_j}$ if the two coupling constants $^1J_{C_iH_i}$ and $^1J_{C_jH_j}$ are known. Since in our case $^1J_{C_iH_i} = {}^1J_{C_jH_j}$ and $\Delta' = {}^1/_2\ {}^1J_{CH}$, eq 7 becomes

$$I_{cross}/I_{reference} = \tanh(\Gamma^c_{C_iH_i,C_jH_jT}) \quad (8)$$

Traces along the proton dimension are shown in Figure 4 for the $4H2'_zC2'_xC1'_y \rightarrow 4C2'_yC1'_xH1'_z$ transfer at each concentration of the protein for the 2′-Ant-Ado. From these peaks the value of the $\Gamma^c_{C1'H1',C2'H2'}$ rate can be obtained, according to eq 7. In Table 1 the extracted rates are reported for each peak at

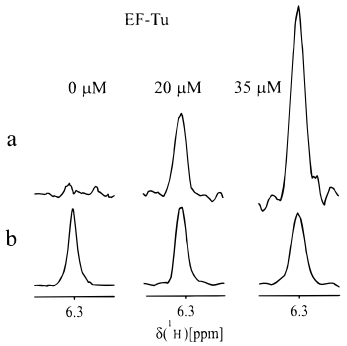

Figure 4. Traces corresponding to the *C*2′−*H*1′ peak are shown for 2′-Ant-Ado: (a) traces extracted from the *quantitative*-Γ-HCCH-cross experiment and multiplied by 4; (b) traces from the *quantitative*-Γ-HCCH-reference-experiment divided by 2. For the 20 μM and 35 μM EF-Tu samples, the *quantitative*-Γ-HCCH -cross-experiment ran 3 times longer than the reference one, which was 7.3 h with a recycling delay of 1.25 s; for the 0 μM EF-Tu sample, the *quantitative*-Γ-HCCH-cross-experiment ran 4 times longer than the reference one, which was 3.5 h with a recycling delay of 2.8 s. The mixing time for the evolution of the double/zero quantum coherences was 25 ms in order to refocus $^1J_{CC}$ couplings. The traces in each column refer to different protein concentrations. All samples were buffered at pH 7.5 (50 mM Na_3BO_3, 10 mM $MgCl_2$, 50 mM KCl).

Table 1. Values of the Cross-Correlated Rates[a]

2′-Ant-Ado	0 μM	20 μM	35 μM
C1′H1′−C2′H2′	0.2	3.7 Hz	7.6 Hz
C3′H3′−C4′H4′	/	/	/

[a] Values, calculated as explained in ref 5, are reported for the two C1′H1′−C2′H2′ and C3′H3′−C4′H4′ moieties of the 2′-Ant-Ado. When both symmetric peaks were available, the average is taken. No value (/) is given when the corresponding peak in the *quantitative*-Γ-HCCH-cross experiment is missing. The $\Gamma^c_{C1'H1',C2'H2'}$ rate for the 2′-Ant-Ado increases with the concentration of the protein as expected.

the three protein concentrations. Transfer via the $\Gamma^c_{C1'H1',C2'H2'}$ rate is observed in the 2′-Ant-Ado at all concentrations of EF-Tu; the value for the Ant-Ado alone is 17 times smaller than that for the 20 μM EF-Tu solution, showing that in this case the term $p_L\Gamma_{ij,L}(\tau_{c,L})$ in eq 3 is negligible.

No analysis of the data regarding the 3′-Ant-Ado is presented here. In this case, strong coupling between the C2′ and the C3′carbons, whose chemical shift difference is approximately 1 ppm, distorts the intensities of the interesting peaks. A structural interpretation of these data would require fitting of experimental signals, which is outside the scope of this paper.

For the 2′-Ant-Ado at all protein concentrations no peak due to transfer via $\Gamma^c_{C3'H3',C4'H4'}$ was observed. The upper limit of the absolute value of the ratio between the $\Gamma^c_{C1'H1',C2'H2'}$ and the $\Gamma^c_{C3'H3',C4'H4'}$ cross-correlated rates can be calculated from the signal-to-noise (S/N) ratio of the peak corresponding to the transfer via $\Gamma^c_{C1'H1',C2'H2'}$, since the nonobservable peak due to the $\Gamma^c_{C3'H3',C4'H4'}$ can at most be as large as the noise. This S/N value has to be scaled down by the ratio between the integrals of the *C*1′−*H*2′ (or *C*2′−*H*1′) and *C*3′−*H*4′ or (*H*3′−*C*4′) peaks in the reference spectrum. This accounts for the different autorelaxation rates and different carbon-coupling topologies in the two *C*1′*H*1′−*C*2′*H*2′ and *C*3′*H*3′−*C*4′*H*4′ moieties, that may cause an intrinsic difference in the volumes of the two corresponding peaks in the cross experiment, even if the $\Gamma^c_{C1'H1',C2'H2'}$ and $\Gamma^c_{C3'H3',C4'H4'}$ cross-correlated rates were the same. The S/N ratio was extracted from the *quantitative*-Γ-HCCH-cross spectrum of the 20 μM EF-Tu·GDP sample. For the 2′-Ant-Ado, one gets

2′-Ant-Ado: $\Gamma^{c}_{C1'H1',C2'H2'}/\Gamma^{c}_{C3'H3',C4'H4'}| > 20, \quad \Gamma^{c}_{C1'H1',C2'H2'} > 0$

If the pseudoration amplitude of the ribose ring χ^{max} is fixed to the most common value of 40° and a single conformation is assumed, this combination of information defines the allowed values for the pseudoration phase *P* (Figure 3), thus determining the conformation of the ribose ring. For the 2′-Ant-Ado the allowed range for *P* is 127°−133°. The conformation of the 2′-Ant-Ado in complex with EF-Tu is then very close to the C2′-endo conformation, confirming what is found by analysis of vicinal spin−spin coupling constants $J_{1'2'}$ for free 2′-Ant-Ado[3], by X-ray crystallography in the complex 3′-Ant-Ado/EF-Tu·GppNHp[6] and in the analogous complexes 3′-Phe-tRNA/EF-Tu·GppNHp and EF-Tu·GTP[7] and what was inferred by transfer NOE in solution.[3a,c] In the crystal structure of the complex between Phe-tRNA and EF-Tu·GTP the adenine ring of the 3′-terminal adenosine is located in a lipophilic pocket of the protein. This can be achieved only when the corresponding terminal ribose is in C2′-endo conformation. This conformation is in addition stabilized by a H-bond interaction between the 2′-OH group of ribose with glutamate-271.[7b] In the case of 2′-Ant-Ado the 2′-OH group is substituted and cannot participate in the same H-bond. The results presented here demonstrate that the C2′-endo ribose conformation which is required for binding to the protein is induced by the aminoacylation of adenosine and does not entirely depend on the H-bond between glutamate-271 and 2′-OH group of the ribose.

If an equilibrium between two different conformers in standard north ($\chi^{max} = 40°$, $P = 18°$) and south ($\chi^{max} = 40°$, $P = 162°$) conformations is assumed, the experimental data are in agreement with the following population distribution: $p_{south} = 66.0$, $p_{north} = 34.0$, which points again to the fact that the 2′-Ant-Ado assumes preferentially a C2′-endo conformation upon binding to EF-Tu·GDP.

In conclusion we have shown that transfer cross-correlated relaxation rates represent a valuable and unique method to obtain angular structural information in ligands weakly bound to macromolecules. We expect this to be a valuable tool in the contextof SAR by NMR[8] techniques and for the study of transient species in enzyme-catalyzed reactions.

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