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Selective NMR Experiments on Macromolecules: Implementation and Analysis of QUIET-NOESY

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The QUIET-NOESY experiment (Zwahlen *et al., J. Am. Chem Soc.* 116, 362–368, 1994) is applied to measure the mobility of the flexible extensions in the large aggregate (800 kDa) of a small heat-shock protein. The proper choices of the experimental protocol and parameters are discussed in order to employ a simplified data analysis procedure. Further experimental verification of the proposed strategy is also presented using the cyclic peptide gramicidin S as a model compound. Under suitable conditions, the determinations based on the analysis of QUIET-NOESY data are affected to a negligible extent by the approximations that are introduced by the proposed approach.

Key Words: **QUIET-NOESY; selective NOE; cross-relaxation rate determination; local mobility; correlation-time determination; small heat-shock proteins; Hsp25.**

INTRODUCTION

The application of selective NMR experiments to biopolymer studies is generally hampered by severe sensitivity losses due to relaxation during the selective pulses and during the time intervals of the sequences. Even when the interpulse and observation intervals can be compressed, relaxation that occurs during the shaped pulses may prove to be too fast to allow adequate sensitivity. For large molecules such as biopolymers at high magnetic fields, these detrimental effects are mainly due to the fast decay of the transverse components of the magnetization, although longitudinal relaxation effects should also be considered (*1, 2*).

Some partial remedies have recently been proposed (*3*), and further progress can be expected from improved pulse-shaping schemes. Relaxation during selective pulses still remains, however, the bottleneck for selective NMR spectroscopy of large biomolecules. Nevertheless, the application of selective NMR methods may provide access to novel experimental approaches in the field. In particular, valuable information is expected to arise from use of QUIET-NOESY (*q*uenching *u*ndesirable *i*ndirect *e*xternal *t*rouble in *n*uclear *O*verhauser *e*ffect *s*pectroscop*y*) (*4*), an experiment which was devised to quench spin diffusion during NOE measurements. Spin diffusion hinders the precise assessment of single cross-relaxation rates in the presence of a network of simultaneous dipolar interactions which determine the dynamics of spin populations of the molecular magnetization reservoir (*5*). As is well known, the phenomenon becomes particularly severe with macromolecules because of their slow tumbling and the complexity of their dipolar network.

The solution offered by QUIET-NOESY relies on isolating the spin group of interest from the whole system of nuclear magnetization. Isolation is achieved in two ways. First, only selective pulses are employed to detect the Overhauser enhancement of a single spin (or group of equivalent spins) upon selective perturbation (inversion) of a dipolar partner. From this point of view, QUIET-NOESY may be regarded as an *evolutionary product* of the selective-inversion experiment, first proposed by Freeman *et al.* in 1974 (*6*) and subsequently repeatedly modified up to the QUICK-NOESY version (*7*) that incorporates shaped selective pulses for inversion and detection. Secondly, in the innovative aspect of the QUIET-NOESY experiment, a doubly selective inversion pulse in the middle of the evolution time isolates the dipolar pair of interest from the bulk magnetization. The isolation is achieved by simultaneous spin-temperature inversion which leads to a back-transfer of the magnetization fluxes between the selected pair and the bulk reservoir. Although the efficiency of a dipolar decoupling between the selected pair and the remainder of the system could be improved by repeated double-inversion pulses (*8*), only minor deviations from ideality are experienced when a single doubly selective inversion pulse is employed (*4*). A thorough analysis (*9*) has shown, in fact, that as long as longitudinal and transverse-relaxation losses during the selective pulses are properly accounted for, the results of QUIET-NOESY experiments lend themselves to accurate quantitation of cross-relaxation rates, even when the data are analyzed by a simple two-site submatrix approach. A more extended treatment is required only if selectivity fails to retrieve single-pair cross-relaxation data, as occurs with poorly resolved dipolar subsystems of three or more spins.

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The feasibility of a quantitative NOE analysis suggests some interesting applications in the area of macromolecular biophysics if selective pulses with a suitable performance could be employed. Such experiments could be utilized for calibration purposes, such as in the determination of accurate internuclear distances or local correlation times in proteins and oligonucleotides. Other applications could include assessing NOEs in complexes of antibodies with small antigens and proteins with DNA or other proteins, or within transient intermediates of partially unfolded proteins, that is, under conditions where spin diffusion may introduce substantial uncertainties in the determination of NOEs, or even casts doubt on the very existence of certain dipolar interactions. The application we present here aims at quantifying the extent of local flexibility in large aggregates of Hsp25, a small heat-shock protein (*10*, *11*).

RESULTS AND DISCUSSION

Theory

The QUIET-NOESY experiment is depicted in Fig. 1a. The mixing time, τ_m, following the selective inversion of the source spin is bisected by a doubly selective inversion pulse which acts on the source and the target spins. Detection is achieved by a selective reading pulse applied to the target spin. The net NOE amplitude is determined by subtraction of the FID resulting from the same scheme, apart from the initial pulse being off-resonance (Fig. 1b). If the separation of the two-spin subsystem from the remainder of the magnetization reservoir is successfully achieved, the experimental data can be analyzed by computing the evolution of the relative populations under the action of the corresponding relaxation super-operator. Expressed in terms of the same mixing-coefficient function as reported for 2D NOESY cross-peaks of two isolated spins, *A* and *B*, that is, the source and the target, respectively, we have (*12*)

$$a_{AB} \propto -\exp(-R_L\tau_m)\cdot[1-\exp(-R_C\tau_m)], \qquad [1]$$

where a_{AB} is the mixing coefficient, and R_L and R_C are the leakage and cross-relaxation rate constants, respectively, as defined by Macura and Ernst (*12*), the pre-exponential intensity factors having been omitted. Equation [1] contains two exponential terms and would therefore require several experiments to solve for R_C. With the inherent precision limits of NMR determinations, the pitfalls of multiexponential fitting should be avoided to enable a reliable estimation of cross-relaxation parameters. A simple way to bypass this problem is to measure the auto- and cross-relaxation terms from the source-spin amplitude by means of the scheme in Fig. 1c. The corresponding mixing coefficient, a_{AA}, can be expressed as (*12*)

$$a_{AA} \propto \exp(-R_L\tau_m)\cdot[1+\exp(-R_C\tau_m)], \qquad [2]$$

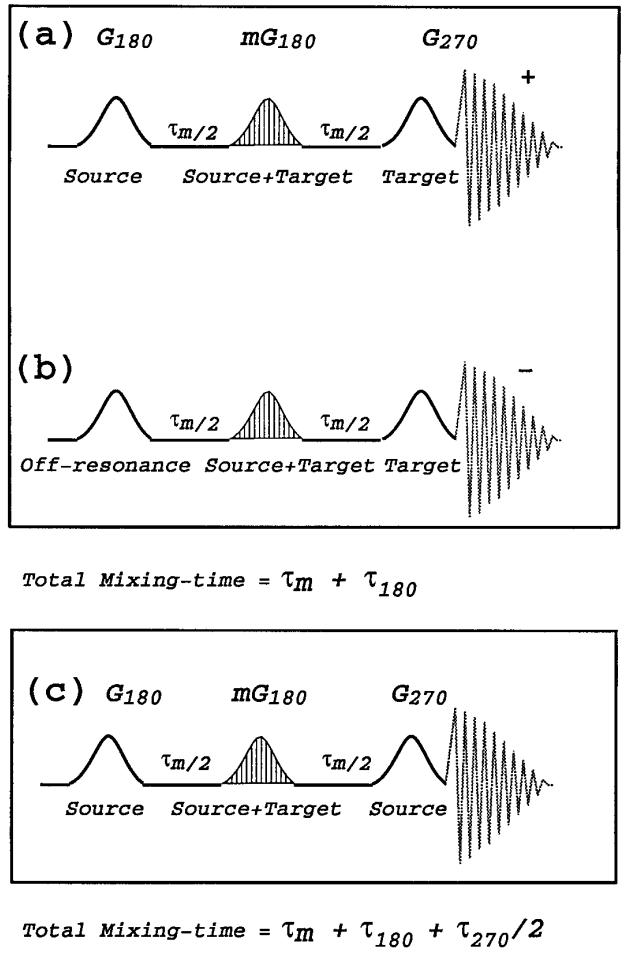


FIG. 1. Pulse-sequence schemes for QUIET-NOESY (*4*). G_{180} and G_{270} are the singly selective Gaussian-shaped pulses with flip angles of 180° and 270°, respectively, applied to either source or target-spin frequency, as indicated. mG_{180} is the audio-modulated doubly selective inversion pulse, applied at the midpoint frequency between the source and target-spin resonances. (a) On-resonance sequence and (b) off-resonance sequence for the measurement of the target-spin NOE. The two FIDs are collected in interleaved mode to minimize effects from instrumental drifts, and their subtraction is performed after correcting the data from experiment (b) for the source-spin transverse attenuation (Eq. [4]). (c) Sequence for the measurement of the source-spin amplitude. According to the discussion of the Theory subsection, the overall value of the mixing time includes the half-durations of the inversion pulses ($\tau_{180}/2$), which have the same time length to ensure a constant excitation bandwidth around the selected resonances. For the sequence in (c), the mixing-time duration also includes the reading-pulse half-duration ($\tau_{270}/2$).

where the same factors as in Eq. [1] have been omitted. The determination of R_C requires fitting the ratio

$$\frac{a_{AB}}{a_{AA}} = -\left[\frac{1-\exp(-R_C\tau_m)}{1+\exp(-R_C\tau_m)}\right], \qquad [3]$$

which implicitly assumes that external relaxation is equal for both *A* and *B* spins. Alternatively, R_C may be determined by solving directly with the data from measurement with a single τ_m value (*13*).

Of course, the two-site submatrix approach implied by Eqs. [1]–[3] loses validity if selectivity is insufficient and fails to isolate properly the auto- and cross-relaxation terms for a single pair of nuclei. Moreover, if markedly different contributions arise throughout τ_m from external relaxation losses, additional exponential terms survive in Eq. [3]. To perform safely a single-exponential fitting, these extra terms should be independently eliminated by also determining a_{BA} and a_{BB} via QUIET-NOESY, or estimated by selective T_1 measurements. Although such corrections are feasible only for individually resolved spin pairs, the reliability of the implied two-site treatment can also be readily assessed from the experimental time course of a single target-to-source ratio, by recasting Eq. [3] into a linear form. Even when a two-site submatrix approximation can be reasonably used, however, the application of Eq. [3] for quantitative analysis is hampered by relaxation effects occurring during the selective pulses. These effects encompass both transverse and longitudinal losses.

Transverse relaxation introduces damping factors at each pulse which, in rough terms, recast the magnetization conditions at the start of each interpulse evolution interval as well as during the pulses themselves. Hence, the mixing coefficients in Eqs. [1] and [2] should be written as a product of contributions over the mixing-time subintervals, with each contribution being taken with the proper damping factor. The calculation of R_C would therefore become more involved because the compact form of Eq. [3] would be lost.

Longitudinal-magnetization losses, on the other hand, would introduce, if neglected, a back-shift in the time scale of the NOE buildup due to auto- and cross-relaxation also occurring during the pulses. These effects were extensively examined by Horita *et al.* (*2*), who estimated the errors affecting the determination of cross-relaxation parameters in transient 1D NOE experiments with a variety of shaped, selective inversion pulses. They showed that a two-site submatrix approximation accounts for the time lag of the NOE buildup by resetting either the starting magnetization conditions or the experimental time variable. Quantitative analysis by this approach leads to satisfactory results when relaxation effects during the shaped pulses are simultaneously minimized with respect to the actual pulse duration and to the sensitivity factor of the relaxation/excitation profile for the selected amplitude-modulation scheme (*1*).

From the viewpoint of the number and type of shaped pulses, the QUIET-NOESY scheme (Fig. 1) is inherently more complex than the transient-NOE experiment with a single selective pulse that was analyzed by Horita *et al.* (*2*). In the QUIET-NOESY experiment, the error accumulated by repeated approximations severely undermines the reliability of a simplified analysis. The issue of cross-relaxation-rate estimation from QUIET-NOESY experiments has been closely examined by Schwager and Bodenhausen (*9*). Their results demonstrate that accurate quantitation can be obtained when the relaxation processes occurring during the selective pulses are explicitly accounted for. The overall calculation yields excellent results simply from considering a 2×2 relaxation matrix. The success of the two-spin treatment reflects the performance quality of the QUIET-NOESY perturbation on the whole magnetization. However, as noted by Schwager and Bodenhausen (*9*), calculations based on the time-lag method may also give comparable results if applied under suitable conditions.

Implementation of the QUIET-NOESY Experiment for Simplified Analysis

Using the previous conclusions (*9*) as a starting point, we decided to explore the feasibility of an easier implementation of the QUIET-NOESY experiment to study biological macromolecules. To minimize the relaxation effects during the inversion pulses, only Gaussian-shaped pulses, G_{180} and G_{270}, were employed because of their favorable duration/bandwidth product, $\Delta t \Delta\Omega$, where Δt is the time length of the pulse and $\Delta\Omega$ is the corresponding excitation bandwidth (*14*). Thus, only 14.4 ms is required by a G_{180} pulse for a bandwidth of 50 Hz (*1*), which is much less than the 66 ms necessary for the same selectivity with a quaternion Gaussian cascade, Q^3_{180}, as used in previous applications of QUIET-NOESY (*4*, *9*). Similar arguments, which entail sacrificing some selectivity to improve sensitivity, also apply for the choice of the reading pulse. Therefore, Gaussian-shaped pulses with a 270° flip angle (G_{270}) and a duration of 26.4 ms were used, which provided the same excitation bandwidth as the inversion pulses (50 Hz) (*1*). With short pulses, the convenient approximation of a separate treatment and correction of longitudinal and transverse relaxation effects becomes less critical. For the longitudinal losses, the results of Horita *et al.* (*2*) suggest that, for a Gaussian inversion, the fraction of the overall pulse length to be considered for the time shift of the cross-relaxation buildup (τ_{int}) approaches the value of 0.5. This *theoretical* value could be intuitively understood to reflect an equivalent division into transverse and longitudinal components during the actual magnetization excursion, inasmuch as the only effective cross-relaxation processes occurring during a Gaussian inversion of a single spin take place in the laboratory frame. Although such an approximation breaks down if T_1 and T_2 diverge substantially, that is, when $T_1/T_2 \geq 100$, it can be reasonably expected to hold true for limited pulse lengths, even with biopolymers. Similar arguments, however, do not apply to a doubly selective Gaussian inversion. In this case, the two spins which undergo the inversion are the dipolar partners whose cross-relaxation the experiment aims at isolating. Since their corresponding magnetizations already have either opposite or parallel polarization along the *z* axis in the schemes of Fig. 1, their mutual dipolar exchange should continue during the entire double-inversion excursion. Only the laboratory-frame cross-relaxation processes should be considered, however, because the ro-

tating-frame transfers are averaged out. In fact, the tranverse cross-relaxation term (σ_t) is nonsecular under the (most common) conditions of biselective inversion of a sufficiently resolved spin pair, that is, with a chemical-shift difference much larger than $1/\sigma_t$ (*15*). On the contrary, under the same conditions, net longitudinal cross-relaxation transfers are expected which can be shown to occur at half the R_C rate of conventional transient-NOE or NOESY experiments (*16*) and can therefore be considered as taking place at full rate or half of the pulse duration (τ_p), that is, $\exp - (R_C \cdot \tau_p/2)$. Hence, only half of the doubly selective pulse length should be included in the τ_m of Eq. [3]. By following the same arguments as Horita *et al.* (*2*), the longitudinal relaxation processes occurring during the G_{270} reading pulses (Fig. 1) should be treated with the same factor as that of the selective inversion pulses, that is, 0.5. However, considering these longitudinal contributions is only necessary when measuring the source-peak amplitude with the scheme of Fig. 1c, because the corresponding contributions to the target spin are already canceled out by difference of the FIDs resulting from the schemes of Figs. 1a and 1b (*9*). In summary, by using only Gaussian-shaped pulses of short duration, the auto- and cross-relaxation contributions arising during a QUIET-NOESY experiment can be reasonably computed by adding to the experimental τ_m value the half-durations of the inversion pulses for the target-spin data and the half-durations of all pulses, that is, inversions and excitation, for the source-spin data.

In the context of the same approach, and with regard to the transverse-relaxation losses, the starting point is the realization that the relative attenuation depends on the tranverse-decay rate of the monitored spin. It has been shown that the tranverse magnetization which is recovered following a G_{270} pulse is a linear function of the initial longitudinal component (*9*), even when all relaxation processes that are occurring are precisely accounted for. Therefore, the transverse-relaxation attenuation during Gaussian pulses may be easily accounted for by means of the T_2 value of the monitored spin, which can be estimated independently, and the limiting τ parameter given by Hajduk *et al.* (*1*). The latter is defined as the fraction of the pulse duration during which the magnetization has to relax to yield the experimental amplitude, assuming that the selective pulse were instantaneous. According to Hajduk *et al.* (*1*), $\tau = 0.490$ and 0.242 for G_{270} and G_{180} pulses, respectively. The extent of transverse relaxation occurring during the pulses in the schemes of Fig. 1 is exactly the same up to the start of acquisition, except in scheme of Fig. 1b, where the damping factor which affects the initial magnetization of the source spin is missing altogether. Hence, the evaluation of a proper difference between the experiments of Figs. 1a and 1b should be performed after correcting the corresponding signal amplitudes accordingly. Since for both schemes the same transverse attenuation is expected from the doubly selective inversion and reading pulses, a difference can be performed after scaling down the result from the experiment of Fig. 1b by the attenuation factor AF that would be observed if the first inversion pulse were applied on-resonance:

$$\mathrm{AF} = \exp - \left(\frac{\text{pulse duration} \cdot \tau}{\text{source spin } T_2} \right). \qquad [4]$$

The resulting difference should represent the extent of pulse-damped cross-relaxation that has occurred throughout the experiment up to the start of acquisition. The overall damping effect from transverse relaxation during pulses, however, may be considered to cancel out upon calculating the ratio of Eq. [3], much as if all the transverse-attenuation coefficients that should update the initial-magnetization condition at each pulse elementary step (*9*) were collected into a single, effective damping factor. The further scaling of the target and source-spin data that would be required in the ratio of Eq. [3] to compensate for the different transverse-damping factors which are introduced by the final reading pulses (applied to either target and source spin in the schemes of Fig. 1) may be avoided if those factors may be considered only negligibly different for the two nuclei. Under conditions of similar transverse-decay rates and limited reading-pulse lengths, the latter approximation should be quite safe.

Pulse-Scheme Symmetry

The presence of auto- and cross-relaxation contributions during the selective pulses, no matter whether they are properly accounted for, entails a loss of symmetry of the ideal QUIET-NOESY scheme of Fig. 1. The doubly selective inversion pulse, in fact, is no longer located at the center of the mixing time if the initial inversion and the final reading Gaussian pulses have different time lengths, as is required to keep the excitation bandwidth constant throughout the experiment. Moreover, the pulse sequence schemes for source and target-spin observation are not equivalent, as far as the total mixing time is concerned, because the extent of auto- and cross-relaxation that takes place during the reading pulse is compensated for when the target-spin data are processed, whereas it has to be explicitly considered with the source-spin data. The former asymmetry may be simply handled by introducing compensation intervals following or prior to the doubly selective inversion pulse, according to Fig. 2. As a result, the double inversion is placed exactly midway with respect to the actual NOE buildup. Removal of the latter asymmetry, on the other hand, may be achieved by decreasing the $\tau_m/2$ intervals by $(\tau_{270} - \tau_{180})/2$ in the measurement of the source-spin decay (Fig. 2c), where τ_{270} and τ_{180} are the time lengths of the initial inversion and final detection pulses, respectively. This modification is necessary to avoid fitting the target-to-source ratios (Eq. [3]) with different values of the total mixing times, which would leave a double exponential in the denominator of Eq. [3].

Application of QUIET-NOESY to Aggregates of a Small Heat-Shock Protein

Small heat-shock proteins (Hsps) are a group of ubiquitous proteins that are expressed in large quantities in response to cellular stress (e.g., heat, denaturants, various disease states). Mouse Hsp25 is a protein of 205 amino acids that aggregates into stable oligomers with an average mass of around 800 kDa. In this form Hsp25 exhibits chaperone ability, whereby it is capable of complexing and stabilizing proteins that are partially unfolded and about to precipitate out of solution (reviewed in Ref. *11*).

Hsp25 is not amenable to detailed high-resolution NMR analysis because its extensive aggregation leads to prohibitively slow rotational diffusion in solution (estimated overall tumbling time $\cong$ 0.2 μs). ^{1}H NMR spectra of the Hsp25 aggregate (Fig. 3) revealed, however, the presence of a flexible extension which, through conventional two-dimensional NMR analysis, was shown to arise from the last 18 amino acids of the protein (Arg188 to Lys205) that adopted little preferred conformation (*17*). Using the NOESY cross-peak ratio of the two α–β connectivities of Pro194 as a probe, an evaluation of the flexibility of the entire extension was also attempted (*17*). Within the framework of the isolated spin-pair approximation and linear buildup assumption, a value of 0.71 ns was obtained for the correlation time (τ_c) of the selected internuclear-vector pair, which is close to the τ_c value of 0.46 ns expected for the overall tumbling of an isolated 18-residue peptide under the same conditions. Since the ready detectability and absence of chemical-shift dispersion of resonances in the C-terminal extension of Hsp25 imply substantial flexibility for this region, the experimentally determined value of τ_c appears a reasonable estimate. However, this type of determination, and hence functional implications concerning the role of the C-terminal extension in the chaperone action of Hsp25, might be very much in error if spin diffusion were actively operating. For instance, it is not inconceivable that only the latter part of the C-terminal octadecapeptide is genuinely flexible, with mobility decreasing toward Arg188, which forms the pivotal and anchoring point to the relatively rigid domain core. Another possibility could be the occurrence of a fast exchange between two limiting conformers where the C-terminal fragment either freely protrudes in solution or sticks to the bulk protein. In any such circumstances the observed NOEs would result from spin diffusion leveling out NOE effects among the individual populations. The QUIET-NOESY experiments presented here imply that this is not the case.

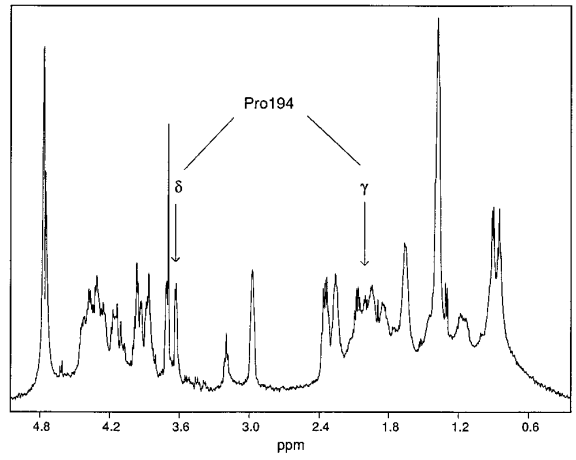
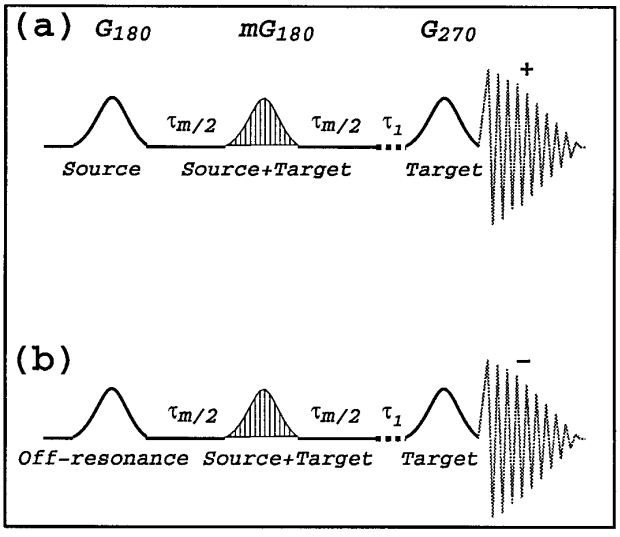
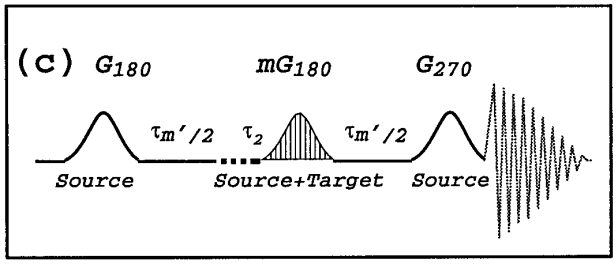


FIG. 3. Aliphatic region of the ^{1}H NMR spectrum of Hsp25 (*10*) in D_2O at 500 MHz and 298 K. The protein aggregates into stable oligomers with an average mass of 800 kDa (*10*) and only resonances from the C-terminal 18-residue fragment are observed (*17*). The resonance pair of Pro194 that was monitored in the QUIET-NOESY experiments is indicated.



$\tau_1 = \tau_{180}/2$

Total Mixing-time = $\tau_m + \tau_{180} + \tau_1$



$\tau_2 = (\tau_{270} - \tau_{180})/2$

Total Mixing-time = $\tau_m' + \tau_{180} + \tau_2 + \tau_{270}/2$

FIG. 2. Pulse-sequence schemes for compensated QUIET-NOESY. (a) On-resonance sequence and (b) off-resonance sequence for the measurement of the target-spin NOE. (c) Sequence for the measurement of the source-spin amplitude (see the caption to Fig. 1 for symbol meanings and processing procedure). The doubly selective inversion pulse is located exactly at the center of the mixing time by introducing the compensation delay $\tau_1 = \tau_{180}/2$ in sequences (a) and (b) and $\tau_2 = (\tau_{270} - \tau_{180})/2$ in sequence (c), where $\tau_{270} > \tau_{180}$ is assumed. The overall mixing-time duration of experiment (c) is made equal to that of experiments (a) and (b) if [$\tau_m'/2 = (\tau m/2 - \tau 2)$ (see text)].

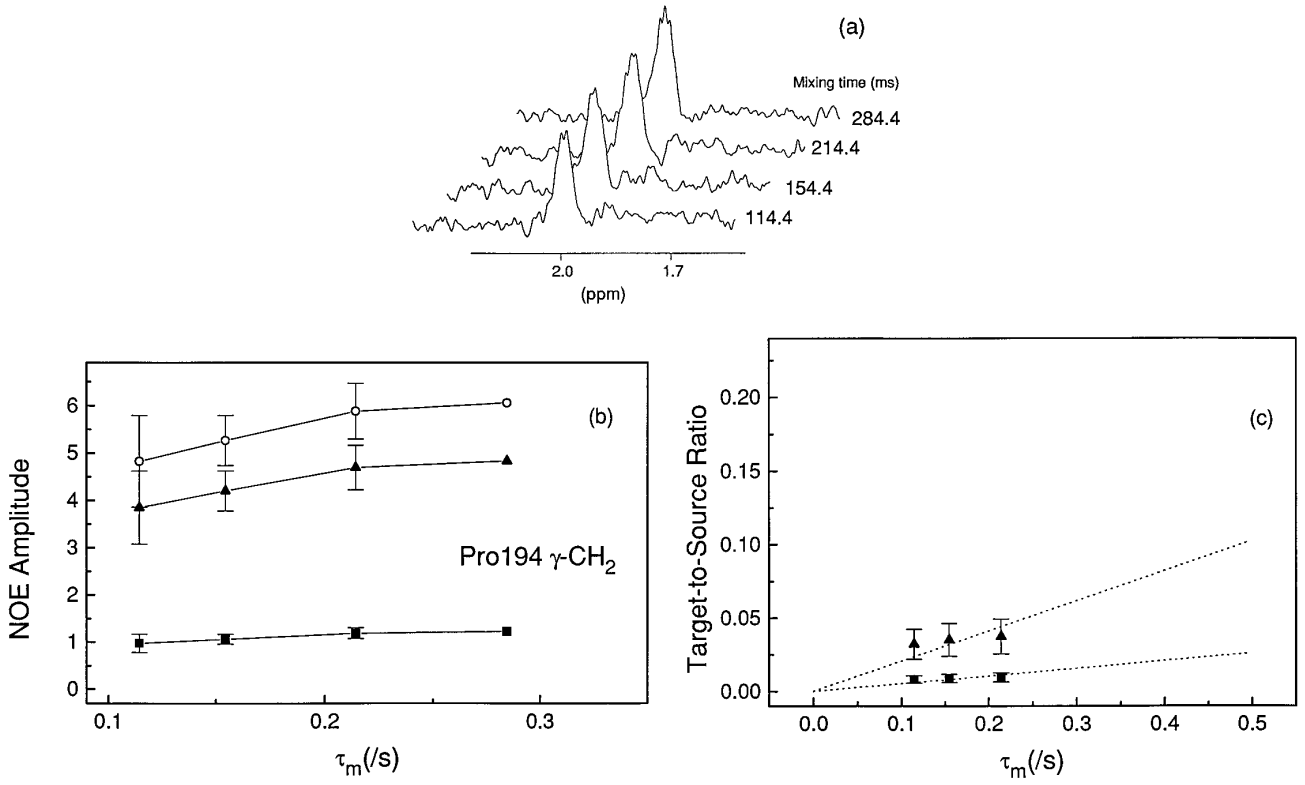


FIG. 4. (a) Target-spin (Pro194 γCH_2) NOE-difference spectra obtained from QUIET-NOESY experiments on Hsp25, according to the scheme of Fig. 1. The reported traces resulted from difference of 512 on-resonance and 512 off-resonance transients, collected in the interleaved mode (see Experimental section). The integrity of the sample was checked following each series of measurements. The data point from the fourth series of experiments was not used for subsequent analysis because the sample appeared degraded by the end of the corresponding measurements (no error bar is, in fact, reported for the relative point in panel (b)). (b) Target-spin QUIET-NOESY amplitudes (arbitrary units) from Hsp25, as a function of total mixing time. The values were calculated after correction of the off-resonance data (Fig. 1b, see text). The overall NOE (empty circles) is split into the two component contributions from the vicinal dipolar interactions of Pro194 δ–γ hydrogen pairs (see text), corresponding to distances of 0.230 nm (solid triangles) and 0.289 nm (solid squares). (c) Target-to-source ratios (Eq. [3]) as a function of total mixing time from QUIET-NOESY measurements on Hsp25. The dotted lines show the fitting curves, whose parameters are listed in Table 1. The symbols correspond to the monitored dipolar interactions of Pro194, as reported in panel (b).

The resonances of the αCH-βCH_2 spin system of Pro194, which had been previously employed to estimate the local τ_c value through 2D NOESY spectra (*17*), are not amenable to safe 1D determinations by QUIET-NOESY since none of these resonances are resolved ($\delta_{H\alpha}$ = 4.39 ppm; $\delta_{H\beta}$ = 1.93 and 2.26 ppm; Fig. 3). In general, the results of a two-site submatrix treatment of QUIET-NOESY data may be adversely affected if a dipolar partner of the selected pair is inadvertedly perturbed by the inversion and/or detection pulses. Although no such problem should be encountered with the α–β hydrogen pairs of Pro194, the lack of resolution prevents any selective observation of the source-spin resonance. However, because of the unique rigidity of its five-membered ring, any of the proline hydrogen pairs may be chosen to extract information on the local motional regime of the peptide backbone. Therefore, we took advantage of the resolved and fairly isolated δCH_2 resonance of Pro194 (Fig. 3) to monitor the δCH_2–γCH_2 dipolar interaction. Each of these two diastereotopic proton pairs exhibits substantially degenerate resonances at 3.62 ppm (δCH_2) and 2.00 ppm (γCH_2), which should give rise to a degenerate pair of vicinal dipolar interactions, corresponding to internuclear separations of 0.230 and 0.289 nm, assuming an average, unpuckered geometry of the proline ring (*18*).

By using the (resolved) δCH_2 resonance as source spin and the (unresolved) γCH_2 resonance as target spin, QUIET-NOESY experiments were performed according to the procedure of Fig. 1, under the conditions described in the Experimental section. Typical difference spectra, obtained with the schemes of Fig. 1a and 1b, are reported in Fig. 4a, where the quality of selective-spectroscopy data from the large-molecular-weight aggregate of Hsp25 can be appreciated. The corresponding NOE-buildup curve is given in Fig. 4b, where the overall NOE time course is also split into the two component contributions, as calculated from the inverse-sixth-power ratio of the intervening nuclear separations. This linear assumption is consistent within the context of our QUIET-NOESY analysis if one considers that (i) the effects from spin diffusion should be expectedly reduced, if not removed, by the experimental protocol, and (ii) the strongest cross-relaxation transfers occur to the same extent within each of the unresolved γ and δ pairs of Pro194 and, therefore, do not affect the amplitude of the resolved NOE transfers. Only the points from the first three

TABLE 1
Cross-Relaxation Rate Constants, R_C ($R_C = 2\sigma$) Determined from QUIET-NOESY Data, and Correlation-Time Values, τ_c, Calculated from the Corresponding R_C Parameters after Imposing the Reported Internuclear Separations

Experiment	Source spin	Target spin	R_C (s^{-1})	τ_c (ns)	Distance (nm)
		Hsp25 Pro194			
Uncompensated	proS-δ/proR-δ	proS-γ/proR-γ	-0.41 ± 0.08	0.73 ± 0.08	0.230
	proS-δ/proR-δ	proR-γ/proS-γ	-0.10 ± 0.02	0.73 ± 0.08	0.289
		Gramicidin S			
Uncompensated	Leu αCH	DPhe NH	$-1.20_5 \pm 0.04_6$[a]	1.05 ± 0.03	0.210
Compensated	Leu αCH	DPhe NH	$-1.25_1 \pm 0.04_4$[a]	1.08 ± 0.03	0.210

[a] Final dropped digits were kept only for comparative purposes and should not be considered significant figures.

series of measurements could be used for the subsequent analysis. The sample appeared in fact degraded by the end of the fourth series of measurements, as inferred from the observation of additional resonances that were ascribed to partial unfolding of the aggregate subunits. No such feature had been previously detected on inspection of the control spectra that were regularly collected at the end of each series of measurements. Figure 4c shows the fitting of the target-to-source ratios (Eq. [3]) from the reliable data points. From *S/N* ratio evaluations, the experimental uncertainties in the intensity values were estimated to range between 10 and 20% and to propagate on the calculated intensity differences and ratios, as well as on the fitting parameters, to variable extents, with an average value of approximately 15%. The target-to-source ratios were calculated after accounting for the attenuation factor of Eq. [4], upon evaluation of the target-spin difference intensities, with source-spin $T_2 = 95.4$ ms. The latter was quickly estimated by means of a spin-echo (CPMG) experiment. Since the measurements were performed using the schemes of Fig. 1, no compensation delay was introduced to correct for the asymmetry of the mixing-time intervals flanking the doubly selective inversion pulse. The half-durations of the singly and doubly selective inversions were included, however, in the overall mixing-time variable that was considered for the fitting, whereas the contributions arising during the selective reading pulse to the source-spin intensity were neglected. The relevant results are listed in Table 1. Although the overall motional freedom of the C-terminal fragment of Hsp25 is limited by the large aggregate to which it is attached, the conformational space that is accessible to Pro194 should be quite extended due to the independent torsions of at least 6 (ϕ, ψ) pairs which separate this residue from Arg188 (the first mobile, fully detectable residue of Hsp25 C-terminal fragment). This torsional independency and the wide motional span thereof are consistent with the disordered conformation that was observed in the fragment (*17*) and do not conflict with the possible occurrence of either a mobility gradient along the same fragment or a conformational exchange between a free, disordered state and a more compact arrangement relative to the protein aggregate. By assuming, therefore, isotropic motion, a value of 0.73 ± 0.08 ns is extracted from the fitted cross-relaxation values of the Pro194 δ–γ proton pairs of Hsp25, which is in agreement, within the experimental error, with the value of 0.71 ns obtained from our previous 2D NOESY determinations for the α–β proton pairs of the same residue (*17*). Such an agreement rules out any possibility of large differences in external relaxation of the selected pair of spins because the intervening dipolar exchanges with the bulk magnetization reservoir should have differred appreciably under selective and nonselective experimental protocols. Since spin diffusion, if active, should have been substantially quenched in the QUIET-NOESY experiment, the close agreement of the present result with that obtained from conventional NOESY confirms that the C-terminal extension of Hsp25 is genuinely flexible and supports the conclusions thereof.

QUIET-NOESY Control Determinations with Gramicidin S

The limited stability of the Hsp25 sample did not allow us to collect more points, or to perform the experiments according to the compensated protocol of Fig. 2. To assess the actual improvements introduced by the latter protocol and, ultimately, to test further the quantitative reliability of the result obtained with Hsp25, QUIET-NOESY experiments were repeated with the cyclic decapeptide gramicidin S (*19*). This molecule, in fact, is expected to tumble with a similar rate as the C-terminal extension of Hsp25. In gramicidin S we focused on the sequential-NOE interaction between Leu αCH and DPhe NH. The distance between these two hydrogens is 0.219 nm, according to the coordinates that were reported by Dygert *et al.* (*20*) for the calculated model. However, as previously noticed (*21, 22*), Dygert's model is slightly different from the crystal structure reported by Hull *et al.* (*19*). Several 1D and 2D NOE determinations on gramicidin S, performed in DMSO-d_6 under a variety of temperature and concentration conditions, have been reported (*13, 23–26*). These data suggest that, in solution, the actual Leu αCH–DPhe NH distance may be smaller than the corresponding distance in the model. We imposed therefore a separation of 0.210 nm to extract the τ_c value for the (isotropic) motion of the intervening internuclear vector from the

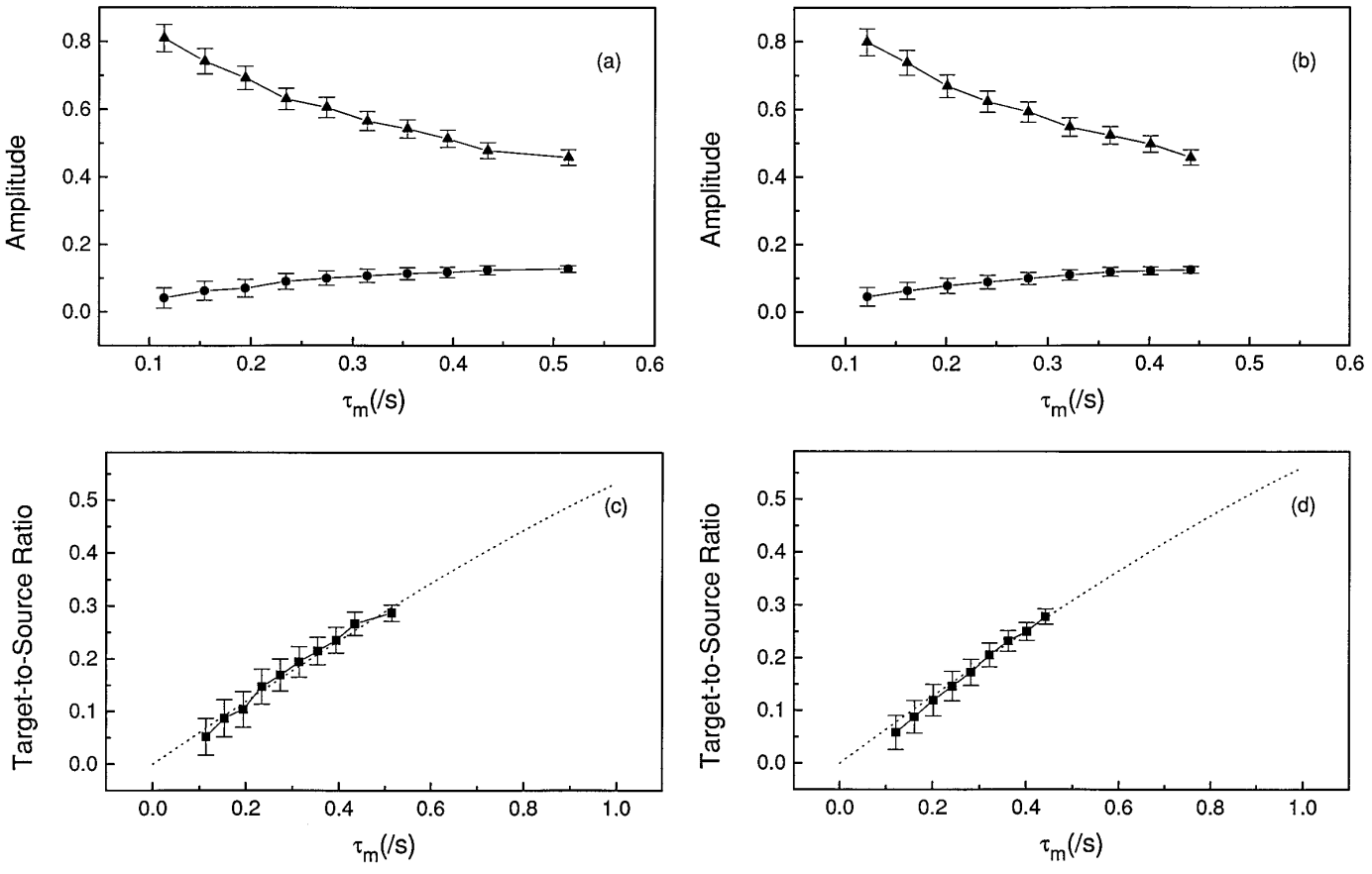


FIG. 5. Source-spin (triangles) and target-spin (circles) amplitudes (arbitrary units) as a function of total mixing time from (a) uncompensated and (b) compensated QUIET-NOESY experiments on gramicidin S. The sequential NOE between Leu αCH (source spin) and DPhe NH (target spin) was selectively observed. The corresponding target-to-source ratios (Eq. [3]) from data in (a) and (b) are shown in panels (c) and (d), respectively, as a function of total mixing time. The dotted lines show the fitting curves whose parameters are listed in Table 1.

cross-relaxation rate estimated by QUIET-NOESY measurements. The experiments were first executed with the uncompensated mode of Fig. 1 and then with the compensated mode of Fig. 2, with the same pulse lengths and selectivity as employed with Hsp25. The raw target-spin (DPhe NH) data were corrected for the source-spin (Leu αCH) attenuation factor (Eq. [4]), prior to difference evaluation. An estimate of the T_2 for the Leu αCH was obtained from the ratio of the steady-state signal amplitudes that were measured by nonselective 90° and selective G_{270} pulses, according to

$$A_S = A_{NS} \cdot \exp\left[-\tau_{270} \cdot \frac{1}{2} \left(T_{1SS}^{-1} + T_2^{-1}\right)\right] \qquad [5]$$

where A_S and A_{NS} are the recovered amplitudes following selective and nonselective pulses, respectively, τ_{270} is the duration of the G_{270} pulse, and T_{1SS}^{-1} is the selective longitudinal relaxation rate. With a specific measurement, the last parameter was estimated to be 0.56 ± 0.01 s, by single-exponential fitting of the initial-recovery data. From Eq. [5], a T_2 value of 60 ms was obtained for Leu αCH, which through Eq. [4] enabled computation of the AF values for the G_{180} and G_{270} pulses (0.944 and 0.806, respectively). Figures 5a and 5b show the plots of target and source-spin peak amplitudes as a function of the total mixing time, which were retrieved from the uncompensated and compensated schemes, respectively. The corresponding profiles for the target-to-source ratios are respectively depicted in Figs. 5c and 5d, where the fitting curves are also shown. The relative fitting parameters are given in Table 1. Within the experimental error, no major difference is observed between the resulting cross-relaxation rate parameters, that is, $-0.60_3 \pm 0.02_3$ s^{-1} from the uncompensated experiments and $-0.62_5 \pm 0.02_2$ s^{-1} from the compensated ones. As expected, a substantial identity is also obtained for the τ_c values that were calculated from the cross-relaxation rate constants of Table 1, after imposing the specific distance. The most precise determinations, however, should come from the compensated experiments. These results definitely demonstrate that, for the motional regime of the selected internuclear vector of gramicidin S, only minor deviations are introduced by the mixing-time asymmetry of the

TABLE 2

Nonselective (T_{1NS}), Singly Selective (T_{1SS}), and Doubly Selective (T_{1DS}) Longitudinal Relaxation Times of Gramicidin S DPhe NH, with Cross-Relaxation Rate σ and Correlation-Time τ_c Determined for the Dipolar Interaction with Leu αCH

T_{1NS} (s)[a]	T_{1SS} (s)[b]	T_{1DS} (s)[b]	σ (s^{-1})[c]	τ_c (ns)[d]
0.96 ± 0.01 (1.04 ± 0.04)	0.420 ± 0.006	0.58 ± 0.01	$-0.64_1 \pm 0.07_6$	$0.99_5 \pm 0.03_2$

[a] Estimated from fitting of all recovered points. The value obtained from single-exponential fitting of the initial-50-ms data is given in parentheses.
[b] Estimated from single-exponential fitting of the initial-50-ms recovery.
[c] Calculated from ($T_{1DS}^{-1} - T_{1SS}^{-1}$). Final dropped digits were kept only for comparative purposes and should not be considered significant figures.
[d] Calculated from the ratio T_{1NS}/T_{1SS} using the initial points (up to 50 ms) of the recovery curves. Final dropped digits were kept only for comparative purposes and should not be considered significant figures.

QUIET-NOESY schemes of Fig. 1, under the chosen experimental conditions. Such an asymmetry, however, may lead to nonnegligible errors if short mixing times are employed, even when spin diffusion is virtually absent as in our experimental conditions (up to 30–40% of the determined cross-relaxation rate for a duration of the first pulse comparable to the evolution half-period and some 10–20% uncertainty on the experimental target-to-source ratio). For the addressed cases, the limited difference between compensated and uncompensated experiments that is observed with gramicidin S should also apply to the results obtained with Hsp25. This adds quantitative confidence to the conclusion reached for the mobility of the C-terminal extension in the Hsp25 aggregate.

The reliability of the σ and τ_c figures calculated from QUIET-NOESY data on gramicidin S can be tested by comparison with the corresponding estimates from the longitudinal relaxation rates of DPhe NH. The latter parameters were estimated in independent determinations of singly selective (T_{1SS}^{-1}), doubly selective (T_{1DS}^{-1}), and nonselective (T_{1NS}^{-1}) longitudinal relaxation rates, in the limit of initial-rate approximation (*27*). Cross-relaxation rates may be evaluated from the difference of doubly and singly selective longitudinal relaxation-rates (*28*). Correlation times may be more conveniently calculated from T_{1NS}/T_{1SS} rather than T_{1DS}/T_{1SS} ratios, when outside the extreme-narrowing limits. Both determinations are independent of imposed internuclear distances. As a matter of fact, the single-exponential fitting of the data points collected within 50 ms from the end of the inversion pulses gave a σ value of $-0.64_1 \pm 0.07_6$ s^{-1} and a τ_c of $0.99_5 \pm 0.03_2$ ns (Table 2), values which are close, within experimental error, to the QUIET-NOESY results (Table 1).

CONCLUSIONS

We have shown that a simplified approach can be successfully employed for the implementation and analysis of QUIET-NOESY experiments. By choosing appropriate pulse shapes and lengths to minimize relaxation losses, and a suitable pair of dipolar-coupled nuclei to avoid perturbing other dipolar partners, a very simple experimental protocol and data-analysis procedure can be applied. Provided the selectivity is sufficient for isolating the spin pair of interest, the approximations which are introduced for a two-site analysis are safe and lead to deviations below the experimental uncertainties, in the absence of appreciable differences in external relaxation within the considered nuclear pair. Errors arising from the latter source can be corrected for when both the source and target spin resonances are resolved, without losing the convenience of a single-exponential fitting. Otherwise, a more extended treatment including all terms of the involved dipolar subset is necessary. According to the proposed implementation, in addition to the QUIET-NOESY series of measurements, an estimate of the transverse-relaxation losses during the selective pulses is required to correct for the amplitude attenuations of the first inversion and, possibly, the final reading steps. The original QUIET-NOESY protocol (*4*) can be easily modified to compensate for the mixing-time asymmetry about the doubly selective inversion (Figs. 1 and 2), although minor differences in results are expected from the compensated and uncompensated schemes when short selective pulses are used, compared to the overall mixing period, and spin diffusion is not extremely severe. With a further modification, nonselective reading pulses might also be introduced which would bear all the advantages of removing the relaxation contributions associated with a selective detection pulse, and the need to set different compensation intervals in the target and source-spin observation sequences (Fig. 2). Unfortunately, using nonselective pulses may introduce difference artifacts or even undermine the feasibility of the experiments if an intense solvent peak were present in the spectrum.

EXPERIMENTAL

Recombinant mouse Hsp25 was prepared as described by Engel *et al.* (*10*). A solution of Hsp25 in D_2O (CIL), 0.5 mM on a subunit basis, with 20 mM phosphate and 0.02% NaN_3, pH* (uncorrected meter reading) 7, was used for the measure-

ments. Control experiments were performed on a 30-mM solution of the cyclic, symmetric decapeptide cyclo(Pro-Val-Orn-Leu-DPhe)$_2$, gramicidin S (Sigma), in deuterated dimethylsulfoxide (DMSO-d_6, CIL). All ^{1}H NMR spectra were acquired at 500 MHz and 298 K with a Bruker AM500 spectrometer equipped with a selective-excitation unit. Gaussian-shaped selective pulses, with flip angles of 180° and 270° (*29*) and corresponding durations of 14.4 and 26.4 ms, were employed for inversion and excitation purposes, respectively. Spectra were typically acquired over 256 points with a sweep width of 500 Hz and 64 to 512 transients. A long relaxation delay of 5–10 s was always allowed to restore the equilibrium magnetization. Difference spectra for the measurement of the cross-relaxation term were obtained, following appropriate corrections (see earlier discussion), through subtraction of on-resonance from off-resonance spectra, which had been both collected in an interleaved mode with eight scans/loop. Doubly selective inversion of a pair of resonances was achieved by superposition of a cosinusoidal audio-frequency modulation onto the Gaussian-modulated amplitude of an inversion pulse applied halfway between the selected pair of resonances (*4*). Nonselective (NS), singly selective (SS), and doubly selective (DS) longitudinal-relaxation-rate measurements were also performed on gramicidin S, using a conventional inversion–recovery sequence, where, whenever needed, the initial inversion pulse was replaced with a singly or doubly selective Gaussian inversion pulse of 14.4 ms. Selective relaxation rates were obtained by exponential fitting of the data points collected within 50 ms from inversion. For nonselective relaxation rates, the fitting was extended to the entire data set. Fitting of the inversion-recovery and QUIET-NOESY data was performed by using a standard PC software-package (Axum 5.0, Mathsoft, Cambridge, MA).

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