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Separation of intramolecular NOE and exchange peaks in water exchange spectroscopy using spin-echo filters

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Summary

A technique for separating intramolecular NOE and solvent-proton exchange peaks in exchange spectroscopy is demonstrated. This method utilizes the large differences in relaxation and coupling properties of water and macromolecules to separate the two effects. The spin-echo filter consists of a water-frequency selective 90° pulse followed by a spin-echo sequence. If the echo time is sufficiently long, protein resonances (e.g. $C^{\alpha}H$ protons) excited by the selective pulse are removed due to their much shorter T_2 values and J-coupling evolution. By combining the filter with exchange spectroscopy (EXSY) or water exchange (WEX) filter experiments, exchange peaks can be selectively observed. In this paper the filter is combined with a modified version of the WEX filter (WEX II filter) with 1D and 2D detection and applied to a zinc finger peptide and to staphylococcal nuclease, allowing estimation of the contribution of intramolecular NOEs to the exchange spectra.

The study of exchangeable protons by NMR can provide important insight into conformational and dynamic properties of macromolecules. When exchange is slow on the NMR time scale, deuterium substitution studies can be successfully applied (Englander and Kallenbach, 1984). For faster exchange, 1D and 2D proton magnetization transfer methods are necessary. Among those methods are 1D approaches, such as saturation transfer and inversion transfer experiments (Forsen and Hoffman, 1963; Redfield et al., 1975; Schwartz and Cutnell, 1983), two-dimensional exchange spectroscopy (EXSY) (Jeener et al., 1979) and, more recently, water exchange (WEX) filter sequences (Mori et al., 1994,1996). The inversion transfer method has also been applied to multidimensional spectroscopy (Gemmecker et al., 1993; Grzesiek and Bax, 1993b; Kriwacki et al., 1993).

In many of the above methods, water magnetization is perturbed and subsequent effects on exchanging sites are observed. However, because water magnetization is encoded based on its chemical shift either by chemical shift selective pulses or a chemical shift encoding period, exchange processes from water to the macromolecule cannot be distinguished from NOEs of nuclei that are in the same chemical shift range as water, e.g. $C^{\alpha}H$ protons. In order to separate these processes, several methods have been proposed. Among those are observing pH dependency (Spera et al., 1991) and comparing NOESY and ROESY spectra (Otting and Wüthrich, 1989). The latter approach provides potent ways to distinguish various sources of solvent–macromolecule interactions depending on the correlation time (Otting and Wüthrich, 1989; Clore et al., 1990; Otting et al., 1991a,b; Grzesiek and Bax, 1993a,b). However, information about the correlation time has to be available to quantify the contribution of intramolecular NOEs to exchange peaks. Although it is often possible to assume the slow correlation time limit for large molecules ($ROE = -2 \times NOE$) (Grzesiek and Bax, 1993b), this assumption may not hold for small peptides or at higher temperatures. Another effective separation method is purging of ^{13}C-bound protons before mixing (Gemmecker et al., 1993; Grzesiek and Bax, 1993b). However, this method requires isotropic ^{13}C labeling. In this communication, we demonstrate that the contribution of intramolecular NOEs to exchange intensities can be

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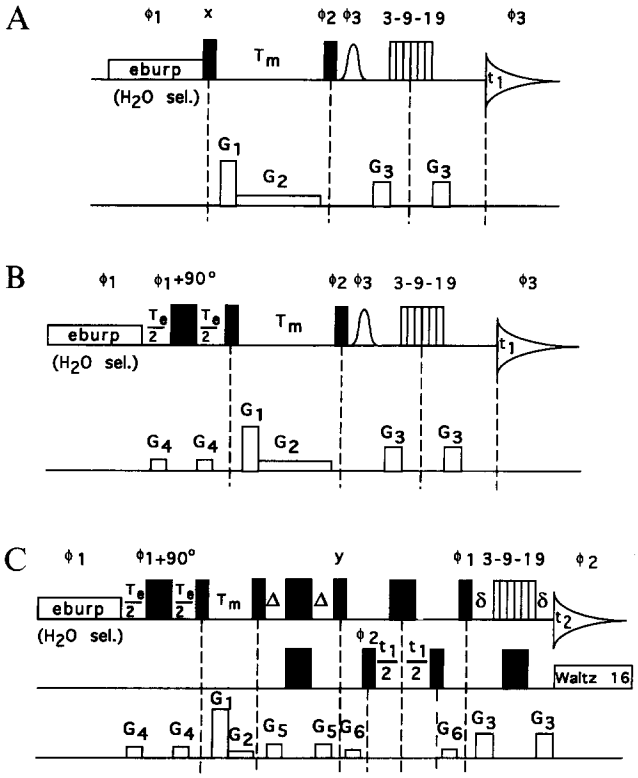

Fig. 1. Pulse sequences for the experiments described. Wide and narrow squares are 180° and 90° hard pulses, respectively. Phases of rf pulses are along x, unless otherwise specified. (A) WEX II filter combined with WATERGATE detection. The first 90° pulse (eburp, 16 ms) is selective to water. The second and third 90° pulses are nonselective and define the mixing time (T_m). Non-zero order magnetization during T_m is removed by a gradient pulse G_1 (1 ms×9 G/cm). The rest of T_m is filled with a weak gradient (G_2: 0.1 G/cm) to prevent radiation damping. The two different gradient pulses (G_1 and G_2) are required during T_m because G_2 is not sufficiently strong to effectively remove non-zero order magnetization at short T_m. The fourth rf pulse is a water-selective flip-back pulse (Gaussian function, 4 ms). The WATERGATE detection consists of a 3-9-19 pulse (Sklenář et al., 1993) and a pair of gradients G_3 (1 ms×11 G/cm). Phases ϕ_1, ϕ_2 and ϕ_3 are {x,–x,x,–x}, {x,x,–x,–x} and {x,–x,–x,x}, respectively. (B) 1D WEX II filter with spin-echo filter. G_4 is 1 ms×1.5 G/cm and is applied at the beginning and the end of T_e to avoid radiation damping. Other parameters are identical to those of Fig. 1A. (C) WEX II-FHSQC (^{15}N) with the spin-echo filter. Phases of ϕ_1 and ϕ_2 are {x,x,–x,–x} and {x,–x,x,–x}, respectively. G_5 is 1 ms×2.5 G/cm. A small gradient G_6 (0.5–1 ms×1–3 G/cm) is recommended to remove radiation damping during t_1. The periods δ and Δ are tuned to $1/4J_{NH}$. Other parameters are identical to those in Fig. 1A.

effectively reduced by inserting a spin-echo sequence prior to the mixing time. In this so-called spin-echo filter, protein magnetization is selectively eliminated because of its short T_2 and J-coupling evolution. This technique can be applied to existing 1D and multidimensional water exchange spectroscopy methods such as EXSY or WEX filters. As a demonstration, the spin-echo filter is combined with 1D and 2D versions of the recently designed WEX II filter (Fig. 1A (Mori et al., 1996)). At normal predelays, the WEX II filter displays at least a factor of two to three improvement in sensitivity compared to the original WEX filter, because water magnetization is not destroyed during the sequence and gradient coherence selection has been replaced by a two-step phase cycle. Despite the removal of gradient selection for the exchanging protons, clean coherence selection is retained because of an improved inversion pulse (a combined soft 90°/hard 90° pulse) in which cancellation errors by phase cycling are minimized by dephasing the protein magnetization during mixing. Contrary to conventional selective inversion methods, this improved inversion pulse also achieves excellent definition of the mixing time. As a first demonstration of a spin-echo-filtered exchange experiment, the amide proton exchange and $C^\alpha H$ NOEs in a small peptide (consensus zinc finger peptide CP-1) (Krizek et al., 1991) and a protein (staphylococcal nuclease) are separated.

Figure 1A shows the 1D WEX II filter sequence combined with WATERGATE detection, by which exchange can be measured with EXSY resolution in a 1D experiment (Mori et al., 1996). Briefly, water magnetization is selectively excited by the first excitation pulse and flipped to the longitudinal axis at the start of the mixing time T_m, where magnetization transfer from water to exchangeable peaks takes place. During T_m, a large gradient crusher removes higher order coherences, while a series of small gradients avoids radiation damping at longer T_m values (Wider et al., 1994). After T_m, water magnetization is selectively flipped back to the z-axis to avoid interscan water saturation (Grzesiek and Bax, 1993a). The residual water magnetization in the transverse plane is suppressed by the WATERGATE sequence (Piotto et al., 1992; Sklenář et al., 1993).

One problem with the WEX II filter sequence, as well as with most of the other conventional exchange techniques, is that the first water-selective pulse excites not only water magnetization but also other resonances close to the water frequency, typically $C^\alpha H$ protons in protein studies. The subsequent NOE from $C^\alpha H$ to the exchanging sites may contaminate the water–protein exchange spectrum. However, we will show that the shorter T_2 of $C^\alpha H$ compared with water and the $C^\alpha H$ J-coupling evolution can be used to remove $C^\alpha H$ contributions prior to the mixing time. Figure 1B shows the WEX II filter combined with a spin-echo filter. During T_e, the excited protons originate either from water or from $C^\alpha H$ protein protons at the water frequency. For long T_e, the intramolecular NOE is selectively suppressed. One important precaution in spin-echo-filtered WEX experiments is that a pair of gradients (G_4) has to be applied at the beginning and the end of the T_e period to prevent water magnetization loss by radiation damping. The strength of G_4 has to be minimum (not more than 2–5 G/cm×1 ms) to avoid diffusion signal losses. The extension of the WEX II method to two dimensions is simply attained by replacing WATERGATE detection with any 2D sequence, for instance the HSQC method (Fig. 1C). For the HSQC part

of the sequence, the FHSQC (Mori et al., 1995) approach is used to avoid water saturation.

The 1D version of the spin-echo-filtered WEX II filter was tested on an 8 mM solution of a zinc finger consensus peptide (CP-1, MW: 3.0 kDa) at pH 7.0 and 22 °C (Krizek et al., 1991). Figure 2 shows spectra recorded by (A) a simple WATERGATE sequence, and (B) the WEX II filter at $T_m = 200$ ms. Peaks observed in Fig. 2B are either from H_2O-NH exchange or $C^{\alpha}H$-NH NOEs and the peak intensities are proportional to the sum of the two effects. Figure 2C shows the results of adding a spin-echo filter with $T_e = 40$ ms. It can be seen that the Cys^4 NH peak (indicated by a solid arrow) disappears, while all other peaks are retained. This result shows that the Cys^4 NH peak originates from an NOE and not from exchange, and is consistent with the fact that the $C^{\alpha}H$ of Cys^4 has the same chemical shift as the water resonance. The result can be further confirmed using a diffusion filter (Van Zijl and Moonen, 1990; Kriwacki et al., 1993). In this experiment, strong gradients (30 G/cm × 5.5 ms) are used for G_4 to selectively attenuate the water magnetization, while keeping T_e short. The result displayed in Fig. 2D shows that the Cys^4 NH resonance is much less attenuated by the diffusion weighting than other signals, suggesting it originates from protein magnetization. As a second example, we applied a combined 2D WEX II/spin-echo filter to a 1.5 mM solution of ^{15}N-enriched staphylococcal nuclease (MW: 16.8 kDa) at pH 6.15 and 39.5 °C. Figure 3 shows some slices of 2D spectra recorded by the (A) FHSQC, (B) WEX II-FHSQC and (C) spin-echo-filtered WEX II-FHSQC methods. Cross peaks shown in Fig. 3B have contributions of both exchange peaks and intramolecular NOEs, and the changes in relative intensity ratio on going from Fig. 3A to Fig. 3B are determined by the extent of these effects. For example, protons disappearing in Fig. 3B are exchanging slower than the detection limit and/or do not have an NOE from $C^{\alpha}H$. On the other hand, protons showing strong intensities in Fig. 3B experienced rapid exchange and/or NOEs. The changes in intensities on comparing Figs. 3B and 3C, in turn, indicate the contribution of the NOE effect to the total signal intensity in Fig. 3B. It can be clearly seen that some of the signals (e.g. Leu^{14} and Lys^{70}) are entirely due to NOEs (negligible intensity in Fig. 3C).

From the above discussion it follows that, with a proper choice of T_e, the spin-echo-filtered approach gives rise to three types of peaks: (i) pure NOE peaks, which disappear completely upon spin-echo filtering, e.g., Cys^4 in CP-1 (Fig. 2) and Leu^{14} and Lys^{70} in staphylococcal nuclease (Fig. 3); (ii) pure exchange peaks, for which the signal loss with respect to the pure WEX II filter depends only on the T_2 of water, e.g., all proton resonances except Cys^4 in CP-1 and Lys^{116} and the unidentified resonances #1, #2 and #4 in staphylococcal nuclease; and (iii) mixed peaks, e.g., Thr^{33} and resonance #3 in staphylococcal nuclease. The optimum length of T_e depends on the T_2 of solvent water and the T_2 values and J-couplings of the macromolecular C^{α} protons. In order to quantify the extent of these effects, we measured T_2 of water in our solutions of CP-1 and staphylococcal nuclease and in solutions of a small peptide (100 mM glutathione; MW = 307 Da) and a single amino acid (*N*-acetyl alanine, MW = 131 Da). To avoid radiation damping effects, we used a spin-echo sequence with dephase gradients at the beginning and rephase gradients at the end of the echo time. The T_2 values for water were 146 ms (CP-1), 133 ms (SN), 300 ms (glutathione) and 1 s (*N*-acetyl alanine). For all solutions, T_1 of water was of the order of 1–4 s, and a predelay of 20 s was used to avoid saturation. The relatively short T_2 of water in protein solution leads to two kinds of signal losses for the exchange peaks. In addition to simple T_2 signal intensity loss, the water magnetization that is dephased randomly by T_2 relaxation during the spin-echo filter cannot be flipped back to the

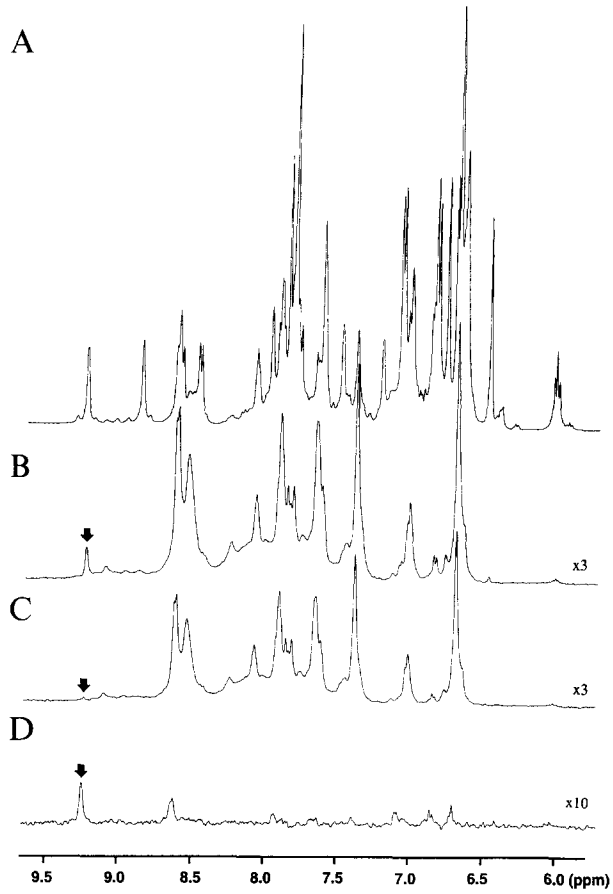

Fig. 2. Amide region spectra of an 8 mM solution of a consensus zinc finger peptide (CP-1, pH 7, 22 °C) recorded by (A) a WATERGATE sequence and (B) the WEX II filter with $T_m = 200$ ms. Spectra (C) and (D) were recorded by using the spin-echo-filtered WEX II sequence with spin-echo filter and diffusion filter, respectively. The peak indicated by the solid arrow (Cys^4) is a pure NOE peak. The conditions used for the spin-echo filter were $T_e = 40$ ms and $G_4 = 1$ ms × 1.5 G/cm. For the diffusion filter, $T_e = 20$ ms and $G_4 = 5$ ms × 30 G/cm were used. The spectral width was 5000 Hz. The acquisition time was 0.5 s, the number of scans was 256, and the prescan delay was 2 s.

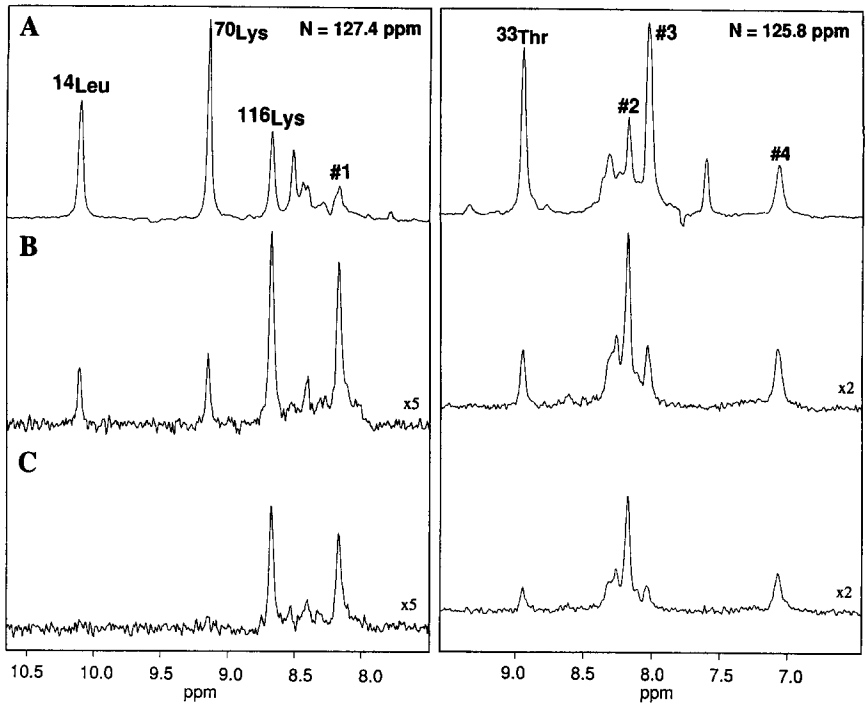

Fig. 3. Two slices of 2D spectra of a 1.5 mM solution of staphylococcal nuclease (pH 6.15, 39.5 °C) recorded by (A) a ^{15}N-FHSQC sequence, (B) the WEX-FHSQC sequence (T_m = 40 ms) and (C) the spin-echo-filtered WEX-FHSQC (T_m = 40 ms, T_e = 40 ms). Spectra on the left show slices at 127.4 ppm, those on the right depict slices at 125.8 ppm in the nitrogen domain. Peaks #1, #2, #3 and #4 are unassigned. The spectral widths were 7500 and 2000 Hz for ^{1}H and ^{15}N, respectively. The acquisition time was 0.128 s and the number of scans was 32 at each increment, with a total of 256 increments. The prescan delay was 2 s.

z-axis, leading to water saturation losses at the short repetition times used. For example, in CP-1 or SN solution, where the T_2 values are 146 and 133 ms, respectively, a spin-echo filter T_e period of 40 ms leads to dephasing of 24–26% of water by T_2 relaxation in each scan. Since T_1 of water is 3.3 s and our interscan delay was 2.25 s, only 50% of the dephased water relaxes during interscan delay; thus, about 10–12% of the dephasing is carried over to the next scan, leading to a total loss of about 35% for pure exchange peaks. The actual loss of water magnetization by the spin-echo filter can be measured from the signal attenuation of rapidly exchanging protons, because k becomes dominant compared to the NOE rate. This is illustrated in Fig. 4 where, for both CP-1 and staphylococcal nuclease, all peaks with exchange rates of more than 15 s^{-1} decrease in intensity by 35 ± 5% at T_e = 40 ms. In fact, when using an interscan delay of 8 s, loss by the spin-echo filter was reduced to about 25%.

When using T_e = 40 ms, C^α protons lose about 65–75% of their signal by relaxation, assuming that the T_2 of a protein is 30–40 ms. Further signal losses occur due to J-coupling evolution into antiphase magnetization. In this respect it is important to realize that $C^\alpha H$ normally has three coupling partners (NH, C^β, and $C^{\beta'}$ protons), and that the dephasing by J-coupling is cumulative.

Experimentally, there are two ways to remove the NOE contribution from exchange spectroscopy using the spin-echo filter. In the first approach, T_e is simply set long enough for NOE contributions to become negligible. The exchange rate can then be estimated by observing the T_m dependence of the spin-echo-filtered WEX II spectra. One obvious drawback of this approach is the loss of sensitivity due to water relaxation and saturation. In the second approach, T_m dependency data are obtained by the original WEX filter sequences and, at a certain T_m, an extra data set is recorded using a spin-echo filter. For the pure exchange peaks, the k values are as determined from the T_m analysis, while the pure NOE peaks can be deleted from the data set. For the mixed peaks, the exchange rate can be determined assuming that the NOE contribution is negligible after the spin-echo filter. Using a short T_m in the spin-echo-filtered data set, it is allowed to assume linear evolution of exchange and NOE signals (initial rate approximation), and the exchange rate can be estimated from the equation:

$$\frac{k}{NOE + k} = \frac{S_{T_e = 40\,ms} / S_{T_e = 0\,ms}}{1 - f_s} \quad (1)$$

where f_s is the saturated fraction of water magnetization (0.35 in this study), and $S_{T_e=40\,ms}$ and $S_{T_e=0\,ms}$ are signal intensities with and without the spin-echo filter. Since the apparent exchange rate (NOE + k) was already determined from the T_m dependency experiment, we can obtain the pure exchange rate. In order to accurately estimate the

exchange rates for the mixed peaks, it is very important to confirm that the NOE contribution is actually negligible after the spin-echo filter. This can be done by carrying out at least two experiments with different T_e (e.g., 40 and 60 ms). If the NOE contribution is negligible at both T_e values, the ratio in Eq. 1 should remain unchanged.

There are several points that should be addressed. First, the spin-echo-filter approach is only accurate when the relative sizes of exchange (k) and NOE differ sufficiently. The extent of the NOE effect can be estimated from the signal attenuation of pure NOE peaks by spin-echo filtering. For example, for staphylococcal nuclease, the highest value of the NOE rate was about 3.7 s^{-1}. Because the range of exchange rates we measure by the WEX filter is 1–100 s^{-1}, we can safely neglect the effect of NOE if more than 90% of α-proton magnetization is dephased after the spin-echo filter. Second, because the method relies on the difference in T_2 relaxation between water and the material under study, it is not suitable to distinguish exchange and NOE processes in small molecules. Third, the spin-echo-filtered signal may still include contributions from intermolecular NOEs between free water and protein (Otting et al., 1991a,b), and from spin diffusion of exchanging peaks. However, the former effect is considered to be small compared to exchange, and the latter effect can be minimized by using short mixing times. Finally, a technical consideration is important as well. The use of gradient pulses can cause time shifts of spin echoes when some residual gradients are present. This time shift leads to imperfect refocussing of the signal and concomitant imperfect inversion of water magnetization prior to mixing, resulting in signal loss. For example, if the first selective 90° and hard 180° pulses are applied along x and y, water magnetization should refocus along –y. The subsequent 90° hard pulse along x or –x is supposed to rotate the magnetization along –z or z, respectively. However, if the magnetization is not completely aligned along –y due to imperfect refocussing, only part of the magnetization is converted to the longitudinal axis. In order to avoid the echo shift, the performance of the gradient pulse has to be checked in the context of a separate spin-echo sequence. It should also be pointed out that, depending on the NMR system, locking may interfere with the signal phase when the lock is lost by the application of a gradient pulse. It is therefore recommended to apply a lock-hold during the sequence and acquisition and to apply locking during the predelay.

In summary, we have shown that it is possible to distinguish exchange and intramolecular NOE processes in a quantitative manner by using a spin-echo filter. This filter can be combined with multidimensional exchange spectroscopy methods such as the WEX filters or EXSY.

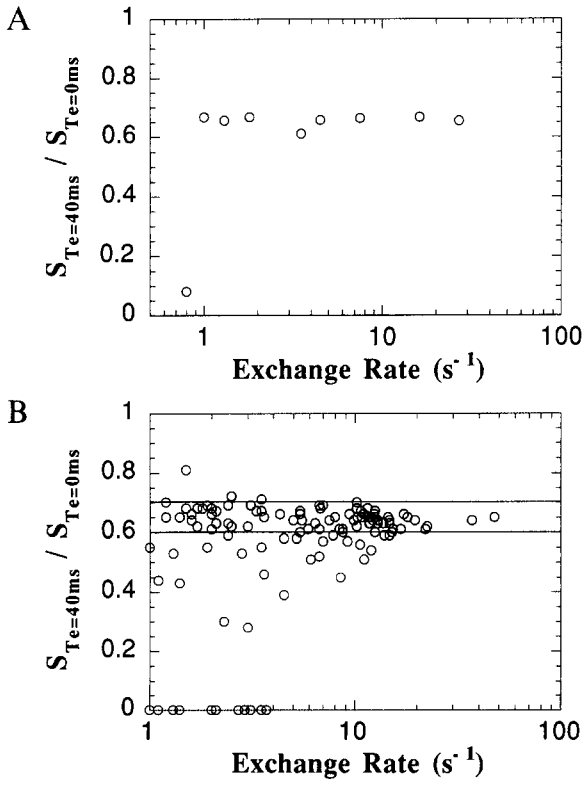

Fig. 4. Relative signal intensities ($S_{T_e=40ms}/S_{T_e=0ms}$) of the spin-echo-filtered WEX II spectra with respect to the nonfiltered WEX II spectra as a function of amide proton exchange rate for (A) CP-1 and (B) staphylococcal nuclease.

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