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Detection of protein–ligand NOEs with small, weakly binding ligands by combined relaxation and diffusion filtering

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Summary

The use of a diffusion filter is proposed to suppress the NMR signals of small organic compounds in the presence of macromolecules. Combined with a spin-echo relaxation filter, the diffusion filter enables the selective and simultaneous detection of intermolecular solvent–protein NOEs in a straightforward two-dimensional NOESY experiment. Using the intermolecular NOEs observed between *N*,*N*-dimethylformamide (DMF) and hen egg-white lysozyme in an aqueous solution containing 2 M DMF, the binding of DMF at the specificity-determining substrate binding site C of the enzyme was modelled.

The observation that the reactivity and substrate specificity of enzymes can be modulated by organic solvents sparked an interest in understanding the interaction of proteins with organic solvents at atomic detail (Arnold, 1993; Fitzpatrick et al., 1993; Halling, 1994; Wescott and Klibanov, 1994; Yennavar et al., 1995). In addition, the cocrystallization of proteins with a set of simple, low-affinity organic solvent molecules has been proposed as a tool to probe protein surfaces for binding sites with the different functional groups presented by various organic solvents (Allen et al., 1996; Mattos and Ringe, 1996). A knowledge of the binding preferences of different functional groups would enable the design of molecules containing several of the functional groups in correct spatial orientation to represent ligands of increased affinity.

In principle, NMR spectroscopy is particularly well suited for the identification of preferential contact sites of organic molecules on protein surfaces: in solution, the entire protein surface can be studied without interference from protein–protein contacts in a crystal lattice, site-specific binding constants can be measured by monitoring chemical shift changes as a function of ligand concentration, and intermolecular NOEs can be used to model the orientation of the ligand molecules at the binding sites (Liepinsh and Otting, 1997).

Since most organic solvents bind with very low affinities, molar concentrations of the protonated solvents are needed for the observation of intermolecular ^{1}H-^{1}H NOEs. At the same time, most proteins require the presence of water to prevent denaturation and maintain solubility. Previously used ligand molecules gave rise to only one or two ^{1}H NMR signals, which could be suppressed by a single spin-lock purge pulse (Otting et al., 1991) while suppressing the water signal by presaturation (Liepinsh and Otting, 1994,1997). The observation of intermolecular NOEs with different, nondegenerate sets of proton resonances in a ligand molecule would provide more detailed information on the ligand binding mode. This communication demonstrates the use of a diffusion filter (Van Zijl and Moonen, 1990; Wider et al., 1994; Wu et al., 1995) for the simultaneous suppression of multiple solvent/ligand resonances, enabling the simultaneous measurement of intermolecular ligand–protein NOEs with all ligand resonances.

Figure 1 shows the NOESY pulse sequence supplemented by a diffusion filter after the mixing time and a spin-echo relaxation filter (Mori et al., 1996) before the evolution time t_1. The diffusion filter consists of a pair of pulsed field gradients (PFGs) after the mixing time to defocus the magnetization and a second pair of PFGs before the acquisition time to refocus the magnetization. Each pair of PFGs is arranged in a PFG-180° pulse-PFG sandwich, where the 180° pulses are placed so that the chemical shift evolution is refocused by the start of the acquisition time t_2. Since the magnetization is transverse

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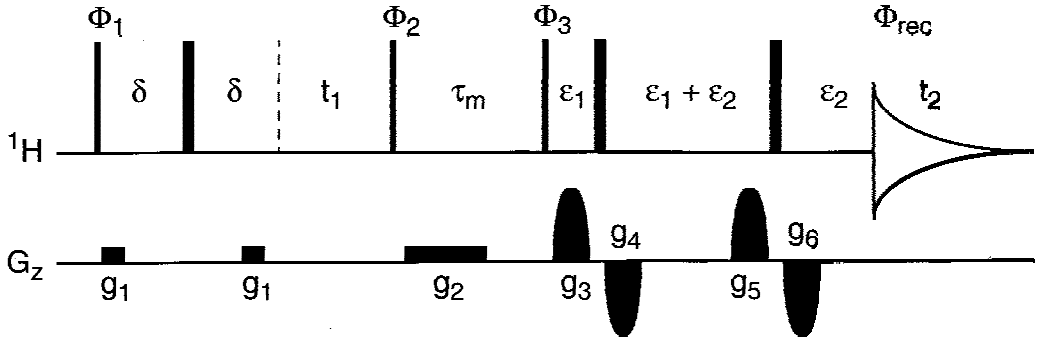

Fig. 1. Two-dimensional NOESY pulse sequence used for the selective observation of solvent–protein cross peaks in solvent mixtures of water and small organic molecules. The 90° and 180° pulses are identified by narrow and wide bars, respectively. All pulses are applied with phase x unless indicated differently. The PFGs g_3 to g_6 are shaped to reduce eddy currents, with rising and falling flanks following sine and cosine functions, respectively, during 2×16% of the total pulse length. Amplitudes and durations of the PFGs: $g_1 = 1$ G/cm, 2 ms; $g_2 = 3.5$ G/cm, 7 ms; $g_3 = 45$ G/cm, 3.5 ms; $g_4 = -45$ G/cm, 3 ms; $g_5 = 45$ G/cm, 3.35 ms; $g_6 = -45$ G/cm, 3.15 ms. Filter delays: $\delta = 100$ ms; $\varepsilon_1 = 3.5$ ms; $\varepsilon_2 = 6$ ms. Phase cycle: $\Phi_1 = 4(x,-x)$; $\Phi_2 = 2(x,x,-x,-x)$; $\Phi_3 = 4(x),4(-x)$; $\Phi_{rec} = x,-x,-x,x,-x,x,x,-x$. The phase cycle is extended twofold by inverting the signs of all gradients. States-TPPI is applied to Φ_1 and the first 180° pulse.

during the diffusion filter, the total filter delay is chosen as short as possible to minimize scalar coupling evolution and the loss of protein magnetization by transverse relaxation. The minimum duration of the filter delay is determined by the gradient strength and duration required to obtain a significant suppression of the solvent signals. Since the suppression effect relies on the incomplete refocusing of magnetization of rapidly diffusing molecules, the signals from smaller molecules are suppressed the best.

Compared to single PFG pulses, PFG pulses of opposite polarity in PFG-180°-PFG sandwiches have the advantage of compensating for eddy currents with time constants much longer than the duration of the PFG pulses (Wider et al., 1994; Wu et al., 1995). Yet, to make use of the coherence order selection properties of the PFGs, it is advantageous to apply them with nonidentical amplitudes or durations. Otherwise, imperfect refocusing by the 180° pulses results in noticeable phase distortions, or additional phase cycling of the 180° pulses must be used. A small recovery delay before the acquisition time suppresses the effects from residual eddy currents.

The spin-echo relaxation filter before the evolution time is optional. Its purpose is to suppress the protein signals by T_2 relaxation while retaining the signals from the more

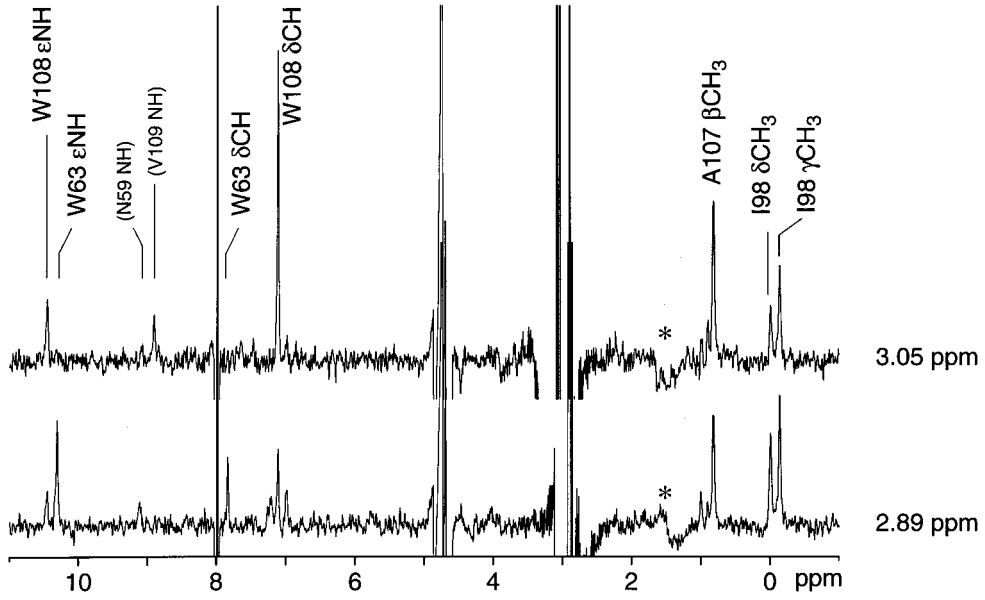

Fig. 2. Cross sections through a 2D NOESY spectrum recorded with a 7.8 mM aqueous solution of HEWL containing 2 M DMF and 7% (v/v) D_2O at 36 °C. Experimental parameters: $t_{1max} = 28.9$ ms, $t_{2max} = 115.5$ ms, mixing time $\tau_m = 100$ ms, 2.8 s interscan relaxation delay, total experiment time 16 h. The cross sections were taken along the F2 frequency axis at the F1 chemical shifts of the DMF methyl groups (3.05 and 2.89 ppm). The most intense NOE cross peaks are labelled with the protein assignments. Tentative assignments are in brackets. Asterisks identify baseline distortions from the PFGs in the diffusion filter (see text). The spectrum was recorded at a ^1H frequency of 600 MHz on a Bruker DMX-600 NMR spectrometer with a GREAT 1/10 gradient unit. A triple-resonance probehead equipped with a self-shielded gradient coil was used.

slowly relaxing ligand molecules. In this way, intermolecular ligand–protein NOEs can be observed without interference from intraprotein NOEs. The spin-echo relaxation filter is effective only if bound and free ligand molecules are in rapid exchange and the free ligand is in large excess. Since scalar couplings within the ligand evolve during the filter delay, the spin-echo relaxation filter is most useful for resonances with no or very small coupling constants. Defocusing and refocusing the magnetization by PFGs at the start of the filter delay and before the evolution period reduces signal losses by radiation damping and suppresses artifacts from imperfections of the refocusing 180° pulse. These PFGs must not be too strong to avoid loss of magnetization by diffusion of the solvent molecules during the filter delay. If deemed necessary, radiation damping during the evolution time t_1 can be further suppressed using a weak bipolar gradient (Sklenář, 1995).

The experiment of Fig. 1 was used to record intermolecular NOEs between *N*,*N*-dimethylformamide (DMF) and hen egg-white lysozyme (HEWL) in an aqueous solution containing 2 M DMF. The parameters of the diffusion filter were adjusted to result in a more than 200-fold suppression of the DMF signals and a more than 1000-fold suppression of the water resonance. The diffusion filter decreased the signals of HEWL about three- to fivefold. The spin-echo relaxation filter resulted in virtually complete suppression of the protein signals, whereas the methyl resonances of DMF were reduced by about 25%.

Figure 2 shows the cross sections taken through the two-dimensional NOESY spectrum along the F2 frequency axis at the F1 chemical shifts of the DMF methyl resonances. These cross sections contain the intermolecular cross peaks between DMF and the protein. Residual signals from DMF and H_2O appear at 8.0, 4.8, 3.0 and 2.9 ppm. The baseline distortions around 1.3 ppm (marked with asterisks in Fig. 2) arose from the PFGs in the diffusion filter by an unexplained mechanism. These artifacts were present also when recovery delays of 1 ms were inserted after the PFGs g_3 and g_5 (Fig. 1). Using a different probehead, the artifacts appeared at shifted frequencies. They were significantly reduced by adding two experiments recorded with opposite polarity of all PFGs. No intermolecular NOEs between the DMF formyl proton and HEWL could be detected.

The cross peaks were assigned to specific protons of HEWL by comparison with NOESY and TOCSY spectra recorded with the diffusion filter but without the spin-echo relaxation filter. The same protein protons were found to be involved in NOEs with the DMF methyl groups as in earlier experiments with methanol, isopropanol, DMSO, acetone, acetonitrile, cyclopropane and ethylene (Liepinsh and Otting, 1997; Otting et al., 1997). All these protein protons line the substrate binding site C of HEWL, which accommodates an *N*-acetyl group of the natural substrate, *N*-acetylglucosamine-containing carbohydrates. The difficulty of observing intermolecular NOEs with most of the solvent-exposed HEWL protons may be attributed to little restricted diffusion of the DMF molecules near most of the protein surface, corresponding to too short residence times for effective NOE interactions (Jóhannesson et al., 1997; Otting et al., 1997).

Based on the intermolecular NOEs, the binding mode of DMF at site C was modelled using one of the crystal structures of HEWL (Fig. 3). In comparing the NOEs with both methyl groups of DMF, the different amounts of proton magnetization present for the two methyl groups at the start of the NOE mixing time were taken into account. This correction was needed, because the relaxation times of the different DMF resonances are not identical, leading to different relaxation during the spin-echo relaxation filter, and because the repetition rate of the NOESY experiment was faster than the T_1 relaxation times of the solvent, leading to incomplete recovery of equilibrium magnetization. A one-dimensional spectrum

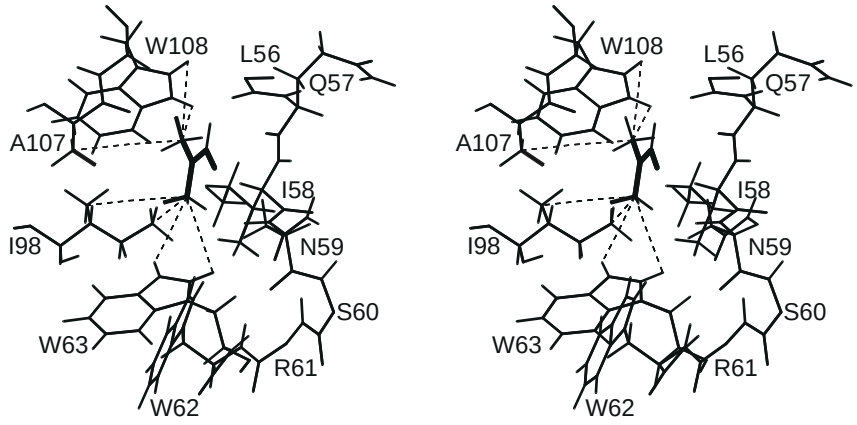

Fig. 3. Stereoplot of DMF bound to site C of HEWL. Protein coordinates were taken from Cheetham et al. (1992; PDB accession code 1HEW). The residues Leu[56] to Trp[63], Ile[98], Ala[107] and Trp[108] are shown. For residues Leu[56], Ser[60] and Arg[61] only the backbone atoms are displayed. The intermolecular NOEs observed with DMF are identified by dotted lines. For protein protons showing NOEs to both DMF methyl groups, only the stronger NOE is displayed. The heavy atoms of DMF are drawn with bold lines. The figure was produced using the program MOLSCRIPT (Kraulis, 1991).

recorded with the spin-echo filter of Fig. 1 after a 90° pulse, using dummy scans and the repetition rate of the NOESY experiment, showed a 22% attenuation of the signal of the methyl group at 2.89 ppm, which is trans with respect to the formyl proton, compared to the signal of the cis-methyl group at 3.05 ppm.

All NOEs assigned in Fig. 2 can be explained by a single DMF molecule. The model of Fig. 3 was obtained using the program MIDAS (Ferrin et al., 1988) by interactively fitting the DMF molecule into the binding site C of one of the crystal structures of HEWL (Cheetham et al., 1992) so that, for each protein proton involved in NOEs with both DMF methyl groups, the stronger NOE corresponds to the shorter distance. The model provides a good fit of the van der Waals surfaces of both molecules. NOEs between the formyl proton of DMF and the protein protons would not be expected. The model does not predict the NOE observed between the resonances of the DMF methyl group at 2.89 ppm and Trp^{108} $H^{\varepsilon 1}$ (Fig. 2). This may be explained by a certain degree of mobility of the DMF molecule at the binding site. Recent ^2H relaxation dispersion measurements showed that the molecular order parameter of a dimethylsulfoxide (DMSO) molecule bound at site C is significantly reduced at 27 °C (Jóhannesson et al., 1997), although DMSO binds with higher affinity at site C than DMF (1.7 M^{-1} (Liepinsh and Otting, 1997) versus 0.5 M^{-1} at 36 °C). The mobility of the bound DMF molecule would also explain the apparent absence of spin diffusion between the methyl groups of DMF despite the relatively long mixing time used (100 ms). For example, the NOE with Trp^{63} $H^{\varepsilon 1}$ was observed for the cis- but not for the trans-methyl group of DMF (Fig. 2). A NOESY experiment recorded with 60 ms mixing time yielded an intensity ratio of about 10:1 between the Trp^{108} $H^{\varepsilon 1}$ diagonal peak and the Trp^{108} H^{δ}-$H^{\varepsilon 1}$ cross peak. Therefore, even the cross peak between the DMF methyl group at 3.05 ppm and Trp^{108} $H^{\varepsilon 1}$ in Fig. 2 cannot exclusively arise from spin diffusion from the intense DMF-Trp^{108} H^{δ} NOE.

The binding affinity of DMF was determined from the chemical shift changes of the Trp^{108} $H^{\varepsilon 1}$ resonance observed upon titration of a 1 mM aqueous solution of HEWL with DMF. The starting solvent was 90% H_2O/10% D_2O at 36 °C, pH 3.8. DMF was added in 10 steps up to a final concentration of 6 M. The concentrations of DMF were checked by comparing the intensities of the DMF signals with resolved HEWL signals and the binding constant calculated as described previously (Liepinsh and Otting, 1994). For accurate chemical shift measurements at high DMF concentrations, one-dimensional NMR spectra were recorded with the diffusion filter of Fig. 1.

The diffusion filter is most effective in suppressing the resonances of small, rapidly diffusing solvent molecules. Although it involves significant losses in protein magnetization, it is attractive because it combines an easy experimental setup with the simultaneous observation of intermolecular NOEs with all ligand resonances and an excitation profile in the detection dimension which is uniform unless the protein resonances relax with significantly different rates during the filter delay. It can be used with any type of one-, two- or multi-dimensional NMR experiment. If concomitant sensitivity losses can be accepted, the solvent resonances can, in principle, be suppressed to the level of white noise. The spin-echo relaxation filter is very effective in selecting slowly relaxing magnetization. Together, both filter elements provide practical tools for the observation of intermolecular NOEs between protein protons and the protons of small, weakly binding ligands and solvent molecules present in high concentrations.

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