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WaterLOGSY as a method for primary NMR screening: Practical aspects and range of applicability

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Abstract

WaterLOGSY represents a powerful method for primary NMR screening in the identification of compounds interacting with macromolecules, including proteins and DNA or RNA fragments. Several relay pathways are used constructively in the experiment for transferring bulk water magnetization to the ligand. The method is particularly useful for the identification of novel scaffolds of micromolar affinity that can be then optimized using directed screening, combinatorial chemistry, medicinal chemistry and structure-based drug design. The practical aspects and range of applicability of the WaterLOGSY experiment are analyzed in detail here. Competition binding and titration WaterLOGSY permit, after proper correction, the evaluation of the dissociation binding constant. The high sensitivity of the technique in combination with the easy deconvolution of the mixtures for the identification of the active components, significantly reduces the amount of material and time needed for the NMR screening process.

Introduction

NMR is a powerful method for identifying compounds that interact with macromolecules, including proteins and DNA or RNA fragments (Feeney et al., 1979; Lian et al., 1993). Over the last few years, NMR screening has gained importance in target-directed drug discovery programs (Shuker et al., 1996; Hajduk et al., 1997a). Several methods have been proposed for screening mixtures of compounds against the target of interest (Hajduk et al., 1997b; Lin et al., 1997a,b; Meyer et al., 1997; Chen and Shapiro, 1998; Stockman, 1998; Fejzo et al., 1999; Henrichsen et al., 1999; Klein et al., 1999; Mayer and Meyer, 1999, 2001; Moore, 1999; Chen and Shapiro, 2000; Jahnke et al., 2000, 2001). One of these techniques is the Water-LOGSY (Water-Ligand Observed via Gradient SpectroscopY) experiment (Dalvit et al., 2000) where the large bulk water magnetization is partially transfered via the protein-ligand complex to the free ligand in a selective manner. In this experiment, the resonances of non-binding compounds appear with opposite sign and tend to be weaker than those of the interacting ligands. The idea for this technique was based on many experimental observations.

Poornima and Dean (1995) have analyzed the X-ray crystallographic structures of the protein-ligand complexes with resolution better than 2 Å and R factor <0.23 for the presence of water molecules at the protein-ligand interface. In all 19 complexes analyzed, water molecules were found linking the ligand to the protein with a maximum of six bridging water molecules found in DHFR complexed to NADPH and in Staphylococcal nuclease complexed with 5′-deoxythymidine. Most of the water molecules (∼80%) involved in bridging interactions make three

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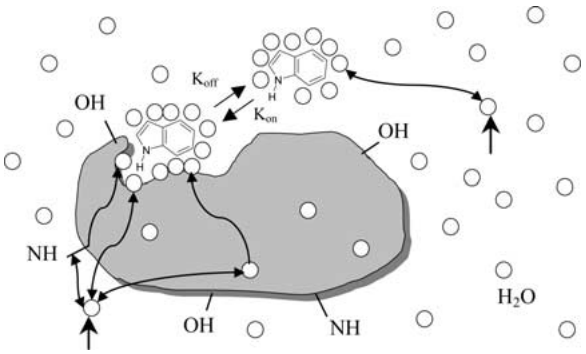

Figure 1. WaterLOGSY principle. The protein is shown with the buried cavities and the active binding site. The ligand is shown in the bound and free states. Excitation of bulk water (circles) is shown with a solid arrow and some of the different magnetization transfer pathways are shown with dashed lines.

or more hydrogen bonds that connect ligand and protein atoms together with other water molecules in the site. These water molecules have a low B factor and therefore play an important role in the stabilization of the protein-ligand interactions.

A general water-release model suggests that the maximum entropic cost for the inclusion of a water molecule at the interface at 298 K is -9 kJ mol^{-1} whereas the maximum enthalpic gain that can be achieved is -16 kJ mol^{-1}, i.e., a single tightly bound water molecule can contribute up to 7 kJ mol^{-1} to the free energy of the complex (Connely, 1997), corresponding to a ten-fold improvement in the binding constant of the ligand at 298 K.

Often, water molecules at protein-ligand interfaces do not satisfy all their hydrogen-bonding capabilities and, in extreme cases such as the Lovastatin-LFA-1 complex, they may be buried between two hydrophobic surfaces (Dalvit et al., 1999). Independent of the number of hydrogen bonds that can be formed, the residence times of water in protein cavities invariably seem to range between a few ns to a few hundred μs (Otting and Wüthrich, 1989; Otting et al., 1991, 1997; Denisov and Halle, 1995; Denisov et al., 1995; Ernst et al., 1995; Halle, 1999; Wiesner et al., 1999), i.e., a time span long compared to the effective correlation time, where intermolecular water-protein NOEs change sign (ca. 0.3 ns at 600 MHz) and short compared to the chemical shift time scale, where a separate resonance for the bound water could be observed (ms). Thus, selective excitation of the water signal followed by NOE mixing effectively transfers magnetization from the bulk water to the protein, which has the same sign as the starting magnetization.

A second important mechanism for magnetization transfer from the water to the protein-ligand complex is by chemical exchange with labile carboxyl, amino, hydroxyl, imidazol, guanidinium and amide protons (Wüthrich, 1986; Otting and Liepinsh, 1995; Liepinsh and Otting, 1996). Like the intermolecular NOE with buried water molecules, this magnetization transfer pathway conserves the sign of the magnetization, i.e., both processes act constructively to transfer magnetization from the bulk water to the protein (Figure 1). The large number of exchangeable protons and buried, yet exchangeable, water molecules in a protein-ligand complex could explain the high sensitivity of the WaterLOGSY experiment for the selective detection of binding ligands, which makes the method a powerful tool for primary screening of compound mixtures by NMR spectroscopy. In the present work, parameters for the WaterLOGSY experiment were optimized for sensitivity. An improved, more sensitive version of the WaterLOGSY experiment was developed, and a method for quantitative estimates of ligand-binding affinities by WaterLOGSY was established.

Material and methods

Expression and purification of cyclinA

A cDNA encoding residues 173–432 of human cyclinA was amplified by the polymerase chain reaction (PCR) and cloned into the N-terminal GST fusion bacterial expression vector, pGEX6P (AmershamPharmacia). GST-cyclinA was expressed in the E. coli host strain BL21trx (Novagen). Cell cultures were grown in LB media to an optical density of 0.8 at 25 °C and induced with 0.1 mM IPTG. Growth was continued overnight at 25 °C. Cells were harvested by low speed centrifugation and lysed by passage through a APV homogenizer. The soluble fraction was applied to glutathione sepharose resin (AmershamPharmacia) and the GST moiety was removed, after extensive washes, by cleavage with PreScission Protease (Pharmacia). The resulting cyclinA in solution was estimated to be 95% pure by SDS-PAGE.

Expression and purification of Cdk2

A human cDNA clone encoding full length cdk2 was amplified by PCR and cloned into a pVL1392 vector (Pharmingen) for expression in HighFive (Invitrogen) insect cells as a GST fusion. GST-Cells were infected with virus at an Moi of 1 for 48 h, at 27 °C. Cells

were harvested and lysed by APV homogenizer. The supernatant was loaded onto a glutathione sepharose (Pharmacia) column and washed with buffer (DPBS, Sigma).

Cdk2/CycA complex formation and purification

The resulting resin, with GST-Cdk2 linked, was incubated for 1 h at 4 °C with the previously purified CycA. After extensive washes and the cleavage with PreScission protease, the complex cdk2/cycA pure at 99% (SDS-PAGE and gel filtration) was obtained.

Fatty acid free human serum albumin (A-3782) was purchased from Sigma and used without further purification.

The NMR samples were in phosphate buffered saline (PBS) pH 7.4. D_2O was added to the solutions (8% final concentration) for the lock signal. The small molecules were prepared in concentrated stock solutions in deuterated DMSO and stored at 253 K.

NMR experiments

All spectra were recorded at 293 K with a Varian Inova 600 MHz NMR spectrometer equipped with a 5 mm triple-resonance inverse probe. For each sample a reference spectrum and a 1D WaterLOGSY spectrum were recorded. The details of the pulse sequence version used for the WaterLOGSY experiment reported here can be found in the literature (Dalvit, 1996, 1998). The first water selective 180° pulse does not require high selectivity and, in our experience, a pulse of 8 to 25 ms length was found to be sufficient. The first two Pulsed Field Gradients (PFGs) have a typical length of 1–2 ms and a power strength of 2–6 G/cm. This strength is sufficient to destroy the unwanted magnetization and, at the same time, it avoids signal losses due to diffusion occuring between the first two PFGs. A weak rectangular PFG is applied during the entire length of the mixing time. A short gradient recovery time of 1–2 ms is applied at the end of the mixing time before the detection pulse. The water suppression in both experiments was achieved with the excitation sculpting sequence (Hwang and Shaka, 1995). The two water selective 180° square pulses and the four PFGs of the scheme were 2.7 and 1 ms, respectively. The gradient recovery time was 0.2 s. The data were collected with a sweep width of 7407 Hz, an acquisition time of 0.648 s, and a relaxation delay of 2.648 s. Prior to Fourier transformation the data were multiplied with an exponential function with a line broadening of 1 Hz.

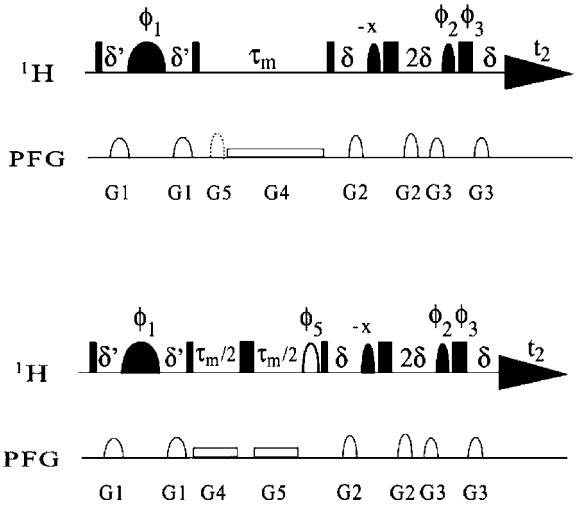

Figure 2. Pulse sequences for the homonuclear one-dimensional ePHOGSY without (upper panel) and with water flip-back pulse (lower panel) used in our studies. The gradient G5 (dashed line) is optional. The scheme in the lower panel differs from the original experiment used for hydration studies by the presence of a hard 180° pulse applied in the middle of the mixing time and a water selective flip-back pulse. The additional hard 180° pulse is necessary for the suppression of artefacts that originate from the almost complete relaxation of the protons of the small molecules during the long mixing period (Stott et al., 1997). For short mixing times typically used in hydration studies its use was clearly not necessary. The phase ϕ_5 is (x, −x) whereas the phases of all the other pulses are the same as previously reported (Dalvit, 1998). The gradients G4 and G5 of different strength are applied during the entire length of the two $\tau_m/2$ periods. In our experience the use of one non-selective 180° pulse in experiments recorded with $\tau_m = 1.2$ to 1.5 s was sufficient for artefacts suppression. If this is not sufficient it is possible to improve the quality of the resulting spectra by using two 180° pulses applied at about 0.3 τ_m and 0.8 τ_m (Stott et al., 1997). In this version of the experiment ϕ_5 has to be changed to (−x, x). The additional 180° pulse at $\tau_m/2$ could be applied also in the scheme without flip-back pulse (upper panel), but does in this case not noticeably improve the performance of the experiment. A $T_{1\rho}$ filter can be applied before the double-spin echo scheme as discussed in the text.

Results and discussion

The simplest version of the WaterLOGSY experiment starts with selective water excitation. Several methods have been proposed for selectively and efficiently exciting the water signal (Otting, 1997; Wider, 1998; Melacini et al., 1999a,b). ePHOGSY, which is based on a water-selective 180° refocusing pulse between two pulsed field gradients (Figure 2), is one of the technically most robust schemes to achieve selective water excitation. In particular, ePHOGSY effectively defocuses the magnetization of all resonances that are not near the water chemical shift, which provides the basis for a low level of artifacts. The improved Wa-

terLOGSY scheme of Figure 2 (lower panel) uses a 180° inversion pulse in the middle of the mixing time to avoid the recovery of equilibrium magnetization of the protein and ligand protons (Stott et al., 1997). This pulse is necessary to maintain the level of artifact suppression at the long mixing times that we found to be optimum for sensitivity. Combined with the water-selective flip-back pulse (Grzesiek and Bax, 1993) at the end of the mixing time, the sensitivity can be improved about 1.2 to 1.3 fold over that of the original experiment as seen in the example of Figure 3. Following the NOE mixing time, the double-spin echo scheme (Hwang and Shaka, 1995) provides excellent water suppression and suppresses partially the signals of the protein resonances by transverse relaxation. For small and medium size proteins the double-spin echo is not sufficient to destroy completely the protein signals. In this case it is sufficient to introduce in the pulse sequence a $T_{1\rho}$ filter (Ernst et al., 1987) before the acquisition period.

As the WaterLOGSY experiment starts from water magnetization, the optimum repetition rate of the original pulse scheme without water flip-back depends on the T_1 relaxation time of the water protons which in the case of the Human Serum Albumin (HSA) -L-Trp complex was measured to be 2.6 s. The water flip-back version of Figure 2 alleviates this relaxation time dependence by keeping a large fraction of the water magnetization along the +z axis during the acquisition and repetition delay periods. This allows a faster repetition rate and reduces problems of water signal saturation.

The optimum mixing time depends on the size of the complex. During the mixing time the water magnetization that has migrated to the protein is transferred via direct or relay processes to the ligand. For large proteins the relay process known also as spin-diffusion is very efficient due to the fast flip-flop transitions (Kalk and Berendsen, 1976; Stoesz and Redfield, 1978). In this case even short mixing times suffice to spread the magnetization through the entire protein.

In the case of a water molecule which is rigidly buried at the protein-ligand interface, the magnetization transfer rate σ_{wp} by the intermolecular NOE to the protein-ligand complex can be described by (Otting, 1997; Halle et al., 1999):

$$\sigma_{wp} = \frac{\gamma^4\hbar^2\left[\mu_0/(4\pi)\right]^2}{10r_{wp}^6}\frac{\tau_r\tau_p}{\tau_r+\tau_p}\left\{\frac{6}{\left[1+\frac{4\omega_0^2\tau_r^2\tau_p^2}{(\tau_r+\tau_p)^2}\right]}-1\right\} \quad (1)$$

where τ_r and τ_p are the residence time of water within the protein and the rotational correlation time of the protein, respectively, r_{wp} is the distance between water and protein protons, ω_0 is the Larmor frequency. The other symbols have their usual meanings. Figure 4 shows a simulation of the intermolecular NOEs as a function of the water residence time and the protein correlation time. In the simulation we have assumed a r_{wp} of 2.5 Å and a Larmor frequency of 600 MHz. It is evident that the intermolecular NOE is very weak and positive for water molecules with residence time < 300 ps. For residence times longer than 300 ps the NOEs change sign and increase in magnitude. In addition, the magnetization transfer is more efficient for large proteins with long rotational correlation time. The protein correlation time can also be increased by decreasing the temperature, by increasing the solution viscosity or by coupling the protein to a solid matrix.

In contrast to protein or DNA hydration studies by intermolecular NOEs, where short mixing times are employed in order to avoid problems originating from spin-diffusion and to distinguish intermolecular NOEs from exchange-relayed NOEs, WaterLOGSY constructively uses all magnetization transfer processes in order to maximize magnetization transferred to the ligand. Therefore, the experiments are recorded with long mixing times. Figure 5 shows the signal of human serum albumin (HSA) in the ePHOGSY experiment (recorded without $T_{1\rho}$ filter) as a function of the mixing time. The protein signals increase rapidly and reach a maximum with a mixing time of ~1.2 s. The fast build-up curve is followed by a slow decay at long mixing times.

Application of the WaterLOGSY experiment to two ligands (E and F) complexed with the cyclin dependent kinase 2 (cdk2) protein (Mw ~34000) is shown in Figure 6. The resulting spectra are devoid of artefacts and the water resonance is remarkably well suppressed. The positive signals originate from the two ligands, while the negative signals originate from small molecules that do not interact or interact

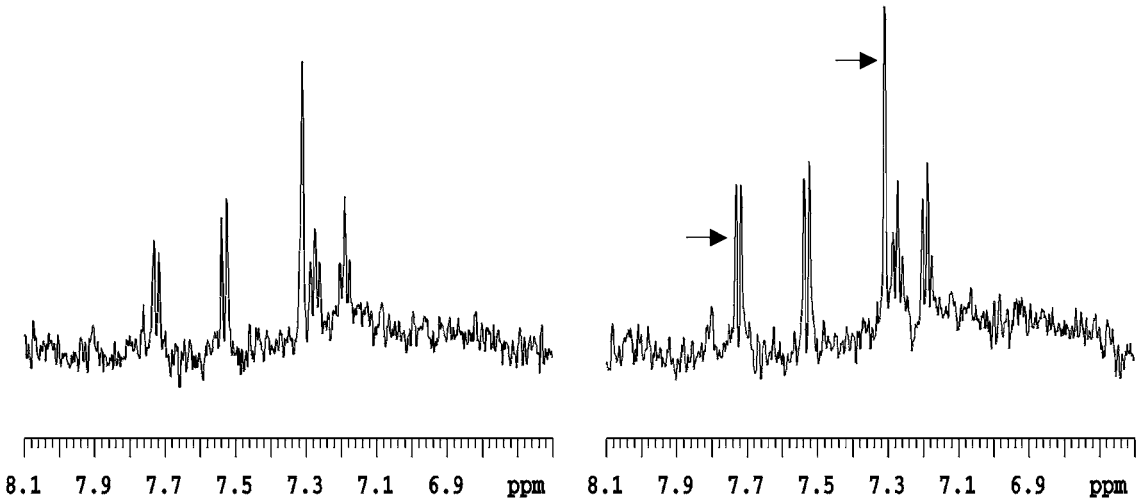

Figure 3. One-Dimensional WaterLOGSY spectra recorded for a 10 μM Human Serum Albumin (HSA) solution in the presence of 200 μM L-Trp. The spectra were recorded with the pulse sequences of Figure 1 without (left) and with (right) the water flip back pulse. The spectra were recorded with 1600 scans, 2 s repetition time and 2.3 s mixing time. The length of the water selective 180° pulse and 90° flip-back pulse was 12.5 and 4 ms, respectively. The arrows in the spectrum on the right indicate the intensity of the corresponding signals in the spectrum on the left.

only very weakly with the protein. The S-CH_3 signal of compound E and the CH_3 signal of DMSO, as a function of the mixing time length, are shown in the insert. Although the signal of DMSO has not reached maximum intensity even at 2.5 s the signal of the ligand reaches its maximum at ∼2.0 s. The ligand signal intensity reaches its maximum value at longer delays when compared to the protein signals (see Figure 5). This is due to the longer spin-lattice (T_1) relaxation of the protons of the small molecules.

Although the selective water excitation scheme also excites some of the protein resonances at the water frequency, the signals for the ligands observed in the WaterLOGSY spectrum originate exclusively from the excitation of bulk water. This can be appreciated in Figure 7 where WaterLOGSY spectra for the complex of HSA with L-tryptophan recorded in H_2O and D_2O are shown. Although the signals of the ligand and protein are visible in the spectrum recorded in H_2O these signals are absent in the WaterLOGSY experiment recorded in D_2O.

Clearly, the excitation of the protein resonances at the water frequency by the ePHOGSY excitation scheme is efficiently compensated by the rapid decay of this magnetization due to the short T_2 relaxation times of the protein protons. No false positives would be expected even in cases where resonances of the small compounds overlap with the water resonance and get excited by the ePHOGSY scheme; in this situation, intramolecular NOEs within the small compound would give signals in the WaterLOGSY spectrum, which are strong and positive only for ligands that bind to the protein. However, this statement is valid only when the ratio ligand/protein is not very large.

Titration and competition binding experiments are also possible with WaterLOGSY. These experiments can be recorded to extract an approximate value for the binding constant of the ligand. However, particular care must be taken in the analysis of the titration experiments, since two off-setting effects are responsible for the signal intensity in the WaterLOGSY spectra.

Expression of the signal intensity I for the ligand proton *i* as a function of the different effects is given, in first approximation, by the equation:

$$I \propto [PL]\left(\sum_j \sigma_{ij}^{bound} + \sum_k \sigma_{ik} + \sum_w \sigma_{iw}^{bound}\right) + [L]\left(\sum_j \sigma_{ij}^{free} + \sum_w \sigma_{iw}^{free}\right) \quad (2)$$

where [PL] and [L] are the bound and free ligand concentrations, respectively. The two concentrations are related to each other via the equation: $[L] = [L_{tot}] - [PL]$ where $[L_{tot}]$ is the total ligand concentration. The indices j are ligand exchangeable protons, k are protein protons near ligand and w are water molecules near ligand. A large dissociation constant K_D and high ligand concentration L_{tot} result in

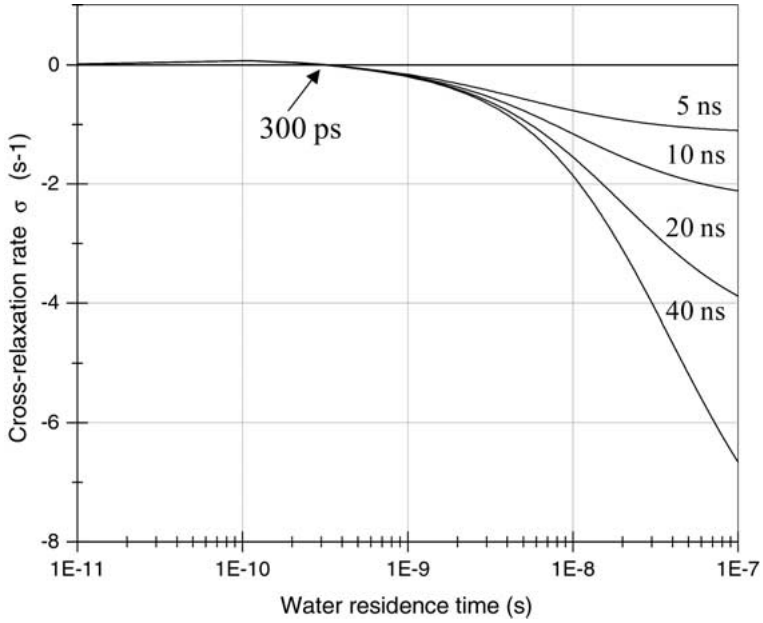

Figure 4. Intermolecular cross relaxation between water and protein-ligand complex according to Equation 1 as a function of water residence time (x axis). The simulation was performed for a Larmor frequency of 600 MHz, using a r_{wp} of 2.5 Å. Simulations were performed for different rotational correlation times of the protein (values indicated with the curves).

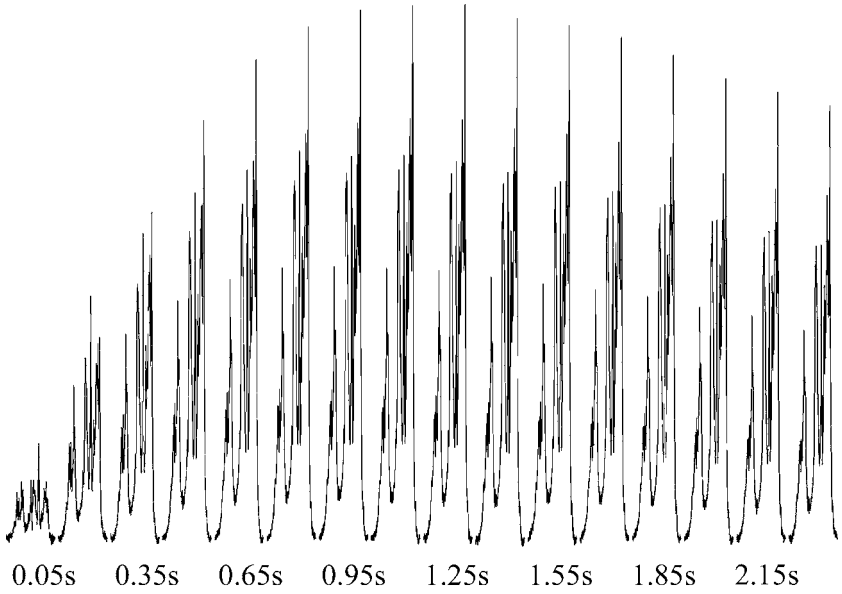

Figure 5. One-Dimensional NOE-ePHOGSY spectra for HSA as a function of the mixing time. The entire spectrum is displayed. The protein was 100 μM in PBS (8% D_2O). The 180° water selective pulse was 25 ms long and the length of the first two PFGs was 2 ms. A total of 800 scans were recorded with a repetition delay of 3.65 s. The spectra were obtained with the pulse sequence of Figure 2 by incrementing the length of the mixing time in 0.15 s increment.

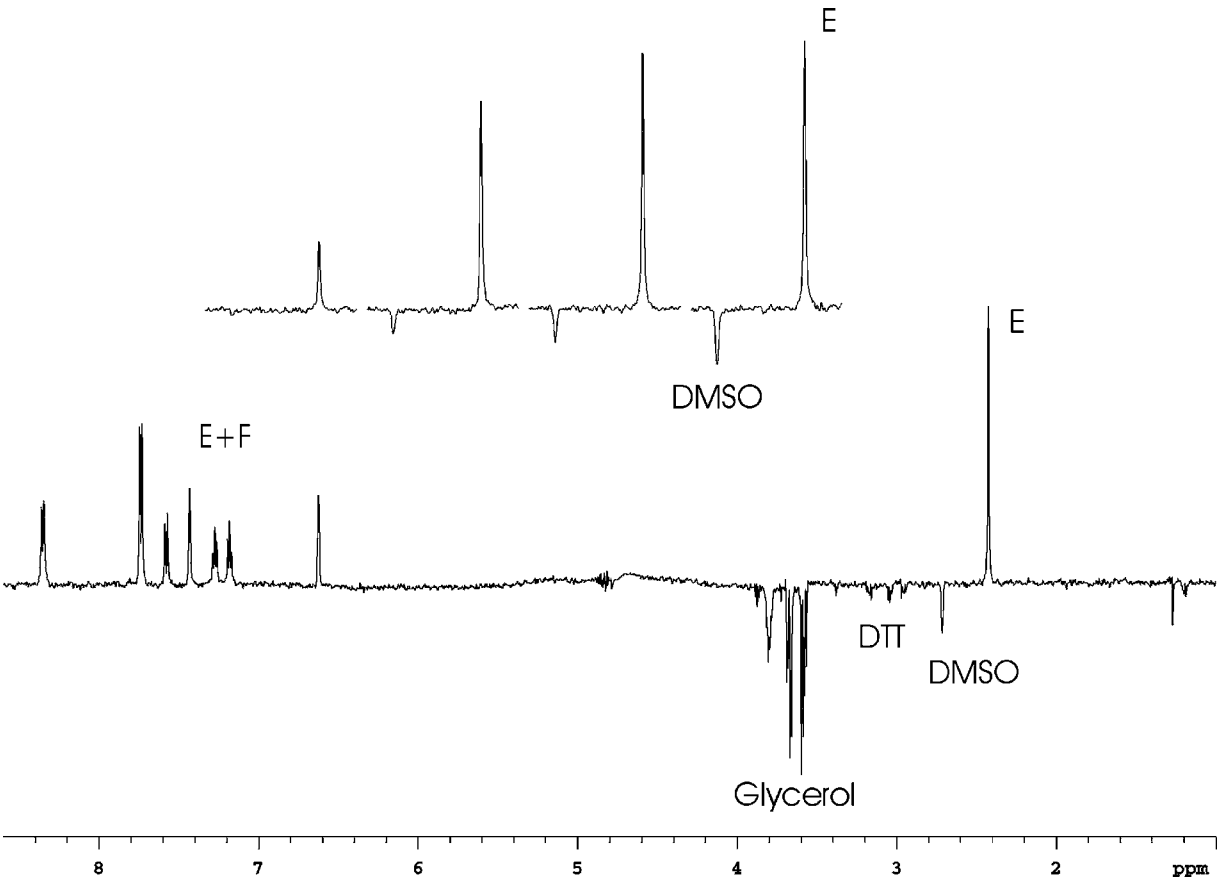

Figure 6. One-Dimensional WaterLOGSY for ligands E and F (200 μM) in the presence of 10 μM cdk2. The spectrum was recorded with 512 scans. The relaxation and mixing times were 2.6 and 2.0 s, respectively. Positive and negative signals identify cdk2 binding and non-interacting molecules, respectively. Glycerol was present at mM concentration. The insert shows expansions of the spectral region between 2.3 and 2.8 ppm which were measured by WaterLOGSY experiments with mixing times of, respectively, 0.2, 0.8, 1.2 and 2.5 s (from left to right).

small fraction of bound ligand $[PL]/[L_{tot}]$. Therefore, a high ligand/protein ratio will render the second term of Equation 2 more significant, in particular if the free ligand is highly hydrated and if the ligand proton monitored is very close in space to a ligand exchangeable proton. This is the case for example for the C2-H proton of tryptophan. Figure 8 shows the experimental WaterLOGSY signal intensity (circles) as a function of the ligand concentration for the C2-H resonance of tryptophan in the presence of 10 μM HSA. The initial build-up phase is followed by a decrease in signal intensity at high ligand/protein ratio. The decrease in signal intensity is due to the second term of Equation 2 that, at high concentrations of free ligand, becomes predominant.

The contribution of the second term of Equation 2 can be corrected for by subtracting the result of WaterLOGSY experiments recorded for the ligand in the absence of the protein. The absence of aggregation at high ligand concentration can be verified by recording two spectra at low and high ligand concentration. If aggregation is absent, both experimental points should lie on a straight line crossing the origin (triangles in Figure 8). The WaterLOGSY signals for the ligand in the presence of the protein can then be corrected by subtracting the value of the ligand signals recorded in the absence of the protein. The resulting corrected data (squares in Figure 8) are now lying on a conventional dose response curve and, assuming a simple binding mechanism, the data can be fitted to the equation:

$$I = \frac{-I_{max}}{1 + \left(\frac{L}{K_D}\right)} + I_{max} \quad (3)$$

where I_{max} is the maximum WaterLOGSY signal, K_D is the dissociation binding constant and L is the free ligand concentration.

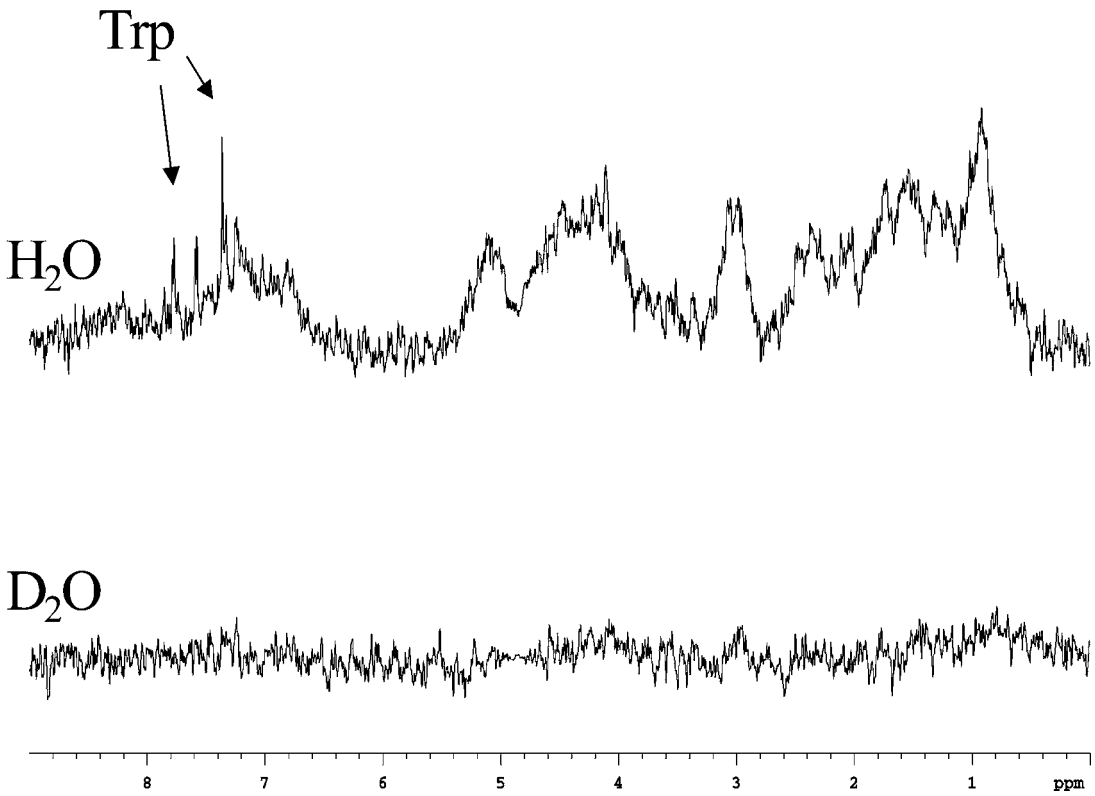

Figure 7. One-Dimensional WaterLOGSY spectra recorded for a 10 μM HSA solution in the presence of 100 μM L-Trp in H_2O (upper trace) and D_2O (lower trace). The spectra were recorded with 1024 scans and a 2.65 s repetition time. Other parameters are the same as described in Figure 6.

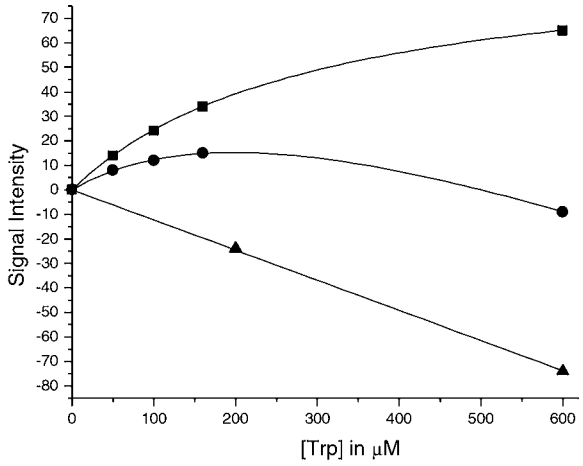

Figure 8. WaterLOGSY signal intensity for the C2-H resonance of L-Trp as a function of the ligand concentration. The experiments were recorded with 2048 scans and a 3.65 s repetition time. The triangles and circles are experimental points recorded in the absence and presence of 10 μM HSA in PBS, respectively. The square points are the intensities difference of the WaterLOGSY spectra recorded in the presence and absence of the protein. The curves represent the best fits for the data. The signal intensity is on an arbitrary scale.

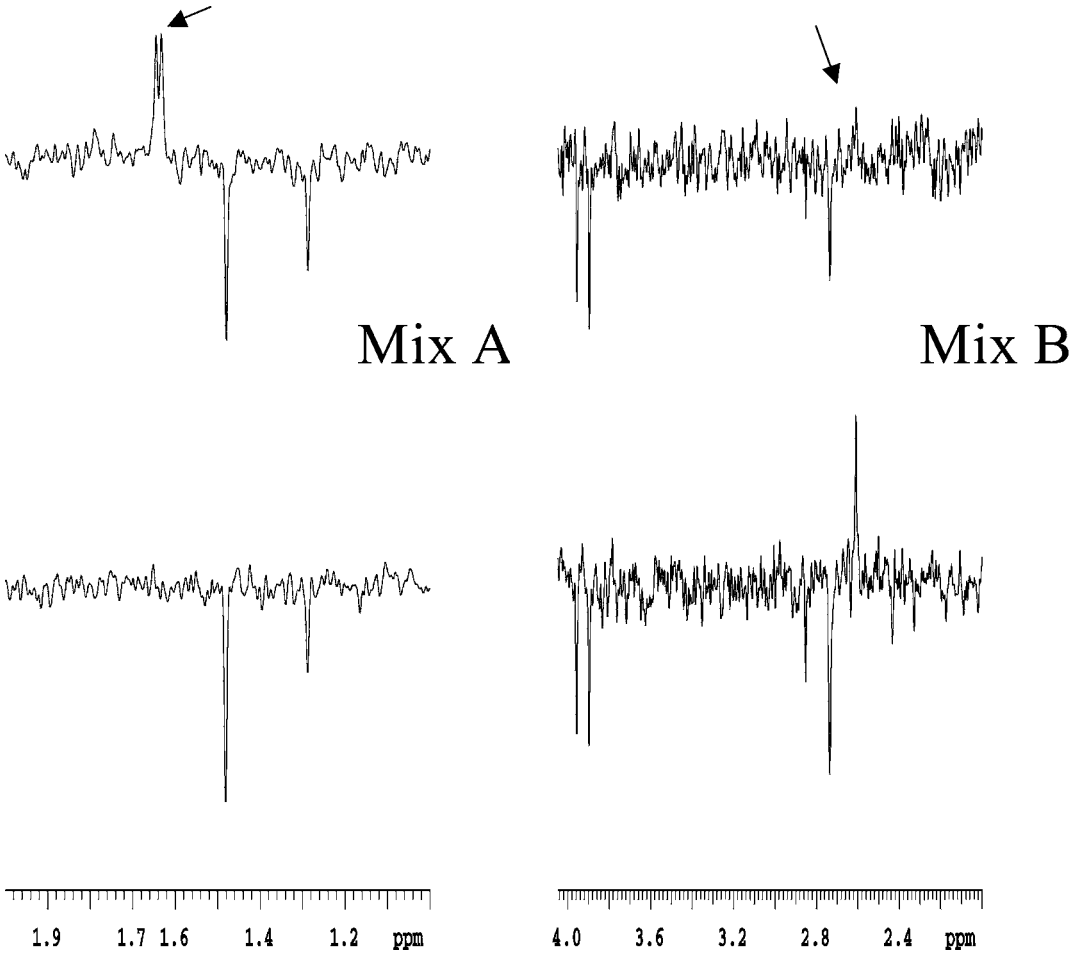

Figure 9. Competition binding experiments for two different four compound mixtures (mix A left, mix B right) with the cdk2/cycA complex in the presence of the inhibitor flavopiridol. The protein and mixture concentrations were 1.5 and 40 μM, respectively. A total of 2048 scans were recorded for each spectrum with a repetition time of 2.65 s and a mixing time of 1.5 s. Expanded spectral regions without (upper traces) and with flavopiridol (lower traces) are displayed. The ratio protein/flavopiridol is close to 1.

In this particular case the K_D obtained was 292 μM ± 7 μM. Alternatively, the experimental points (circles) can be fitted without correction for the free ligand, using the equation:

$$I = \frac{-I_{max}}{1 + \left(\frac{L}{K_D}\right)} + I_{max} - aL \qquad (4)$$

where a is the slope of the straight line originating from the ligand hydration in the absence of protein. The K_D measured with Equation 4 is 369 μM ± 84 μM. Despite the small number of experimental points (four) and the large number of parameters (three) to be fitted by Equation 4, a value similar to the one extracted with the experimental correction is obtained. The K_D value reported in the literature for L-Trp bound to HSA is 100 μM and was determined by equilibrium dialysis (McMenamy and Oncley, 1958; Bertuzzi et al., 1997). Our K_D value is of the same order of magnitude which is acceptable in many practical situations, considering the few number of data points required and the selectivity of the NMR experiment.

Another method for extracting the binding constant is with competition binding experiments carried out with titration of an inhibitor of known binding constant (Cheng and Prusoff, 1973). These experiments can also be used in a qualitative way simply to confirm that the NMR hits are displaced in the presence of known inhibitors. This is demonstrated in Figure 9 for a mixture of compounds with cdk2/cycA complex in the absence and in the presence of the strong inhibitor

flavopiridol (K_D in the low nM range). The methyl group signal of the NMR hit in mixture A, indicated by the arrow, is absent in the spectrum recorded in the presence of flavopiridol. The displacement of this compound from the protein results from either direct competition for the binding site of flavopiridol or from an allosteric effect originating from substantial structural and/or dynamic changes of the protein associated with the binding of flavopiridol. An allosteric effect is clearly observed for the compound indicated by an arrow in Figure 9 for mixture B. The signal, virtually absent in the spectrum recorded without flavopiridol, appears in the spectrum recorded with flavopiridol indicating that the presence of the strong inhibitor affects the binding of the compound to the cdk2/cycA complex.

Conclusion

The WaterLOGSY experiment represents a powerful NMR approach for primary screening of compounds for binding to the target of interest in the μM range. The method requires only limited amount of protein and therefore allows the screening of proteins that cannot be expressed in large quantities. Deconvolution of the mixtures for the identification of the active components is straightforward with WaterLOGSY. The experiment is not limited to the interactions of small molecules with proteins, but can be used efficiently also in the identification of molecules interacting with DNA or RNA fragments. Recently, the WaterLOGSY method has been applied successfully to screen the SHAPES library against the P456 domain of the self-splicing Group I intron ribozyme from Tetrahymena thermophila (Moore, 2000; Williamson, 2000).

A drawback of the method, as with all screening techniques that detect the ligand resonances, is its inability to detect strongly binding ligands with slow dissociation rates since the ligand is in high excess. With this method, we have been able to identify compounds with K_D values in the 100 nM range (data not shown). However, this is possible only with diffusion-controlled association rate constants or with electrostatically enhanced rate constants (Vijayakumar et al., 1998). With a diffusion-limited on rate of 10^8 M^{-1} s^{-1} and with a K_D value of 100 nM, the residence time of the ligand within the protein is about 100 ms. However, the main goal of our screening approach and the follow-up experiments is to identify several different scaffolds of micromolar affinity (Lepre et al., 2000) that can be then optimized using directed screening, combinatorial chemistry and structure-based drug design.

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