Journal of Biomolecular NMR, **18:** 65–68, 2000.
KLUWER/ESCOM

Identification of compounds with binding affinity to proteins via magnetization transfer from bulk water*

Claudio Dalvit**, Paolo Pevarello, Marco Tatò, Marina Veronesi, Anna Vulpetti & Michael Sundström
Pharmacia, Viale Pasteur 10, I-20014 Nerviano (MI), Italy

Received 21 June 2000; Accepted 26 June 2000

Key words: bulk water, magnetization transfer, NMR screening, protein hydration, protein–ligand interactions

Abstract

A powerful screening by NMR methodology (WaterLOGSY), based on transfer of magnetization from bulk water, for the identification of compounds that interact with target biomolecules (proteins, RNA and DNA fragments) is described. The method exploits efficiently the large reservoir of H_2O magnetization. The high sensitivity of the technique reduces the amount of biomolecule and ligands needed for the screening, which constitutes an important requirement for high throughput screening by NMR of large libraries of compounds. Application of the method to a compound mixture against the cyclin-dependent kinase 2 (cdk2) protein is presented.

Over the last few years, screening by NMR has emerged as a potent method for the identification of small molecules that bind to a protein drug target (Shuker et al., 1996; Hajduk et al., 1997a, b; Lin et al., 1997; Meyer et al., 1997; Moore, 1998; Stockman, 1998; Fejzo et al., 1999; Henrichsen et al., 1999). Although this methodology suffers from its intrinsic low sensitivity and therefore requires significantly more protein material than other screening methods, the results obtained with screening by NMR are more reliable as many artifacts observed with other methods are avoided.

Several NMR parameters can be used for monitoring molecules interacting with a protein (Feeney et al., 1979; Lian et al., 1993). The most frequently used NMR experiments are the two-dimensional $^1H/^{15}N$ HSQC (when ^{15}N labelled protein is available) and the T_2 filter one-dimensional experiments where spectra for a compound mixture are recorded in the absence and presence of the target biomolecule. More recently, several methods have been proposed allowing the identification of the ligands in a compound mixture without the need to record the reference spectra. These experiments utilize the magnetization transfer from the protein to the ligand (Chen and Shapiro, 1998; Klein et al., 1999; Mayer and Meyer, 1999) or from the ligand to the protein (Chen and Shapiro, 2000). In the experiment proposed by Meyer and co-workers (Klein et al., 1999; Mayer and Meyer, 1999) i.e., the steady state NOE experiment, a difference spectrum is generated from a spectrum recorded with saturation of a protein resonance (or resonances) and a normal spectrum (with off-resonance saturation). With on-resonance saturation the magnetization is efficiently transferred via flip-flop transitions (Kalk et al., 1976; Stoesz et al., 1978) throughout the entire protein and to the compounds interacting with the macromolecule. The authors showed that the method, applied to compound mixtures and proteins dissolved in D_2O, is very powerful for the identification of ligands.

Water plays a pivotal role in the protein–ligand, protein–protein and protein–DNA recognition mechanisms. It has been noticed in many hydration NMR studies of protein–ligand complexes that several H_2O molecules are present at the interface (Otting, 1997 and references therein). For example, a layer of H_2O was detected around Lovastatin, a low molecular

*This work has been presented at the SAPIO Meeting, Advancements in Biomolecular NMR, Florence (Italy), 19 February 2000.
**To whom correspondence should be addressed. E-mail: claudio.dalvit@eu.pnu.com

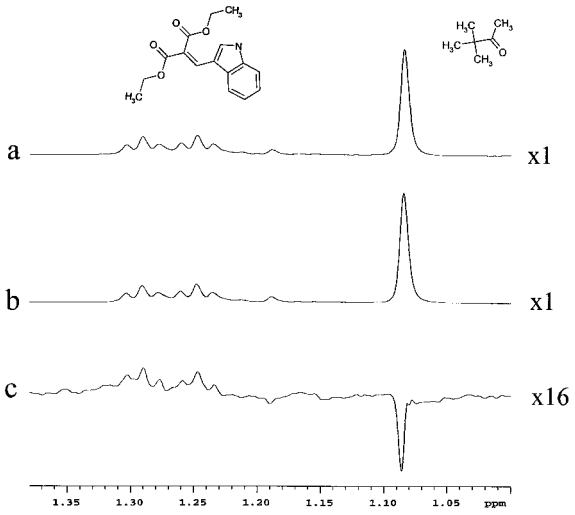

Figure 1. Expanded region of the 1D ^{1}H WaterLOGSY spectrum of a 10 compound mixture (see text) in the presence of the protein cdk2 recorded with a 40 Hz and 2 s long RF presaturation field applied off-resonance (a) and at the H_2O chemical shift (b). (c) Difference spectrum obtained by subtracting spectrum (b) from spectrum (a). Human cdk2 protein was expressed in Sf9 insect cells using a recombinant baculovirus encoding cdk2. The NMR sample was in Phosphate Buffered Saline (PBS) (8% D_2O) and the protein concentration was 10 μm. The spectra have been recorded at Te = 19 °C with a Varian Inova 600 MHz spectrometer. The H_2O solvent suppression was achieved with the H_2O excitation sculpting sequence (Hwang and Shaka, 1995). A total of 256 scans were recorded for each spectrum (a,b). The chemical structures of the two molecules are depicted. Positive and negative signals in (c) identify cdk2 interacting and not interacting molecules, respectively.

weight natural product, interacting with the protein LFA-1 (Dalvit et al., 1999). The observed intermolecular water–ligand NOEs were negative, indicating that the residence time of these bound H_2O molecules is longer than ∼1 ns (Otting and Wüthrich, 1989; Otting et al., 1991). These NOEs were detected even at short mixing times and, based on the 3D protein–ligand structure, could not be ascribed to magnetization relay processes arising from exchangeable protons or a spin diffusion mechanism. It is interesting to notice that these water molecules were not detected in the X-ray structure of the I-domain of LFA-1 complexed with Lovastatin (Kallen et al., 1999). Two possible explanations for these NMR experimental observations can be given: (i) A shell of bound or 'squeezed' H_2O is present at the interface between protein and ligand; or (ii) an extensive network of hydrogen-bonded water molecules with a long residence time surrounds the free ligand. In the latter case the water–ligand NOEs appear negative due to the modulation arising from the bound state of the ligand.

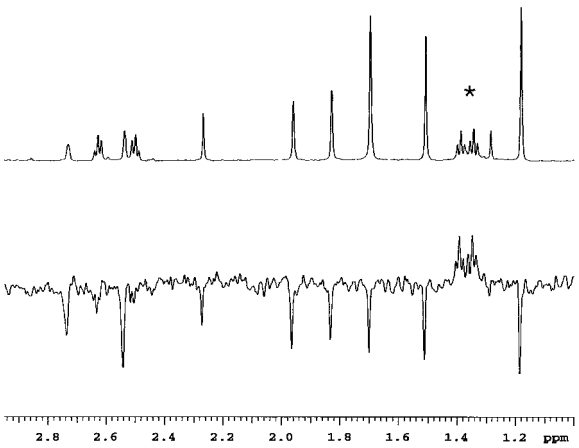

Figure 2. One-dimensional reference (upper) and WaterLOGSY with NOE-ePHOGSY (lower) spectra recorded for the 10-compound chemical mixture in the presence of 10 μm cdk2. The WaterLOGSY and the reference spectra were recorded at Te = 17 °C with 256 and 128 scans, respectively. The H_2O solvent suppression in both experiments was achieved with the H_2O excitation sculpting sequence (Hwang and Shaka, 1995). The WaterLOGSY was recorded with a 38 ms long 180° H_2O selective Gaussian pulse. This pulse can be set also to only 10 to 20 ms length, because no high selectivity is required. The relaxation and mixing times were 2.6 and 2 s, respectively. Positive and negative signals in the lower spectrum identify cdk2 interacting and not interacting molecules, respectively. The asterisk indicates the methyl group resonances of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate.

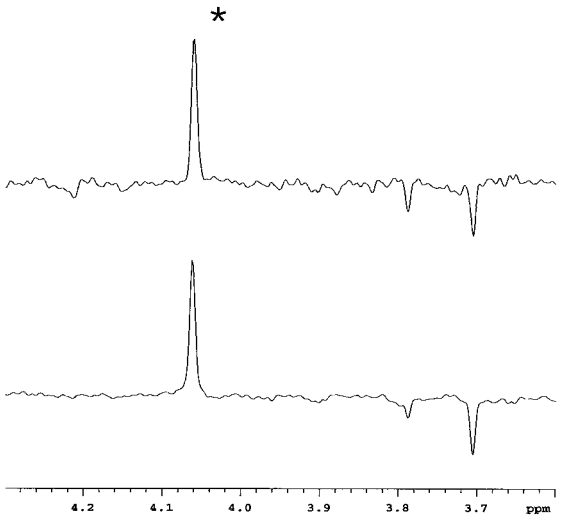

Figure 3. Expanded region of 1D WaterLOGSY with NOE-ePHOGSY (lower) and ROE-ePHOGSY (upper) spectra for the 10-compound mixture in the presence of 10 μm cdk2. The spectra were recorded at Te = 17 °C with 1024 scans and with 2.6 s relaxation delay. The mixing and spin-lock times were 2 and 0.3 s, respectively. The signal at 4.06 ppm, labelled with an asterisk, originates from an exchangeable proton resonance.

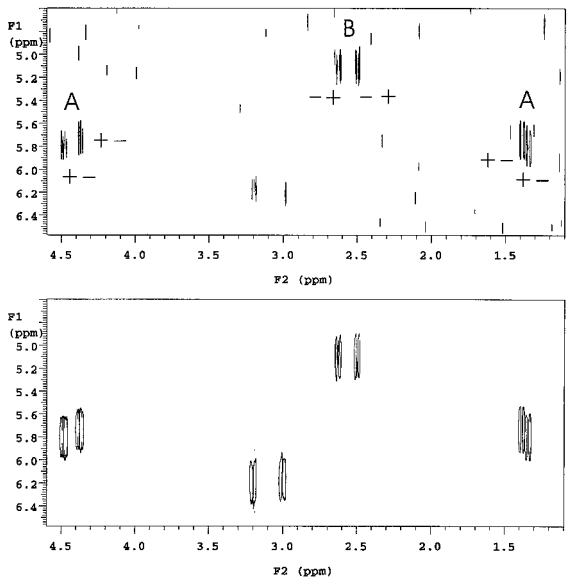

Figure 4. Expanded region of the WaterLOGSY ^{1}H 2D PFG DQ spectra of the 10-compound mixture with cdk2. The spectra above and below were obtained respectively by subtracting and adding the two spectra recorded with H_2O and an off-resonance presaturation rf field of 40 Hz and length 2 s. The 45°/135° version of the experiment was recorded at Te = 19 °C with pulsed field gradients tilted at the magic angle for better solvent suppression. The excitation DQ period was 41 ms long and 16 scans were recorded for each of the 128 t_1 increments. In the difference spectrum the cross peaks of the two CH_3-CH_2 moieties (labelled A) of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate have opposite sign when compared to the cross peaks of the CH_2-CH_2 moiety (labelled B) of mono-methyl succinate.

Based on all these observations we propose here the use of bulk H_2O for the detection of molecules interacting with a protein. Two different classes of experiments can be used for this purpose i.e. a steady state NOE experiment with on-resonance saturation applied at the water chemical shift or a NOE experiment with selective inversion of the H_2O signal and with a long mixing time. Numerous schemes have been devised for selective water excitation (Otting, 1997 and references therein). A member of this type of experiments is the NOE-ePHOGSY and related experiments (Dalvit and Hommel, 1995; Dalvit, 1996; Melacini et al., 1999a,b).

The saturation of water yields the following effects: (i) saturation of some of the αH protein resonances, (ii) complete saturation of the fast exchanging NH and OH protons of the protein and small molecules resonating at the H_2O chemical shift, (iii) partial or total saturation of rapidly exchanging NH and OH protons of the protein and small molecules resonating at a chemical shift different from H_2O, (iv) magnetization transfer from bulk water to bound water located in different cavities of the protein, and (v) magnetization transfer from bulk water to the squeezed water at the protein–ligand interface. Inversion of most of this magnetization is achieved in the NOE-ePHOGSY experiment with the exception, in large biomolecules, of the αH protein signals resonating at the H_2O chemical shift (i). The acquisition of these experiments is technically demanding when working in H_2O. Often the effects observed in the difference spectra are very small. Radiation damping and demagnetizing field mechanisms originating from bulk water can introduce artifacts and mask the small effects (Sobol et al., 1998; Price, 1999). However, it is possible to overcome these problems by properly using pulsed field gradients. We propose the name WaterLOGSY (Water-Ligand Observation with Gradient SpectroscopY) for these experiments used for detection of ligands via bulk water. Figure 1 shows the principle of the experiment recorded with steady state NOE applied to a mixture of 10 low molecular weight compounds (concentration 100 μm) in the presence of 10 μm of cyclin-dependent kinase 2 (cdk2) protein (Mw ∼34 kDa). The molecules of the mixture are 3-methylenecyclopropane-trans-1,2-dicarboxylic acid, mono-methyl succinate, s-benzylthioglycolic acid, 3,3-dimethylacrylic acid, 1,2,4-triazole, 5,5-dimethyl-2-4-oxazolidinedione, 2,2-dimethyl-1,3-dioxane-4,6-dione, fluoroacetamide, pinacolone and ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate. The expanded region contains only the two methyl group signals (1.29 and 1.25 ppm) of the indole derivative and the methyl t-butyl signal (1.08 ppm) of pinacolone. The spectra in (a) and (b) were recorded with water and off-resonance saturation, respectively. A weak positive NOE effect (negative signal) for pinacolone and a weak negative NOE effect (positive signals) for the indole derivative are observed in the difference spectrum (Figure 1c). Pinacolone does not interact with the protein and therefore displays a positive NOE with H_2O whereas the indole derivative that interacts with the protein (measured Ki is in the high μm range) displays a negative NOE stemming from the effects associated to the saturation of bulk H_2O, as described above.

The 1D WaterLOGSY experiments with the H_2O presaturation scheme can give rise to small artifacts originating from the difference spectroscopy method. However, the version with the NOE-ePHOGSY scheme is completely devoid of artifacts. Even very

weak effects can be analyzed with confidence. This can be appreciated in Figure 2. Our 10 small molecules mixture contains NMR signals consisting mostly of sharp singlets. Comparison of the 1D NOE-ePHOGSY (lower spectrum) with the 1D reference spectrum (upper spectrum) allows easy identification of the only molecule interacting with the protein. The measuring time of the WaterLOGSY spectrum of Figure 2 was only 20 min. The quality of the spectra obtainable with the NOE-ePHOGSY scheme and the sensitivity of the experiment have allowed application of the method to protein concentrations as low as a few hundred nM (data not shown). The exchangeable proton resonances, when visible, will also appear as positive peaks in the WaterLOGSY experiments. These peaks usually can be easily recognized in the spectrum. However, if doubts remain it is sufficient to record the WaterLOGSY experiment with the ROE-ePHOGSY scheme for the unambiguous identification of the exchangeable resonances. Figure 3 shows application of this strategy to our mixture. The positive peak at 4.06 ppm observed in the WaterLOGSY with NOE step (lower spectrum) does not originate from a ligand of cdk2, but it is simply an exchangeable proton resonance as confirmed by the WaterLOGSY experiment with ROE step (upper spectrum).

The WaterLOGSY schemes (either with H_2O presaturation or NOE-ePHOGSY) can be also used in 2D experiments (DQ, TOCSY, etc.). Use of WaterLOGSY in the ^1H 2D PFG DQ experiment applied to our compound mixture is shown in Figure 4. The signals of the CH_3-CH_2 moiety of the cdk2 ligand ethyl alpha-(ethoxycarbonyl)-3-indoleacrylate and the signals of the CH_2-CH_2 moiety of mono methyl succinate are visible in this expanded spectral region (lower spectrum). These signals are also visible in the DQ difference spectrum (upper spectrum). However, the signals of the ligand are easily recognized because they have opposite sign when compared to the signals of mono methyl succinate.

In summary, we have shown that by using the large reservoir of bulk H_2O magnetization it is possible to detect via different transfer mechanisms small molecules that interact with a target biomolecule (proteins, DNA or RNA fragments). The method, like all the techniques based on ligand resonance observation, has the disadvantage that it does not provide information about the ligand binding site. Despite this drawback the technique represents a rapid means for ligand identification.

References

Chen, A. and Shapiro, M.J. (1998) *J. Am. Chem. Soc.*, **120**, 10258–10259.

Chen, A. and Shapiro, M.J. (2000) *J. Am. Chem. Soc.*, **122**, 414–415.

Dalvit, C. (1996) *J. Magn. Reson.*, **B112**, 282–288.

Dalvit, C., Cottens, S., Ramage, P. and Hommel, U. (1999) *J. Biomol. NMR*, **13**, 43–50.

Dalvit, C. and Hommel, U. (1995) *J. Magn. Reson.*, **109**, 334–338.

Feeney, J., Batchelor, G., Albrand, J.P. and Roberts, G.C.K. (1979) *J. Magn. Reson.*, **33**, 519–529.

Fejzo, J., Lepre, C.A., Peng, J.W., Bemis, G.W., Ajay, Murcko, M.A. and Moore, J.M. (1999) *Chem. Biol.*, **6**, 755–769.

Hajduk, P.J., Olejniczak E.T. and Fesik, S.W. (1997b) *J. Am. Chem. Soc.*, **119**, 12257–12261.

Hajduk, P.J., Sheppard, D.G., Nettesheim, D.G., Olejniczak, E.T., Shuker, S.B., Meadows, R.P., Steinman, D.H., Carrera, G.M., Jr., Marcotte, P.M., Severin, J., Walter, K., Smith, H., Gubbins, E., Simmer, R., Holzman, T.F., Morgan, D.W., Davidsen, S.K., Summers, J.B. and Fesik, S.W. (1997a) *J. Am. Chem. Soc.*, **119**, 5818–5827.

Henrichsen, D., Ernst, B., Magnani, J.L., Wang, W.-T., Meyer, B. and Peters, T. (1999) *Angew. Chem. Int. Ed. Engl.*, **38**, 98–102.

Hwang, T.-L. and Shaka, A.J. (1995) *J. Magn. Reson.*, **A112**, 275–279.

Kalk, A. and Berendsen, H.J.C. (1976) *J. Magn. Reson.*, **24**, 343–366.

Kallen, J., Welzenbach, K., Ramage, P., Geyl, D., Kriwaki, R., Legge, G., Cottens, S., Weitz-Schmidt, G. and Hommel, U. (1999) *J. Mol. Biol.*, **292**, 1–9.

Klein, J., Meinecke, R., Mayer, M. and Meyer, B. (1999) *J. Am. Chem. Soc.*, **121**, 5336–5337.

Lian, L.Y. and Roberts, G.C.K. (1993) In *NMR of Macromolecules* (Ed., Roberts, G.C.K.), Oxford University Press, Oxford, pp. 153–182.

Lin, M., Shapiro, M.J. and Wareing, J.R. (1997) *J. Am. Chem. Soc.*, **119**, 5249–5250.

Mayer, M. and Meyer, B. (1999) *Angew. Chem. Int. Ed. Engl.*, **38**, 1784–1788.

Melacini, G., Boelens, R. and Kaptein, R. (1999a) *J. Biomol. NMR*, **13**, 67–71.

Melacini, G., Boelens, R. and Kaptein, R. (1999b) *J. Biomol. NMR*, **15**, 189–201.

Meyer, B., Weimar, T. and Peters, T. (1997) *Eur. J. Biochem.*, **246**, 705–709.

Moore, J.M. (1999) *Analyt. Biotechnol.*, **10**, 54–58.

Otting, G. (1997) *Progr. NMR Spectrosc*, **31**, 259–285.

Otting, G., Liepinsh, E. and Wüthrich, K. (1991) *Science*, **254**, 974–980.

Otting, G. and Wüthrich, K. (1989) *J. Am. Chem. Soc.*, **111**, 1871–1875.

Price, W.S. (1999) In *Annual Reports on NMR Spectroscopy* (Ed., Webb, A.), Academic Press, New York, NY, Vol. 38, pp. 289–354.

Shuker, S.B., Hajduk, P.J., Meadows, R.P. and Fesik, S.W. (1996) *Science*, **274**, 1531–1534.

Sobol, A.G., Wider, G., Iwai, H. and Wüthrich, K. (1998) *J. Magn. Reson.*, **130**, 262–271.

Stockman, B.J. (1998) *Progr. NMR Spectrose*, **33**, 109–151.

Stoesz, J.D. and Redfield, A.G. (1978) *FEBS Lett.*, **91**, 320–324.