Detecting Binding Affinity to Immobilized Receptor Proteins in Compound Libraries by HR-MAS STD NMR

Jens Klein, Robert Meinecke, Moriz Mayer, and Bernd Meyer*

Institute for Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

Received March 8, 1999
Revised Manuscript Received April 12, 1999

NMR experiments based on saturation transfer difference (STD) NMR spectroscopy allow direct identification and structure elucidation of substances with binding activity from mixtures.[1] Here we demonstrate that STD NMR can be used as a fast and sensitive screening method to characterize binding affinity in mixtures, e.g., combinatorial libraries, in heterogeneous samples if high resolution magic angle spinning (HR-MAS) NMR techniques are utilized. Screening a mixture of seven oligosaccharides for affinity to wheat germ agglutinin (WGA) immobilized to controlled pore glass (CPG) revealed only resonances of *N,N*′-diacetylchitobiose, a disaccharide that binds to WGA. Nonbinding compounds gave no response in the STD spectrum because their signals canceled out. A reference experiment proved the absence of unspecific interactions between the solid support and the components of the library.

New developments in combinatorial chemistry allow the production of many compounds in a short time and a great variety,[2] such that the characterization of biological activity in combinatorial libraries has become rate-limiting. Screening experiments based on NMR spectroscopy allow the identification and structural elucidation of substances with binding activity directly from mixtures of compounds.[1,3−5] Therefore, they possess advantages compared with other screening methods such as ELISA, RIA, immunoblotting, or affinity chromatography. NOE pumping,[5a] affinity NMR,[5b,5c] transferred nuclear Overhauser enhancement (tr-NOE)[6−8] and, to an even greater extent, the recently published STD NMR method[1] are highly sensitive because they can utilize a large excess of unlabeled ligands in the presence of relatively small amounts of receptor molecule. There is no need to isolate substances from mixtures because the bioactive component(s) can easily be identified (screening) and, at the same time, the regions of the ligands directly interacting with the receptor can be assigned (epitope mapping).

Intermolecular spin diffusion and saturation transfer difference spectra have long been used for structure elucidation and the study of macromolecule−ligand interactions.[9,10] As a new approach, STD NMR can be used to screen substance libraries for biological activity.[1] Selective saturation of resonances of a receptor protein leads to fast intramolecular magnetization transfer that spreads the saturation efficiently over the entire protein. Intermolecular cross-relaxation transfers saturation from the macromolecular receptor to bound ligands. Saturation is transferred into solution through fast exchange of ligand molecules from the bound to the free state where the saturation-transfer effect is detected (Figure 1). Subtraction of the spectrum with saturation of the protein from another spectrum without saturation yields the final STD NMR spectrum that cancels all resonances but those from species with binding affinity. Neither variations in composition and concentration of library components nor the ratio of free to bound ligand molecules are critical in STD NMR. The difference mode ensures that only nuclei of molecules that were at one time bound to the receptor contribute to the STD spectrum eliminating the risk of detecting false positives. Furthermore, STD NMR can be applied to almost all types of NMR spectra to identify structurally complex components from ligand libraries and to obtain information about the parts of the ligand in direct contact with the receptor. The utility of NMR based screening methods would be greatly enhanced if insoluble receptor proteins in heterogeneous systems could be analyzed. Susceptibility-matched high-resolution magic angle spinning (HR-MAS) probes allow recording of high-resolution NMR spectra in heterogeneous systems.[11−13]

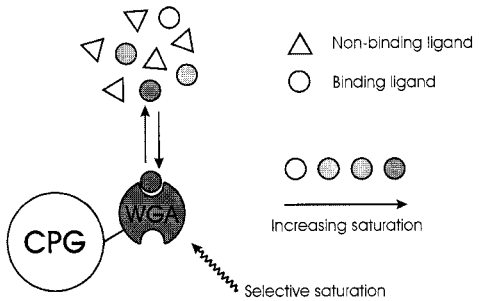


Figure 1. Scheme displaying the saturation transfer between a receptor protein and ligand molecules. Protein resonances are selectively saturated indicated by the shading of the receptor. Resonances of small molecules are not directly affected by the applied selective pulse. Ligand molecules interacting with the receptor (circles) are saturated by intermolecular saturation transfer. A fast exchange equilibrium between receptor bound ligand and the free state allows the detection of these molecules in solution. The degree of saturation of the binding molecules lags behind the one of the receptor and increases gradually as indicated by the different shadings of the binding molecules.

As a model system, we analyzed the binding affinity to wheat germ agglutinin (WGA) coupled to a solid support. WGA is a 36-kDa protein possessing binding sites for oligomers of *N*-acetylglucosamine, with strongest binding of the di- or trisaccharide.[14] WGA was coupled to a variety of solid phases, e.g., agaroses, dextrans, celluloses, controlled pore glasses (CPG), and Toyopearl, of which CPG turned out to have the best performance for detecting binding by HR-MAS methods.

The conventional ^{1}H HR-MAS NMR spectrum of a mixture of 7 oligosaccharides[15] in suspension with WGA coupled to CPG is shown in Figure 2a. It is crowded, and especially the hump region is characterized by severe overlap of resonances such that

* Corresponding author. E-mail: Bernd.Meyer@sgi1.chemie.uni-hamburg.de. Tel: +49 40 42838 5913. Fax: +49 40 42838 2878.

(1) Mayer, M.; Meyer, B. *Angew. Chem.* **1999**, in press.
(2) Gordon, E. M.; Gallop, M. A.; Patel, D. V. *Acc. Chem. Res.* **1996**, *29*, 144−154.
(3) Meyer, B.; Weimar, T.; Peters, T. *Eur. J. Biochem.* **1997**, *246*, 705−709; German Patent 19649359; Intl. Patent pending.
(4) (a) Hajduk, P. J.; Olejniczak, E. T.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 12257−12261; (b) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1537.
(5) (a) Chen, A.; Shapiro, M. J. *J. Am. Chem. Soc.* **1998**, *120*, 10258−10259; (b) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Org. Chem.* **1997**, *62*, 8930−8931; (c) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Am. Chem. Soc.* **1997**, *119*, 5249−5250.
(6) Ni, F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 517−606.
(7) (a) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402−417; (b) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1983**, *53*, 423−442.
(8) Zvi, A.; Feigelson, D. J.; Hayek, Y.; Anglister, J. *Biochemistry* **1997**, *36*, 8619−8627.
(9) Akasaka, K. *J. Magn. Reson.* **1979**, *36*, 135−140.
(10) Reid, D. G.; Salisbury, S. A.; Williams, D. H. *Biochemistry* **1983**, *22*, 1377−1385.
(11) (a) Fitch, W. L.; Detre, G.; Holmes, C. P.; Shoolery, J. N.; Keifer, P. A. *J. Org. Chem.* **1994**, *59*, 7955−7956; (b) Keifer, P. A. *Drugs Future* **1998**, *23*(3), 301−317; (c) Keifer, P. A.; Baltusis, L.; Rice, D. M.; Tymiak, A. A.; Shoolery, J. N. *J. Magn. Reson, Ser. A* **1996**, *119*, 65−75.
(12) Doskocilová, D.; Schneider, B. *Chem. Phys. Lett.* **1970**, *6*(4), 381−384.
(13) Zelaya, F. O.; Crotier, S.; Dodd, S.; McKenna, R.; Dodrell, D. M. *J. Magn. Reson., Ser. A* **1995**, *115*, 131−136.
(14) Wright, C. S.; Kellogg, G. E. *Protein Sci.* **1996**, *5*, 1466−1476.
(15) The oligosaccharide library contained the following: 1.5 μmol of *N,N*′-diacetylchitobiose and 2.1 μmol of the nonbinding sugars maltose, D-lactose, sucrose, cellobiose, trehalose, and D-raffinose.
(16) Habeeb, A. F. S. A.; Cassidy, H. G.; Singer, S. J. *Biochim. Biophys. Acta* **1958**, *29*, 587−593.

10.1021/ja990706x CCC: $18.00
Published on Web 05/22/1999

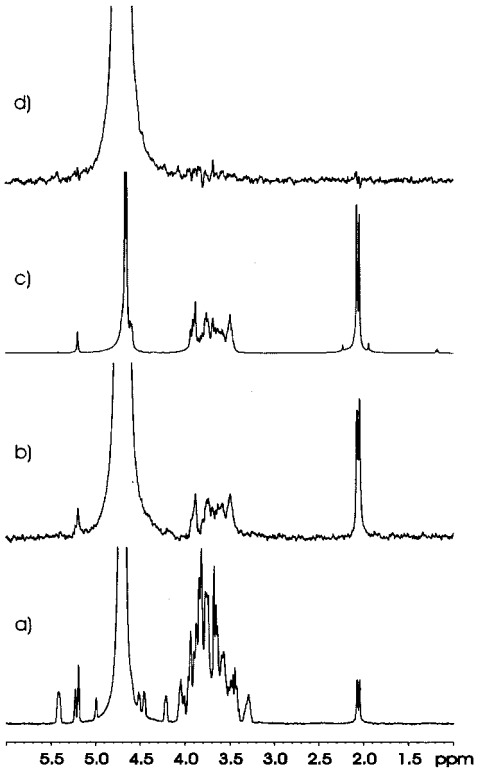
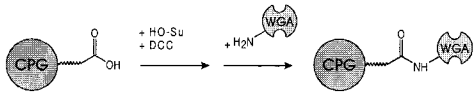


Figure 2. Identification of binding affinity on immobilized WGA by 1D ^{1}H HR-MAS STD NMR. (a) Spectrum of a mixture of seven oligosaccharides in the presence of WGA derivatized succinamidopropyl-CPG beads showing all signals of the components. (b) STD spectrum of a mixture of seven oligosaccharides in the presence of WGA derivatized succinamidopropyl-CPG beads showing the cancellation of all signals but those of *N*,*N*′-diacetylchitobiose. (c) Spectrum of *N*,*N*′-diacetylchitobiose given for reference purposes. (d) STD spectrum of a mixture of seven oligosaccharides in the presence of succinamidopropyl-CPG beads showing that no unspecific binding is present and, therefore, all signals are canceled.

Scheme 1. Scheme showing the coupling of the WGA protein to succinamidopropyl-CPG beads by *N*-hydroxysuccinimide (HO-Su)/*N*,*N*′-dicyclohexylcarbodiimide (DCC) activation.



individual saccharides cannot be identified. In contrast, the STD HR-MAS NMR spectrum (Figure 2b) contains only the resonances of *N*,*N*′-diacetylchitobiose and no signals of the other members of the library. This shows that only resonances from molecules with binding affinity show up in the STD spectrum because they have been partially saturated by intermolecular magnetization transfer from the protein. For comparison, the ^{1}H NMR spectrum of pure *N*,*N*′-diacetylchitobiose is given (Figure 2c). The STD spectrum of the saccharide library with unconjugated CPG is shown (Figure 2d) to prove that unspecific binding does not occur. STD NMR yields fast information about the binding species with one-dimensional spectra taking less than 5 min to record.

To immobilize WGA (Scheme 1), 32 mg (107 μL) of succinamidopropyl-CPG (pore diameter, 100 nm) was succinylated.[16] After washing and drying, the succinamidopropyl-CPG beads were activated using *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide in dioxane.[17] Protein immobilization was achieved by adding a solution of 10 mg of WGA (Vector, USA) dissolved in 0.5 mL of buffer (100 mM sodium bicarbonate, 500 mM sodium chloride, pH 8.5) to the activated beads.[18] The coupling reaction was terminated after 24 h, and the glass beads were washed and remaining activated groups hydrolyzed (1 M glycine solution in 100 mM sodium bicarbonate buffer, pH 7.0, hydrolysis overnight). The concentration of active binding sites was approximately 60 nmol per 100 μL settled solid-phase beads. NMR samples were prepared using 4-mm ZrO_2 HRMAS rotors. Samples contained 95−100 μL of settled CPG. Appropriate amounts of sugar were added to give a 35-fold excess of each nonbinding sugar (2.1 μmol per 100 μL) and a 25-fold excess of the binding sugar (1.5 μmol per 100 μL) compared to the number of active binding sites. Phosphate buffer in D_2O (pD, 7.0) was given to the suspension to yield a 50 mM buffer solution. Spectra were recorded at 306 K and MAS spinning rates of 4000 Hz. To saturate the protein selectively, a total saturation time of 2.04 s was applied, consisting of a pulse train of 40 Gaussian bell shaped selective pulses of 50-ms length, each, separated by a 1-ms delay. A 10-ms spin lock pulse was utilized to remove residual protein resonances. Difference spectra were obtained by internal alternated subtraction with appropriate phase cycling using a frequency list for *on resonance* and *off resonance* irradiation, 4000 and 20 000 Hz, respectively.

The utility of the other solid phases investigated is limited either by a high flexibility of tethers linked to the solid phase which caused broad, intensive lines in some spectra[19] or by unspecific interactions that occurred between ligand molecules and the surface of the bead. In contrast the STD NMR spectra obtained with succinamidopropyl-derivatized CPG were highly reproducible and yielded the correct responses compared with other binding assays. However, even this derivatized CPG reproducibly interacted unspecifically with small molecules of less than about 250 Da, such as monosaccharides. None of the larger molecules such as di- or higher oligosaccharides tested in this work gave evidence for nonspecificity. Therefore, succinamidopropyl-derivatized CPG represents a suitable solid support for investigating receptor−ligand interactions using immobilized proteins, although low molecular mass ligands of the size of monosaccharides cannot be used together with this CPG as solid support. Current work is under way to remove this obstacle.

The application of saturation transfer difference NMR spectroscopy to heterogeneous samples provides an excellent way of obtaining binding information about receptor proteins that cannot be handled in solution either because of solubility problems or a possible change in conformation or binding ability. Furthermore, the conjugation of the protein to an insoluble matrix facilitates the recovery of the receptor from the ligand. Employing this technique to substance libraries offers a convenient, fast, and sensitive method for the identification of biologically active components with the extra benefit of gaining structural information on the bound species and the binding epitope. If more than one ligand is present in the mixture, their relative binding strengths can be assessed semiquantitatively by integrating their signals (to be published). As it was shown, this method is applicable even in the presence of a large excess of nonbinding components. Reference experiments using plain, nonreceptor bound-solid phase must be used to verify true ligand−receptor binding and rule out unspecific binding to the solid phase. In addition, unspecific binding to the protein can be ruled out if a tight-binding ligand is available and added to the mixture to suppress the STD effects. The method is limited to ligands with significantly longer T_2 relaxation times than the immobilized receptor. So far, succinamidopropyl-derivatized controlled pore glass has been found to work best, combining a high capacity for immobilization of receptor protein, mechanical stability required for magic angle spinning experiments, and high inflexibility of side chains to avoid resonances of the solid support in the spectrum with a minimal tendency toward unspecific interaction with molecules of the ligand mixture.

Acknowledgment. Support by the BMBF, Bruker Analytik GmbH, and DFG, Grants SFB 470/B2 and GRK 464, is gratefully acknowledged.
JA990706X

(17) Cuatrecasas, P.; Parikh, I. *J. Biochem.* **1972**, *11*, 2291−2299.
(18) Matsumoto, I.; Mizuno, Y.; Seno, N. *J. Biochem. (Tokyo)* **1979**, *85*, 1091−1098.
(19) Keifer, P. A. *J. Org. Chem.* **1996**, *61*, 1558−1559.