JOURNAL OF MAGNETIC RESONANCE **131,** 336–338 (1998)
ARTICLE NO. MN971376

Isotope-Filtered Affinity NMR

Nina Gonnella,* Mengfen Lin,† Michael J. Shapiro,†,[1] James R. Wareing,‡ and Xiaolu Zhang*

Department of Protein Structure, †Department of Analytics, and ‡Department of Metabolic and Cardiovascular Diseases, Preclinical Research, Novartis Pharmaceuticals Corporation, 556 Morris Avenue, Summit, New Jersey 07901

Received September 24, 1997; revised December 22, 1997

A double-editing pulse sequence has been developed that allows the direct observation of protein binding ligand(s) from a mixture of compounds. This technique should aid the discovery of lead pharmaceutical compounds. The proton NMR signals from protein and the nonbinding ligands are simultaneously eliminated using ^{13}C isotope editing and PFG diffusion-edited NMR. This new experiment is demonstrated using $^{13}C/^{15}N$-labeled stromelysin catalytic domain (SCD). © 1998 Academic Press

Key Words: **Diffusion NMR; affinity NMR; ^{13}C isotope editing; pulse sequence; protein binding.**

The ability to screen multiple compounds simultaneously complements the increased productivity in synthesis obtained from combinatorial chemistry (*1*). Utilization of mixtures of compounds requires a method for determining which molecule in the mixture is responsible for the desired effect (*2*). Methods that identify active components of mixtures without the need for deconvolution could eliminate ''false positives'' and greatly reduce the effort required to analyze mixtures.

An NMR method for identifying synthetic precursors from a library of low molecular weight ligands using ^{15}N-labeled protein has been recently reported (*3*). The binding affinity is determined by the ^{15}N or ^{1}H NMR chemical shift changes in the protein upon the binding of the ligand. The method, which at present is limited to biomolecules of molecular weight around 30 KDa or smaller, promises to be a valuable tool in the drug discovery process.

An alternate method for obtaining binding information would be to look at the ligand instead of the receptor. Recently we have demonstrated a useful technique for addressing molecular recognition termed ''affinity NMR'' (*4*). Molecules that are interacting with the receptor can be differentiated by NMR from noninteracting molecules based on diffusion coefficients. This method is reminiscent of physical separation of mixtures by affinity chromatography. We have shown that the diffusion coefficient of a small molecule bound to a ''receptor'' in solution is significantly different from the small compound alone. Diffusion encoded spectroscopy (DECODES), which involves the use of pulse field gradients (PFG) and TOCSY, simplifies the identification of the interacting molecule (*5*).

A potential advantage of the diffusion NMR method over the chemical shift method is that binding is detected by direct observation of the ligand NMR spectrum and not inferred from changes in the spectrum of the macromolecule. A further advantage of this method is that the identity of the bound ligand can be obtained directly without the need for a deconvolution step. A potential problem with affinity NMR is that the signals of the receptor are always present in the diffusion-edited spectrum and this complication can hamper the interpretation of the data. While DECODES is effective for systems involving small molecular receptors, for larger systems like proteins this method may not be suitable.

In order to extend affinity NMR to protein/ligand interactions, we have developed a new experiment involving isotope (*6*) and diffusion-edited spectroscopy (*7*) to suppress the ^{1}H NMR signals from the protein and also eliminate the signals arising from the nonbinding ligands. The pulse sequence is shown in Fig. 1.

Based on previous studies (*7, 8*) those molecules that have

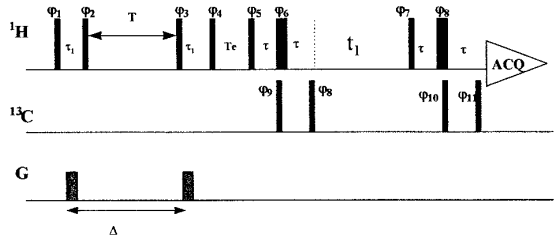


FIG. 1. Pulse scheme of the LED-[F1-C,F2-C] -COSY, where narrow and wide bars represent 90° and 180° flip angles, respectively. $\varphi_1 = 2(x, -x), 2(y, -y), 2(-x, x), 2(-y, y)$; $\varphi_2 = 2(-x), 2(x), 2(-y), 2(y)$; $\varphi_3 = 4(-x), 4(-y), 4(y), 4(-y)$; $\varphi_4 = x, -x, -x, x, y, -y, -y, y, -x, x, x, -x, -y, y, y, -y$; $\varphi_5 = x, y, -x, -y$; $\varphi_6 = 4(y), 4(-y)$; $\varphi_7 = 8(x), 8(y), 8(-x), 8(-y)$; $\varphi_8 = 32(x), 32(-x)$; $\varphi_9 = 16(x), 16(-x)$; $\varphi_{10} = 8(x), 8(-x)$; $\varphi_{11} = x$; $\varphi_{12} = 4(x, -x), 4(-x, x)$. $\tau = 3.4$ ms ($\frac{1}{2}J_{CH}$). Te is the eddy current delay time, Δ is the diffusion delay time, and τ_1 is the decay for eddy current dissipation.

[1] To whom correspondence should be addressed.

1090-7807/98 $25.00

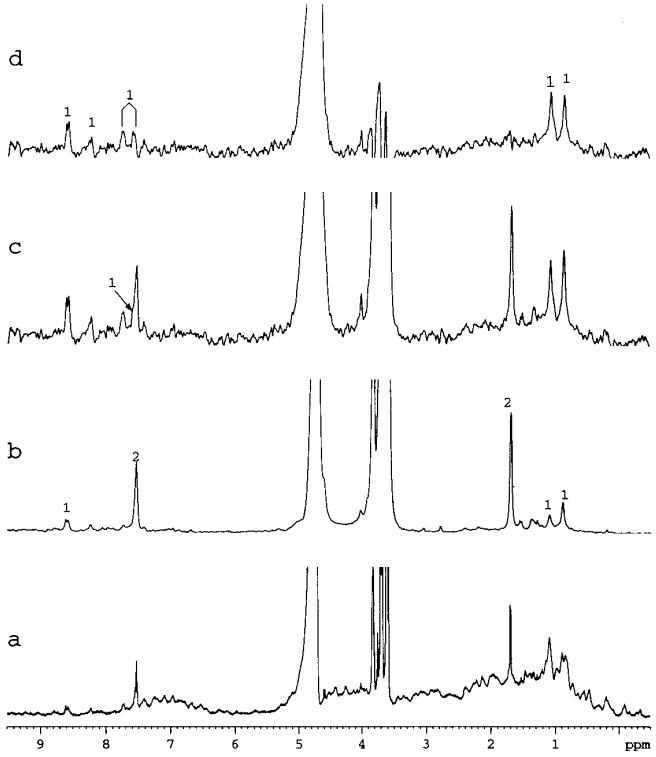
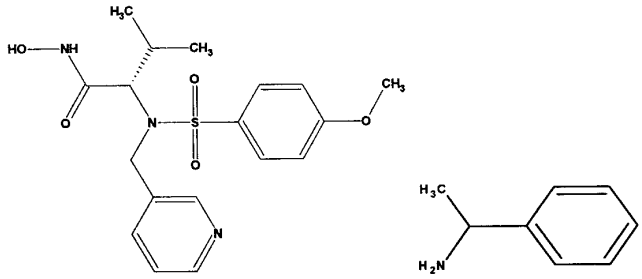


FIG. 2. (a) The ^{1}H NMR spectrum of $^{13}C/^{15}N$-labeled SCD with a 0.3 mM of a known inhibitor **1** and 1 mM of an inert compound **2** in D_2O with presaturation of the HOD signal; (b) the ^{1}H NMR spectrum with ^{13}C-isotope editing and a weak gradient; (c) the ^{1}H NMR spectrum with ^{13}C-isotope editing and a strong gradient; and (d) the difference spectrum (c − b) using the double-editing pulse sequence. Resonances arising from compounds **1** and **2** are indicated.

faster translational diffusion coefficients will be edited out of the spectrum. This concept is demonstrated by using the 1D version of this pulse sequence where the delay time t_1 was 3 μs. The first part of the sequence is the LED sequence for diffusion editing (*8*), the second part is for carbon-13 editing (*6*).

The data are shown in Fig. 2. Spectrum 2a shows the ^{1}H NMR spectrum for $^{13}C/^{15}N$-labeled SCD with a known inhibitor **1** (*9*) having a Ki of 13 nM and an ''inert'' compound **2,**



1 **2**

and Spectrum 2b the ^{1}H NMR spectrum after ^{13}C-isotope editing and using a weak gradient. The latter shows that the pulse sequence adequately removes the signals arising from the labeled protein while signals from the unlabeled substrates are observed. Figure 2c is the ^{1}H NMR spectrum with ^{13}C-isotope editing and using a strong gradient and shows that the signals from **2** decrease at a much faster rate than those of **1.** It can be seen in Fig. 2d that after subtraction of 2c (after scaling the inert signal intensity) from 2b, the double editing pulse sequence affords the direct observation of binding ligand **1.**

As a complementary tool to ''SAR by NMR'', affinity NMR and isotope-edited affinity NMR should be valuable tools in drug discovery.[2] The practical applicability of this and any method involving protein/ligand interactions relies on the ability of the protein to handle the relatively high total concentration of the ligand molecules and the potential problems with nonspecific binding and substrate solubility. We are currently investigating the utility of the affinity NMR method for the screening of larger mixtures in order to identify compounds that bind to biomolecular targets.

ACKNOWLEDGMENTS

The authors acknowledge the help of Jim Koehn and Naveen Pathek for their help with protein purification.

REFERENCES

1. (*a*) A. Furka, F. Sebestyan, M. Asgedom, and G. Dibo, *Int. J. Peptide Protein Res.* **37,** 487 (1991). (*b*) F. Sebestyen, G. Dibo, A. Kovacs, and A. Furka, *Med. Chem. Lett.* **3,** 413 (1993). (*c*) C. T. Dooey, N. N. Chung, B. C. Wilkes, P. W. Schiller, J. M. Bidlack, G. W. Pasternak, and R. A. Houghten, *Science* **266,** 2019 (1994). (*d*) K. Burgess, *J. Med. Chem.* **37,** 2985 (1994). (*d*) E. Erb, K. D. Janda, and S. Brenner, *Proc. Nat. Acad. Sci. USA* **91,** 11,422 (1994). (*e*) D. J. Ecker, T. A. Vickers, R. Hanecak, V. Driver, and K. Anderson, *Nucl. Acids Res.* **21,** 1853 (1993). (*f*) R. Fathi, M. J. Rudolph, R. G. Gentles, R. Patel, E. W. MacMillan, M. S. Reitman, D. Pelham, and A. F. Cook, *J. Org. Chem.* **61,** 5600 (1996).

2. (*a*) R. S. Youngquist, G. R. Fuentes, M. P. Lacey, and T. Keough, *J. Am. Chem. Soc.* **117,** 3900 (1995). (*b*) X. Cheng, R. Chen, J. E. Bruce, B. L. Schwartz, G. A. Anderson, S. A. Ofstadler, D. C. Gale, R. D. Smith, J. Gao, G. B. Sigal, M. Mammen, and G. M. Whitesides, *J. Am. Chem. Soc.* **117,** 8859 (1995). (*c*) Y-H. Chu, Y. M. Dunayevsky, D. P. Kirby, P. Vouros, and B. L. Karger, *J. Am. Chem. Soc.* **118,** 7827 (1996).

[2] All NMR experiments were carried out at 310 K on a Bruker DMX-500 NMR spectrometer equipped with a Acustar II pulse field gradient accessory. PFG-NMR spectra, containing 0.3 mM of protein and 0.3 mM of **1** and 1 mM of **2** in D_2O, were acquired using our modified pulse sequence. The data were collected using 1.0-ms gradient pulses with a gradient strength of 10 G/cm for the weak gradient and 54 G/cm for the strong gradient, and a 0.15-s delay between the two gradient pulses. All spectra were collected with 512 scans and were processed using a 10-Hz exponential decay function.

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

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

5. M. J. Shapiro and M. Lin, *J. Org. Chem.* **61,** 7617 (1996).

6. M. Ikura and A. Bax, *J. Am. Chem. Soc.* **114,** 2433 (1992).

7. M. Lin, M. J. Shapiro, and J. R. Wareing, *J. Org. Chem.* (1997), in press.

8. (*a*) K. F. Morris and C. S. Johnson, Jr., *J. Am. Chem. Soc.* **114,** 3139 (1992). (*b*) K. F. Morris and C. S. Johnson, Jr., *J. Am. Chem. Soc.* **115,** 4291 (1993). (*c*) K. F. Morris, P. Stilbs, and C. S. Johnson, Jr., *Anal. Chem.* **66,** 211 (1994). (*d*) S. J. Gibbs and C. S. Johnson, Jr., *J. Magn. Reson.* **93,** 395 (1991). (*e*) M. Lin, D. A. Jayawickrama, R. A. Rose, J. A. Delvisio, and C. K. Larive, *Anal. Chim. Acta* **307,** 449 (1995). (*f*) P. Stilbs, K. Paulsen, and P. C. Griffiths, *J. Chem. Phys.* **100,** 8180 (1996).

9. (*a*) A. L. Marcy, L. L. Eiberger, R. Harrison, H. K. Chan, N. L. Hutchinson, W. K. Hagmann, P. M. Cameron, D. A. Boulton, J. D. Hermes, *Biochemistry* **30,** 6476 (1991). (*b*) L. J. MacPherson and D. T. Parker, U.S. Patent 5,455,258 (1995). (*c*) N. C. Gonnella, Y. Li, X. Zhang, and C. G. Paris, *Biorg. Med. Chem.* **5,** 2193–2201 (1997).