One-Dimensional Relaxation- and Diffusion-Edited NMR Methods for Screening Compounds That Bind to Macromolecules

Philip J. Hajduk, Edward T. Olejniczak, and Stephen W. Fesik*

Contribution from the Pharmaceutical Discovery Division, D-47G, AP10, Abbott Laboratories, Abbott Park, Illinois 60064

Received May 16, 1997. Revised Manuscript Received September 9, 1997[⊗]

Abstract: Two one-dimensional ^{1}H NMR techniques for efficiently screening libraries of compounds for binding to macromolecules are described that exploit the changes in relaxation or diffusion rates of small molecules which occur upon complex formation. The techniques are demonstrated by detecting ligands that bind to the FK506 binding protein and the catalytic domain of stromelysin in the presence of compounds that do not bind to these proteins. These one-dimensional methods detect complex formation between a ligand and a macromolecule and thus eliminate false positives often observed with other techniques. In addition, since these methods monitor signals of the uncomplexed compound rather than the bound ligand or macromolecule, ligands for macromolecules of unlimited size can be detected. Furthermore, active compounds can be directly identified from a mixture, significantly reducing the time and material needed for screening large libraries of compounds.

Introduction

A critical aspect of the drug discovery process is the ability to reliably detect and identify small molecules which bind to macromolecular targets. Current high-throughput screening strategies have enabled the testing of large chemical libraries and have been very effective at identifying useful lead compounds. In some cases, however, the molecules identified in these screens do not bind to the target of interest. Instead, they may trigger the assay due to an artifact of the method of detection (e.g., color or fluorescence) or by binding to other components of the assay system. In addition, compounds are usually tested as mixtures to efficiently screen large numbers of molecules. This approach is especially important for screening the large libraries produced by combinatorial chemistry. The screening of mixtures requires the subsequent identification of the active component, which can be difficult and time consuming. Methods for directly identifying compounds that bind to macromolecules in the presence of a mixture of nonbinding compounds could significantly reduce the number of "false positives" and eliminate the need for deconvoluting active mixtures.

NMR is an excellent method for identifying compounds that bind to macromolecules and could potentially be used to screen large libraries of compounds. Many NMR-sensitive parameters change upon complex formation, such as chemical shifts, relaxation rates, and diffusion rates.[1] Monitoring the amide chemical shift changes of ^{15}N-labeled proteins has become an important tool for characterizing the interactions between proteins and a wide variety of ligands, including nucleic acids,[2] peptides,[3] and small molecules.[4] In fact, we recently described a method for identifying small molecules that bind to proximal subsites on a protein by observing the amide chemical shift changes of a protein which occur upon ligand binding.[5] These compounds, which bind weakly to the protein, are subsequently linked together to produce high affinity ligands. By using this method (called SAR by NMR), high affinity ligands for the FK506 binding protein[5] and the catalytic domain of the matrix metalloproteinase stromelysin[6] have been rapidly discovered. However, the application of this technique in its current form is limited to small proteins that can be ^{15}N-labeled and obtained in large quantities.

Relaxation and diffusion rates have also been widely used in the study of molecular recognition, and a variety of protein−protein and protein−ligand complexes have been investigated with these techniques.[7,8] In addition, the NMR signals of small molecules have been selectively observed[9] or suppressed[10,11]

* To whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.

(1) Otting, G. *Curr. Opin. Struct. Biol.* **1993**, *3*, 760−768.

(2) (a) Görlach, M.; Wittekind, M.; Beckman, R. A.; Mueller, L.; Dreyfuss, G. *EMBO J.* **1992**, *11*, 3289−3295. (b) Ramesh, V.; Frederick, R. O.; Syed, S. E. H.; Gibson, C. F.; Yang, J.-C.; Roberts, G. C. K. *Eur. J. Biochem.* **1994**, *225*, 601−608.

(3) (a) Hensman, M.; Booker, G. W.; Panayotou, G.; Boyd, J.; Linacre, J.; Waterfield, M.; Campbell, I. *Protein Sci.* **1994**, *3*, 1020−1030. (b) Emerson, S. D.; Madison, V. S.; Palermo, R. E.; Waugh, D. S.; Scheffler, J. E.; Tsao, K.-L., Kiefer, S. E.; Liu, S. P.; Fry, D. C. *Biochemistry* **1995**, *34*, 6911−6918. (c) Swanson, R. V.; Lowry, D. F.; Matsumura, P.; McEvoy, M. M.; Simon, M. I.; Dahlquist, F. W. *Nature Struct. Biol.* **1995**, *2*, 906−910.

(4) (a) Kallen, J.; Spitzfaden, C.; Zurini, M. G. M.; Wider, G.; Widmer, H.; Wüthrich, K.; Walkinshaw, M. D. *Nature* **1991**, *353*, 276−279. (b) Zheng, J.; Cahill, S. M.; Lemmon, M. A.; Fushman, D.; Schlessinger, J.; Cowburn, D. *J. Mol. Biol.* **1996**, *255*, 14−21.

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

(6) 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.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 5818−5827.

(7) (a) Gibbs, S. J.; Johnson, C. S. *J. Magn. Reson.* **1991**, *93*, 395−402. (b) Altieri, A. S.; Hinton, D. P.; Byrd, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 7566−7567.

(8) (a) Wemmer, D.; Williams, P. G. *Meth. Enzymol.* **1994**, *239*, 739−767. (b) London, R. E. *J. Magn. Reson. Ser. A* **1993**, *104*, 190−196. (c) Fejzo, J.; Lepre, C. A.; Peng, J. W.; Su, M. S.-S.; Thomson, J. A.; Moore, J. M. *Protein Sci.* **1996**, *5*, 1917−1921. (d) Bertini, I.; Luchinat, C.; Pierattelli, R.; Vila, A. J. *Eur. J. Biochem.* **1992**, *208*, 607−615. (e) Bertini, I.; Luchinat, C.; Pierattelli, R.; Vila, A. *Inorg. Chem.* **1992**, *31*, 3975−3979. (f) Chen, A.; Wu, D.; Johnson, C. S., Jr. *J. Phys. Chem.* **1995**, *99*, 828−834. (g) Lennon, A. J.; Scott, N. R.; Chapman, B. E.; Kuchel, P. W. *Biophys. J.* **1994**, *67*, 2096−2109. (h) Liu, M.; Nicholson, J. K.; Lindon, J. C. *Anal. Chem.* **1996**, *68*, 3370−3376.

(9) Rabenstein, D. L.; Nakashima, T.; Bigam, G. *J. Magn. Reson.* **1979**, *34*, 669−674.

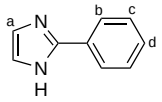
S0002-7863(97)01596-5 CCC: $14.00

in the presence of macromolecules by exploiting differences in their relaxation or diffusion rates. Even very small differences in diffusion rates can be exploited, as has recently been demonstrated in the analysis of small molecule interactions in organic solvents.[12] However, no relaxation- or diffusion-based strategies have been described for efficiently screening large libraries of compounds for binding to macromolecules.

Here we describe two NMR-based methods for screening compounds that bind to proteins or other macromolecules. The methods exploit the changes in either the relaxation rates or diffusion rates of a small compound which occur upon binding to the biomolecule and thus eliminate the need for isotopically labeled targets. Due to the method of detection and the lack of components in the mixture often required by other assays, the identification of false positives caused by mechanisms unrelated to ligand binding are minimized. Furthermore, since these methods rely on detecting the NMR signals of uncomplexed molecules rather than bound ligands or macromolecules, the techniques are applicable even to very large systems.

Results and Discussion

Relaxation-Edited Detection of Ligand Binding. The ability to identify compounds that bind to macromolecules by using a one-dimensional relaxation-edited approach is demonstrated with the FK506 binding protein (FKBP).[13] A mixture of nine compounds was prepared containing 2-phenylimidazole (**1**), which binds to FKBP with an affinity of 200 μM,[5] and

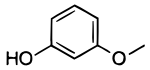
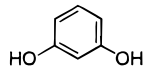
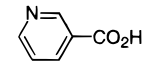
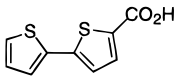
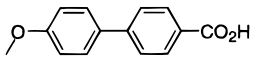
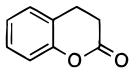
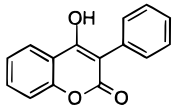
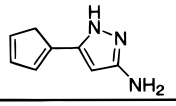


1

eight compounds (**3**−**10**, Table 1) that do not bind to the protein. Since the relaxation-edited approach exploits the rapid relaxation of the macromolecule and bound ligands, a CPMG spin-lock[14] time was needed that would reduce or eliminate the signals of the protein and bound ligands, without significantly affecting the signals of unbound molecules. Figure 1 depicts a plot of protein and free ligand signal intensity as a function of spin-lock time. From these data, average proton T_2 relaxation times of 0.04 and 2.1 s were obtained for FKBP and **1**, respectively. Thus, for a spin-lock time of 400 ms, greater than 99% of the protein signal intensity was eliminated, whereas the ^{1}H NMR signals corresponding to **1** were only reduced by 17%.

To detect ligand binding with this approach, several relaxation-edited ^{1}H NMR spectra were acquired with a CPMG spin-lock time of 400 ms. First, a relaxation-edited spectrum of the test compounds in the absence of FKBP was obtained (Figure 2A). The signals corresponding to all of the compounds in the mixture (**1**, **3**−**10**) appear in this spectrum. Next, relaxation-edited spectra of FKBP alone and the test compounds in the presence of FKBP were obtained and subtracted to produce a spectrum (Figure 2B) that only contains the resonances of those compounds whose transverse relaxation rates were not reduced in the presence of FKBP.[15] A comparison of the spectra in parts A and B of Figure 2 indicates that ligand signals have disappeared in the presence of the protein, which are clearly visualized by subtracting these two spectra (Figure 2C).[16] From this difference spectrum, the compound that binds to FKBP can be readily identified from an analysis of the chemical shifts which correspond to those of the free molecule (see reference

Table 1. Inactive Compounds[a] Tested Against FKBP and Stromelysin

Compound No.	Structure
3	
4	
5	
6	
7	
8	
9	
10	

[a] Compounds were considered inactive based on the absence of amide chemical shift changes of ^{15}N-labeled protein (using ^{15}N-HSQC spectra) upon addition of ligand up to concentrations of 1 mM.

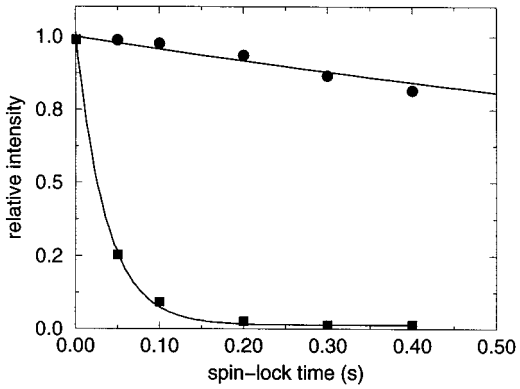


Figure 1. Intensity decay rates of signals from FKBP (filled squares) and **1** (filled circles) in the absence of protein. Data points are an average of all signals of **1** and five signals from FKBP. The solid curves are best fit simulations of the data with use of the relationship $M(t) = \exp(-t/T_2)$.

(10) (a) Larive, C. K.; Rabenstein, D. L. *Magn. Reson. Chem*. **1991**, *29*, 409−417. (b) Rabenstein, D. L.; Isab, A. A. *J. Magn. Reson*. **1979**, *36*, 281−286.

(11) Ponstingl, H.; Otting, G. *J. Bio. NMR* **1997**, *9*, 441−444.

(12) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Am. Chem. Soc*. **1997**, *119*, 5249−5250.

(13) Liu, J.; Farmer, J. D., Jr.; Lane, S.; Friedman, J.; Weismann, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807.

(14) Meiboom, S.; Gill, D. *Rev. Sci. Instrum*. **1958**, *29*, 688.

(15) Although the overall signal intensity of FKBP was reduced by greater than 99% in the relaxation-edited spectrum, a few residual peaks remained. Thus, a relaxation-edited spectrum of FKBP alone was subtracted from the relaxation-edited spectrum of the compounds in the presence of the protein.

(16) Narrow Lorenztian line shapes (as expected for low molecular weight compounds) can often give rise to dispersion signals in difference spectra. For the compounds tested against FKBP, Gaussian apodization of the free induction decay essentially eliminated the dispersion signals in the difference spectra, resulting in clearly identifiable resonances for the active compound. However, as a result of the line broadening caused by the apodization, the spin−spin splittings for these compounds were not observed.

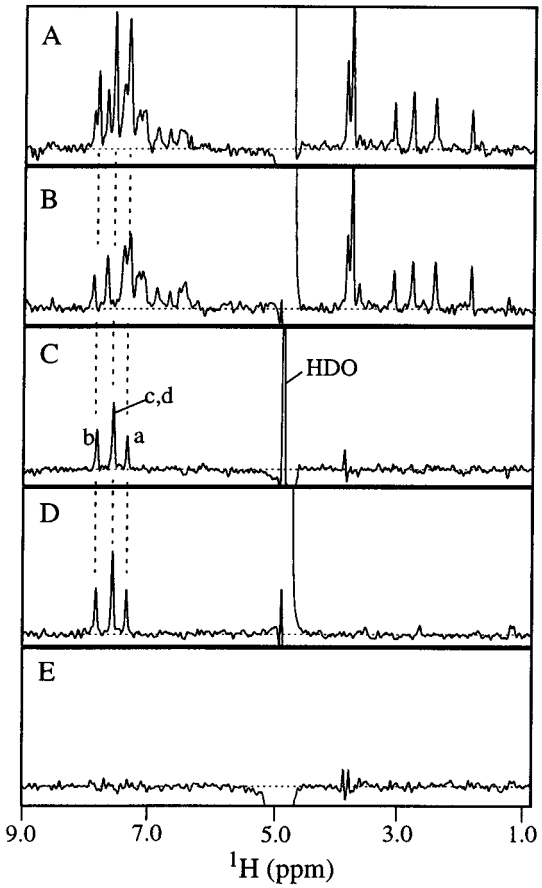


Figure 2. Analysis of ligand binding to FKBP with use of a relaxation-edited approach. (A) Relaxation-edited ^{1}H NMR spectrum of a mixture of nine compounds (**1**, **3**−**10**) in the absence of FKBP. (B) Relaxation-edited ^{1}H NMR spectrum of a mixture of nine compounds (**1**, **3**−**10**) in the presence of FKBP after correcting for residual protein signals by subtracting an analogous spectrum of FKBP alone. (C) A difference spectrum obtained by subtracting the spectrum in B from A. The resonances of **1** occur at 7.89 (b), 7.63 (overlap of c and d), and 7.41 ppm (a) in the absence of protein and are indicated by the vertical dashed lines. (D) A reference spectrum of **1** alone. (E) A difference spectrum obtained in an analogous fashion to the spectrum shown in C, but on a mixture of eight compounds (**3**−**10**) which do not bind to FKBP. All relaxation-edited spectra utilized a CPMG spin-lock time of 400 ms.

spectrum, Figure 2D).[17] Figure 2E depicts a control experiment performed on a separate mixture containing only compounds **3**−**10**, which do not bind to FKBP. In this spectrum, no ligand resonances are observed.

By using the relaxation-edited approach, a wide range of ligand affinities can be monitored. Knowing the approximate values for the transverse relaxation rates of the uncomplexed compounds and the macromolecule, the length of the spin-lock time can be selected to optimize the NMR experiments for detecting ligand binding within a range of target affinities. Short spin-lock times will preferentially detect high-affinity ligands, while longer spin-lock times can be used to include the detection of weaker binding ligands. An optimal screening strategy would employ multiple spin-lock times for each sample to allow the detection of both high and low affinity ligands.

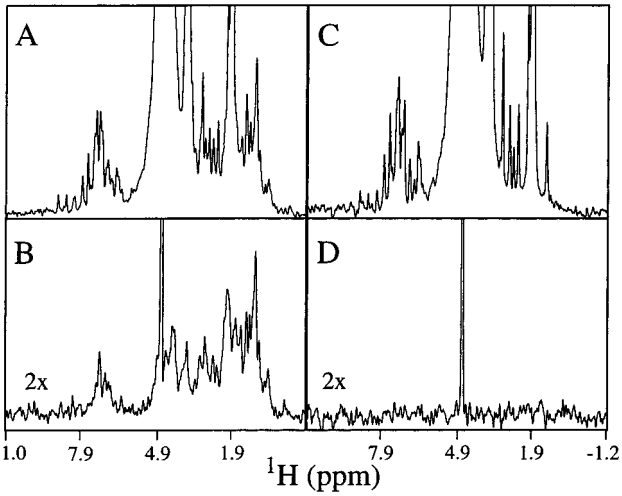
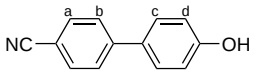


Figure 3. PFG-STE spectra of (A, B) stromelysin and a mixture of compounds **3**−**10** and (C, D) compounds **3**−**10** in the absence of protein. The spectra shown in parts A and C were acquired by using gradient strengths of 1.2 G/cm, while those shown in parts B and D were acquired by using gradient strengths of 48 G/cm and have been scaled by a factor of 2.

Diffusion-Edited Detection of Ligand Binding. The utility of diffusion editing is illustrated in the identification of small molecules that interact with the catalytic domain of the matrix metalloproteinase stromelysin.[18] For these experiments, a mixture of nine compounds was prepared containing 4-cyano-4′-hydroxybiphenyl (**2**) and eight compounds (**3**−**10**, Table 1)



2

which do not bind to the protein. In the presence of aceto-hydroxamic acid, **2** binds to stromelysin with a dissociation constant of approximately 20 μM.[19] Since these experiments employed a pulsed field gradient stimulated echo (PFG-STE) sequence,[7] appropriate gradient strengths were determined which could discriminate between the uncomplexed test compounds and the protein. At a low gradient strength of 1.2 G/cm, the NMR signals of the protein and the compounds are observed (Figure 3, spectra A and C). However, at a high gradient strength of 48 G/cm, the signals from the free compounds are completely eliminated (Figure 3D), while those from the protein are only reduced by 50% (Figure 3B).

To identify compounds that bind to stromelysin from a mixture of nonbinding compounds with use of a diffusion-edited approach, several PFG-STE spectra were recorded and analyzed. First, a PFG-STE spectrum of the test compounds in the absence of protein (Figure 4A) was acquired at a low gradient strength (1.2 G/cm). At this gradient strength, the resonances from all of the compounds (**2**−**10**) are observed. Next, PFG-STE spectra of the test compounds in the presence of stromelysin were obtained at low and high gradient strengths (analogous to the spectra shown in parts A and B of Figure 3) and subtracted to produce a spectrum (Figure 4B) that only contains the signals of compounds that do not bind to the protein.[17] By subtracting the spectra shown in parts A and B of Figure 4,[16] the ^{1}H NMR

(17) The spin-lock times and gradient strengths used in the examples presented here resulted in complete elimination of the bound ligand signals. Hence, only positive peaks appear in the final difference spectra at the chemical shifts of the uncomplexed ligand (Figures 2C and 4C). Incomplete reduction of the bound ligand signals will give rise to negative peaks in the difference spectrum at the chemical shifts of the ligand in the presence of the protein.

(18) Beckett, R. P.; Davidson, A. H.; Drummond, A. H.; Huxley, P.; Whittaker, M. *Drug Discovery Today* **1996**, *1*, 16−26.

(19) Acetohydroxamic acid and **2** bind to stromelysin in a cooperative fashion, with the hydroxamate chelating the catalytic zinc and **2** occupying the S1′ subsite (see ref 6). This illustrates the fact that these relaxation- and diffusion-edited methods can be used to detect ligand binding to macromolecules even in the presence of ligands which bind to other sites.

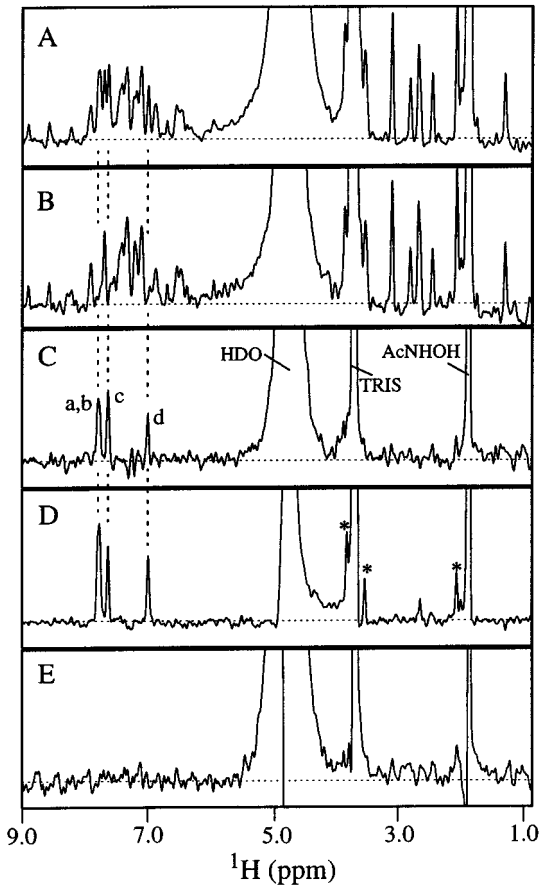


Figure 4. Analysis of ligand binding to the catalytic domain of stromelysin by using a diffusion-edited approach. (A) PFG-STE spectrum of a mixture of nine compounds (**2**−**10**) in the absence of stromelysin with use of low gradient strengths. (B) PFG-STE spectrum of a mixture of nine compounds (**2**−**10**) in the presence of stromelysin with use of a low-gradient strength, after removal of protein signals by subtracting a PFG-STE spectrum of the same sample obtained at high gradient strengths. (C) A difference spectrum obtained by subtracting the spectrum in B from that in A. The resonances of **2** occur at 7.84 (overlap of a and b), 7.70 (c), and 7.06 ppm (d) in the absence of protein and are indicated by the vertical dashed lines. The signals from TRIS (3.74 ppm) and acetohydroxamic acid (AcNHOH, 1.94 ppm) were significantly attenuated in the difference spectrum, but not eliminated. (D) A reference spectrum of **2** alone. Signals arising from impurities in the buffer are indicated with an asterisk (*) and do not appear in the difference spectrum (Figure 2C) since they do not bind to the protein. (E) A difference spectrum obtained in an analogous fashion to the spectrum shown in C, but on a mixture of eight compounds (**3**−**10**) which do not bind to stromelysin.

signals of the compound that binds to stromelysin are readily identified from an analysis of the chemical shifts which correspond to those of the free molecule (see reference spectrum, Figure 4D). No ligand signals were observed in a control experiment performed on a separate mixture containing only compounds **3**−**10**, which do not bind to stromelysin (Figure 4E).

Conclusions

The one-dimensional relaxation- and diffusion-edited strategies described here have several advantages over conventional screening methods and other NMR-based approaches for identifying ligands for macromolecules. Since ligand binding is directly detected, the observation of false positives is minimized. In addition, due to the capability of the methods to identify ligands within a mixture, the need for deconvolution of mixtures to identify active components is eliminated. Although a recently described diffusion-based method has been used to identify a small compound in a mixture that binds to another small molecule dissolved in an organic solvent,[12] this approach is not readily applicable to large biomolecules, where the resonances of the bound ligand may be broadened or obscured by those of the macromolecular target. The one-dimensional screening methods described here overcome these limitations by removing the signals of the biomolecule and selectively observing only the signals of the uncomplexed ligands. These methods also have advantages over current NMR-based screening strategies which rely on observing the ^{15}N/^{1}H amide chemical shift changes that occur upon ligand binding. Since only the signals from the ligands are observed, the need for isotope labeling of the bimolecule is eliminated. In addition, unlike methods which require well-resolved, observable resonances of the macromolecule, the one-dimensional techniques can be applied to very large biomolecules. In fact, larger target molecules should improve the methodology, since the differences in relaxation and diffusion rates between the free and bound ligand will be even greater. Furthermore, less protein is required for the one-dimensional approaches versus the previously described methods that require the acquisition of heteronuclear correlation spectra. In the examples presented, only 50−100 μM protein was required, while concentrations of more than 300−500 μM are typically used in 2D ^{15}N-HSQC experiments.[20] Although the one-dimensional relaxation- and diffusion-edited strategies are not able to identify ligand binding sites, they are extremely useful for rapidly identifying compounds that bind to macromolecules and should thus extend the utility and applicability of NMR as a tool in drug research.

Experimental Section

All spectra were recorded at 298 K on a Bruker DMX 500 MHz NMR spectrometer. Compounds **1**−**9** (Aldrich) and **10** (Lancaster) were used as obtained without further purification. The samples for FKBP contained 50 μM protein and 50 μM of each ligand in a 95% D_2O buffered solution[21] (50 mM PO_4, 100 mM NaCl, pH 6.5). The samples for the catalytic domain of stromelysin contained 100 μM protein and 100 μM of each ligand in a 95% D_2O buffered solution[21] (5 mM TRIS, 20 mM $CaCl_2$, 5 mM acetohydroxamic acid, pH 7.0). FKBP and stromelysin were cloned, expressed, and purified as previously described.[6,22]

Relaxation-Edited NMR Experiments. The NMR experiments utilized a $[D-90_x-(\Delta-180_y-\Delta)_n-P_y-\text{acquire}]$ pulse sequence, where $D = 1.8$ s (preacquisition delay), $\Delta = 2$ ms, $n = 100$ (for a total spin-lock time of 400 ms), and $P_y = 100$ μs (purge pulse). The total recycle time was 2.7 s. The data were collected with a sweep width of 8333.3 Hz and 256 scans. Processing was performed with in-house written software with a Gaussian apodization function (3 Hz line-broadening, Gaussian shift 0.01) over 2048 complex points and zero-filling to 4096 complex points before Fourier transformation. Spectral differencing was performed manually to allow for small variations in the volume

(20) The low concentrations of protein used in the one-dimensional experiments significantly reduce potential chemical shift changes of the compounds which may occur in the presence of protein. This is important to minimize subtraction artifacts in the difference spectra.

(21) The experiments on FKBP and stromelysin were performed in 95% D_2O buffer. While the water signal is suppressed in PFG-STE spectra acquired at high gradient strengths, alternate suppression strategies are required for the relaxation-edited spectra and the PFG-STE spectra acquired at low gradient strengths. Several methodologies exist for suppression of the solvent signal, including presaturation and water flip-back methods. However, for the purposes of subtracting spectra acquired on dilute solutions, we found that a high level of D_2O was the most robust approach for removal of the water signal.

(22) Meadows, R. P.; Nettesheim, D. G.; Xu, R. X.; Olejniczak, E. T.; Petros, A. M.; Holzman, T. F.; Severin, J.; Gubbins, E.; Smith, H.; Fesik, S. W. *Biochemistry* **1993**, *32*, 754−765.

of the protein solution and ligand mixture. Scaling factors ranged from 0.93 to 1.0 and were optimized for the removal of both residual protein signals and the signals from the mixture of compounds in the absence and presence of protein.

Diffusion-Edited NMR Experiments. The pulse sequence used for diffusion-edited detection of bound ligands was the LED sequence[7a] with gradient lengths of 1 ms, gradient recovery times of 500 ms, a diffusion delay time of 400 ms, and a longitudinal eddy-current delay (LED) of 60 ms. A three-pulse train was employed prior to the LED sequence to equalize the total field gradients during the two transverse evolution periods.[7a] A pre-acquisition delay of 1 s was used and the total recycle time was 2.6 s. Data were collected with gradient strengths of 1.2 (256 scans) and 48 G/cm (512 scans) to differentiate between free and bound ligand signals. Additional scans were acquired for the NMR experiments employing higher gradient strengths because of the inherently lower signal to noise. Processing was performed with in-house written software with a Gaussian apodization function (3 Hz line-broadening, Gaussian shift 0.01) over 2048 complex points and zero-filling to 4096 complex points before Fourier transformation. Spectral differencing was performed manually. For the removal of protein and bound ligand signals, scaling factors ranging from 0.63 to 0.87 were optimized for the removal of protein signals. For the subtraction of signals from the mixture of compounds in the absence and presence of protein, scaling factors ranging from 0.95 to 0.98 were used (after removal of protein signals).

Acknowledgment. We would like to thank Lora Tucker-Garcia and William Howe for help in preparation of the samples and Dr. Heng Liang for helpful discussions.

JA9715962