Expedited Articles

High-Throughput Nuclear Magnetic Resonance-Based Screening

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A high-throughput screening strategy is described that involves the acquisition of two-dimensional $^{15}N/^{1}H$ correlation spectra in less than 10 min on 50 μM protein samples using cryogenic NMR probe technology. By screening at these concentrations, small organic molecules can be tested in mixtures of 100, which dramatically increases the throughput of the NMR-based assay. Using this strategy, libraries of more than 200 000 compounds can be tested in less than 1 month. There are many advantages of high-throughput NMR-based screening compared to conventional assays, such as the ability to identify high-affinity ligands for protein targets with no known function. This suggests that the method will be extremely useful for screening the large number of targets derived from genomics research.

Introduction

A critical aspect of the drug discovery process is the identification of potent lead compounds that can be optimized through medicinal chemistry and/or structure-based design approaches. Although high-throughput screening of large libraries of compounds can yield suitable leads, these assays often require extensive development. Furthermore, false positives can result from artifacts of the detection method (e.g., colorimetric or fluorimetric detection) or from compounds which interact with other components of the assay system rather than the target of interest. The ideal assay system would be simple to implement, composed of only a few components, amenable to all classes of molecules, and capable of rapidly screening large libraries of compounds.

NMR-based screening in which changes in the $^{15}N/^{1}H$ amide chemical shifts are detected upon the addition of ligand has led to the discovery of potent lead compounds for several protein targets[1−3] using a technique called SAR by NMR. This method was successful even in cases where conventional screening methods have failed to produce leads.[2,3] The key to the success of SAR by NMR is the simplicity of the NMR-based assay (involving only the protein and test compound), the elimination of background signals (due to the ^{15}N spectral editing), and the ability to differentiate different binding sites on the protein surface. However, the technique is limited by the low sensitivity of the NMR experiment. High protein concentrations (>0.3 mM) must be used in order to acquire $^{1}H/^{15}N$ HSQC spectra in a reasonable period of time (10−30 min). In addition, because changes in the protein signals are detected, the test compounds must also be screened at these concentrations. This limits the number of compounds that can be tested in each mixture, since it is critical to keep the total concentration of added molecules at a reasonable level (∼5−10 mM). In earlier studies, compounds were screened at a concentration of 1 mM each in mixtures of 10, allowing a maximum throughput of 1000 compounds/day. In addition to solubility considerations of the individual compounds at these concentrations, this low screening rate precludes using SAR by NMR as a high-throughput screening method.

One approach to increasing compound throughput is to increase the number of test compounds in each mixture. In this case, the small molecule concentration and thus the protein concentration must be decreased while maintaining rapid data collection. Unfortunately, lower protein concentrations would significantly increase the time required for the acquisition of each $^{1}H/^{15}N$ HSQC spectrum, offsetting the gains in throughput that can be achieved using larger mixture sizes. Here we describe a strategy which overcomes these limitations and allows for the rapid NMR-based screening of very large libraries of compounds. This is made possible by the dramatic improvements in signal-to-noise that can be achieved using recent advances in cryogenic NMR probe technology.

Results and Discussion

New cryogenic NMR probe technology, in which the preamplifier and radio frequency coils of the probe are cooled to low temperatures,[4−6] can significantly increase the signal-to-noise in NMR spectra. Using these probes, called CryoProbes,[6] suitable $^{1}H/^{15}N$ HSQC spectra can now be rapidly obtained on protein samples as low as 50 μM, as shown in Figure 1A. This is not possible using

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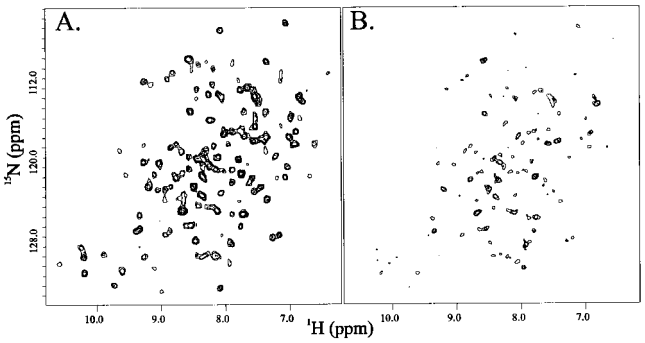

Figure 1. Sensitivity-enhanced ^{1}H/^{15}N HSQC spectra[13] acquired in 10 min on a 50 μM sample of the catalytic domain of stromelysin using (A) a dual (^{15}N/^{1}H) inverse CryoProbe (Bruker) equipped with a lock and a *z*-gradient and (B) a conventional triple-resonance (^{13}C/^{15}N/^{1}H) inverse probe (Nalorac). Based on peak volumes, an average gain in signal-to-noise of 2.4 was realized with the CryoProbe as compared to the conventional TXI probe.

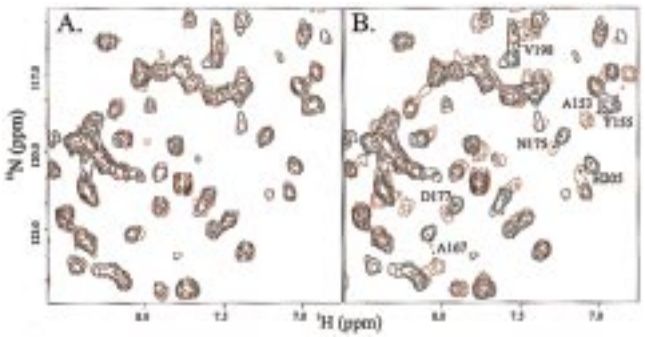

Figure 2. Expansions of ^{1}H/^{15}N HSQC spectra using a CryoProbe on 50 μM samples of stromelysin (A) in the absence (black contours) and presence (red contours) of a mixture of 100 known nonbinders and (B) in the absence (black contours) and presence (red contours) of a mixture of 100 known nonbinders plus 3-[4-(4-cyanophenyl)phenoxy]propanohydroxamic acid.[2] Compounds were tested at a concentration of 50 μM each.

a conventional probe (Figure 1B). As a result of this new technology, compounds can now be screened at a concentration of 50 μM each in mixtures of 100, keeping the total concentration of the added molecules at a practical level (5 mM) while increasing the throughput of the assay by 10-fold. The ability to apply NMR-based screening using low protein concentrations and mixtures of 100 compounds is demonstrated using the catalytic domain of stromelysin.[2] Upon the addition of a mixture of 100 compounds known not to bind to stromelysin, no chemical shift changes are observed relative to a reference spectrum (Figure 2A). However, upon the addition of a mixture of these same 100 compounds but now containing 3-[4-(4-cyanophenyl)phenoxy]propanohydroxamic acid (a known inhibitor of stromelysin),[2] large, unambiguous chemical shift changes are observed (Figure 2B). These spectra demonstrate that large mixtures of compounds containing an active compound can be reliably distinguished from a mixture of inactive compounds.[7]

As a result of lowering the concentration of the protein and test compound, the stringency of the NMR-based assay also changes dramatically. Figure 3 compares the stringency levels for NMR-based screening at protein/ligand concentrations of 0.5 and 0.05 mM. For ligands

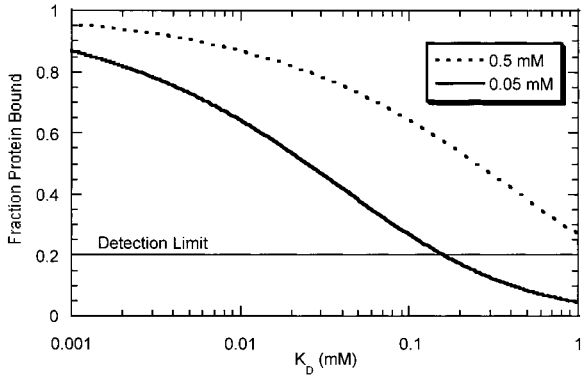

Figure 3. Theoretical occupancy levels of a target protein as a function of ligand K_D under the conditions of 0.5 (thick dotted line) and 0.05 (thick solid line) mM ligand and protein. The detection limit for ligands in fast exchange[9] (thin solid line) is set at 20% of full occupancy.

in fast exchange with the protein,[8] chemical shift changes corresponding to approximately 20% of the protein in the bound form can be observed. Therefore, by screening at a protein and ligand concentration of 0.5 mM, ligands with dissociation constants in the millimolar range can be readily detected. While this is suitable for screening libraries composed of small molecular fragments that exhibit very weak binding, this stringency level is not appropriate for high-throughput screening of large libraries due to the large numbers of hits that would be obtained and require deconvolution. However, by reducing the protein and ligand concentrations to 0.05 mM, only ligands with dissociation constants better then approximately 0.15 mM will give rise to observable chemical shift changes. Thus, the hit rate for large libraries is reduced to levels that will not require extensive deconvolution of mixtures.

Conclusions

Using this screening strategy, more than 10 000 compounds can be screened in a single day. Taking into consideration the time required for hit deconvolution,[7] this indicates that libraries of more than 200 000 compounds can be screened in less than a month. Although one-dimensional proton NMR methods have been reported[9−12] which could potentially reduce the time required for screening even further, we have found that compound insolubility, broad NMR line widths of the ligand, and nonspecific chemical shift changes in the ligand resonances upon the addition of target reduce the reliability and usefulness of these one-dimensional techniques.

The method described here is limited to moderately small (<40 kDa) proteins that can be ^{15}N-labeled. However, there are many advantages of high-throughput NMR-based screening compared to conventional assays. Due to the ^{15}N spectral editing, no background signals from the test compounds are observed, resulting in the reliable and robust detection of ligand binding. In contrast to other methods of detection (e.g., mass spectroscopy, fluorescence, UV, etc.) which may not be amenable to many classes of compounds, virtually any compound can be tested using the NMR-based approach. Another significant advantage of the NMR assay is the ability to determine the binding site location from an analysis of the amide chemical shift changes. This

allows structure−activity relationships to be analyzed for compounds that bind to the same site. In addition, the NMR-based assay is very simple and consists only of the target of interest and the test compounds. This results in an identical assay from target to target, reducing the need to invest time in developing difficult or elaborate functional assays. In fact, unlike many other assays, knowledge of the protein's function is not required. This feature of the method will be especially important for screening potential protein targets derived from the enormous number of DNA sequences now available from genomics research. Even before the function of the protein is known, high-affinity ligands for these potential targets can be discovered by this screening strategy and tested in other systems to aid in target selection. The ability to apply high-throughput NMR-based screening techniques to large compound libraries is therefore expected to greatly facilitate the drug discovery process.

Experimental Section

NMR Experiments. The catalytic domain of stromelysin was expressed and purified as previously described.[2] NMR samples were composed of uniformly ^{15}N-labeled stromelysin at 0.05 mM in an H_2O/D_2O (9:1) solution containing 50 mM Tris, 20 mM $CaCl_2$, pH 7.0. Sensitivity-enhanced ^{1}H/^{15}N HSQC spectra[13] were acquired at 303 K on Bruker DRX500 spectrometers using either a conventional ^{1}H/^{13}C/^{15}N triple resonance probe (Nalorac) or a dual ^{1}H/^{15}N CryoProbe (Bruker). All experiments were acquired with 8 scans, 32 complex points, and a recycle time of approximately 1 s. Linear prediction to 64 complex points was performed in the indirect dimension before Fourier transformation. A mixture of 100 compounds known not to bind to stromelysin was prepared by mixing equal volumes of 100 mM DMSO stocks of the individual compounds. A mixture of these same 100 compounds and the stromelysin inhibitor 3-[4-(4-cyanophenyl)phenoxy]propanohydroxamic acid (IC_{50} = 25 nM)[2] was also prepared. The mixtures were added to the stromelysin samples so that each compound had a final concentration of 0.05 mM.

CryoProbe. The Bruker CryoProbe system is comprised of two main units: the probe assembly, which consists of the cold radio frequency coils, radio frequency circuitry, and preamplifiers for ^{1}H and ^{2}H, and a remotely installed CryoPlatform cooling system. The cooling system is connected to the CryoProbe with a vacuum insulated transfer line. The principal component of the CryoPlatform is a Gifford-McMahon Cryocooler which cools the helium gas in a closed-cycle loop to temperatures of about 20 K. All cooling parameters of the CryoProbe system were permanently stabilized and controlled by dedicated control electronics.

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(7) Active compounds can be identified from the active mixture of 100 by deconvolution first as 10 mixtures of 10 compounds each, followed by the testing of the individual compounds in the active mixture of 10. Thus, an additional 20 experiments must be performed to identify the active ligand from a mixture, requiring approximately 5 h of experimental time. These additional experiments should comprise only a small percentage of the time required for screening.

(8) For ligands in fast exchange on the NMR time scale (dissociation constants typically greater than 20−50 μM), the observed chemical shift will be the population-weighted average of the chemical shifts for the free and bound states. Ligands with significantly better affinities for the protein can exhibit intermediate-exchange (K_D < ∼20−50 μM) or slow-exchange (K_D < ∼1 μM) dynamics, in which cases the protein resonances can broaden or split into discrete signals for the free and bound states. In these regimes, ligand binding is still readily detected by the extensive perturbations of the protein resonances. However, quantitative determination of the dissociation constants by NMR is more difficult, and only estimates can generally be given.

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