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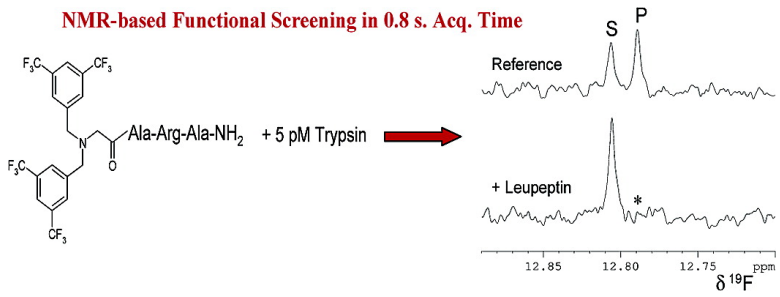

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Sensitivity Improvement in ^{19}F NMR-Based Screening Experiments: Theoretical Considerations and Experimental Applications

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Abstract: NMR-based binding and functional screening performed with FAXS (*f*luorine chemical shift *a*nisotropy and e*x*change for *s*creening) and 3-FABS (*t*hree *f*luorine *a*toms for *b*iochemical *s*creening) represents a potential alternative approach to high-throughput screening for the identification of novel potential drug candidates. The major limitation of this method in its current status is its intrinsic low sensitivity that limits the number of tested compounds. One approach for overcoming this problem is the use of a cryogenically cooled ^{19}F probe that reduces the thermal noise in the receiver circuitry. Sensitivity improvement in the two screening techniques achieved with the novel cryogenic ^{19}F probe technology permits an increased throughput, detection of weaker binders and inhibitors (relevant in a fragment-based lead discovery program), detection of slow binders, and reduction in protein and substrate consumption. These aspects are analyzed with theoretical simulations and experimental quantitative performance evaluation. Application of 3-FABS combined with the cryogenic ^{19}F probe technology to rapid screening at very low enzyme concentrations and the current detection limits reached with this approach are also presented.

Introduction

Over the past decade, since the first application of NMR-based screening to a drug discovery project,[1,2] there have been important technological and technical advances related to this methodology. Several review articles covering the theory and application of NMR-based screening have appeared in the literature.[3−15] Many industries and academic laboratories are investing in this technology for identifying and optimizing novel drug candidates or for performing follow-up screens to confirm high-throughput screening (HTS) hits. NMR-based screening can be used to run binding and functional assays. In addition, it provides accurate measurements of the dissociation binding constant and inhibitory activity of the identified binders and inhibitors, respectively, allowing the construction of a meaningful structure−activity relationship (SAR) table. Two of these experiments, recently introduced—FAXS (*f*luorine chemical shift *a*nisotropy and e*x*change for *s*creening)[16] and 3-FABS (three *f*luorine *a*toms for *b*iochemical *s*creening)[17]—utilize ^{19}F NMR spectroscopy as the detection method. These approaches, as previously explained, have some important advantages when compared to other technologies. Although ^{19}F NMR-based screening scores high in quality because it is less subject to artifacts, it suffers from its intrinsic low sensitivity. It cannot compete with the high sensitivity of fluorescence spectroscopy[18]

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or scintillation proximity assay (SPA),[19] the techniques of choice often used in HTS. To increase the throughput of ^{19}F NMR-based screening, it is therefore necessary to improve the sensitivity of the technique. One proposed approach is the use of substrates and spy molecules with magnetically equivalent multiple fluorine atoms.[20] A similar technique has been used in ^{19}F NMR imaging.[21] A second approach for achieving this goal is the use of cryogenic probe technology[22] optimized to ^{19}F detection. The gain in sensitivity obtained with this technology has other beneficial effects that go beyond the increased throughput. It allows the detection of weaker binders and inhibitors (relevant in a fragment-based lead discovery approach), the identification of slow binders, and a significant reduction in protein and substrate consumption. These aspects are analyzed and discussed in detail with theoretical simulations using realistic parameter sets. Application of 3-FABS with the new cryogenic ^{19}F probe technology to rapid screening at a very low enzyme concentration is presented. A quantitative performance evaluation and the establishment of detection limits reached with this technology are also described.

Results and Discussion

Sensitivity Issue. A thorough analysis of the advantages achieved with a gain in sensitivity in the FAXS and 3-FABS is discussed in the following sections.

FAXS. FAXS[16] is a ligand-based binding competition screening experiment[23−25] that utilizes a weak affinity spy molecule containing a CF_3 or CF moiety and ^{19}F as the nucleus of detection. A library of fluorine-containing molecules that are well characterized, are chemically stable, and have high aqueous solubility is first tested in mixtures against the receptor of interest for the identification of potential spy molecules. The FAXS approach has some distinct advantages. The absence of overlap permits the screening of large chemical mixtures and allows for automated analysis of the spectra. The large chemical shift anisotropy (CSA) of fluorine results in a large difference in line width for the spy molecule in the free and bound states, especially at the high magnetic fields currently used. This phenomenon, combined with the large exchange contribution, allows for the selection of a weak-affinity spy molecule, thus resulting in both a lower binding affinity threshold for the identified NMR hits and a lower protein consumption.

The observed parameter of the spy molecule, typically the ^{19}F signal intensity after a spin−echo filter, is studied in the absence and in the presence of a competing molecule. Sometimes the signal intensity is compared to the signal intensity of a fluorine-containing control molecule present in the solution or to an electronically generated signal of a defined frequency, line width, and amplitude, known also as an ERETIC signal.[26]

As it was previously demonstrated, the fraction of spy molecule bound to the receptor, $[EL]/[L_{TOT}]$ (where [EL] and $[L_{TOT}]$ are the bound and total spy molecule concentration, respectively), in the presence of a competing molecule can be calculated from a graph constructed with titration experiments performed at different protein concentrations and knowing a priori the dissociation binding constant of the spy molecule. A measurement performed at a single competing molecule concentration allows the determination of the binding constant of the identified hit. A more complete analysis requires the acquisition of several experimental points at different competing molecule concentrations.

The fraction of spy molecule bound to the receptor, $[EL]/[L_{TOT}]$, in the presence of a competing molecule is then given by the equation[27−29]

$$\frac{[EL]}{[L_{TOT}]} = \frac{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a}{3K_D + 2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a} \quad (1)$$

where

$$\theta = ar\cos\left[\frac{-2a^3 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}}\right] \quad (2)$$

$$a = K_D + K_I + [L_{TOT}] + [I] - [E_{TOT}] \quad (3)$$

$$b = \{[I] - [E_{TOT}]\}K_D + \{[L_{TOT}] - [E_{TOT}]\}K_I + K_D K_I \quad (4)$$

$$c = -K_D K_I [E_{TOT}] \quad (5)$$

K_D and K_I are the dissociation binding constants of the spy and competing molecules, respectively, $[L_{TOT}]$ and [I] are the concentrations of the spy and competing molecules, respectively, and $[E_{TOT}]$ is the concentration of the protein.

Figure 1 shows a simulation performed with eq 1 representing a typical experimental condition in a FAXS experiment where the ratio $[L_{TOT}]/[E_{TOT}] = 50$ and the K_D of the spy molecule is 10 μM. It is evident that if the competing molecule is a strong binder (low K_I), then a low concentration can displace completely the spy molecule. When the competing molecule is a weak binder, it is necessary to use a high concentration to achieve a detectable reduction in the fraction of bound spy molecule. The fraction of bound spy molecule in the FAXS experiments, as it can be appreciated in Figure 1, is small due to the weak affinity of the spy molecule and its high excess compared to the protein concentration. These experimental conditions are different when compared to a fluorescence polarization (FP) assay, where the fraction of bound spy molecule is high and the ratio $[L_{TOT}]/[E_{TOT}] < 1$ in order to reduce interferences originating from the fluorescence of the free spy molecule.

Figure 2 shows the fraction of bound spy molecule as a function of K_D and $[L_{TOT}]$. This is calculated with eq 1 by setting [I] to 0. As it is evident, the use of a lower spy molecule concentration, possible with a sensitivity improvement in the technique, results in a higher fraction of bound spy molecule.

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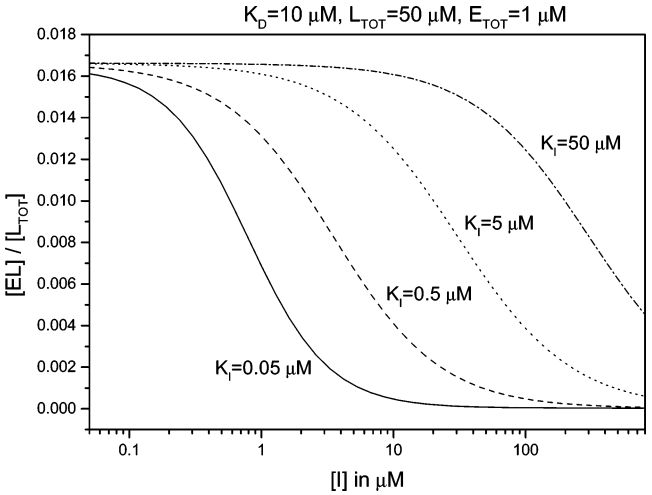

Figure 1. Simulation performed with eq 1, showing the fraction of bound spy molecule as a function of the concentration of the competing molecule [I] for different values of the binding constant K_I of the competing molecule. The K_D and the concentration of the spy molecule were 10 and 50 μM, respectively, and the concentration of the protein was 1 μM.

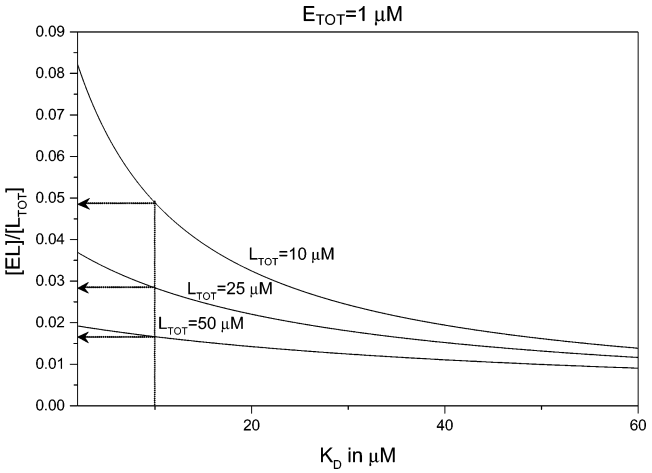

Figure 2. Simulation performed with eq 1 and [I] = 0, showing the fraction of bound spy molecule [EL]/[L_{TOT}] as a function of K_D and [L_{TOT}]. The protein concentration was 1 μM. The vertical line is drawn at K_D = 10 μM. For this value, [EL]/[L_{TOT}] is 0.049, 0.029, and 0.017 for [L_{TOT}] of 10, 25, and 50 μM, respectively.

For example, with a K_D of 10 μM, the [EL]/[L_{TOT}] is 0.017 with [L_{TOT}] of 50 μM, but it becomes 0.049 with [L_{TOT}] of 10 μM. The increase in [EL]/[L_{TOT}] results in a more sensitive method, thereby extending its application to small proteins and requiring a shorter T_2 filter. However, as it has been demonstrated previously, it is possible with FAXS to work at a very low fraction of bound spy molecule. In this case the protein concentration can be lowered and the sensitivity improvement further reduces the protein consumption necessary for the FAXS run.

In the competition binding experiments, a percentage of reduction of the fraction of bound spy molecule is typically defined as the threshold above which the molecules are considered as hits. Figure 3 shows a diagram of the percentage of reduction of the fraction of bound spy molecule as a function of the K_I of the competing molecule and the concentration of the spy molecule. In a typical FAXS run we can set the detection limit of the experiments to 30%, which is easily achievable with ^{19}F NMR due to the large difference in the transverse relaxation

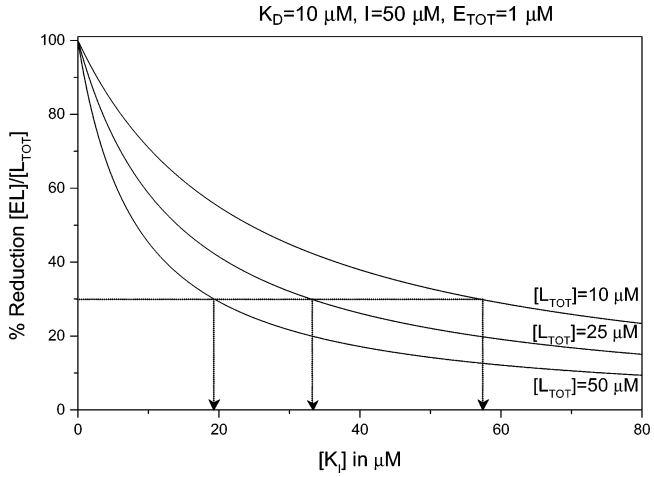

Figure 3. Simulation performed with eq 1, showing the percentage reduction of the fraction of bound spy molecule as a function of the binding constant K_I of the competing molecule for different concentrations of the spy molecule. The K_D of the spy molecule was 10 μM, and the concentration of the competing molecule and protein was 50 and 1 μM, respectively. The horizontal line drawn at 30% reduction in [EL]/[L_{TOT}] represents a conservative detection limit of a FAXS run. For this value, the detection limit of K_I is 57, 33, and 19 μM for [L_{TOT}] of 10, 25, and 50 μM, respectively.

T_2 between the free and bound states. The concentration of the tested molecules is 50 μM, and for a spy molecule with a K_D of 10 μM it is possible to detect binders with $K_I \leq 19$ μM if we use [L] = 50 μM, but the limit is extended to $K_I \leq 57$ μM if we use [L] = 10 μM. Therefore, it is evident that the lower spy molecule concentration feasible with the sensitivity increase allows the detection of weaker binders. This is important in fragment-based lead discovery programs at academic institutions and small pharmaceutical companies where no HTS screening capacity is available. In this approach it is not the throughput that is relevant, but the possibility of detecting chemical fragments that bind weakly to the receptor.

3-FABS. 3-FABS[17] is a robust functional assay that identifies inhibitors of an enzymatic reaction and measures with high accuracy their IC_{50} values (i.e., the inhibitor concentration at which 50% inhibition of the enzymatic reaction is achieved). The substrate is tagged with a CF_3 moiety, and fluorine NMR spectroscopy is used as the method of detection.

If the substrate has a low Michaelis constant (K_M), it is not practicable to use a high substrate concentration, as this would limit detection only to very strong inhibitors. An improvement in sensitivity allows a reduction in the concentration of the substrate used in the experiments, therefore making possible the detection of weak- and medium-affinity inhibitors.

Another experimental situation where the improvement in sensitivity is useful is in the kinetic measurements. Some inhibitors display slow kinetics deriving from a slow on-rate. An on-line (real-time) kinetics measurement allows the identification and characterization of these “slow binders”. However, these experiments require the rapid acquisition of spectra at different lag times from the start of the reaction. This is clearly possible only when the sensitivity of the experiment is high.

As is the case with other techniques used in HTS, two experimental factors can be used to define the quality of a 3-FABS run. These are the signal-to-noise ratio, S/N, defined as

$$\mathrm{S/N} = \frac{U - L}{\sqrt{\sigma_U{}^2 + \sigma_L{}^2}} \tag{6}$$

and the quality factor Z', defined as[30]

$$Z' = 1 - \left(\frac{3\sigma_U + 3\sigma_L}{U - L}\right) \tag{7}$$

where U and L represent the two plateaus (intensities of the substrate or product signals) obtained from the 3-FABS experiments with 0 and 100% inhibition and where σ_U and σ_L are their standard deviations. The equations (6) and (7) apply also to the FAXS experiments where L and U correspond to the intensity of the spy molecule signal with 0 and 100% displacement, respectively. The best conditions for the screening are achieved when the S/N ratio is high and when the quality factor lies between 0.5 and 1. These conditions can be reached by improving the sensitivity of the experiment.

Finally, to perform the screening in the initial linear region of the enzymatic reaction, it is sometimes necessary to quench the reaction when only a small fraction of substrate has been transformed to product. In this case the sensitivity improvement makes possible the detection of the miniscule amount of product.

How To Improve the Sensitivity. The use of a CF_3-containing spy or substrate molecule provides high sensitivity, but it is not always sufficient as discussed above. A 2- to 4-fold sensitivity improvement is obtained with the use of magnetically equivalent multiple CF_3 fragments properly introduced in the substrate or spy molecule.[20] In this case the increase in S/N is achieved by enhancing the difference $U - L$ in eq 6. However, it is not always possible to use such an approach because the introduction of the multiple CF_3 chemical moiety in the substrate or spy molecule either can require troublesome chemical synthesis or could interfere with the binding to the receptor and with the enzymatic reaction. In addition, the presence of multiple CF_3 groups renders the molecule more hydrophobic, therefore introducing solubility and aggregation problems.

Another approach for improving the sensitivity is the use of cryogenic probe technology optimized for ^{19}F detection. In this case the increase in S/N is accomplished by reducing σ_U and σ_L in eq 6. The new 5-mm SEF Z-Gradient ^{19}F{^{1}H−^{2}H} CryoProbe developed by Bruker is equipped with cryogenically cooled preamplifiers for all nuclei (^{19}F, ^{1}H, and ^{2}H). This results in a 4-fold sensitivity gain for ^{19}F with respect to that possible with a conventional fluorine observe probe-head and also a 2-fold sensitivity gain for ^{1}H compared to that possible with a conventional inverse probe-head. The high sensitivity of the ^{2}H lock channel allows for optimal stability and long-time performance even in perturbed environments. Fully software controlled automatic tuning and matching (ATM) is available for all nuclei. Gradient shimming, together with ATM, allows full automation and high-throughput NMR screening at optimal performance levels without manual interference.

Figure 4 shows the experimental sensitivity improvement in the 3-FABS experiment achieved with the new cryogenic probe technology. The CF_3-tagged substrate, phosphorylated by the cyclic AMP-dependent human protein kinase A (PKA), is present at a concentration of only 5 μM. The experimental gain

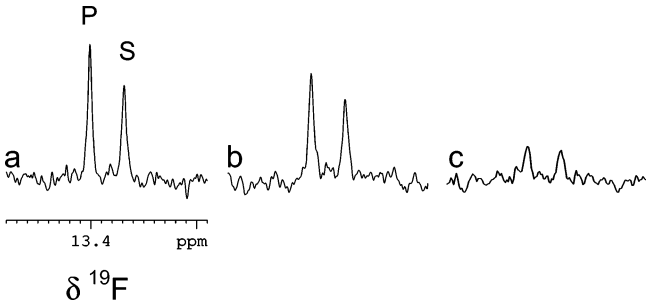

Figure 4. ^{19}F NMR spectra of only 5 μM AKTide[31] CF_3-tagged peptide (ARKRERAF(3-CF_3)**S**FGHHA) recorded with a SEF CryoProbe (a) with and (b) without proton decoupling and (c) with a conventional ^{19}F-selective probe. The spectra were recorded at 20 °C with 256 scans and 2.8 s repetition time (12 min total acquisition time). The enzymatic reaction was performed with 5 nM PKA in the presence of 60 μM ATP, 1 mM DTT, 5 mM $MgCl_2$, and 50 mM Tris pH 7.5. The reaction was quenched after 3 h and 20 min with 20 μM staurosporin. Substrate and product of the kinase reaction (phosphorylated peptide) are indicated with the letters S and P, respectively. The signal intensities are (a) 1.2, (b) 1, and (c) 0.3.

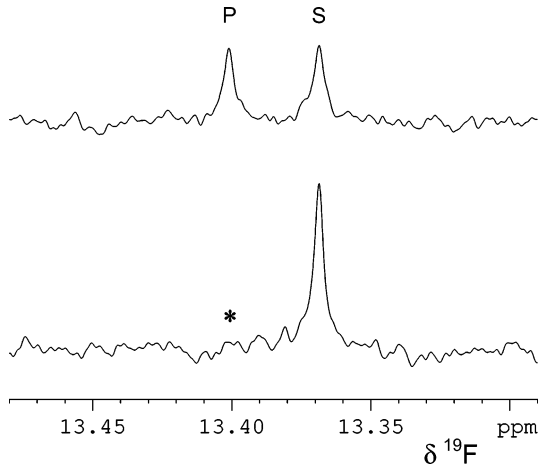

Figure 5. ^{19}F NMR spectra of 15 μM CF_3-tagged peptide (ARKRERAF-(3-CF_3)**S**FGHHA) recorded with a SEF CryoProbe. The spectra were acquired at 20 °C with 16 scans and 2.8 s repetition time (45 s total acquisition time). The enzymatic reaction was performed with 5 nM PKA in the presence of 60 μM ATP, 1 mM DTT, 5 mM $MgCl_2$, and 50 mM Tris pH 7.5. Substrate and product of the reaction (phosphorylated peptide) are indicated with the letters S and P, respectively. In the presence of 20 μM *N*-(2-{[(2*E*)-3-(4-bromophenyl)prop-2-enyl]amino}ethyl)isoquinoline-5-sulfonamide, known as H89 (lower spectrum), no product formation is observed.

in sensitivity obtained with the cryogenically cooled probe when compared to the sensitivity of a conventional probe is about 3-fold. An additional 20% sensitivity improvement is attained with the use of composite pulse proton decoupling, as shown in Figure 4a. This is simply due to the presence of small unresolved heteronuclear long-range ^4J scalar couplings between the aromatic protons H2 and H4 and the CF_3 of the modified phenylalanine residue. A typical screening run with 3-FABS in the presence of 5 nM PKA and 15 μM CF_3-tagged substrate, recorded with the cryogenically cooled probe, is shown in Figure 5. Only 16 scans, amounting to an acquisition time of 45 s, were necessary for recording the spectra, thus allowing the rapid collection of many samples.

It was demonstrated that 3-FABS is a reliable and robust approach.[17] This was ascribed to the simplicity of the assay and

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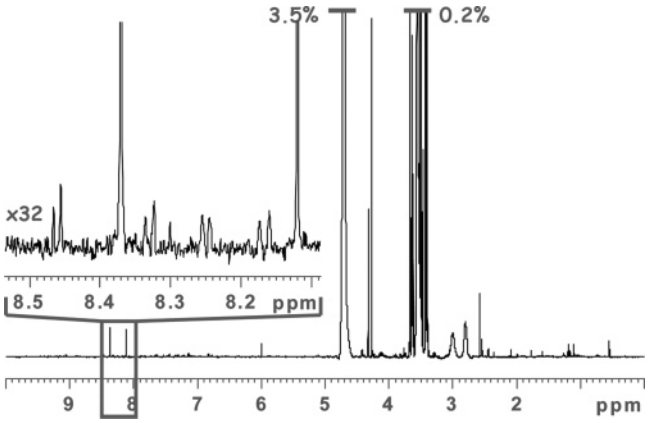

Figure 6. 1D ^{1}H presaturation (using a composite read pulse) experiment acquired with 64 scans (4 min experimental time) at 14.09 T (600 MHz) with the 5 mm SEF ^{19}F {^{1}H-^{2}H} Z-Gradient CryoProbe. The strong signal originates from the Tris buffer present at a concentration of 50 mM. The values at the position of the two strongest peaks, residual water signal and Tris signal, correspond to the percentage of the total signal intensities displayed in the plotted spectrum. The inset shows a zoom-in of the aromatic spectral region displaying some signals of the inhibitor H89 and ATP.

to the absence of interferences with the detection system. However, the main advantage of the 3-FABS approach compared to other techniques used in HTS is the direct measurement of the sample concentration, sample purity, compound identity, solubility in aqueous solution, aggregation state, and chemical stability. The characterization of these properties is paramount for deriving accurate values of IC_{50} and for identifying false positives and potential false negatives. These measurements are obtained with the acquisition of 1D ^{1}H spectra and sometimes 1D WaterLOGSY (for determining the aggregation state) of the identified hits. The spectra are recorded in the presence of a water-soluble reference molecule of known concentration. The SEF CryoProbe allows the automated acquisition with high sensitivity of the ^{1}H spectra after the acquisition of the ^{19}F spectra. A typical 1D ^{1}H presaturation experiment acquired with 64 scans (4 min experimental time) is shown in Figure 6. The inset shows a zoom-in on the aromatic spectral region containing the signals of the screened molecule *N*-(2-{[(2*E*)-3-(4-bromophenyl)prop-2-enyl]amino}ethyl)isoquinoline-5-sulfonamide, known as H89.

The use of magnetically equivalent multiple CF_3 moieties in combination with the cryogenically cooled ^{19}F probe results in a large improvement of the sensitivity of 3-FABS, allowing high throughput. This can be appreciated in the screening run shown in Figure 7, performed with trypsin and the N-capped polyfluorinated glycine peptide PFG-ARA-NH_2. The spectra were recorded with a single scan, corresponding to 0.8 s acquisition time. In this case it is evident that the throughput with 3-FABS is not limited by the acquisition time. The bottleneck is represented by the time required to introduce the sample in the magnet, and the time to shim the magnet. Improvements in the speed of these operations will significantly increase the throughput achievable with 3-FABS.

The sensitivity gain obtained with the SEF CryoProbe allows also the detection of a small amount of product formation. This is relevant when the enzyme is present at very low concentration, for a cell-based 3-FABS application, or for a potential diagnostic utilization of 3-FABS. An example of a 3-FABS run performed at an enzyme concentration of only 200 fM is shown in Figure 8. Despite the low amount of the cleaved peptide (~500 nM), it is possible to perform the screening and identify inhibitors in a reasonable experimental time. In this particular case, only 0.235 μg of enzyme would be needed for screening 100 000 single compounds. This compares favorably with the amount of enzyme used by other techniques applied to HTS.

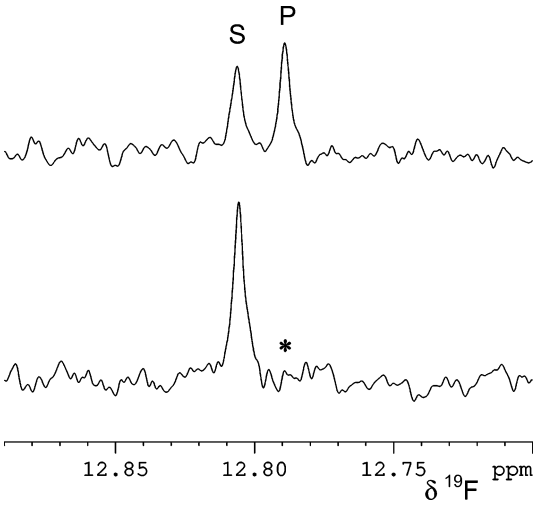

Figure 7. 3-FABS of 10 μM PFG-ARA-NH_2 recorded with a SEF CryoProbe. The spectra were acquired with 1 scan and 0.8 s total experimental time. The enzymatic reaction was performed with 5 pM trypsin in 50 mM Tris pH 7.5. Substrate and product of the reaction (cleaved peptide) are indicated with the letters S and P, respectively. The reaction was stopped after 22 h. In the presence of 20 μM leupeptin (lower spectrum), no product formation is observed.

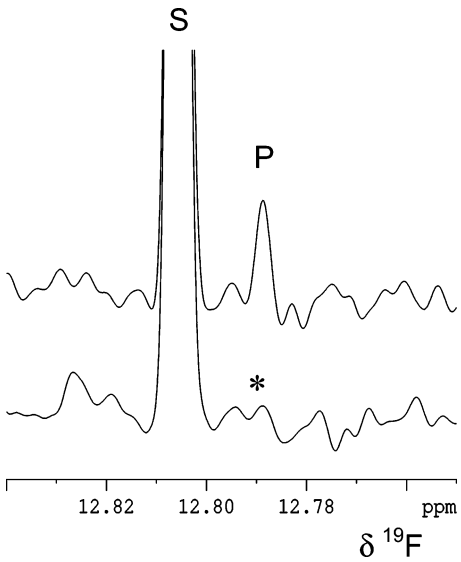

Figure 8. 3-FABS of 10 μM PFG-ARA-NH_2 recorded with a SEF CryoProbe. The enzymatic reaction was performed with only 200 fM trypsin in 50 mM Tris pH 7.5. The spectra were recorded at 20 °C with 256 scans and 2.8 s repetition time (12 min total acquisition time). Substrate and product of the reaction (cleaved peptide) are indicated with the letters S and P, respectively. The tiny amount (~500 nM) of product is clearly visible in the upper spectrum, whereas in the presence of 20 μM leupeptin (lower spectrum), no product formation is observed.

Conclusion

The sensitivity improvement in the ^{19}F NMR detection experiments described in this work has pushed the limit of the NMR-based screening technology. It is now possible to perform robust and reproducible screening of many samples in a short

time with very low enzyme consumption. The flexibility of the novel SEF CryoProbe with automated acquisition of both ^{19}F and ^{1}H spectra permits identification of reliable hits, accurate determination of their K_D or IC_{50} values, and characterization of their chemical and physical properties. The quality of these data is fundamental for the selection and optimization of meaningful lead compounds, thus avoiding squandered time and resources in the pursuit of unsuitable molecules.

Material and Methods

The peptide ARKRERAF(*3-CF*$_3$)SFGHHA was synthesized at Bachem. The tetrapeptide PFG-ARA-NH_2 (PFG, polyfluorinated glycine, or [bis(3,5-bis-trifluoromethyl-benzyl)amino]acetic acid) was synthesized according to the procedures described previously.[20] The Ser/Thr cyclic AMP-dependent protein kinase A (PKA) was purchased from PanVera (Invitrogen, P2912), and the protease trypsin was purchased from Roche (Roche Molecular Biochemical catalog no. 1418475).

The NMR spectra were recorded at 20 °C with a Bruker 600 MHz NMR spectrometer operating at a ^{19}F Larmor frequency of 564 MHz. The data were recorded with a spectral width of 12 000 Hz, an acquisition time of 0.8 s, and a relaxation delay of 2.8 s. Chemical shifts are referenced to trifluoroacetic acid. The simulations were performed using the Origin 5.0 and Microsoft Excel software packages.

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