Communication

2-Fluoro-ATP as a Versatile Tool for F NMR-Based Activity Screening

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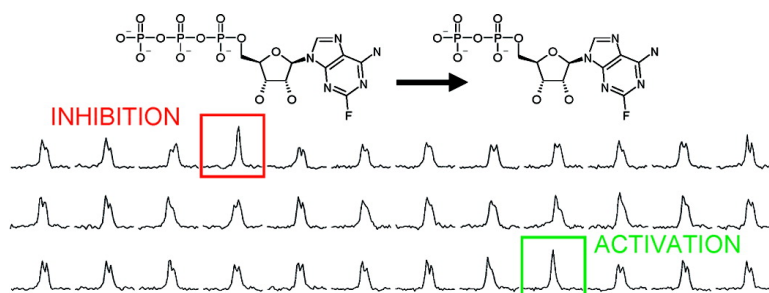
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2-Fluoro-ATP as a Versatile Tool for ^{19}F NMR-Based Activity Screening

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^{19}F NMR-based methods have been shown to be useful in direct binding screening experiments,[1,2] competition binding screening experiments,[1] and activity-based screening assays.[3] In the case of direct binding experiments, the large ^{19}F chemical shift dispersion results in virtually no resonance overlap for mixtures of up to 10 fluorine-containing compounds. In the cases of competition binding and activity assays, only a small percentage of fragments in a typical screening library will contain fluorine. Interference with the ^{19}F signal of interest (site probe, substrate, or product) is expected to be even less of an issue. For these two experiment types, the availability of a fluorine-containing site probe or substrate molecule is the only prerequisite. Suitable molecules can be identified by screening libraries of fluorine-containing compounds, carrying out similarity searches around known ligands or substrates, or synthesizing fluorine-containing analogues of known ligands or substrates. However, because enzymes catalyze a diverse set of reactions, a large variety of fluorinated substrates would need to be identified to target each one separately. A more streamlined approach would be to develop a small number of fluorinated substrates that are applicable to numerous enzymes. Because many enzymes utilize ATP as a substrate, a fluorine-containing ATP analogue would find great utility as a versatile tool for ^{19}F NMR-based activity screening.

Nicotinamide adenine dinucleotide synthetase (NadE), which catalyzes the ATP-dependent conversion of nicotinic acid adenine dinucleotide to nicotinamide adenine dinucleotide (NAD), was chosen first to explore the suitability of fluorine-containing ATP analogues because we previously developed a ^{1}H NMR-based activity assay for this enzyme.[4] The downfield H2 singlet resonance at 9.22 ppm of the product NAD was used for monitoring the reaction. Even in this best-case scenario, however, overlaps with a fragment ^{1}H resonance complicated or precluded analysis for about 5% of the reaction mixtures. Complications from fragment ^{1}H resonance overlap are expected to be even more problematic in systems where substrate or product signals do not have such characteristically resolved chemical shifts, thus preventing the application of ^{1}H NMR-based activity assays in these systems.

The first fluorine-containing ATP analogue tested was 2′-fluoro-ATP. Unfortunately, as shown in Figure 1B, this analogue did not serve as a substrate for NadE as evidenced by the lack of 2′-fluoro-AMP formation. However, it did function as a substrate for 3-phosphoinositide dependent kinase 1 (PDK1) as shown in Figure 1C, and for UDP-*N*-acetylmuramic acid/L-alanine ligase (MurC) (data not shown). These results indicate that the 2′-fluoro-ATP analogue will be useful in a number of systems, but that it is not suitable as a universal tool for ATP-requiring enzymes.
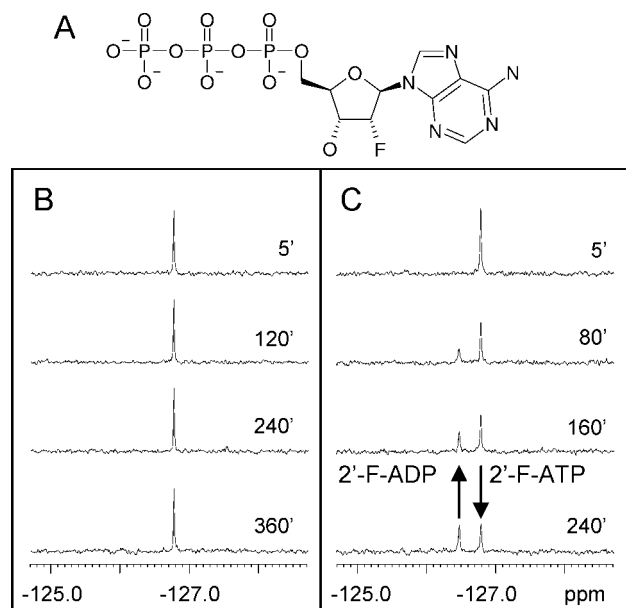

Figure 1. (A) Structure of 2′-fluoro-ATP. Time courses of the NadE (B) and PDK1 (C) reactions using 2′-fluoro-ATP as the substrate. The NadE reaction contained 190 nM *S. aureus* NadE, 2 mM NH$_4$Cl, 100 μM nicotinic acid adenine dinucleotide and 200 μM 2′-fluoro-ATP (TriLink Biotechnologies, San Diego, CA) and was buffered with 60 mM deuterated[5] Tris at pH 8.0 containing 10 mM MgCl$_2$ and 10 mM KCl. The PDK1 reaction contained 1 μM human PDK1, 100 μM PDKtide (Upstate, Lake Placid, NY), and 200 μM 2′-fluoro-ATP and was buffered with 25 mM deuterated Tris at pH 7.5 containing 100 mM NaCl, 1 mM MgCl$_2$, and 2 mM TCEP. The 471 MHz ^{19}F{^{1}H-decoupled} NMR spectra were collected at 25 °C on a Bruker DRX spectrometer equipped with a conventional 5 mm SEF probe optimized for ^{19}F detection. Data sets are the average of 32 scans (2 min measurement time). ^{19}F chemical shifts were referenced to internal 50 μM trifluoroethanol.

The second analogue tested was 2-fluoro-ATP. As shown in Figure 2B, this analogue does function as a substrate for NadE as evidenced by the formation of 2-fluoro-AMP. It was also shown to function as a substrate for PDK1 as shown in Figure 2C, and for thymidylate kinase (TMK) (data not shown). Previous biochemical studies have also shown that 2-fluoro-ATP can serve as a substrate for the enzymes adenylate kinase, hexokinase, pyruvate kinase, and the myosin ATPase,[6] as well as for creatine kinase and RNA polymerase.[7] These eight enzymes along with NadE are representative of three of the six enzyme subclasses, namely the transferases, hydrolases, and ligases. This suggests that the 2-fluoro-ATP analogue is suitable as a universal tool for ATP-requiring enzymes. Importantly, 2-fluoro-ATP has been determined to be a valid substrate for a variety of kinases, including both small molecule and protein kinases, suggesting that it may be useful for investigating the large number of pharmaceutically relevant kinases.
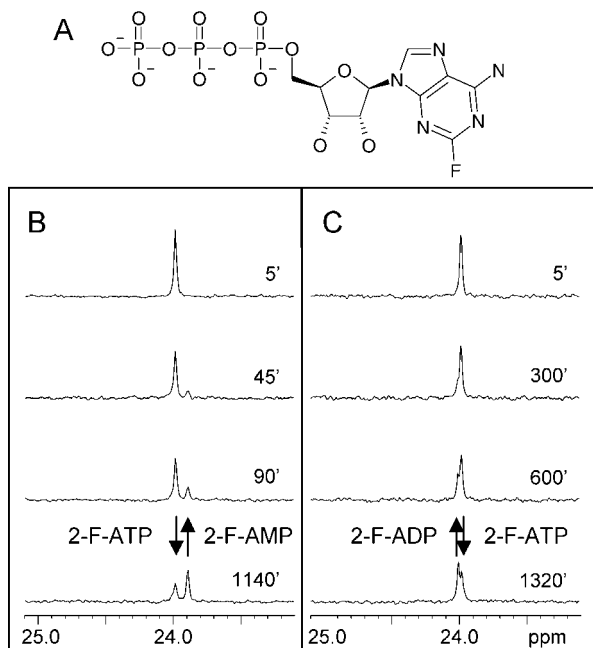
Figure 2. (A) Structure of 2-fluoro-ATP. Time courses of the NadE (B) and PDK1 (C) reactions using 2-fluoro-ATP as the substrate. The NadE reaction contained 190 nM *S. aureus* NadE, 2 mM NH$_4$Cl, 100 μM nicotinic acid adenine dinucleotide, and 200 μM 2-fluoro-ATP (ChemCyte, San Diego, CA or Chemilia, Huddinge, Sweden) and was buffered with 60 mM deuterated Tris at pH 8.0 containing 10 mM MgCl$_2$ and 10 mM KCl. The PDK1 reaction contained 600 nM human PDK1, 100 μM T308tide (Sigma, St. Louis, MO), and 100 μM 2-fluoro-ATP, and was buffered with 25 mM deuterated Tris at pH 7.5 containing 100 mM NaCl, 1 mM MgCl$_2$, and 2 mM TCEP. This reaction proceeds very slowly because of the very high K_m value for the T308tide substrate.[8] ^{19}F{^{1}H-decoupled} NMR spectra were collected as described in Figure 1. Data sets are the average of 128 scans (8 min measurement time) for NadE and 256 scans (16 min measurement time) for PDK1.
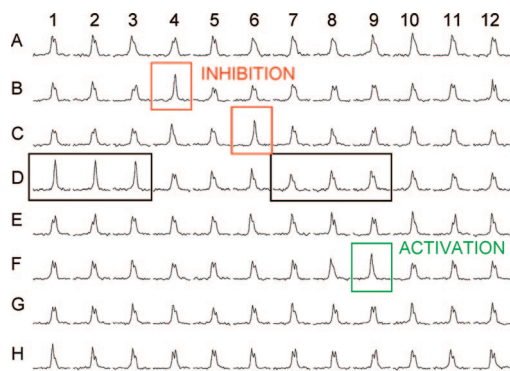

Figure 3. Fragment screening results for PDK1. Reaction conditions were as described in Figure 2C except that 1 μM PDK1 was used and each reaction well contained 10 fragments, each at a concentration of 230 μM. Reactions were initiated in Marsh 1.2 mL microtubes by addition of MgCl$_2$ using a Zymark RapidPlate-96/384, allowed to run for 18 h at 21 °C, and then were quenched by addition of 10 mM EDTA using a Zymark RapidPlate-96/384. Solutions were transferred to Norell 502 NMR tubes using a Gilson GX-271 liquid handler and ^{19}F{^{1}H-decoupled} NMR spectra were collected as described in Figure 2C.

The utility of 2-fluoro-ATP for screening kinases is demonstrated in Figure 3. A library containing 900 fragments, each in mixtures of 10 in 96-well plate format, was screened for the ability to effect the reaction catalyzed by PDK1. Control wells without added MgCl$_2$ (D1–D3) or without fragments (D7–D9) indicate the spectrum expected for no reaction (fully inhibited) or for no effect, respectively. For the no reaction controls, 100%

of the substrate 2-fluoro-ATP signal remains, while for the no effect controls approximately 60% of the substrate is converted to 2-fluoro-ADP under these conditions. The 90 reaction wells that contain fragment mixtures have no confounding ^{19}F signals from the fragments themselves despite the fact that 39% of the wells contain at least one fluorine-containing fragment. Two mixtures in this fragment plate, corresponding to wells B4 and C6, completely inhibited the PDK1 reaction as evidenced by no conversion of the 2-fluoro-ATP signal to 2-fluoro-ADP. Follow-up testing of each compound in mixtures B4 and C6 individually indicated that a single fragment in each mixture was responsible for the inhibition. Interestingly, the mixture in well F9 appeared to activate the PDK1 reaction as evidenced by nearly 100% conversion of the signal to 2-fluoro-ADP. Follow-up testing of each compound in mixture F9 individually indicated that a single fragment was responsible for the activation. Further investigations of these active fragments will be reported elsewhere.

Applications of 2-fluoro-ATP in fragment screening should result in the efficient identification of fragments that inhibit or activate ATP-utilizing enzymes. The method should also find broad utility as a complementary assay to evaluate compounds identified using other screening methods. The throughput described here is sufficiently fast to be useful in a pharmaceutical fragment screening paradigm. When ATP-competitive inhibitors are desired, fragment screens should be run at 2-fluoro-ATP concentrations near the K_m value. It thus may be possible to increase sensitivity and extend the range of applicability using trifluoro-ATP analogues.[3] When targeting other substrate sites or allosteric sites, the concentration of 2-fluoro-ATP can be based on detection while the concentrations of other reaction components are optimized for monitoring interactions at these sites.[3]

Supporting Information Available: One figure detailing the follow up testing of each compound in mixtures B4, C6, and F9 individually in the PDK1 2-fluoro-ATP assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) Although deuterated buffers were used in this work to facilitate related ^{1}H NMR experiments on these systems, their use is not required. In fact, a big advantage to ^{19}F NMR is the ability to collect data on systems that require high concentrations of detergents or other reagents without the expense of deuteration.
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