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J. Am. Chem. Soc., **2005**, 127 (22), 7978-7979• DOI: 10.1021/ja0425942 • Publication Date (Web): 12 May 2005

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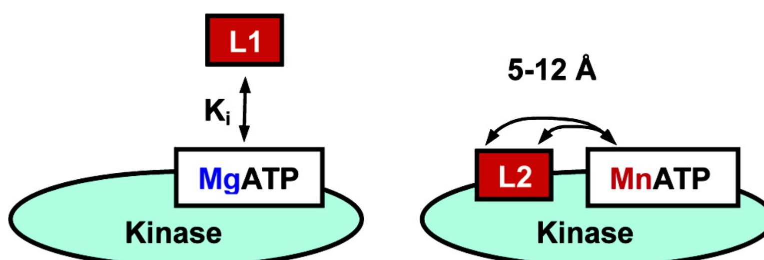

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Published on Web 05/12/2005

Screening of Protein Kinases by ATP-STD NMR Spectroscopy

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Protein kinases are key mediators of cellular function: deregulation of kinase activity has been related to a wide range of diseases. Searching for potent inhibitors that are selective against the more then 500 potential human kinase targets is currently an active area of drug discovery.[1] The identification of novel ATP-competitive kinase inhibitors is nonetheless challenging. Not only are corporate libraries deficient in compounds with kinase binding motifs, but also high throughput kinase assays are susceptible to a high rate of false positives.[2] ATP typically binds to kinases with K_d's of 10−100 μM; purine replacements would be expected to have similar affinities. This affinity range is difficult for many assay formats but ideal for NMR screening methods. While SAR-by-NMR is the best-known of these methods,[3] ligand detected NMR screening methods (such as STD-NMR) are an excellent choice to screen for novel kinase cores.[4] Ligand detected methods typically do not reveal their target binding site, although paramagnetic relaxation enhancement (PRE) has been used to estimate distances between ligands that bind simultaneously to two different sites.[5] Some advantages of STD-NMR and related methods are reduced protein requirements, no restrictions on target size and no requirement of obtaining high quality protein NMR spectra. We report the use of ATP as a site-specific marker in STD-NMR experiments that are designed to test the potency of protein kinase inhibitors. ATP-STD spectra detect NMR signals that originate from target-bound ATP. Reduction of the ATP-STD signal by competitive inhibitor binding permits a direct measurement of the inhibitor K_i with respect to the natural substrate: ATP. We show that by adding $MnCl_2$, ATP is converted into a paramagentic probe[6] from which the proximity of non-ATP competitive inhibitors can be inferred.

ATP-STD NMR requires the collection of 4 simple 1D proton NMR spectra typically consisting of two presaturation experiments, a 2-hr ATP−STD reference and a 2-hr ATP-STD competition experiment. There are 4 objectives to these experiments: quality control, K_i measurement, identification of non-ATP competitive compounds and proximity determination of non-ATP competitive compounds.

(1) Quality control is monitored in two 1D spectra of the protein kinase, ATP, and TSP, with and without inhibitor. Protein aggregation and unfolding in response to compound addition can be directly observed for proteins tested at about 5 μM concentrations. Compound & ATP aliquots are variable and their concentrations should be measured directly relative to a known concentration of TSP (trimethylsilylpropane sulfonate) as an internal reference. Compound impurities and degradation can also be detected. ATP hydrolysis can be monitored by observing chemical shift changes in purine & sugar protons.

(2) Target-ligand interactions are detected and quantified by STD detected competition.[4] ATP and MgATP bind to most active and many inactive kinases. In the absence of protein, magnesium has a K_d of ~38 μM for ATP.[7] When ATP and MgATP bind the target protein kinase with different affinities, the $MgCl_2$ concentration can be adjusted (0−10 mM) to alter ATP-target affinity.[8] We use the adjustability of the ATP-target affinity to optimize the ATP-STD signal and to tune the affinity range of the ATP/inhibitor competition experiment (See Supporting Information). A typical STD spectrum from target-bound ATP yields peaks from the purine H8 and H2 protons and the sugar H1′ proton. Binding an ATP competitive inhibitor reduces the ATP−STD signal (Figure 1). Simultaneous reduction of the ATP epitope and appearance of a compound STD epitope suggests ATP competitive binding from which K_i's can be calculated.[9] Active kinases can be tested with ATP (no Mg^{2+}), nonhydrolyzable ATP analogues; AMP and ADP can also be used.

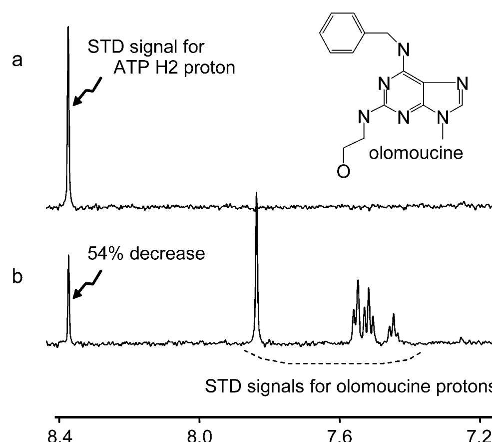

Figure 1. (a) H2 ATP-STD peak for MgATP (200 μM ATP; 1 mM $MgCl_2$) bound to 5 μM ERK2. Strong ATP-STD peaks for H8 (8.63 PPM) and H1′ (6.26 PPM) are also present but not shown. (b) STD-NMR spectrum after 170 μM olomoucine is added to **1a**. Appearance of olomoucine STD peaks indicates that it binds to ERK2. Reduction of the ATP-STD peak indicates that olomoucine competes with MgATP for ERK2 with an estimated K_i of ~30 μM.

(3) The ATP site can be blocked with a high affinity inhibitor to distinguish competitive from non-ATP competitive inhibitors. Addition of staurosporine (which is ATP competitive and has a low nanomolar K_d for most Ser/Thr kinases) eliminates the STD peaks for ATP and for competitive inhibitors such as olomoucine. The STD signals of non-ATP competitive compounds are not reduced upon staurosporine addition.

(4) A site-specific paramagnetic probe can be used to determine the proximity of non-ATP competitive compounds. The MnATP probe is formed by binding Mn^{2+} to ATP (Figure 2). The relaxation of ATP protons is strongly enhanced by Mn^{2+} binding even in the absence of the protein kinase. Upon binding to the protein kinase, MnATP enhances the relaxation of protein and ligand protons in and around the ATP site. PRE of target-bound ligand protons is conveniently monitored in STD-NMR spectra. PRE is active throughout the STD data collection. The protein-bound ligand protons are, however, saturated in the on-resonance (transfer) data collection. PRE therefore affects ligand protons primarily during the off-resonance (reference) data collection. The STD ligand epitope decreases due to PRE which has an r^{-6} dependence on the proton-Mn^{2+} distance.

We demonstrate the use of the MnATP proximity probe on the catalytic domain of the Ser/Thr kinase MEK1. The crystal structure of the MEK1−MgATP−PD318088 ternary complex (Figure 3a)

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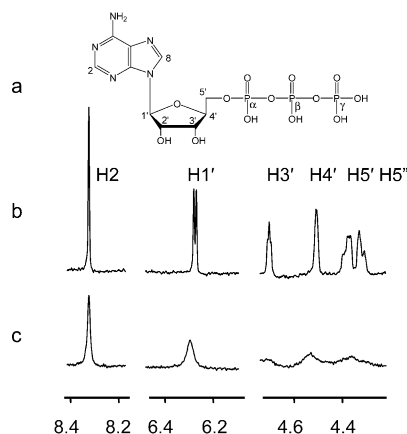

Figure 2. (a) Structure of ATP. Divalent metals bind between the $\beta-\gamma$ and $\alpha-\gamma$ phosphates (b) The ^{1}H NMR spectrum of 200 μM ATP and 1 mM $MgCl_2$. Mg^{2+} binds ATP with a K_d ~38 μM.[7] (c) The ^{1}H NMR spectrum from **2b** after the addition of 100 μM $MnCl_2$. Mn^{2+} binds ATP (K_d = 13 μM)[10] in the absence of protein kinase forming a paramagnetic probe: MnATP. The relaxation of ATP protons depends on the ^{1}H−Mn distance and on the fraction of Mn^{2+} that is ATP-bound. Relaxation rates of ATP protons are enhanced by 4−50 s^{-1} for ^{1}H−Mn distances of ~5−12 Å. Similar enhancements are expected for protein, ATP and ligand protons that are proximal to kinase-bound MnATP.

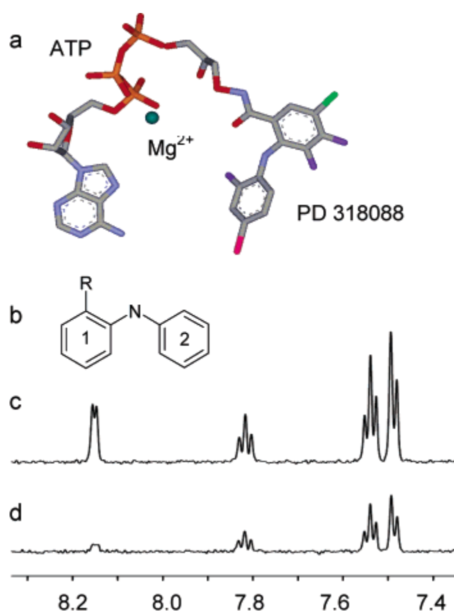

Figure 3. (a). X-ray crystal structure of the ternary complex of a potent diphenylamine inhibitor (PD 318088), MgATP and MEK1 (from patent EP 1321518). PD 318088 is a non-ATP competitive MEK1 inhibitor. It binds to a nearby pocket that is 7−9 Å from the Mg^{2+} atom. (b) Structure of a simple phenylamine. (c) STD signals from compound **3b** bound to MgATP−MEK1. (d) Addition of $MnCl_2$ to **3c** reduces the MEK1-bound STD signals of ring 1 and 2 protons indicating that compound **3b** binds MEK1 near MnATP. The binding orientation of compound **3b** is suggested by a greater reduction of ring 1 protons versus ring 2 protons.

shows the binding site of PD 318088 to be proximal to ATP. The binding of related phenylamines (compound **3b**) to MEK1 in the presence of MgATP is indicated by strong STD signals in Figure 3c**.** Staurosporine does not block the MEK1-binding of **3b** indicating that it is not ATP competitive. (see Supporting Information). The STD epitope for all protons of compound **3b** are reduced by the addition of the MnATP probe (Figure 3d) suggesting the close proximity of compound **3b** to MEK1-bound MnATP. The contribution of nonspecific Mn^{2+}-protein binding can be assessed by monitoring STD changes as a function of ATP addition to a solution already containing the $MnCl_2$ (see Supporting Information). Nonspecific Mn^{2+} binding is minimized by keeping [$MnCl_2$] < 100 μM. Protons from rings 1 and 2 of **3b** are relaxed at different rates from which the orientation of **3b** with respect to the MEK1-bound MnATP can be estimated. In principle, precise metal-nuclei distances can be calculated from PRE data.[11,12] Precise distance calculations are complicated in our experiments due fractional occupations from Mg^{2+} and Mn^{2+} competition for ATP; MgATP and MnATP competition for the enzyme, the limited lifetime of the enzyme-MnATP-compound complex, positional uncertainty of the ATP-bound manganese atom due to ATP flexibility and the possibility of multiple Mn^{2+} binding sites to enzyme-bound ATP. We consequently use Mn-enhanced relaxation as a qualitative distance marker to assess the proximity of compounds to target-bound MnATP.

ATP-STD NMR is simple, versatile, cost-effective and robust. This method can be used with virtually any target that binds a nucleotide. Enzyme activity is not required for detection; fully active as well as inactive enzymes can be tested. K_i's from 20 nM to 5 mM can be determined. Use of the MnATP probe gives this assay the powerful feature of providing qualitative structural information for non-ATP competitive compounds**.**

Acknowledgment. We thank Dr. Jennifer Gesell, Dr. Kate Smith, and Tom Hesson for supplying MEK1 and ERK2.

Supporting Information Available: Additional information on binding of non-ATP competitive compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0425942