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Fluorine NMR reporter for phosphate anions†

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A fluorine-labelled zinc(II)-dipicolylamine coordination complex reports the presence of phosphate anions in aqueous solution, especially pyrophosphate and ADP, and is used to monitor the enzymatic hydrolysis of ATP.

^{19}F NMR has many attractive features that encourage its use in biomedicine.[1] The ^{19}F nucleus has a natural abundance of 100% and signal sensitivity of 83% relative to ^{1}H NMR. ^{19}F NMR spectra of small molecule samples at submillimolar concentrations can be acquired with high signal to noise. Furthermore, recent breakthroughs in NMR signal enhancement, using techniques that exploit dynamic nuclear polarization, allow detection of ^{19}F signals from samples at submicromolar concentrations.[2] The lack of endogenous fluorine in most biomedical samples eliminates background interference problems, and the wide dispersion of ^{19}F chemical shifts diminishes the chance of complications due to overlapping signals. It is not surprising that ^{19}F NMR is an emerging strategy for magnetic resonance imaging and is often considered for development into drug screening assays.[3]

A requirement for essentially all of these ^{19}F NMR techniques is development of fluorine-labelled reporter molecules. Since many drug structures contain fluorine atoms, it is logical to directly study fluorine-labelled fragments or drug candidates using ^{19}F NMR screening methods that monitor a pharmaceutically relevant protein binding process.[4] In other cases, the ^{19}F label is incorporated within the structure of an enzyme substrate and the change in chemical shift upon enzyme action is detected as an assay output signal.[5] Fluorine-labelled enzyme substrates have also been incorporated into new methods for ^{19}F MRI. The results of enzyme action alter the substrate structure and produce either a change in signal chemical shift or relaxation time.[6] The central concept that is described here is a ^{19}F NMR reporter with ability to undergo a change in chemical shift upon reversible association with a molecular target.

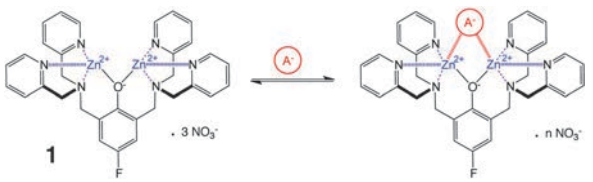

Fig. 1 Reversible association of ^{19}F NMR reporter **1** with a bidentate anion.

The few literature examples of this strategy include ^{19}F labelled reporters that respond reversibly to temperature,[7] pH,[8] metal cations,[9] diol-containing molecules,[10] and protease enzymes.[11]

Herein, we describe the recognition properties of compound **1** (Fig. 1) as the first example of a ^{19}F NMR reporter that can detect the presence of biologically relevant phosphate anions.[12] Compound **1** is a zinc(II)-dipicolylamine (ZnDPA) coordination complex.[13] The organic scaffold is a phenol derivative with two *ortho*-substituted 2,2′-dipicolylamine groups and a fluorine atom attached to the *para* carbon. The oxyanion recognition properties of this class of ZnDPA complexes have been studied quite extensively, and strong association is typically observed with polyanionic phosphate esters.[14] Most notably, optically active versions of these compounds with chromophores appended to the *para* carbon of the phenol scaffold have been observed to undergo red-shifts in absorption maxima.[14b,d,g] A logical explanation for this effect is that phosphate coordination to the zinc cations pushes electron density onto the *para* carbon and into the attached chromophore. If true, we reasoned that a fluorine label on the *para* carbon would exhibit a predictable change in chemical shift upon phosphate binding. Tentative support for this idea was a reported difference in ^{19}F chemical shifts for the zinc and copper salts of compound **1** (measured in the absence of coordinating anions).[15]

Structural evidence for our design concept was gained by elucidating the solid-state complex of reporter **1** bound to dihydrogen phosphate. We discovered that mixing compound **1** with sodium phosphate in water produced single crystals that were amenable to analysis by X-ray diffraction.‡ Refinement of the data produced the molecular structure shown in Fig. 2. The general coordination features and bond lengths agree with

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† Electronic supplementary information (ESI) available: Synthesis, X-ray, and NMR data. CCDC 929896. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3cc42169d

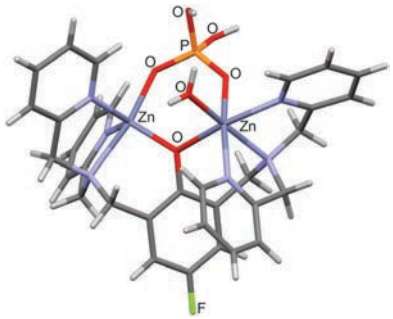

Fig. 2 X-ray crystal structure of **1**$(H_2PO_4)(NO_3)_2{\cdot}H_2O$. The structure omits the two NO_3^- anions for clarity.

related literature structures.[16] As expected, the bidentate dihydrogen phosphate dianion bridges the two zinc cations in **1**. One zinc cation adopts a five-coordinate geometry and the other is six-coordinate with a water molecule occupying the additional coordination site. Related crystal structures in the literature with poylanionic phosphates such as pyrophosphate (PPi) or ADP show tetradentate coordination with four phosphate oxygens coordinated to the two zinc cations.[14b,c,f] Together, the X-ray structures suggest that tetradentate coordination of poylanionic phosphates to compound **1** will push electron density onto the *para* carbon and produce an upfield change in ^{19}F chemical shift for the attached fluorine.

^{19}F NMR titration studies added incremental amounts of oxyanions to separate samples of compound **1** (0.40 mM in 50 mM HEPES buffer, pH = 7.2).§ Shown in Fig. 3 are partial ^{19}F NMR spectra of **1** in the absence and presence of one molar equivalent of different oxyanions. The ^{19}F chemical shift for compound **1** was observed at 21.5 ppm (internal KBF_4 as reference) and there was no detectable signal change produced by addition of P-Ser, P-Tyr, or citrate. Significant changes in chemical shift were produced by addition of PPi, ATP, ADP, phosphate (Pi), and AMP. The anions with high amounts of negative charge produced relatively large upfield changes in chemical shift. The slightly downfield effect produced by AMP is attributed to anisotropic shielding by the proximal adenosine ring. In the case of ATP binding, a broad ^{19}F signal was observed, even when **1** was saturated with ATP, indicating an intramolecular process that exchanges the bound ATP between different phosphate zinc coordination geometries. As expected, binding constants with these polyanionic phosphates were too strong ($K_a > 10^4\ M^{-1}$) for accurate determination by NMR.¶ A further complication preventing NMR measurements of association was the intermolecular exchange broadening that occurred when the ratio of anion to **1** was sub-stoichiometric (see ESI†). Therefore we measured relative anion binding affinities by conducting competition experiments that mixed reporter **1** with different ratios of competing anions. Shown in Fig. 4 are selected spectra for samples that contained equal amounts of two competing anions. Analysis of all the data indicates that **1** has a relative affinity order of PPi > ADP ≈ ATP > Pi.

To demonstrate the potential utility of reporter **1**, we used it to track an enzymatic reaction; namely, the hydrolysis of ATP catalysed by commercially available apyrase.[17] The ^{19}F spectra

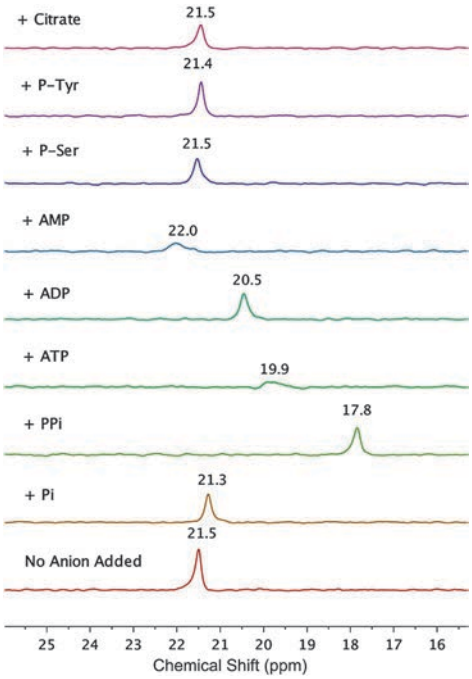

Fig. 3 Partial ^{19}F NMR spectra (564 MHz) of reporter **1** (0.40 mM in HEPES buffer, pH 7.2) with no anion added (*bottom spectrum*) and in the presence of one molar equivalent of the following anions as their sodium salts; phosphate (Pi); pyrophosphate (PPi); ATP; ADP; AMP; *o*-phospho-L-serine (P-Ser); *o*-phospho-L-tyrosine (P-Tyr); citrate.

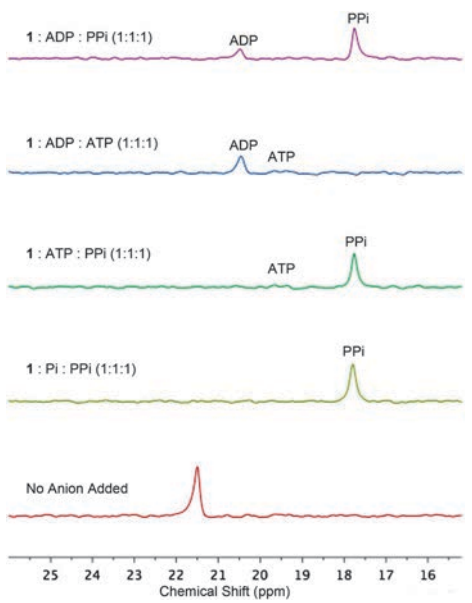

Fig. 4 Partial ^{19}F NMR spectra (564 MHz) of reporter **1** mixed with two competing anions (as their sodium salts). Each of the three components was 0.40 mM in HEPES buffer, pH 7.2.

of reporter molecule **1** in Fig. 5 reflect sequential conversion of ATP into ADP followed by further hydrolysis and production of Pi. The spectra show clearly that the first step in the process (hydrolysis of ATP) is complete after 12 minutes, whereas the second step (hydrolysis of ADP) is much slower and takes well over 41 hours. The high ATPase/ADPase ratio was confirmed by

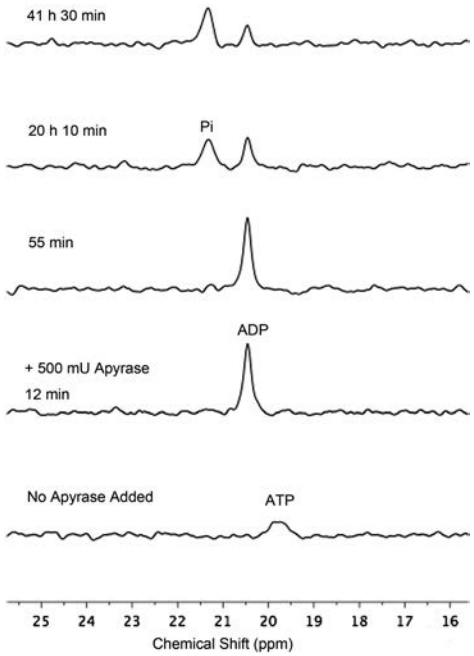

Fig. 5 Partial ^{19}F NMR spectra (564 MHz) of a mixture of reporter **1** and ATP (both 0.40 mM in HEPES buffer, pH 7.2) before addition of apyrase (*bottom spectrum*) and at increasing time points thereafter.

independent experiments that started with a sample of pure ADP (see ESI†) and closely matched the vendor's certification of relative enzyme activities. These enzyme hydrolysis experiments highlight the power of this NMR method to report kinetic information for sequential steps in the same assay sample. In contrast, fluorescent probes that can only report single steps, such as ATP consumption through emission intensity changes, are unable to provide information about any subsequent chemistry.[17] In principle, it should be possible to use compound **1** to monitor other reactions that consume ATP such as kinase catalyzed phosphorylation processes. Compound **1** may also be useful in cell imaging studies of polyphosphate anions. This work was supported by the NIH (GM059078).

Notes and references

‡ A detailed description of the X-ray structure is provided in the ESI.† The crystallographic data is also provided in CCDC 929896.

§ Typical ^{19}F NMR samples contained 0.40 mM of **1** in 0.5 mL of 50 mM HEPES buffer at 22 °C, pH = 7.2 with an external lock sample containing D_2O. The internal reference was KBF_4 (−150.4 ppm relative to $CFCl_3$) and T_1 for the ^{19}F signal of free **1** was measured to be 0.59 ± 0.01 second at 22 °C (operating frequency was 564 MHz). A typical acquisition collected 1000 scans using a 30° pulse and 0.10 second relaxation delay.

¶ A close structural analogue of **1** is reported to bind PPi and Pi with association constants of 6.7 × 10^6 M^{-1} and 1.1 × 10^5 M^{-1}, respectively.[14h]

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