MgF3⁻ and AlF4⁻ Transition State Analogue complexes of Yeast Phosphoglycerate Kinase

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MgF₃⁻ and AlF₄⁻ Transition State Analogue complexes of Yeast
Phosphoglycerate Kinase

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Abstract

The phospho-transfer mechanism of yeast phosphoglycerate kinase (PGK) has been probed through formation of trifluoromagnesate (MgF_3^-) and tetrafluoroaluminate (AlF_4^-) transition state analogue complexes and analyzed using ^{19}F , ^1H waterLOGSY and ^1H chemical shift perturbation NMR spectroscopy. We observed the first ^{19}F NMR spectroscopic evidence for the formation of metal fluoride transition state analogues of yeast PGK and also observed significant changes to proton chemical shifts of PGK in the presence, but not in the absence, of fluoride upon titration of ligands, providing indirect evidence of the formation of a closed ternary transition state. WaterLOGSY NMR spectroscopy experiments using an uncompetitive model were used in an attempt to measure ligand binding affinities within the transition state analogue complexes.

Keywords

Yeast phosphoglycerate kinase, ^{19}F NMR spectroscopy, transition state analogue complex, metal fluoride complexes, MgF_3^- / AlF_4^-

Introduction

Phosphoglycerate kinase (PGK, EC 2.7.2.3) is an important controller of flux within cells for its role in catalyzing the first ATP generating step of glycolysis by transferring the 1-phosphate from 1,3-bisphosphoglycerate (1,3-BPG) onto the β -phosphate of ADP (Scheme 1). Human PGK has two isoforms, PGK1 and PGK2; PGK1 has been implicated in mediating a variety of disease states including cancer,(Zhao et al. 2014; Ameis et al. 2013) whilst PGK2 is involved in spermatogenesis (Yang et al. 2013). In anaerobic pathogens, including Plasmodium and Trypanosoma, PGK has been considered a therapeutic target due to the reliance of these organisms on glycolysis for energy metabolism (Bilsland et al. 2011). Yeast PGK has been examined extensively as a prototypical protein for structure-function studies (Szabo et al. 2008; Varga et al. 2005; Vas et al. 2010). PGK consists of highly conserved C-terminal and N-terminal domains joined by a flexible helix. This helix acts as a hinge, facilitating a transition from an open to a closed conformation upon substrate binding. Domain closure brings the N-terminal domain phosphoglycerate binding site into close proximity to the ADP binding site on the C-terminal domain, thus enabling phosphate transfer. This significant conformational change has been extensively described through the analysis of PGK crystal structures (Bernstein et al. 1997; Zerrad et al. 2011; Zheng et al. 2012; Bernstein et al. 1998). Recently, significant insight into the fundamental mechanism of enzyme-catalyzed phosphoryl transfer has come through the use of metal fluoride transition state analogue complexes (TSAs); these TSA species

exist on a timescale of seconds and can be observed by one-dimensional (1D) ^{19}F NMR spectroscopy (Jin et al. 2014; Griffin et al. 2012; Liu Xiaoxia et al. 2011; Baxter et al. 2010; Bowler et al. 2010; Cliff et al. 2010; Baxter et al. 2006). When assembled into an enzyme active site trifluoromagnesate (MgF_3^-) TSA and tetrafluoroaluminate (AlF_4^-) TSA complexes possess a net charge (MgF_3^- and AlF_4^- TSAs) and adopt geometries (MgF_3^- TSA only) that make them ideal mimics of a transferring phosphoryl group (Scheme 1). Herein, we provide the first description of yeast PGK TSA complexes using complementary NMR spectroscopy experiments: (i) direct observation of the trifluoromagnesate ($\text{PGK}\cdot 3\text{PG}\cdot \text{MgF}_3^-\cdot \text{ADP}$) and tetrafluoroaluminate ($\text{PGK}\cdot 3\text{PG}\cdot \text{AlF}_4^-\cdot \text{ADP}$) TSA complexes by ^{19}F NMR spectroscopy, (ii) determination of the apparent dissociation constants by waterLOGSY NMR using a model derived to evaluate tight-binding ligands in an uncompetitive system, and (iii) determination of the apparent dissociation constants by PGK ^1H NMR chemical shift perturbation.

Materials and Methods

Materials and methods

All materials were purchased from Sigma-Aldrich.

Quantification of yeast PGK by UV absorbance and SDS-PAGE gel densitometry

Yeast PGK (Sigma Aldrich 9001-83-6) was supplied as an ammonium sulfate suspension, therefore, an Amicon Ultra 0.5 mL Centrifugal Filter MWCO 10K was used for buffer exchange and protein concentration. PGK in 50 mM Tris (pH 7.5, Bioshop TRS003.1) or 40 mM KCl (Anachemia AC-7601), 10 mM triethanolamine (TEA, pH 7.1, Sigma Aldrich 102-71-6) was quantified by UV

absorbance at 280 nm using an extinction coefficient of $2.143 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Jakeman et al. 1998). Sample PGK purity was examined by SDS-PAGE and the proportion of PGK determined by densitometry using Scion Image analysis software based on the intensity of PGK band versus all other bands on the SDS-page gel. The PGK concentration was corrected to reflect the proportion of PGK as determined by densitometry. Visual inspection of the 1D ^1H NMR spectrum of PGK was consistent with that previously reported in the literature (Jakeman et. al. 1998).

^{19}F NMR studies

The ^{19}F NMR spectra of $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ TSA and the $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSA (Figure 1) were observed using methods previously described (Cliff et al. 2010). Both samples contained 20 mM DTT (Sigma Aldrich D-0632), 5 mM ADP (Sigma Aldrich A-2754), 10 mM 3PG (Sigma Aldrich P-8877), 5 mM MgCl_2 (Sigma Aldrich M-8266), 10 mM NH_4F (Sigma Aldrich 338869), 10% (v/v) D_2O (Sigma Aldrich 151882), and 50 mM Tris (pH 7.5). The $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ sample also contained 0.09 mM PGK and 3 mM NaN_3 (Fisher S-227). The $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ sample also contained 0.36 mM PGK and 1 mM AlCl_3 (Sigma Aldrich 563919). Spectra were recorded in 5 mm Shigemi Advanced NMR Microtubes using a Bruker AV 500 MHz spectrometer with a BBFO SmartProbe at 300 K. ^{19}F NMR observe experiments were performed with proton decoupling and with background suppression using a composite pulse technique to remove the teflon background arising from the

probe for both $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ and $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ (Cory and Ritchey 1988). The presence of non-enzymatic AlF_x complexes with the same chemical shifts as $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ necessitated ^{19}F saturation experiments which were performed by applying a weak (15 Hz) radio frequency pulse to the target signal for one second prior to each acquisition. The $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ data were acquired for 60,000 scans and the $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ data were acquired for 41,368 scans. Data were processed with line broadening set to 50.

Apparent dissociation constants K_D^{ADP} , $K_D^{\text{F (Mg)}}$, and $K_D^{\text{F (Al)}}$ determined by ^1H waterLOGSY NMR

Spectra were recorded in 5 mm Sample Vault NMR tubes using a Bruker Advance-III 700 MHz spectrometer with a TCI CryoProbe at 300 K. Intensities for ^1H resonances in the absence of yeast PGK were subtracted before fitting. Data fitting to the indicated equations was performed using nonlinear regression software GraFit 5.0 (Erithacus Software). K_D^{ADP} (Scheme 2A) was determined using a previously described waterLOGSY NMR method (Dalvit et al. 2001). ADP was titrated into a solution containing 7 μM PGK, 5 mM 3PG, 5 mM MgCl_2 , 5 mM DTT, 10% (v/v) D_2O , and 50 mM Tris (pH 7.5). A waterLOGSY spectrum was recorded after each ADP addition. K_D^{ADP} was determined by non-linear regression fitting of the NMR signal intensity to a dose-response equation (Equation 1)(Dalvit et al. 2001), where θ is the intensity of the waterLOGSY signal from the ligand in the presence of the enzyme (manually corrected by

subtraction of the signal intensity of free ligand recorded in the absence of enzyme), $\theta_{\max}$ is the maximum intensity of the waterLOGSY signal, S_F is the free ligand, and K_D is the apparent dissociation binding constant.

$$\theta = \frac{-\theta_{\max}}{1 + \left(\frac{S_F}{K_D}\right)} + \theta_{\max} \quad (1)$$

The apparent dissociation constants of the TSA complexes $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ ($K_D^{\text{F (Mg)}}$, Scheme 2B) and $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ ($K_D^{\text{F (Al)}}$, Scheme 2C) were also determined by waterLOGSY NMR. Ammonium fluoride was titrated into a reaction mixture containing 7 μM PGK, 5 mM 3PG, 5 mM MgCl_2 , 5 mM DTT, 10% (v/v) D_2O , 0.5 mM ADP and 50 mM Tris (pH 7.5). AlCl_3 was included at 1 mM when determining $K_D^{\text{F (Al)}}$. A waterLOGSY NMR spectrum was recorded after each ammonium fluoride addition. The obtained ligand intensity data was fit to Equation 9, derived below, by non-linear regression, where $[S_0]$ is the total ADP concentration, $[E_0]$ is the total PGK concentration, $[I_0]$ is the total NH_4F concentration divided by three or four (to reflect that three or four F^- are required for inhibition through TSA formation), $[ES]$ is the concentration of PGK bound ADP, $[ESI]$ is the concentration of enzyme bound TSA, K_D^{S} is the dissociation constant for ADP and K_D^{I} is the dissociation constant for the TSA.

The waterLOGSY signal intensity of a ligand in the absence of inhibitor (θ_-) has been derived from the equilibrium relationship between $[ES]$ and θ_- shown in Equation 2 (Dalvit et al. 2002).

$$\theta_- = \frac{[ES]}{[S_0]} = \frac{[S_0] + [E_0] + K_D - \sqrt{([S_0] + [E_0] + K_D)^2 - 4[S_0][E_0]}}{2[S_0]} \quad (2)$$

It has been demonstrated that waterLOGSY signal intensity can be used to indirectly determine the dissociation constant of a tight-binding ligand

(inhibitor) in a competitive equilibrium system (Dalvit et al. 2002). Here, we derive a similar relationship to describe an uncompetitive system.

$$K_d^s = \frac{([S_0] - [ES] - [ESI]) \cdot ([E_0] - [ES] - [ESI])}{[ES]} \quad (3)$$

$$K_d^i = \frac{([I_0] - [ESI]) \cdot [ES]}{[ESI]} \quad (4)$$

Mass action Equations 3 and 4 were combined to give Equation 5.

$$[ES] + [ESI] = \left(\frac{([S_0] - [ES] - [ESI]) \cdot ([E_0] - [ES] - [ESI])}{K_d^s} \right) \left(1 + \frac{[I_0] - [ESI]}{K_d^i} \right) \quad (5)$$

Signal intensity (θ_+) in an uncompetitive system is determined by contributions from both the concentration of ligand bound to the enzyme (ES) and ligand plus inhibitor enzyme complex (ESI, Equation 6).

$$\theta_+ = \frac{[ES] + [ESI]}{[S_0]} \quad (6)$$

Equation 6 is substituted into Equation 5 to give Equation 7.

$$\theta_+ = \left(\frac{(1 - \theta_+) \cdot ([E_0] - [S_0]\theta_+)}{K_d^s} \right) \left(1 + \frac{[I_0] - [ESI]}{K_d^i} \right) \quad (7)$$

Given the low concentration of enzyme $[E_0]$ relative to $[S_0]$ (ADP) and $[I_0]$ (NH_4F), the assumption that $[I_0] \gg [ESI]$ is imposed, thus $[ESI]$ is negligible (Lin and Riggs 1972). Rearrangement of Equation 7 into the quadratic form (Equation 8) describes the waterLOGSY signal intensity of a ligand in the presence of an uncompetitive inhibitor.

$$\theta_+ = \frac{[S_0] + [E_0] + \frac{K_d^s \cdot K_d^i}{K_d^i + [I_0]} + \sqrt{\left([S_0] + [E_0] + \frac{K_d^s \cdot K_d^i}{K_d^i + [I_0]} \right)^2 - 4[S_0] \cdot [E_0]}}{2[S_0]} \quad (8)$$

The ratio of waterLOGSY signal intensity in the presence and absence of an uncompetitive, tight-binding ligand is shown in Equation 9, which can be solved by nonlinear regression analysis to determine dissociation constant of a tight-binding ligands, K_D^I , when a weaker binding ligand K_D^S is known.

$$\frac{\theta_+}{\theta_-} = \frac{[S_0] + [E_0] + \frac{K_D^S \cdot K_D^I}{K_D^I + [I_0]} + \sqrt{\left([S_0] + [E_0] + \frac{K_D^S \cdot K_D^I}{K_D^I + [I_0]}\right)^2 - 4[S_0] \cdot [E_0]}}{[S_0] + [E_0] + K_D^S + \sqrt{([S_0] + [E_0] + K_D^S)^2 - 4[S_0] \cdot [E_0]}} \quad (9)$$

Measurement of apparent dissociation constant of PGK•3PG•ADP and PGK•3PG•MgF₃⁻•ADP TSAs as determined by ¹H NMR chemical shift perturbation

The apparent dissociation constant for 3PG (K_D^{3PG} , Scheme 4A) was determined by monitoring changes to the ¹H NMR protein chemical shifts as previously described (Jakeman et al. 1998), except that all samples included ADP and MgCl₂. Spectra were taken in 5 mm Sample Vault NMR tubes using a Bruker Advance-III 700 MHz spectrometer with a TCI CryoProbe at 300 K. 3PG was titrated into a reaction mixture containing 0.179 mM PGK, 5 mM ADP, 5 mM MgCl₂, 5 mM DTT, 10 % (v/v) D₂O, 0.04 % (v/v) acetone, 40 mM KCl, and 10 mM TEA (pH 7.1). A second sample was prepared as above with the addition of 5 mM NH₄F. This sample was used to determine an apparent dissociation constant for the formation of the PGK•3PG•MgF₃⁻•ADP TSA complex ($K_D^{3PG(F)}$, Scheme 4b). Chemical shift changes were fitted to Equation 10 using non-linear regression and assuming a 1:1 binding model, where $\Delta\delta H_{max}$ is the maximum chemical shift change, $[S_0]$ is the total substrate concentration, $[E_0]$ is the total

enzyme concentration and K_D is the apparent dissociation constant of the substrate (Jakeman et al. 1998).

$$\Delta \square \square = \frac{\Delta \square \square \square \square}{2} \left(\left(1 + \frac{[\square \square]}{[\square \square]} + \frac{\square \square}{[\square \square]} \right) - \sqrt{\left(1 + \frac{[\square \square]}{[\square \square]} + \frac{\square \square}{[\square \square]} \right)^2 - 4 \frac{[\square \square]}{[\square \square]}} \right) \quad (10)$$

Results and discussion

^{19}F NMR spectroscopy of yeast $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ and $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^-$

•ADP TSAs

Commercial samples of yeast PGK for ^{19}F NMR were mixed with ADP (a reactant) and 3PG (a product) as ligands to prevent catalytic activity, as similarly described for human PGK1 enzyme (Baxter et al. 2008). ^{19}F NMR spectra were recorded to observe the formation of yeast $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ and $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSAs (Scheme 1). The individual fluorides of metal fluoride TSAs are positioned in discrete sites within the enzyme active site such that they do not undergo rotational averaging and therefore appear at distinct chemical shifts (Baxter et al. 2006). The ^{19}F NMR spectrum of the trifluoromagnesate complex contained three distinct resonances, arbitrarily labeled based on chemical shift from left to right F_A , F_B , and F_C , at -153, -156 and -161 ppm, respectively (Fig. 1a), confirming formation of a $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ TSA. Titration of aluminum into this sample resulted in the loss of the signals attributed to the $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ TSA, and the concurrent appearance of four distinct upfield resonances (Fig. 1b). These signals are attributed to the formation of the $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSA with ^{19}F NMR chemical shifts arbitrarily assigned from left to right F_A , F_B , F_C , and F_D ,

at -136, -139, -141, and -146 ppm, respectively. Chemical shifts observed in these TSAs were consistent with the human PGK1•3PG•MgF₃⁻•ADP TSA and with the human PGK1 (Cliff et al. 2010) and *Geobacillus stearothermophilus* (Baxter et al. 2008) PGK•3PG•AlF₄⁻•ADP TSA, as was the observation that formation of the aluminate TSA outcompeted the magnesate TSA. It is perhaps unsurprising that both yeast PGK and human PGK1 are able to stabilize a magnesate TSA in solution as they are highly homologous, having 66 % sequence identity (Altshul et al. 1997), while *G. stearothermophilus* PGK has reduced sequence homology (51%), and has not been observed to form a magnesate TSA complex (Baxter et al. 2008).

Phosphate transfer enzymes are believed to proceed via a trigonal planar phosphoryl (PO₃⁻) transition state (Scheme 2a), to which both the MgF₃⁻ and AlF₄⁻ TSAs are isoelectronic. (Cliff et al. 2010) Additionally, the MgF₃⁻ TSA possesses the same trigonal bipyramidal geometry as a transferring phosphate group, whilst the AlF₄⁻ TSA possesses non-isosteric octahedral geometry. (Cliff et al. 2010) The out competition of an isosteric MgF₃⁻ TSA to a non-isosteric AlF₄⁻ TSA, as observed in the ¹⁹F NMR spectra, suggests that the yeast PGK active site is flexible with respect to geometry. The preferential formation of the non-isosteric AlF₄⁻ complex, in the presence of a five-fold excess of magnesium, may be largely a function of solubility of the free metal fluoride complexes, and is consistent with hPGK. At physiological pH, MgF₃⁻ is largely insoluble, with the major magnesium species present as MgF⁺ and MgF₂, while AlF₄⁻ is highly soluble. (Fovet and Gal 2000) Thus, the observed preferential formation of the

aluminate TSA may be explained by the increased availability of AlF_4^- as compared to MgF_3^- . Furthermore, studies of human PGK1 in which the charge-balance of the enzyme active site has been altered by mutagenesis have provided substantial evidence that charge-retention is prioritized over retention of geometry (Cliff et al. 2010).

The X-ray crystallographic data of highly homologous human $\text{PGK1} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ and $\text{PGK1} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSAs were used to predict the important active site contacts involved in TSA formation in the equivalent yeast PGK complexes (Cliff et al. 2010). In the yeast $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ TSA, non-catalytic Mg^{2+} has trigonal bipyramidal geometry with axial coordination to ADP and 3PG, and equatorial coordination to three fluoride anions. Based on X-ray crystallographic data of the homologous human PGK, the fluorine atoms of MgF_3^- within the active site of yeast PGK are predicted to be coordinated with the catalytic Mg^{2+} , a single water molecule, and K213, G371, R38, K217 and G394. Equivalently, comparative analysis for the yeast $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSA reveals Al^{3+} coordination to 3PG and ADP, and fluorine coordination to two water molecules and K214, G371, R39, K218 and G394.

Apparent dissociation constants of ADP, MgF_3^- and AlF_4^- to yeast PGK as measured by waterLOGSY NMR

The ^{19}F NMR data provided strong evidence for the formation of both the yeast $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ and $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSAs. We next attempted to quantify the apparent dissociation constants associated with each complex using waterLOGSY NMR (Dalvit et al. 2001). An apparent K_D value for the binding of

ADP (K_D^{ADP} , Scheme 2A) was calculated by monitoring the intensity of the ADP proton resonances for three individual ADP protons (Table 1, Fig. 2), with titration of ADP in the presence of PGK. The obtained K_D^{ADP} , $0.56 \text{ mM} \pm 0.12 \text{ mM}$, was comparable in magnitude, to previously recorded values determined by alternate methods including differential absorption (K_D^{ADP} 0.25 mM (Roustan et al. 1973) and 0.19 mM (Wiksell and Larsson-Raznikiewicz 1982)) and gel filtration (K_D^{ADP} 0.25 mM (Tanswell et al. 1974)).

The apparent dissociation constant of 3PG ($K_D^{3\text{PG}}$) could not be accurately determined by waterLOGSY NMR due to difficulties in observing positively phased peaks under the experimental conditions. This suggests poor NOE transfer to this ligand and limits experimental sensitivity. By contrast, the dissociation constant for a β -phosphoglucosyltransferase (β -PGM) MgF_3^- TSA has been proposed to be $\leq 70 \text{ nM}$ (Golicnik et al. 2009). Although high affinity ligands ($K_D < 100 \text{ nM}$) do not show positive binding by waterLOGSY NMR, Davit *et al.* described a method for the indirect detection of tight binding ligands using competition experiments (Dalvit et al. 2001; Dalvit et al. 2002). Herein, we have adapted the technique for the detection of TSAs by employing the assumption that a TSA complex, with a $K_D \sim 70 \text{ nM}$, will behave as a tight-binding ligand. Thus, it was anticipated that the formation of TSA complexes would result in a decrease in intensity of the positively binding resonances from ADP, as fluoride was titrated into the sample due to uncompetitive displacement of ADP from the yeast PGK active site by the tight-binding and long-lived TSA complex. Formation of the metal fluoride complexes within the active site of

PGK is improbable without the prior binding of 3PG, ADP, and catalytic Mg^{2+} to the enzyme, suggesting inhibition by fluoride may be described using an uncompetitive model (Scheme 2B and 2C). Experiments were performed with saturating Mg^{2+} , 3PG, and Al^{3+} (present only for the $\text{PGK} \cdot 3\text{PG} \cdot \text{AlF}_4^- \cdot \text{ADP}$ TSA), and with constant ADP and PGK. Changes in ADP signal intensity were monitored by recording waterLOGSY NMR spectra following each fluoride addition. As predicted, the peak intensities of the ADP protons decreased with increasing fluoride concentration (Fig. 3a-f). The dissociation constants $K_D^{\text{F (Mg)}}$ and $K_D^{\text{F (Al)}}$ (Scheme 2B and 2C) were obtained by fitting the waterLOGSY signal intensity data to a model of uncompetitive ligand binding (for derivation please see ESI) using non-linear regression and solving for the physiologically meaningful root. The $K_D^{\text{F (Mg)}}$ was calculated from the same ADP protons as K_D^{ADP} to give an average $K_D^{\text{F (Mg)}}$ of $0.37 \text{ mM} \pm 0.06 \text{ mM}$ (Table 1). The $K_D^{\text{F (Mg)}}$ values were corrected to reflect the need for three equivalents of NH_4F for one equivalent of TSA. The $K_D^{\text{F (Al)}}$ value was determined as above, except for the addition of 1 mM AlCl_3 at the outset, to give an average $K_D^{\text{F (Al)}}$ of $0.53 \text{ mM} \pm 0.05 \text{ mM}$. The $K_D^{\text{F (Al)}}$ values were corrected to reflect the need for four equivalents of NH_4F for one equivalent of TSA. The H8 adenosine peak intensity did not appear to change with the addition of NH_4F , however, a decrease in peak width was observed (Fig. 3d).

The measured apparent TSA dissociation constants, $K_D^{\text{F (Mg)}}$ and $K_D^{\text{F (Al)}}$, were found to be on the same order of magnitude as K_D^{ADP} . It seems highly unlikely, given the magnitude of the values (high μM), that these accurately

reflect the dissociation constants of the TSAs (Golicnik et al. 2009).

Furthermore, the measured $K_D^{F(AI)}$ is greater than the measured $K_D^{F(Mg)}$. This does not correlate with ^{19}F NMR data in which the $PGK \cdot 3PG \cdot AlF_4^- \cdot ADP$ TSA was observed to outcompete formation of the magnesate TSA complex, even in the presence of five-fold excess magnesium. Inhibition constants by fluoride for β -PGM have been reported in the low millimolar range, and this result was in part attributed to the low formation constant of free MgF_3^- in aqueous solution (Baxter et al. 2006). However, free AlF_4^- is readily observed in solution, and thus, if solubility of the metal fluoride complex was the only factor, we would have obtained a smaller dissociation constant for the $PGK \cdot 3PG \cdot AlF_4^- \cdot ADP$ TSA in comparison to the $PGK \cdot 3PG \cdot MgF_3^- \cdot ADP$ TSA. X-ray crystallography of human PGK1 depicts a conformational change that occurs upon substrate binding, and a further conformational change subsequent to TSA formation such that the structure of $PGK \cdot 3PG \cdot MgF_3^- \cdot ADP$ is more fully closed than $PGK \cdot 3PG \cdot ADP$ (Cliff et al. 2010). This conformational change is suggestive of being a rate-limiting step, and would require a more complex model than the simple uncompetitive model herein applied. Another consideration is the high ligand-to-protein ratio employed in the experiments, may contribute to non-specific binding and thus increase the positive binding signal (Fielding et al. 2005). Although the measured $K_D^{F(Mg)}$ and $K_D^{F(AI)}$ values may not accurately reflect the formation of TSAs, the clear decrease in waterLOGSY signal intensity for ADP protons as a function of increasing fluoride indicates the waterLOGSY experiments did provide evidence of an interaction in solution.

Apparent dissociation constants of 3PG in the presence and absence of fluoride as measured by ^1H NMR chemical shift perturbation

The lack of success using the ligand observe waterLOGSY NMR approach above to measure binding affinities for TSA formation for yeast PGK prompted us to perform a series of titration experiments to observe the global effects of fluoride upon the yeast PGK ^1H NMR spectrum in the presence and absence of PGK•3PG•MgF₃⁻•ADP TSA formation. 3PG was titrated into a solution of ADP, MgCl₂ and PGK and changes to the protein chemical shifts ($\Delta\delta\text{H}$) were monitored by ^1H NMR spectroscopy after each addition. The chemical shifts of PGK protons were observed to change as a function of 3PG concentration (Fig. 4a and 4b). The addition of 3PG induced a $\Delta\delta\text{H}$ for 20 proton resonances (Table 2). We were able to identify three of these resonances as Tyr-380, Phe-342 and His-62 based on assignments previously described in the literature (Fairbrother et al. 1989a). It was possible to quantify the data for these three resonances, whilst the remaining $\Delta\delta\text{H}$ were either too small ($\Delta\delta\text{H}_{\text{max}} < 0.009$ ppm) to accurately calculate a dissociation constant, or individual peaks became obscured by other signals as they shifted. A limitation of our study was the use of commercial yeast PGK which is not isotopically labeled for multi-dimensional NMR experiments. Such labeling may provide additional insight into the conformational changes of yeast PGK upon TSA complexation. His-62 is located within the basic patch, a region of the *N*-terminal domain associated with 3PG binding, whereas, Tyr-380 and Phe-342 are within the *C*-terminal domain. Quantification of 3PG binding to PGK for Tyr-380 and Phe-342 was in

contrast to the data of Fairbrother and Williams (Fairbrother et al. 1989a), where, in the absence of ADP and MgCl_2 which is essential for yeast PGK catalysis, no chemical shift changes were observed for these resonances. That we observed changes to the chemical shift of residues in both the *N*- and *C*- termini as a result of including ADP, is likely an example of interdomain cooperativity and communication and has been observed in *G. stearotheophilus* PGK (Marston et al. 2010). The $\Delta\delta\text{H}$ for Tyr-380, Phe-342 and His-62 was used to fit Equation 10 (Fig. 5a, Table 2) to determine the apparent dissociation constant for the binding of 3PG to yeast PGK ($K_D^{3\text{PG}}$, Scheme 4a) (Jakeman et al. 1998; Fairbrother et al. 1989a). The average $K_D^{3\text{PG}}$, $0.041 \text{ mM} \pm 0.022 \text{ mM}$, was comparable to previously recorded values using an equivalent chemical shift perturbation NMR approach (Jakeman et al. 1998; Fairbrother et al. 1990).

The apparent dissociation constant for the formation of the $\text{PGK} \cdot 3\text{PG} \cdot \text{MgF}_3^- \cdot \text{ADP}$ TSA ($K_D^{3\text{PG}(\text{F})}$, Scheme 2E) was determined as above, with the addition of saturating NH_4F at the outset. The presence of NH_4F alone did not induce a $\Delta\delta\text{H}$ to the yeast PGK ^1H NMR spectrum (Fig. ESI1), which suggested that fluoride did not independently perturb the enzyme. However, the addition of 3PG and NH_4F resulted in a $\Delta\delta\text{H}$ for 52 PGK proton resonances (Fig. 4c and 4d), a two-fold increase in the number of peak shifts observed with the addition of 3PG alone. An increase in the magnitude of the $\Delta\delta\text{H}_{\text{max}}$ values was also observed for these perturbations. Individual resonances were arbitrarily assigned numbers from right to left in the ^1H NMR spectrum (Fig. 4d). A $K_D^{3\text{PG}(\text{F})}$ was calculated for all possible peaks (Table 2, Fig. 5b), including residues 4-

13 which have not been previously reported. Additionally, a $\Delta\delta H$ has not been previously reported for Peaks 5-13 and several of the peaks for which a dissociation constant could not be calculated (Jakeman et al. 1998; Fairbrother et al. 1990; Fairbrother et al. 1989b). There were up to two orders of magnitude variation in the calculated dissociation constants (Table 2), with an average $K_D^{3PG(F)}$ of $0.46 \text{ mM} \pm 0.13 \text{ mM}$, likely indicating that a multitude of factors are being observed through the yeast PGK 1H 1D NMR spectrum changes.

It was anticipated that the PGK•3PG•MgF₃⁻•ADP TSA would form a tighter complex than PGK•3PG•ADP, however, apparent $K_D^{3PG(F)}$ values of residues 4-13 indicated a 10-fold weaker binding than K_D^{3PG} . As with the TSA dissociation constants measured by waterLOGSY, it is likely the measured $K_D^{3PG(F)}$ values do not accurately reflect the formation of the PGK•3PG•MgF₃⁻•ADP TSA. X-ray crystallography data indicates human PGK undergoes conformational change as it transitions from PGK•3PG•ADP to the PGK•3PG•MgF₃⁻•ADP TSA, resulting in a closed conformation at the active site.(Cliff et al. 2010) A $\Delta\delta H$ was observed for a singular methyl resonance upon formation of the yeast PGK•3PG•MgF₃⁻•ADP TSA (Figure 4, Table 2 entry 4). This is consistent with the 1H NMR spectrum of human PGK1 where a singular methyl resonance was shown to shift upon formation of a MgF₃⁻ or AlF₄⁻ TSA (Cliff et al. 2010). Additionally, 1H NMR chemical shift perturbation experiments for the *G. stearothermophilus* TSA showed a $\Delta\delta H$ for residues of the interdomain region that were unaffected by the binding of 3PG or ADP alone.(Marston et al. 2010) We hypothesize that the $\Delta\delta H$ of resonances 4-13 in

the PGK•3PG•MgF₃⁻•ADP TSA may be the result of changes in PGK secondary structure,(Wishart et al. 1991,Sibley et al. 2003)), and solvent exposure (Avbelj et al. 2004) upon formation of the closed active site conformation.

These results provide further evidence for yeast PGK conformational change upon formation of a TSA complex with metal fluorides, that is distinct from the substrate-bound enzyme species that have been examined previously (Fairbrother et al. 1990; Fairbrother et al. 1989b).

Although the $\Delta\delta H$ of resonances 4-13 may be due to overall protein conformational change, the $\Delta\delta H$ of resonances His-62 and Tyr-380 may represent the formation of the TSA directly. Resonances His-62 and Tyr-380 were observed to undergo a $\Delta\delta H$ upon formation of the TSA that was significantly tighter than for 3PG alone (Figure 6a and 6b). The $\Delta\delta H_{\max}$ was reached at a very low concentration of 3PG, suggestive of the observation of a TSA directly. A $K_D^{3PG(F)}$ could not be calculated for these resonances, by fitting Equation 10 to the data using our standard approaches that were constrained by the concentrations(μM range) of yeast PGK suitable for 1D ¹H NMR analysis, due to the extremely tight binding. However, the data for both peaks are highly consistent with a two-state cooperative binding model (Figure 6c) suggested by Marston *et al* (Marston et al. 2010) for *G. stearothermophilus* PGK. In this model the active site of PGK has two conformations in equilibrium (E), one with a weak affinity for ligands (A) and one with a strong affinity for ligands (B). The affinity of the B state to form the TSA is much greater than the affinity for 3PG. At saturation, this results in an approximately equal population of A and B

state when 3PG is the ligand but a total population of the B state as the TSA forms. This difference in binding affinity is reflected by a change to the dissociation curve (Figure 6d and 6e) and curves consistent with the expected trend of this model were observed in the $\Delta\delta H$ of His-62 and Tyr-380 as the TSA is formed upon titration of ADP to form the TSA (Figure 6a and 6b). Although a dissociation constant could not be calculated due to the tight binding, a two-state cooperative binding model suggests $K_D^{3PG(F)}$ is significantly smaller than K_D^{3PG} (Table 2), and K_D values for TSA complexes have been estimated to be as low 10^{-14} M (Marston et al. 2010). However, the average K_D^{3PG} calculated clearly does not accurately reflect the range of K_D^{3PG} values (0.006-0.944) measured. The broad range in K_D^{3PG} values is likely reflective of chemical shift changes that include factors in addition to ligand binding. The observation that one residue from the *N*-terminal domain, His-62, and one residue from the *C*-terminal domain, Tyr-380, fit the two-state cooperative binding model provides the first experimental NMR evidence for yeast PGK to fit this cooperative two-state binding model.

Conclusions

We have presented the first ^{19}F NMR evidence for the existence of magnesium trifluoride and aluminium tetrafluoride TSA analogues for yeast PGK. We have evaluated waterLOGSY NMR and chemical shift perturbation methods to quantify binding of TSA analogues to yeast PGK and developed a waterLOGSY NMR analysis approach for an uncompetitive ligand system. Analysis of the chemical shift perturbation data for two resonances, His-62 within the *N*-

terminal domain, and Tyr-380 within the *C*-terminal domain, provides experimental evidence for a two-state cooperative model for TSA formation.

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Tables

Table 1: Apparent Dissociation Constants Determined Using waterLOGSY NMR

ADP Resonance	Chemical Shift (ppm)	K_D^{ADP} (mM)	$K_D^{F(Mg)}$ (mM) ^a	$K_D^{F(Al)}$ (mM) ^b
H1 of Ribose	6.16	0.43 ± 0.12	0.26 ± 0.04	0.43 ± 0.05
H1 of Ribose	6.17	0.59 ± 0.18	0.3 ± 0.04	0.52 ± 0.05
H2 of Adenine	8.20	0.43 ± 0.03	0.47 ± 0.09	0.62 ± 0.05
H8 of Adenine	8.55	0.8 ± 0.1	0.44 ± 0.05	
Average		0.56 ± 0.12	0.37 ± 0.06	0.53 ± 0.05

^a values have been corrected to reflect three equivalents of NH₄F are required to form one equivalent of TSA.

^b values have been corrected to reflect four equivalents of NH₄F are required to form one equivalent of TSA.

Table 2: Apparent Dissociation Constants Determined Using ¹H NMR Chemical Shift perturbation

PGK Resonance	δH (ppm)	3PG ΔδH _{max} (ppm)	K_D^{3PG} (mM)	3PG(F) ΔδH _{max} (ppm)	$K_D^{3PG(F)}$ (mM)	Residue
1	6.73	0.0204 ± 0.0015	0.104 ± 0.035		«0.104	Tyr-380 ¹¹
2	6.93	0.0097 ± 0.0009	0.001 ± 0.009			Phe-342 ¹¹
3	7.97	0.0199 ± 0.0011	0.018 ± 0.013		«0.018	His-62 ²⁷
4	-0.58			0.0731 ± 0.003	0.944 ± 0.096	
5a	3.29			0.0206 ± 0.002	0.414 ± 0.134	
5b	3.30			0.0208 ± 0.0015	0.433 ± 0.104	
5c	3.31			0.0198 ± 0.0019	0.282 ± 0.107	
6	8.50			0.0151 ± 0.0011	0.102 ± 0.044	
7	9.10			0.0649 ± 0.0056	0.535 ± 0.138	
8	9.36			0.0196 ± 0.001	0.006 ± 0.008	
9	9.41			0.0731 ± 0.004	0.061 ± 0.025	

10	10.16	0.0152 ± 0.0016	0.510 ± 0.161
11	10.60	0.0318 ± 0.002	0.584 ± 0.105
12	10.70	0.0449 ± 0.0047	0.802 ± 0.215
13	11.18	0.1388 ± 0.0129	0.849 ± 0.216
Average		0.041 ± 0.022	0.460 ± 0.13

Figure captions

Scheme 1. A, Conversion of 1,3-bisphosphoglycerate and ADP to 3-phosphoglyceric acid and ATP by PGK. B, PGK•3PG•PO₃[−]•ADP TS. C, PGK•3PG•MgF₃[−]•ADP TSA. D, PGK•3PG•AlF₄[−]•ADP TSA.

Figure 1. ¹⁹F NMR spectra of yeast PGK TSA complexes (a) PGK•3PG•MgF₃[−]•ADP (F_A = -153 ppm, F_B = -156 ppm, F_C = -161 ppm). (b) PGK•3PG•AlF₄[−]•ADP (F_A = -136 ppm, F_B = -138 ppm, F_C = -141 ppm, F_D = -146ppm).

Resonance labels (A-D) were assigned arbitrarily based on chemical shift from left to right and do not indicate corresponding signals for each spectra. The resonance at -120 ppm corresponds to free fluoride.

Figure 2. The waterLOGSY NMR signal intensities of ADP protons. Equation 1 (ESI) was fitted to determine K_D^{ADP} and shown as a best fit curve for H8 (●) and H2 (■) of the adenine moiety and H1 of the ribose moiety. H1 of the ribose moiety appears as a doublet at 6.17 ppm (♦) and 6.16 ppm (+) and thus generated two curves. The values have been corrected for free ADP and as such represent enzyme bound ADP only.

Figure 3. Effect of the formation of the TSAs $\text{PGK}\cdot\text{3PG}\cdot\text{MgF}_3^-\cdot\text{ADP}$ and $\text{PGK}\cdot\text{3PG}\cdot\text{AlF}_4^-\cdot\text{ADP}$ on the interaction of ADP with yeast PGK. H8 (a) and H2 (b) of adenosine, and H1 (c) of ribose was observed by waterLOGSY NMR in the presence of constant PGK, MgCl_2 , ADP, and 3PG, and 0 (■), 0.17 (■), 0.25 (■), 0.42 (■), 1.0 (■), and 1.67 (■) mM NH_4F . H8 (d) and H2 (e) of adenosine, and H1 (f) of ribose was observed by waterLOGSY NMR in the presence of constant PGK, MgCl_2 , 3PG, and AlCl_3 , and 0 (■), 0.19 (■), 0.25 (■), 0.38 (■), 0.75 (■), and 1.25 (■) mM NH_4F . Individual panels have been scaled independently.

Figure 4. ^1H 1D NMR spectrum of yeast PGK and perturbation by 3PG and the TSA $\text{PGK}\cdot\text{3PG}\cdot\text{MgF}_3^-\cdot\text{ADP}$. Spectra were taken in the presence of constant PGK, ADP, MgCl_2 and (a) 0 mM 3PG, (b) 1.5 mM 3PG, (c) 5 mM NH_4F and (d) 5 mM NH_4F and 3 mM 3PG. Peaks whose $\Delta\delta\text{H}$ with the addition of 3PG ($\downarrow$) and peaks for which a $K_D^{3\text{PG}}$ could be calculated (*) are highlighted. Individual sections have been scaled independently.

Figure 5. Effect of 3PG and the TSA $\text{PGK}\cdot\text{3PG}\cdot\text{MgF}_3^-\cdot\text{ADP}$ on the $\Delta\delta\text{H}$ of yeast PGK. The $\Delta\delta\text{H}$ of PGK was plotted as a function of the ratio of 3PG:PGK with constant ADP, MgCl_2 and 0 (a) or 5 (b) mM NH_4F using non-linear regression of Equation 10. The $\Delta\delta\text{H}$ have been measured individually for (a) peaks 1 (■), 2 (■), 3 (■), and (b) peaks 4 (■), 5 (■), 6 (■), 7 (■), 8 (■), 9 (■), 10 (■), 11 (■), 12 (■) and 13 (■). The $\Delta\delta\text{H}$ for 4a, 4b, and 4c have been averaged to 4 for clarity.

Figure 6. Effect of 3PG and the TSA $\text{PGK}\cdot\text{3PG}\cdot\text{MgF}_3^-\cdot\text{ADP}$ on the $\Delta\delta\text{H}$ of yeast PGK. The $\Delta\delta\text{H}$ of PGK was plotted as a function of 3PG concentration with constant ADP, MgCl_2 , and 0 (○) and 5 (●) mM NH_4F and Equation 10 fitted to

the data using non-linear regression. The $\Delta\delta H$ have been measured individually for resonance His-62 (a) and Tyr-380 (b). The two-state cooperative binding model (c), in which the active site of a protein can adopt two conformations which are in equilibrium (E) in the absence of ligand. The (A) state has a weak affinity for ligands (L, panel (d)) and the (B) state has a strong affinity for ligands (e). The affinity of the B state for 3PG is much weaker than the affinity for the TSA. At 3PG saturation the population of PGK in the A and B states is approximately equal (d) but at TSA saturation the population of PGK is almost entirely in the B state (e).

Draft

Scheme 1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6