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Mechanistic Insights into F₄₂₀-Dependent Glucose-6-Phosphate Dehydrogenase using Isotope Effects and Substrate Inhibition Studies

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Abstract

F₄₂₀-dependent glucose-6-phosphate dehydrogenase (FGD) is involved in the committed step of the pentose phosphate pathway within mycobacteria, where it catalyzes the reaction between glucose-6-phosphate (G6P) and the F₄₂₀ cofactor to yield 6-phosphogluconolactone and the reduced cofactor, F₄₂₀H₂. Here, we aim to probe the FGD reaction mechanism using dead-end inhibition experiments, as well as solvent and substrate deuterium isotope effects studies. The dead-end inhibition studies performed using citrate as the inhibitor revealed competitive and uncompetitive inhibition patterns for G6P and F₄₂₀ respectively, thus suggesting a mechanism of ordered addition of substrates in which the F₄₂₀ cofactor must first bind to FGD before G6P binding. The solvent deuterium isotope effects studies yielded normal solvent kinetic isotope effects (SKIE) on k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ for both G6P and F₄₂₀. The proton inventory data yielded a fractionation factor of 0.37, suggesting that the single proton responsible for the observed SKIE is likely donated by Glu109 and protonates the cofactor at position N1. The steady state substrate deuterium isotope effects studies using G6P and G6P-d₁ yielded KIE of 1.1 for both k_{cat} and $k_{\text{cat}}/K_{\text{m}}$, while the pre-steady state KIE on k_{obs} was 1.4. Because the hydride transferred to C5 of F₄₂₀ was the one targeted for isotopic substitution, these KIE values provide further evidence to support our previous findings that hydride transfer is likely not rate-limiting in the FGD reaction [1].

Graphical abstract

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Supporting information: Wild-type FGD single turnover plots at various % D₂O (SI Figure 1), Wild-type FGD apparent rate constants at various %D₂O (SI Table 1), LC-MS-IT-TOF chromatograms of enzymatically synthesized substrates (SI Figure 2).

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Introduction

F₄₂₀-dependent glucose-6-phosphate dehydrogenase (FGD) is an essential pentose-phosphate pathway enzyme that catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone within mycobacteria (Figure 1) [2]. The enzyme utilizes G6P to transfer a hydride to the C5 position on the *si*-face of the oxidized cofactor, producing reduced F₄₂₀ within the cell. Recent studies by Jirapanjawan *et al.* suggest that the reduced F₄₂₀ cofactor is essential for protecting mycobacteria against diverse environmental and antimicrobial agents [3]. More specifically, FGD appears to be the only enzyme within mycobacteria that produces the reduced F₄₂₀ cofactor [3] and is therefore essential for the persistence of environmental and pathogenic mycobacteria, including *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis disease (TB) [3].

The crystal structure confirms that FGD is a 78 kDa homodimer with one F₄₂₀ molecule per subunit [4]. The proposed FGD reaction mechanism based on this crystal structure suggested that His40 acts as an active site base, while Glu109 donates a proton to the F₄₂₀ cofactor, and Trp44 aids in stabilizing reaction intermediates [4]. However, there was no experimental evidence to support the FGD proposed mechanism. In our previous investigations of the FGD mechanism, we converted His40 to alanine (H40A), in order to discern if it is the active site base. We also converted Glu109 to glutamine (E109Q) to investigate whether it was the active site acid, and converted Trp44 to alanine (W44A), phenylalanine (W44F) and tyrosine (W44Y) to study its functionality [1]. Binding studies on these FGD variants suggested that these residues do not function in G6P binding, but that they do play a role in F₄₂₀ cofactor binding. Additionally, our steady state kinetic analysis of these variants suggests that the aromaticity of Trp44 is essential for FGD activity and that His40 and Glu109 play important roles in catalysis [1]. Further analysis of the H40A and E109Q variants, using pH profiles, suggested that His40 does not serve as the active site base, while Glu109 is the active site acid [1]. While this work provided insights into the key residues involved in FGD catalysis, much of the mechanistic details of the reaction is yet to be investigated.

For example, studies using isotopic labeling are informative given the multistep nature of enzymatic reactions, and are therefore effective methods for determining enzymatic mechanisms. Isotopic substitution *via* a deuterium or another heavy atom could change the rate of a specific step in a mechanism. Such studies can also help to determine whether an enzymatic reaction is stepwise or concerted [5, 6]. Additionally, solvent deuterium isotope studies can be useful in determining how many protons are in flight during turnover.

Studies of this kind have been performed with NADP⁺-dependent glucose-6-phosphate dehydrogenase (G6PD) in which glucose-6-phosphate-d₁ (G6P-d₁) and ¹³C-labeled G6P at the C-1 position were used to determine the magnitude of the isotope effect within the enzyme [7]. Steady state experiments were conducted with these substrates and with the natural unlabeled G6P substrate. The data showed an increase in the apparent V/K values with the deuterated substrate, which was characteristic of a mechanism in which all isotope effects are attributed to the same step of a reaction [7]. The isotopically labeled substrate experiments showed an isotope effect of $Dk = 5.27 \pm 0.29$, while in D₂O the number reduced to 3.66 ± 0.29 . The data suggest that there is a coupled motion effect when more than one hydrogen is in motion in the transition state [7]. Such studies have not previously been conducted with FGD nor any other F₄₂₀ dependent enzyme. Therefore, the goal of the present study is to use isotopic substitution to observe the isotope effect on the hydride transfer step using steady state and pre-steady state kinetic methods. We have also performed solvent isotope effects experiments using D₂O in order to determine the number and possible origin of protons in flight during turnover. Finally, since previous steady state kinetic studies could not deduce the order of substrate addition [1], an additional goal of this work is to use dead-end inhibition studies and steady state kinetics to determine the order of addition for G6P and the F₄₂₀ cofactor.

Materials and Methods

Reagents

G6P was purchased from USB Corporation, unless otherwise specified. The F₄₂₀ cofactor with a tail length of 6-9 γ -glutamate residues was prepared as previously described [8]. Activated carbon, ATP disodium salt (98%), sodium citrate, magnesium chloride and Tris buffer were purchased from Fisher Scientific. D₂O, deuterium chloride, D-Glucose and D-glucose-1-d₁ (98 atom % D) were purchased from Sigma Aldrich. Hexokinase was purchased from Worthington Biochemical Corporation. The glucose-6-phosphate assay kit was purchased from ScienCell Research Laboratories.

FGD expression and purification

The wild-type FGD enzyme used for all experiments presented in this work was expressed and purified as previously described [1, 9].

Dead-end inhibition studies using citrate

The wild-type FGD steady state kinetics experiments were performed using a Cary 100 UV-Visible spectrophotometer in a Starna Cells Spectrosil[®] Quartz sub-micro cell (160 μ l nominal volume). Citrate was chosen as the inhibitor for the dead-end inhibition studies [4].

The experiments were conducted at 22 °C in 50 mM Tris-HCl, pH 7.0 buffer containing 0, 2, 4, 8, or 10 mM sodium citrate. The wild-type FGD steady state kinetics experiments performed in the absence of citrate have previously been published elsewhere [1]. The inhibition pattern with respect to G6P concentration was determined at the various citrate concentrations using a constant F₄₂₀ concentration of 20 µM while varying the G6P concentrations (8 – 250 µM). The inhibition pattern with respect to F₄₂₀ concentration was determined at the various citrate concentrations using a constant G6P concentration of 2 mM while varying the F₄₂₀ concentrations (1 – 10 µM). Each reaction was initiated by adding wild-type FGD to a final concentration of 25 nM and the reduction of the F₄₂₀ cofactor was followed at 420 nm.

For a visual inspection of the inhibition patterns, plots of $1/k$ vs $1/[S]$ were fitted to a double reciprocal function (Equation 1) using SigmaPlot (Systat Software, Inc., San Jose, CA, USA) version 13.0.

$$1/k = (K_m/(k_{cat}[S])) + 1/k_{cat} \quad \text{Equation 1}$$

where, k is the rate constant obtained by dividing the initial rate by the enzyme concentration, k_{cat} is the turnover number, K_m is the Michaelis-Menten constant, and $[S]$ is concentration of either G6P or F₄₂₀. Multidimensional fits of k vs $[S]$ were also performed for the G6P and F₄₂₀ inhibition curves using SigmaPlot version 13.0 Enzyme Kinetics Module (Systat Software, Inc., San Jose, CA, USA). The G6P inhibition data fit best to the competitive inhibition model (Equation 2) while the F₄₂₀ inhibition data fit best to the uncompetitive inhibition model (Equation 3).

$$k = k_{cat}/(1 + (K_m/[S]) * (1 + I/K_I)) \quad \text{Equation 2}$$

$$k = k_{cat}/(1 + I/K_I + K_m/[S]) \quad \text{Equation 3}$$

Solvent deuterium isotope effects studies

Steady state kinetics—The effects of solvent on the steady state kinetics parameters of wild-type FGD were investigated by performing the experiments at 22 °C, in 50 mM Tris (pH 7.0) dissolved in H₂O or 50 mM Tris (pD 7.0) dissolved in 100% D₂O. The experiments performed in H₂O have previously been published elsewhere [1]. The D₂O buffer was prepared by deuterium chloride titration of the 50 mM Tris solution to pH 6.6, using the correction pD = pH + 0.4 [10, 11]. The kinetic parameters with respect to G6P were determined using a constant F₄₂₀ concentration of 20 µM and varying G6P concentrations from 0.008 - 2 mM, while the kinetic parameters with respect to the F₄₂₀ cofactor were determined using a constant G6P concentration of 2 mM and varying F₄₂₀ concentrations from 1 – 10 µM. Each reaction was initiated by adding wild-type FGD to a final

concentration of 25 nM and 420 nm wavelength was used to monitor the reduction of the F₄₂₀ cofactor. Plots of rate constant *vs* substrate concentration were fitted to a hyperbolic function (Equation 4) using SigmaPlot (Systat Software, Inc., San Jose, CA, USA) version 13.0.

$$k = k_{\text{cat}}[S]/(K_m + [S]) \quad \text{Equation 4}$$

where, *k* is the rate constant obtained by dividing the initial rate by the enzyme concentration, *k*_{cat} is the turnover number, *K*_m is the Michaelis-Menten constant, and [S] is concentration of either G6P or F₄₂₀. For each varied substrate, the solvent kinetic isotope effect (SKIE) was obtained by dividing the *k*_{cat} with respect to H₂O by the *k*_{cat} with respect to D₂O.

Proton inventory studies

Proton inventory studies were conducted using pre-steady state kinetic experiments under single turnover conditions. The rapid kinetic experiments were performed on a Hitech Scientific DX2 stopped-flow spectrophotometer at 22 °C in 50 mM Tris (pH 7.0) buffer dissolved in varying D₂O concentrations (0-100%). The experiments performed in 0% D₂O were performed in H₂O buffer and have previously been published elsewhere [1]. The 20, 40, 60, and 80% D₂O buffers were obtained by mixing appropriate volumes of 50 mM Tris in H₂O (0% D₂O, pH 7.0) with 50 mM Tris in D₂O (100% D₂O, pD 7.0). To ensure that the pD for each prepared D₂O solution remained at pD 7.0, the pD was measured using a pH electrode with the correction pD = pH + 0.4 [10, 11]. For each buffer composition, a solution of 100 μM FGD was pre-incubated with 10 μM F₄₂₀ and then mixed rapidly with an equal volume solution of 1 mM G6P solution. The final concentrations for the single turnover kinetics were 50 μM FGD, 5 μM F₄₂₀, and 500 μM G6P. The single turnover plots of absorbance at 420 nm *vs* time were fitted to Equation 5 using SigmaPlot (Systat Software, Inc., San Jose, CA, USA) version 13.0,

$$A = A_1 * e^{-k_{\text{obs}} * t} + c \quad \text{Equation 5}$$

where *A* is the absorbance at time *t*, *A*₁ is the initial absorbance at time *t* = 0, *k*_{obs} is the apparent rate constant, *t* is the reaction time, and *c* is the absorbance at infinite time. A linear plot of the proton inventory was analyzed using the simplified version of the Gross-Butler equation (Equation 6) [12, 13]

$$k_n/k_0 = (\Phi^T - 1)n + 1 \quad \text{Equation 6}$$

where *k*_n is the apparent rate constant at a given D₂O concentration, *k*₀ is the apparent rate constant in H₂O, *n* is the mole fraction of D₂O and Φ^T is the fractionation factor of a proton

in transition state [13, 14]. Assuming the hydrogen bond in which this proton participates to be linear, the hydrogen bond length (HBL) between the two proton wells was then calculated using Equations 7 and 8, where $x_{\min}$ is the distance between two proton wells, $2 \text{ \AA}$ is the value of two covalent bond lengths, and Φ^T is the fractionation factor [15-18].

$$\text{HBL} = \Delta x_{\min} + 2 \text{ \AA} \quad \text{Equation 7}$$

$$\Delta x_{\min} = 0.2220 + 1.1924(\Phi^T) - 1.3347(\Phi^T)^2 + 0.6080(\Phi^T)^3 \quad \text{Equation 8}$$

Kinetic deuterium isotope effect studies

G6P and G6P-d₁ synthesis—The deuterated substrate G6P-d₁ was enzymatically synthesized following a previously published protocol [7]. The reaction contained 30 mM D-glucose-1-d₁, 50 mM ATP, 500 U hexokinase and 50 mM MgCl₂ in a total volume of 50 mL. The reaction proceeded overnight at 4 °C and the pH was maintained at pH 8.0 using 2 N KOH. The reaction was then terminated by removing the hexokinase using an Amicon PM Ultrafiltration unit (30 kDa MWCO). The filtrate containing the deuterated substrate was then further purified using 3 cycles of activated charcoal treatment to remove excess ATP and ADP, followed by a NA⁺ Chelex-100 column treatment to remove the Mg²⁺ ions. The purified G6P-d₁ was then precipitated using rotary evaporation and ethanol addition. For fair comparison between the deuterated and protiated substrates, the G6P used for the kinetic isotope effect studies was also synthesized using the procedure above, except for the 30 mM D-glucose-1-d₁ being substituted for 30 mM D-glucose. The molar masses of the synthesized G6P and G6P-d₁ substrates were then confirmed using LC-MS IT-TOF. Both synthesized G6P and G6P-d₁ substrates were dissolved in filtered deionized water and the concentrations of the stocks determined using the ScienCell Research Laboratories G6P assay kit. The concentration used for the mass determination was 1 mM for each of the substrates. Data were acquired using the Shimadzu LC-MS-IT-TOF mass spectrometer (MS) and recorded in negative electrospray (ESI) mode at an ESI interface voltage of -3.7 kV. The LC direct injection elution program was run for 1 minute and the MS chromatograms were recorded from 100 m/z. The data was analyzed using LCMS Solution Software (Shimadzu Corp., Kyoto, Japan) version 3.70.390.

G6P and G6P-d₁ steady state kinetic experiments—The steady state kinetic experiments were performed and analyzed as discussed in the solvent isotope effects section of this manuscript. All the reactions were performed at 22 °C in 50 mM Tris-HCl, pH 7.0 buffer. The kinetic parameters were only determined with respect to the synthesized substrates, using a constant F₄₂₀ concentration of 20 μM and varying the G6P or G6P-d₁ concentrations from 0.008 – 0.4 mM. The final FGD concentration used in each reaction was 25 nM. The hyperbolic steady state kinetic plots of rate constant vs substrate concentration were fitted to Equation 4 using SigmaPlot (Systat Software, Inc., San Jose,

CA, USA) version 13.0. The kinetic isotope effect (k_H/k_D) was obtained by dividing the k_{cat} with respect to G6P (k_H) by the k_{cat} with respect to G6P-d₁ (k_D).

G6P and G6P-d₁ pre-steady state kinetic experiments—The pre-steady state kinetic experiments were performed under single turnover conditions as detailed in the solvent isotope effects section of this manuscript. All the reactions were performed at 22 °C in 50 mM Tris-HCl, pH 7.0 buffer. 100 μM wild-type FGD was pre-incubated with 10 μM F₄₂₀ in the reaction buffer and then mixed rapidly with an equal volume solution of 1 mM G6P or G6P-d₁ solution. The final concentrations in the single turnover reactions were 50 μM wild-type FGD, 5 μM F₄₂₀, and 500 μM G6P or G6P-d₁. The single turnover plots of absorbance at 420 nm vs time displayed exponential decay and were fitted to Equation 5 using SigmaPlot (Systat Software, Inc., San Jose, CA, USA) version 13.0. The kinetic isotope effect was obtained by dividing the k_{obs} with respect to G6P by the k_{obs} with respect to G6P-d₁.

Results

Dead-end inhibition studies using citrate

The Lineweaver-Burk plot of $1/k$ vs $1/[G6P]$ at a fixed concentration of F₄₂₀ and various fixed concentrations of citrate reveals competitive inhibition with respect to G6P (Figure 2A). The multidimensional fit of the G6P data to the competitive inhibition model (Figure 2B) yielded a G6P K_m of 26 ± 2 μM and citrate K_i of 0.84 ± 0.05 μM.

When the concentration of F₄₂₀ was varied at various fixed citrate concentrations and a constant G6P concentration, the Lineweaver-Burk plot of $1/k$ vs $1/[F_{420}]$ revealed an uncompetitive inhibition pattern of citrate with respect to F₄₂₀ (Figure 3A). The multidimensional fit of the F₄₂₀ data to the uncompetitive inhibition model (Figure 3B) yielded an F₄₂₀ K_m of 0.89 ± 0.08 μM and citrate K_i of 7.3 ± 0.4 μM.

Solvent deuterium isotope effect studies

Steady state kinetics—The magnitude of SKIE in the wild-type FGD reaction was determined by performing steady state kinetic experiments by varying the G6P or F₄₂₀ concentration in reaction buffer containing either D₂O or H₂O. The hyperbolic steady state kinetics plots of rate constant vs substrate concentration were fitted to Equation 4 to obtain the kinetic parameters. When G6P was the varied substrate (Figure 4A), the SKIE on the k_{cat} was 3.1 (Table 1). The SKIE on the k_{cat} when F₄₂₀ was the varied substrate (Figure 4B) was 2.6, making it comparable to the G6P SKIE within error (Table 1). The SKIE on the k_{cat}/K_m was 4.6 and 2.6 with respect to G6P and F₄₂₀ respectively (Table 1). The wild-type FGD steady state kinetics figures and parameters for experiments performed in H₂O have previously been published elsewhere [1].

Proton inventory studies—Rapid mixing experiments under single turnover conditions were performed to obtain proton inventory data. The wild-type FGD k_{obs} value in H₂O (0% D₂O) has previously been reported elsewhere [1]. The single exponential decay curves of absorbance at 420 nm vs time were fitted to Equation 5 (SI Figure 1) to obtain the k_{obs} value

at each concentration of D₂O (SI Table 1). Each of the k_{obs} values were divided by the k_{obs} value at 0% D₂O and plotted vs the mole fraction of D₂O and then fitted to Equation 6 to obtain the proton inventory parameters (Figure 5). The fractionation factor, Φ^T calculated from the proton inventory curve was 0.37, while the hydrogen bond length calculated from Equations 7 and 8 was 2.5 Å.

Kinetic deuterium isotope effect studies

G6P and G6P-d₁ molar masses—For the sake of proper comparison, both the protiated G6P and deuterated G6P-d₁ substrates were synthesized enzymatically following the same protocol. The molar masses of the synthesized G6P-d₁ and G6P were confirmed using the Shimadzu LC-MS-IT-TOF mass spectrometer and recorded in both positive and negative electrospray (ESI) mode. The negative ESI mode chromatograms revealed the predicted molar masses of 259.0225 g/mol and 260.0300 g/mol for the G6P (SI Figure 2A) and G6P-d₁ (SI Figure 2B), respectively.

G6P and G6P-d₁ steady state kinetic experiments—To determine the kinetic isotope effect on the wild-type FGD reaction, the steady state kinetic experiments were performed with a constant F₄₂₀ concentration, while varying either the G6P or G6P-d₁ substrate concentration. The hyperbolic curves of the rate constant vs substrate concentration were fitted to Equation 4 to obtain the Michaelis-Menten kinetic parameters (Figure 6). The wild-type FGD K_m and k_{cat} values were very similar for the G6P and G6P-d₁ substrates, resulting in an overall kinetic isotope effect of 1.1 with respect to both k_{cat} and k_{cat}/K_m (Table 2).

G6P and G6P-d₁ pre-steady state kinetic experiments—The kinetic isotope effect on the FGD reaction was also investigated by performing rapid mixing experiments under single turnover conditions. The exponential decay curves of absorbance at 420 nm vs time were fitted to Equation 5 to obtain the k_{obs} values (Figure 7). A kinetic isotope effect of 1.4 was obtained by dividing the k_{obs} with respect to G6P by the k_{obs} with respect to G6P-d₁ (Table 2).

Discussion

FGD is an enzyme that enhances the persistence of *M. tuberculosis*, catalyzing the committed step of the pentose-phosphate pathway which produces pentose phosphate sugars used for nucleotide biosynthesis within the bacterium. Additionally, it appears that FGD is the only *M. tuberculosis* enzyme that produces reduced F₄₂₀ cofactor, which is essential for detoxification processes within the bacterium [3]. While the *M. tuberculosis* FGD strictly uses the F₄₂₀ cofactor for catalysis [2], the human G6PD enzyme uses either NAD⁺ or NADP⁺ [19, 20]. Since *M. tuberculosis* causes TB by incubating within humans, the specificity of FGD for the F₄₂₀ cofactor is a unique characteristic that can be further explored for therapeutic purposes without compromising the human G6PD. In order for FGD to be considered as a potential therapeutic target, it is important to understand the mode of action of this enzyme. However, there is still very little that is known about the FGD reaction mechanism. The purpose of this present work is to provide further insight into

the FGD reaction mechanism by deducing the order of substrate addition using dead-end inhibition studies. We also present deuterium solvent and kinetic isotope effects studies which are useful in determining the number of protons in flight during catalysis and can also be used to determine the rate-limiting step of an enzymatic reaction.

Dead-end inhibition studies with competitive inhibitors can be used to determine whether an enzymatic mechanism is random or ordered, and also to verify the order of substrate addition for enzymes that use two substrates [21, 22]. In 1973, glucoseamine-6-phosphate, a G6P substrate analog, was identified as a competitive inhibitor of G6PD from yeast and rat liver cells [23]. Since then, dead-end inhibition studies have been conducted to investigate the reaction mechanism of other G6PD enzymes using glucoseamine-6-phosphate as the inhibitor [24-26]. However, due to the fact that FGD is unique in its use of the F_{420} cofactor, and because glucoseamine-6-phosphate has only previously been shown to inhibit NAD^+ or $NADP^+$ dependent G6PD, we did not use glucoseamine-6-phosphate in our studies. The FGD crystal structure solved previously lead to the discovery that citrate is a competitive inhibitor of FGD with respect to the G6P substrate [4]. Therefore, the inhibition experiments presented here were performed in the presence of citrate, in an attempt to form an FGD: F_{420} :citrate dead-end complex. The inhibition patterns obtained from the dead-end inhibition experiments eliminate a rapid equilibrium random mechanism of substrate addition. In a random mechanism, citrate would be able to bind to both free FGD and the FGD: F_{420} complex, resulting in non-competitive and competitive inhibition with respect to F_{420} and G6P, respectively. However, citrate was found to be a competitive inhibitor with respect to G6P (Figure 2) and an uncompetitive inhibitor with respect to F_{420} (Figure 3), implying that citrate can only bind to the FGD: F_{420} complex. This supports a mechanism of ordered addition of substrates, in which the F_{420} cofactor must bind to FGD first before G6P can bind [21, 24, 27].

Product inhibition studies can also be utilized to investigate enzyme reaction mechanisms [21], and such work has been conducted previously, using NADPH for other G6PD enzymes [24-26, 28, 29]. In the case of FGD, we were unable to perform product inhibition studies with the $F_{420}H_2$ product because it is not as readily available as NADPH. We were also unable to perform product inhibition studies with 6-phosphogluconolactone product due to its unstable nature in solution, which gives it a very short half-life (~ 1.5 s) [30].

Steady-state kinetic experiments performed in Tris buffer dissolved in either D_2O or H_2O revealed the magnitudes of deuterium SKIE on the wild-type FGD k_{cat} and k_{cat}/K_m , with respect to both G6P and F_{420} . All experiments were performed at pH/pD 7.0 which was chosen based on previously published wild-type FGD pH profiles that revealed a pH independence of k_{cat} at pH 7.0 [1]. Regardless of which substrate was varied, the SKIE observed for all four kinetic parameters were normal since all the values obtained were greater than 1 (Table 1). These SKIE values all fall within the range of 1.5-4, which is usually observed in general acid-base catalysis derived from a single transition state site such as a catalytic proton bridge [31]. Since a single proton transferred from an oxygen atom usually yields SKIE ~ 1.5 [32], our SKIE results suggest that a proton transferred from an oxygen atom probably plays a significant role in the rate-limiting step of the FGD reaction.

Proton inventory experiments were performed using rapid mixing experiments under single turnover conditions, to further investigate the effect of solvent-derived protons and to determine the number of solvent protons in transition in the isotope-sensitive steps of the FGD reaction. The proton inventory curve fitted to Equation 6 was linear (Figure 5), suggesting that a single solvent-exchangeable proton was likely involved in the transition state and was responsible for the observed overall SKIE [13, 14, 33]. Fractionation factors obtained from proton inventory curves are often used to characterize hydrogen bonds, where fractionation factors less than 1 are usually attributed to short and strong hydrogen bonds [34]. The fractionation factor, $\Phi^T = 0.37$ obtained from the proton inventory curve was significantly less than 1 and almost near the minimum Φ^T value of 0.16 [17, 34], which is consistent with a proton on a carboxylic acid involved in a short and strong hydrogen bond [35, 36]. We can, therefore, propose that this solvent-exchangeable proton is the proton on Glu109 that protonates the cofactor at position N1 [13, 33]. The hydrogen bond length for the Glu109-COOH $\cdots$ F₄₂₀-N1 interaction as calculated from the measured fractionation factor was 2.5 Å, which is much less than the typical van der Waals contact distance of 2.9 Å between nitrogen and oxygen, indicating short, strong hydrogen bond formation in the FGD mechanism which plausibly serves to increase the enzyme's catalytic efficiency.

Primary deuterium kinetic isotope effects on the FGD reaction were also investigated by using enzymatically-synthesized deuterated and protiated G6P in both steady state and pre-steady state kinetic experiments. The G6P hydrogen atom targeted for deuterium substitution was the C1-H, which is the hydride that is transferred to the C5 position of the F₄₂₀ cofactor during catalysis [4]. Deuterium KIE values of 1.1 were observed with respect to both k_{cat} and k_{cat}/K_m (Table 2). Previous kinetic and mechanistic studies performed by our group showed that hydride transfer is likely not the rate-limiting step in the FGD reaction [1]. The results of our deuterium KIE experiments provide further evidence that hydride transfer is not rate-limiting, since there were no drastic effects on the reaction rates in both the steady state and pre-steady state kinetic parameters (Table 2). We can also conclude that the deuterium KIEs presented here are observed, rather than intrinsic, since the isotope-sensitive hydride transfer step is not rate-limiting [37, 38]. It is plausible that the intrinsic deuterium KIE is masked in k_{cat} because the slower rate-limiting step occurs before or after the isotope-labeled hydride transfer step. Given the sizeable magnitude of the SKIE, it is likely that a proton transfer is involved in the rate-limiting step of the FGD reaction. This further implies that the rate-limiting step could be the first step of the FGD reaction where a base abstracts a proton from C1-OH of G6P, or the final step of the reaction where a proton is transferred from Glu109 to position N1 of the cofactor.

In 1982, the Cleland group utilized a combination of ¹³C-labeling with deuterated and protiated G6P substrates in the investigation of the intrinsic KIE on *L. mesenteroides* G6PD which led to the conclusion that ¹³C and deuterium isotope effects contribution at C1 of G6P occur concomitantly during the reaction [7]. In 1984, further studies on isotope effects by the Cleland group on the same enzyme provided evidence of tunneling in hydrogen motion, due to the first deuterium substitution decreasing the KIE of subsequent deuteration [39]. Based solely on the magnitudes of the deuterium KIE (Table 2), we cannot confidently deduce whether or not tunneling occurs within FGD since we were not able to measure the intrinsic KIE.

Scheme 1 represents the proposed mechanism of FGD based upon the crystal structure solved by Bashiri *et al.* as well as our previously published kinetic studies and our work presented here [1, 4]. The oxidized F₄₂₀ cofactor must first bind to the FGD active site, followed by G6P binding (Scheme 1). We have not at this point been able to deduce the identity of the active site base. Therefore, it is represented as “B” in scheme 1. However, the base would then abstract a proton from G6P, facilitating the transfer of a hydride from the substrate to the oxidized F₄₂₀, thereby reducing it. F₄₂₀ accepts a proton from Glu109 and gets converted to F₄₂₀H₂. The final product, 6-Phosphogluconolactone, along with the reduced cofactor, then leaves the active site, producing free FGD.

In summary, this work provides further significant insights into the FGD reaction. We have presented the first evidence of an ordered mechanism of substrate addition for FGD, in which G6P can only bind to the FGD active site after the F₄₂₀ cofactor has been bound. The solvent isotope effects studies also revealed a proton in flight that is likely from Glu109 and protonates the cofactor at position N1 in the last step of the FGD catalysis. Finally, the primary kinetic isotope effects studies yielded small KIE, implying that hydride transfer is not the rate-limiting step of the FGD reaction, and pointing towards a rate-limiting step that involves a proton transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Oyugi MA, Bashiri G, Baker EN, Johnson-Winters K. Investigating the reaction mechanism of F₄₂₀-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium tuberculosis*: Kinetic analysis of the wild-type and mutant enzymes. *Biochem.* 2016; 55:5566–5577. [PubMed: 27603793]
2. Purwantini E, Daniels L. Purification of a novel coenzyme F₄₂₀-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium smegmatis*. *J Bacteriol.* 1996; 178:2861–2866. [PubMed: 8631674]
3. Jirapanjawat T, Ney B, Taylor MC, Warden AC, Afroze S, Russell RJ, Lee BM, Jackson CJ, Oakeshott JG, Pandey G, Greening C. The redox cofactor F₄₂₀ protects mycobacteria from diverse antimicrobial compounds and mediates a reductive detoxification system. *Appl Environ Microbiol.* 2016; 82:6810–6818.
4. Bashiri G, Squire CJ, Moreland NJ, Baker EN. Crystal structures of F₄₂₀-dependent glucose-6-phosphate dehydrogenase FGD1 involved in the activation of the anti-tuberculosis drug candidate PA- 824 reveal the basis of coenzyme and substrate binding. *J Biol Chem.* 2008; 283:17531–17541. [PubMed: 18434308]
5. Cleland WW. The use of isotope effects to determine enzyme mechanisms. *J Biol Chem.* 2003; 278:51975–51984. [PubMed: 14583616]

6. O'Leary MH. Multiple isotope effects on enzyme-catalyzed reactions. *Ann Rev Biochem Biophys Acta*. 1989; 58:377–401.
7. Hermes JD, Roeske CA, O'Leary MH, Cleland WW. Use of multiple isotope effects to determine enzyme mechanisms and intrinsic isotope effects. Malic enzyme and glucose 6-phosphate dehydrogenase. *Biochem*. 1982; 21:5106–5114. [PubMed: 7138850]
8. Bashiri G, Rehan AM, Greenwood DR, Dickson JM, Baker EN. Metabolic engineering of cofactor F₄₂₀ production in *Mycobacterium smegmatis*. *PLoS One*. 2010; 5:e15803. [PubMed: 21209917]
9. Bashiri G, Squire CJ, Baker EN, Moreland NJ. Expression, purification and crystallization of native and selenomethionine labeled *Mycobacterium tuberculosis* FGD1 (Rv0407) using a *Mycobacterium smegmatis* expression system. *Prot Expr Purif*. 2007; 54:38–44.
10. Mikkelsen K, Nielsen SO. Acidity measurement with the glass electrode in H₂O and D₂O mixtures. *J Phys Chem*. 1960; 64:632–637.
11. Glasoe PK, Long FA. Use of glass electrodes to measure acidities in deuterium oxide. *J Phys Chem*. 1960; 64:188–191.
12. Karpefors M, Ädelroth P, Brzezinski P. The onset of the deuterium isotope effect in cytochrome c oxidase. *Biochem*. 2000; 39:5045–5050. [PubMed: 10819969]
13. Kresge AJ. Solvent isotope effect in H₂O-D₂O mixtures. *Pure Appl Chem*. 1964; 8:243–258.
14. Schowen KB, Schowen RL. Solvent isotope effects of enzyme systems. *Meth Enzymol*. 1982; 87:551–606. [PubMed: 6294457]
15. Harris TK, Mildvan AS. High-precision measurement of hydrogen bond lengths in proteins by nuclear magnetic resonance methods. *Prot Struct Fun Gen*. 1999; 35:275–282.
16. Harris TK, Zhao Q, Mildvan AS. NMR studies of strong hydrogen bonds in enzymes and in a model compound. *J Mol Struct*. 2000; 552:97–109.
17. Mildvan AS, Massiah MA, Harris TK, Marks GT, Harrison DHT, Viragh C, Reddy PM, Kovach IM. Short, strong hydrogen bonds on enzymes: NMR and mechanistic studies. *J Mol Struct*. 2002; 615:163–175.
18. Wei, Y., McDermott, AE. Modeling NMR chemical shifts: gaining insights into structure and environment. Oxford University Press; Cary NC: 1999.
19. Kotaka M, Gover S, Vandeputte-Rutten L, Au SWN, Lams VMS, Admas MJ. Structural studies of glucose-6-phosphate and NADP⁺ binding to human glucose-6-phosphate dehydrogenase. *Acta Crystallogr, Sect D: Biol Crystallogr*. 2005; 61:495–504. [PubMed: 15858258]
20. Au SWN, Gover S, Lam VMS, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP⁺ molecule and provides insights into enzyme deficiency. *Struct*. 2000; 8:293–303.
21. Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or products: II. Inhibition: Nomenclature and theory. *Biochim Biophys Acta*. 1963; 67:173–187. [PubMed: 14021668]
22. Fromm HJ. The use of alternative substrates in studying enzymic mechanisms involving two substrate. *Biochim Biophys Acta*. 1964; 81:413–417. [PubMed: 14170314]
23. Bessell M, Thomas T. The effect of substitution at C-2 of D-glucose 6-phosphate on the rate of dehydrogenation by glucose 6-phosphate dehydrogenase (from yeast and from rat liver). *Biochem J*. 1973; 131:83–89. [PubMed: 4578852]
24. Kanji MI, Toews ML, Carper WR. A kinetic study of glucose-6-phosphate dehydrogenase. *J Biol Chem*. 1976; 251:2258–2262. [PubMed: 4440]
25. Shreve DS, Levy HR. Kinetic mechanism of glucose-6-phosphate dehydrogenase from the lactating rat mammary gland: Implications for regulation. *J Biol Chem*. 1980; 255:2670–2677. [PubMed: 7358698]
26. Wang XT, Shannon WN, Veronica MS, Engel PC. Recombinant human glucose-6-phosphate dehydrogenase: Evidence for a rapid-equilibrium random-order mechanism. *Eur J Biochem*. 2002; 269:3417–3424. [PubMed: 12135480]
27. Cleland, WW. Steady state kinetics in the enzymes. 3rd. Academic Press; New York NY: 1970.
28. Ulusu NU, Tandogan B. Purification and kinetics of sheep kidney cortex glucose-6-phosphate dehydrogenase. *Comp Biochem Physiol*. 2006; 143:249–255.

29. Ulusu NU, Tandogan B, Tezcan FE. Kinetic properties of glucose-6-phosphate dehydrogenase from lamb kidney cortex. *Biochimie*. 2005; 87:187–190. [PubMed: 15760711]
30. Horecker BL, Smyrniotis PZ. Reversibility of glucose-6-phosphate oxidation. *Biochim Biophys Acta*. 1953; 12:98–102. [PubMed: 13115418]
31. Schowen KB, Limbach HH, Denisov GS, Schowen RL. Hydrogen bonds and proton transfer in general-catalytic transition-state stabilization in enzyme catalysis. *Biochim Biophys Acta*. 1999; 1458:43–62.
32. Quinn, DM., Sutton, LD. Theoretical basis and mechanistic utility of solvent isotope effects. CRC Press; Boca Raton FL: 1991.
33. Quinn, DM. Theory and practice of solvent isotope effects. CRC Press; Boca Raton FL: 2005.
34. Kreevoy MM, Liang TM. Structures and isotopic fractionation factors of complexes. *J Am Chem Soc*. 1980; 102:3315–3322.
35. Cleland WW. Low-barrier hydrogen bonds and enzymatic catalysis. *Arch Biochem & Biophys*. 2000; 382:1–5. [PubMed: 11051090]
36. Cleland WW. The low-barrier hydrogen bond in enzymic catalysis. *Adv Phys Org Chem*. 2010; 44:1–17.
37. Northrop DB. The expression of isotope effects on enzyme-catalyzed reactions. *Ann Rev Biochem*. 1981; 50:103–131. [PubMed: 7023356]
38. Cook PF, Cleland WW. Mechanistic deductions from isotope effects in multireactant enzyme mechanisms. *Biochem*. 1981; 20:1790–1796. [PubMed: 7013799]
39. Hermes JD, Cleland WW. Evidence from multiple isotope effect determinations for coupled hydrogen motion and tunneling in the reaction catalyzed by glucose-6-phosphate dehydrogenase. *J Am Chem Soc*. 1984; 106:7263–7264.

Abbreviations

FGD	F ₄₂₀ -dependent glucose-6-phosphate dehydrogenase
G6P	glucose-6-phosphate
G6P-d₁	glucose-6-phosphate-d ₁
G6PD	glucose-6-phosphate dehydrogenase
F₄₂₀ cofactor	7,8-didemethyl-8-hydroxy-5-deazariboflavin-5'-phosphoryllactyl(glutamyl) _n glutamate
TB	Tuberculosis
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
LC-MS-IT-TOF	liquid chromatography mass spectrometry-ion trap-time of flight
SKIE	solvent kinetic isotope effect
<i>k</i>_{cat} (app)	apparent turn-over number
<i>K</i>_m (app)	apparent Michaelis-Menten constant
<i>k</i>_{cat}/<i>K</i>_m	catalytic efficiency

k_{obs}

apparent rate constant

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Figure 1.

The first committed step of the pentose-phosphate pathway that is catalyzed by FGD. The glucose-6-phosphate reacts with the oxidized F_{420} cofactor to yield 6-phosphogluconolactone and the reduced cofactor, $F_{420}H_2$. A hydride transfer occurs from C1 of G6P to C5 of the oxidized F_{420} cofactor. A final protonation step at N1 of F_{420} yields $F_{420}H_2$ [2].

Figure 2.

Inhibition pattern of wild-type FGD by citrate with G6P as the variable substrate. The steady state kinetics experiments were carried out in 50 mM Tris-HCl (pH 7.0) at 22 °C. The wild-type FGD and F_{420} concentrations were held constant at 25 nM and 20 μM respectively. The G6P and citrate concentrations were varied from 8 – 250 μM and 2 – 10 mM respectively. The rate constants were obtained by dividing initial rates by enzyme concentration. **A:** The double reciprocal plots of $1/k$ vs $1/[G6P]$. The symbols represent an average of two measurements of rate constants at various concentrations of G6P and citrate. The solid lines represent the fits to Equation 1 at various citrate concentrations. **B:** Multidimensional fit of k vs $[G6P]$ fitted to the competitive inhibition model. The symbols represent an average of two measurements of rate constants at various concentrations of G6P and citrate. The solid lines represent the fits to Equation 2 at various citrate concentrations.

Figure 3.

Inhibition pattern of wild-type FGD by citrate with F_{420} as the variable substrate. The steady state kinetics experiments were carried out in 50 mM Tris-HCl (pH 7.0) at 22 °C. The wild-type FGD and G6P concentrations were held constant at 25 nM and 2 mM respectively. The F_{420} and citrate concentrations were varied from 1 – 10 μM and 2 – 10 mM respectively. The rate constants were obtained by dividing initial rates by enzyme concentration. **A:** The double reciprocal plots of $1/k$ vs $1/[F_{420}]$. The symbols represent an average of two measurements of rate constants at various concentrations of F_{420} and citrate. The solid lines represent the fits to Equation 1 at various citrate concentrations. **B:** Multidimensional fit of k vs $[F_{420}]$ fitted to the uncompetitive inhibition model. The symbols represent an average of two measurements of rate constants at various concentrations of F_{420} and citrate. The solid lines represent the fits to Equation 3 at various citrate concentrations.

Figure 4.

Wild-type FGD steady state kinetics plots for solvent isotope experiments. The reactions were carried out at 22 °C in 50 mM Tris-HCl dissolved in D₂O (pD 7.0). The reduction of the F₄₂₀ cofactor was tracked at 420 nm to obtain the initial rates of the reactions. The rate constants were obtained by dividing the initial rates by the FGD concentration. **A:** Plots of rate constant *vs* G6P concentration for D₂O. The solid circles represent an average of two values of rate constants at various concentrations of G6P and the solid line represents fit to Equation 4. **B:** Plot of rate constant *vs* F₄₂₀ concentration for D₂O. The solid circles represent an average of two values of rate constants at various concentrations of F₄₂₀ and the solid line represents fit to Equation 4. The error bars represent standard deviations from duplicate reactions of each varied substrate concentration. Similar curves for experiments performed in H₂O (pH 7.0) have previously been published elsewhere [1].

Figure 5.

Proton inventory curve for wild-type FGD. The single turnover stopped-flow experiments were performed in diode array mode at 22 °C in 50 mM Tris-HCl dissolved in varying concentrations of D₂O (pD 7.0). 50 μM wild-type FGD was pre-incubated with 5 μM F₄₂₀ and then rapidly mixed with 500 μM G6P. The exponential decay curves for the reduction of the F₄₂₀ cofactor at 420 nm were fitted to Equation 5 to yield the apparent rate constants (k_n) at varying D₂O concentrations. The k_n values were then divided by the apparent rate constant in pure H₂O (k_0). The k_n/k_0 ratios were then plotted vs the mole fractions of D₂O (*n*) and then fitted to Equation 6. The error bars in the plots represent standard deviations obtained from duplicate measurements of each variable substrate concentration.

Figure 6.

Wild-type FGD steady state kinetics plots with respect to enzymatically synthesized G6P and G6P-d₁ substrates. The reactions were carried out at 22 °C in 50 mM Tris-HCl (pH 7.0). The reduction of the F₄₂₀ cofactor was followed at 420 nm to obtain the initial rates of the reactions. The rate constants were obtained by dividing the initial rates by the FGD concentration. **A:** Plot of rate constant vs G6P concentration. The solid circles represent an average of two values of rate constants at various concentrations of G6P and the solid line represents the fit to Equation 4. **B:** Plot of rate constant vs G6P-d₁ concentration. The solid circles represent an average of two values of rate constants at various concentrations of G6P-d₁ and the solid line represents the fit to Equation 4. The error bars represent standard deviations from duplicate reactions of each varied substrate concentration.

Figure 7.

Wild-type FGD pre-steady kinetics plots of absorbance at 420 nm *vs* time. The single turnover experiments were carried out in diode array mode, using 50 mM Tris-HCl (pH 7.0) buffer at 22 °C. **A:** protiated substrate experiment containing 50 μ M wild-type FGD, 5 μ M F_{420} and 500 μ M G6P. The solid circles represent an average of three absorbance values at various times and the solid line represents the fit to Equation 5. **B:** deuterated substrate experiment containing 50 μ M wild-type FGD, 5 μ M F_{420} and 500 μ M G6P- d_1 . The solid circles represent an average of three absorbance values at various times and the solid line represents the fit to Equation 5.

Scheme 1. FGD proposed mechanism

Wild-type FGD solvent isotope effects on apparent steady state kinetics parameters. The reactions were carried out at 22 °C in 50 mM Tris-HCl dissolved in either D₂O (pD 7.0) or H₂O (pH 7.0). The reactions were monitored by following the reduction of the F₄₂₀ cofactor at 420 nm. The steady state kinetic parameters k_{cat} and K_m were obtained by fitting the kinetic data to Equation 4. The uncertainty in each kinetic parameter is the standard error from least-squares fits of the kinetic data to the appropriate equation.

Table 1

	$G6P k_{cat} (app)^a$ (s ⁻¹)	$F_{420} k_{cat} (app)^a$ (s ⁻¹)	$G6P K_m (app)^a$ (μM)	$F_{420} K_m (app)^a$ (μM)	$G6P k_{cat}/K_m^b$ (M ⁻¹ s ⁻¹)	$F_{420} k_{cat}/K_m^b$ (M ⁻¹ s ⁻¹)
H ₂ O ^c	1.4 ± 0.1 ^c	1.4 ± 0.3 ^c	22 ± 2 ^c	2.6 ± 0.3	(6.0 ± 0.5) × 10 ⁴ ^c	(6.0 ± 1.0) × 10 ⁵ ^c
D ₂ O	0.45 ± 0.01	0.54 ± 0.02	36 ± 5	2.4 ± 0.2	(1.3 ± 0.5) × 10 ⁴	(2.3 ± 0.2) × 10 ⁵
D ₂ O _{KIE}	3.1 ± 0.2	2.6 ± 0.6	ND ^d	ND ^d	4.6 ± 2.0	2.6 ± 0.5

^aThe apparent kinetic parameters presented here are reasonable approximations of the true values.

^bThe uncertainties for the catalytic efficiency values were calculated using the formula $\{((K_m (err)/K_m)^2 + (k_{cat} (err)/k_{cat})^2)^{1/2} * (k_{cat}/K_m)\}$, where $K_m (err)$ and $k_{cat} (err)$ represent the uncertainties in the individual K_m and k_{cat} values.

^cThese values have previously been reported elsewhere [1].

^dNot determined.

Table 2

Wild-type FGD deuterium isotope effects on steady state and pre-steady state kinetics parameters. The experiments were carried out in 50 mM Tris-HCl (pH 7.0) at 22 °C by varying the concentration of either G6P or G6P-d₁ substrates. The reactions were monitored by following the reduction of the F₄₂₀ cofactor at 420 nm. The steady state and pre-steady state kinetic parameters were obtained by fitting the appropriate kinetic data to Equation 4 and Equation 5, respectively. The uncertainty in each kinetic parameter is the standard error from least-squares fits of the kinetic data to the appropriate equation.

	Steady state kinetics				Pre-steady state kinetics	
	k_{cat}^{a} (s ⁻¹)	K_m^{a} (μM)	k_{cat}/K_m^b (M ⁻¹ s ⁻¹)	k_H/k_D	$(k_{cat}/K_m)_H/(k_{cat}/K_m)_D$	k_H/k_D
G6P	1.40 ± 0.03	44 ± 4	(3.2 ± 0.1) × 10 ⁴	1.1 ± 0.1	1.1 ± 0.1	78 ± 8
G6P-d ₁	1.30 ± 0.05	46 ± 6	4 (2.8 ± 0.2) × 10 ⁴			56 ± 1

^aThe apparent kinetic parameters presented here are reasonable approximations of the true values.

^bThe uncertainties for the catalytic efficiency values were calculated using the formula $\{((K_m^{err})/K_m)^2 + (k_{cat}^{err}/k_{cat})^2\}^{1/2} * (k_{cat}/K_m)$, where K_m^{err} and k_{cat}^{err} represent the uncertainties in the individual K_m and k_{cat} values.