Kinetic studies on the catalytic mechanism of F_{420}-dependent glucose-6-phosphate dehydrogenase

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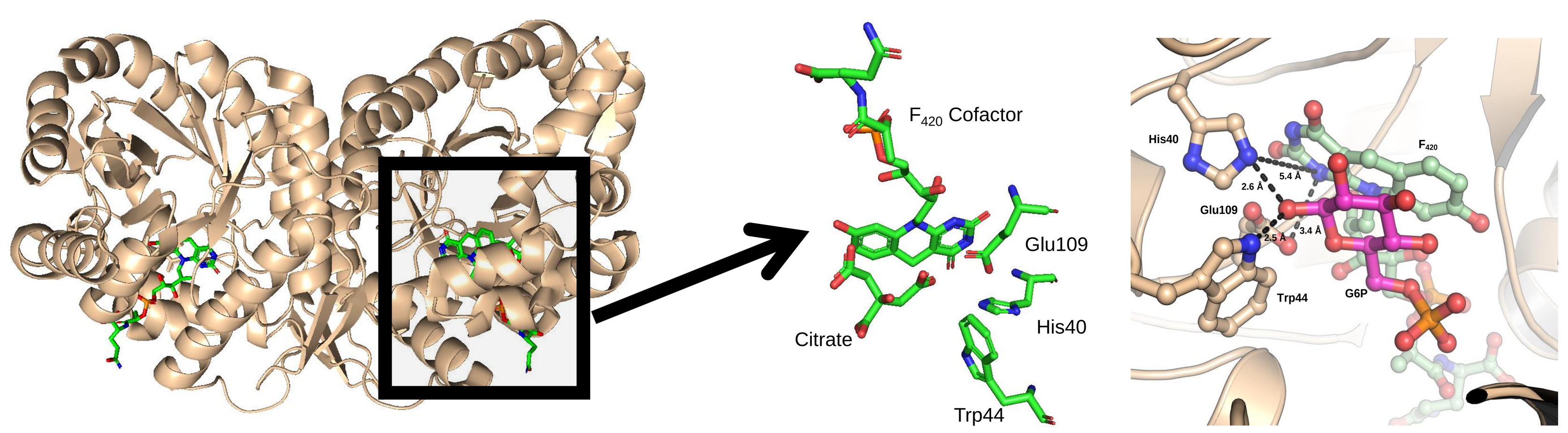
Abstract

F_{420}- dependent glucose-6-phosphate dehydrogenase (FGD) is an important enzyme found in *Mycobacteria tuberculosis*, the causative agent of tuberculosis disease. FGD catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone, using F_{420} cofactor as a hydride transfer acceptor. Understanding the FGD mechanism is vital to the design of new drugs for the treatment of multiple drug resistant and extreme drug resistant forms of tuberculosis disease. To this end, we have created a series of active site variants (H40A, H40Q, H260A , H260N, Glu13A, Glu13Q and Glu109A) and obtained the substrate binding affinities, determined the pre steady-state and steady state kinetic parameters and studied the order of substrate addition along with the pH- rate profiles for *wt*FGD and FGD variants. The binding studies suggest that these conserved amino acids are involved F_{420} binding but are not involved in G6P binding. The steady-state experiments suggest that the studied residues are important in catalysis due to decreased catalytic activity. Based upon the inhibition studies we have determined that FGD follows a sequential mechanism in which F_{420} binds first followed by G6P. The pH profiles along with the PROPKA calculations suggest that Glu13 and His40 function as a catalytic dyad, with His40 donating a proton to Glu13. His40 can then act as an active site base, abstracting a proton from G6P facilitating the reduction of the F_{420} cofactor. The global analysis of the pre steady-state kinetic data suggest that hydride transfer is not rate-limiting in catalysis, and that the mechanism follows a fast chemistry and slow product release with observable intermediates.

Background

Tuberculosis disease (TB) is considered one of the top 10 causes of deaths worldwide. According to the World Health Organization, a total of 1.6 million individuals die from TB between 2018-22 [WHO, 2022]. *Mycobacterium tuberculosis* is the causative agent of TB. Over time, patients can develop multiple-drug resistant (MDR) and extreme drug resistant (XDR) forms of TB disease, making it extremely difficult to treat. FGD, present in this bacterium is targeted to develop therapeutics against these difficult cases. For example, Nitroimidazopyran exhibits bactericidal activity against *M. tuberculosis*. This molecule is activated by a mechanism dependent on the reduced F_{420} cofactor, which is produced during FGD catalysis. Therefore, our understanding of the FGD structure, mechanism, and role in the bacteria have great medical relevance and will add knowledge to the field of this lesser known cofactor.

FGD Crystal Structure



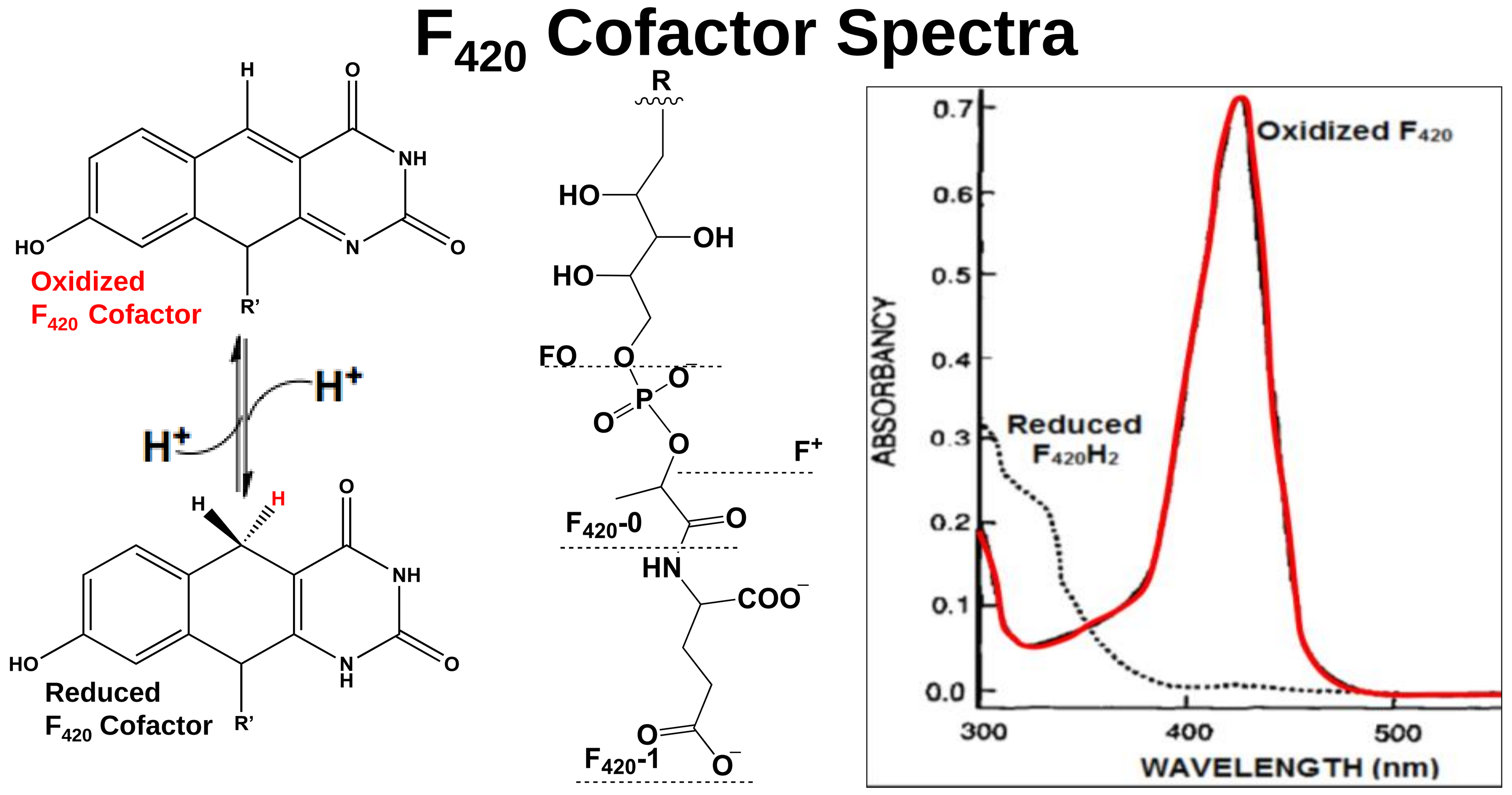
78 kDa Homodimer
α_8/β_8 TIM Barrel
One F_{420} molecule per monomer

Active site

Proposed G6P binding site

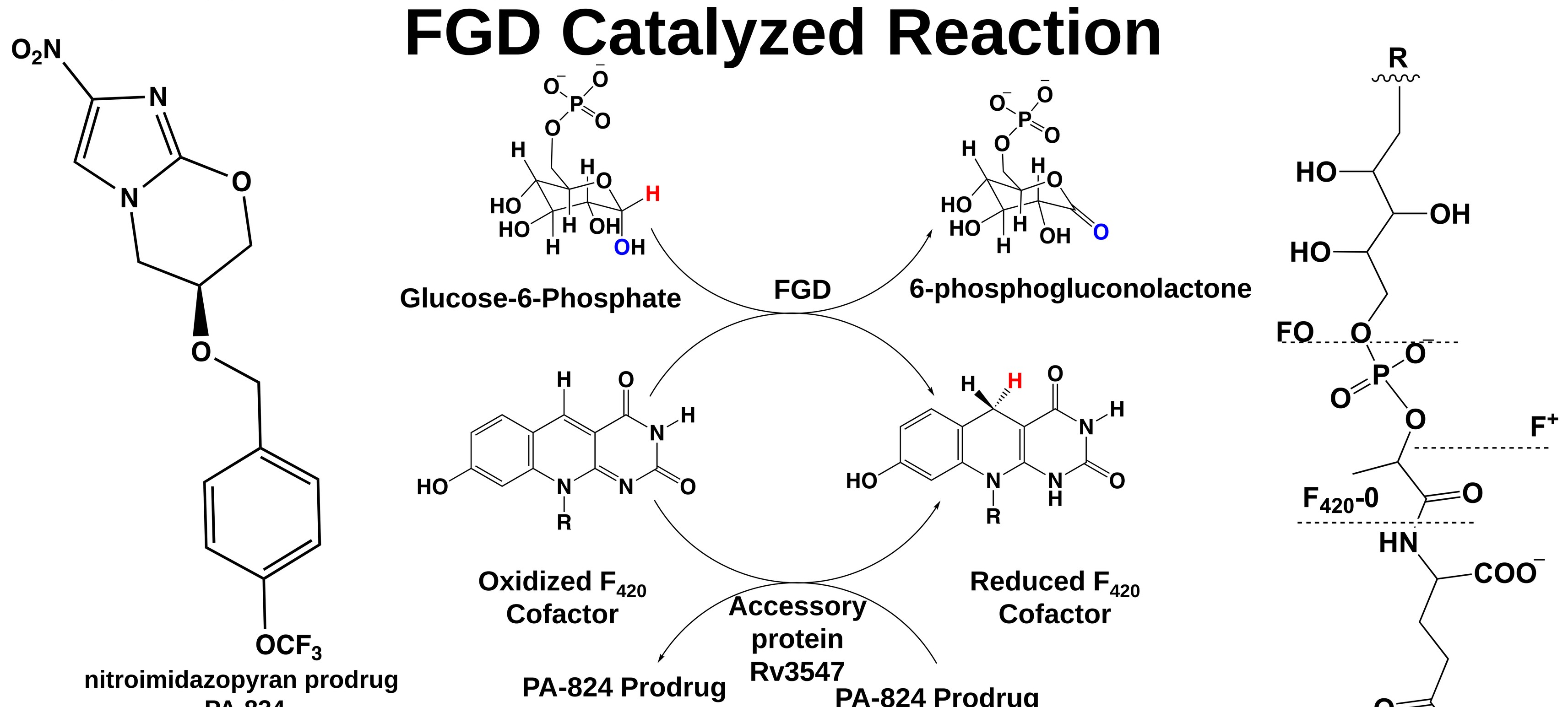
Bashiri, G.; Squire, C. J.; Moreland, N. J., and Baker, E. N.; *J Biol Chem*, *2008*, *283*, 17531-17541.

F_{420} Cofactor Spectra



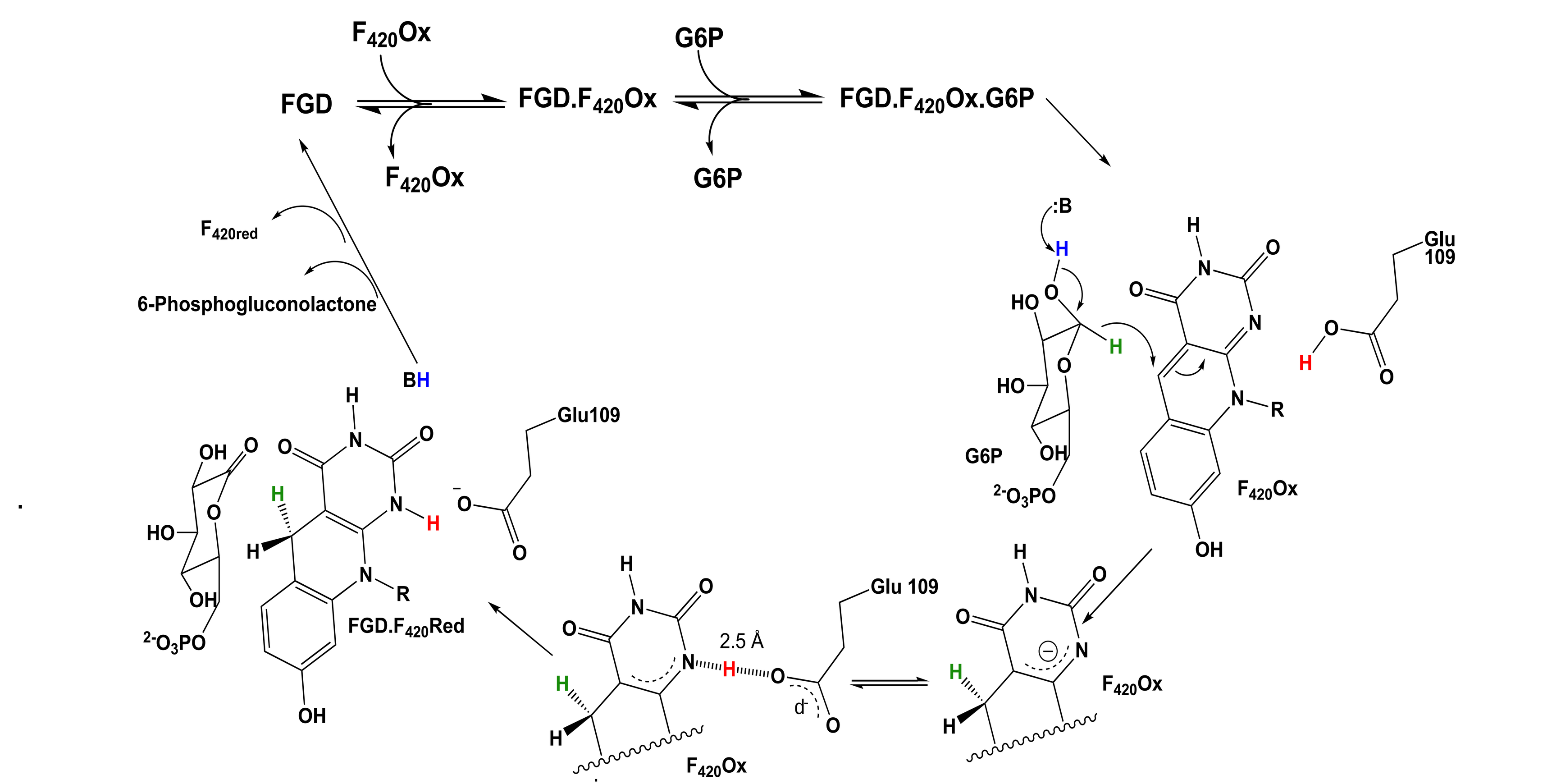
Cheesman, P.; Toms-Wood, A.; Wolfe, R. S. *J. Bacteriol.* **1972**, 112, 527-31.

FGD Catalyzed Reaction



Bashiri, G.; Squire, C. J.; Moreland, N. J., and Baker, E. N.; *J Biol Chem*, *2008*, *283*, 17531-17541.

Updated Mechanism



Oyugi, M. A.; Bashiri, G.; Baker, E. N.; Johnson-Winters, K. Mechanistic Insights into F_{420}-Dependent Glucose-6-Phosphate Dehydrogenase using Isotope Effects and Substrate Inhibition Studies. *Biochem. Biophys. Rep.* **2018**, 1866: 387-395.

FGD Binding Studies

$$\boxed{E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES} \xrightarrow{k_{cat}} P$$

EXPERIMENTAL CONDITIONS

F_{420} binding: $[F_{420}]$ = 0 µM to 5.5 µM

[FGD] = 350 nM 50 mM Tris, pH 7.0

G6P binding: [G6P] = 0 µM to 0.73 µM

[FGD] = 350 nM, 50 mM Tris, pH 7.0

λ_{ex} = 290 nm; λ_{em} = 340 nm

Enzyme	G6P K_d (µM)	F_{420} K_d (nM)
*wt*FGD[a]	**14 ± 4**	**71 ± 37**
H40A	16 ± 3	194 ± 19
H40Q	54 ± 6	249 ± 111
H260A	7 ± 1	138 ± 29
H260N	49 ± 7	33 ± 8
E13A	15 ± 6	339 ± 70
E13Q	38 ± 10	258 ± 84

[a]Oyugi, M. A.; Bashiri, G.; Baker, E. N.; Johnson-Winters, K., Investigating the reaction mechanism of F_{420}-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium tuberculosis*: kinetic analysis of the wild-type and mutant enzymes, **2016**, *Biochemistry*, 55,5566-5577

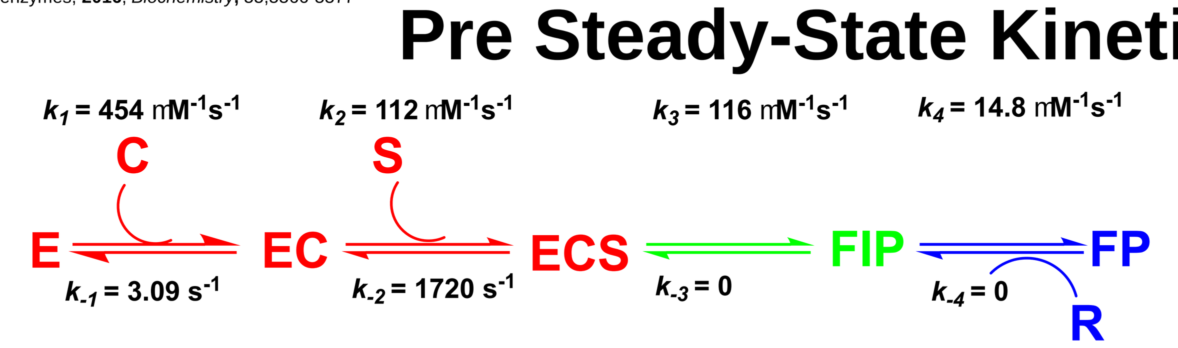
Steady-State Kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \boxed{ES \xrightarrow{k_{cat}} P}$$

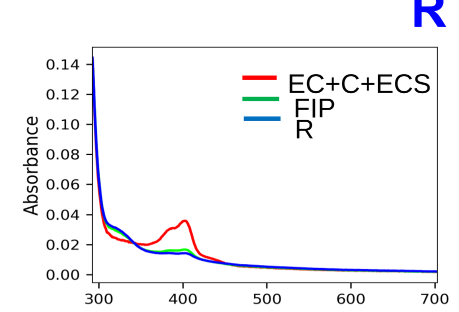
Enzyme	G6P k_{cat} (s^{-1})	G6P K_m (µM)	G6P k_{cat}/K_m ($M^{-1}s^{-1}$)	F420 k_{cat} (s^{-1})	F420 K_m (µM)	F420 k_{cat}/K_m ($M^{-1}s^{-1}$)
*wt*FGD[a]	1.4 ± 0.1	22 ± 2	(6.0 ± 0.5) × 10^4	1.4 ± 0.3	2.6 ± 0.3	(6.0 ± 1.0) × 10^5
H40A[a]	0.0016 ± 0.0001	27 ± 5	(5.0 ± 1.0) x 10^2	0.0020 ± 0.0001	8.0 ± 2.0	(4.0 ± 0.6) × 10^2
H40Q	0.0040 ± 0.0001	0.35 ± 0.05	(1.1 ± 0.2) x 10^1	0.0060 ± 0.0003	2.2 ± 0.5	(2.7 ± 0.6) × 10^2
H260A	0.060 ± 0.002	0.0050 ± 0.0005	(1.2 ± 0.1) x 10^2	0.070 ± 0.004	0.180 ± 0.014	(3.9 ± 0.3) × 10^2
H260N	0.080 ± 0.002	0.3 ± 0.1	(2.6 ± 0.8) x 10^2	0.070 ± 0.005	0.50 ± 0.02	(1.4 ± 0.1) x 10^1
E13A	0.0020 ± 0.0001	55 ± 9	(3.6 ± 0.6) x 10^2	0.0030 ± 0.0007	12 ± 1	(2.5 ± 0.6) × 10^1
E13Q	0.097 ± 0.003	0.17 ± 0.03	(5.7 ± 0.1) x 10^3	0.090 ± 0.002	0.10 ± 0.05	(9.0 ± 0.4) x 10^2

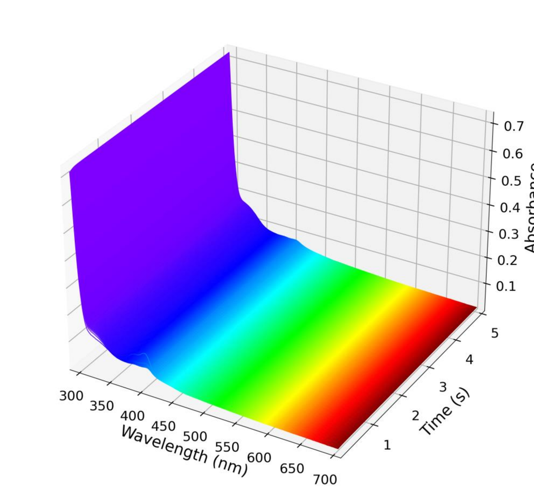
[a]Oyugi, M. A.; Bashiri, G.; Baker, E. N.; Johnson-Winters, K., Investigating the reaction mechanism of F_{420}-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium tuberculosis*: kinetic analysis of the wild-type and mutant enzymes, **2016**, *Biochemistry*, 55,5566-5577

Pre Steady-State Kinetics

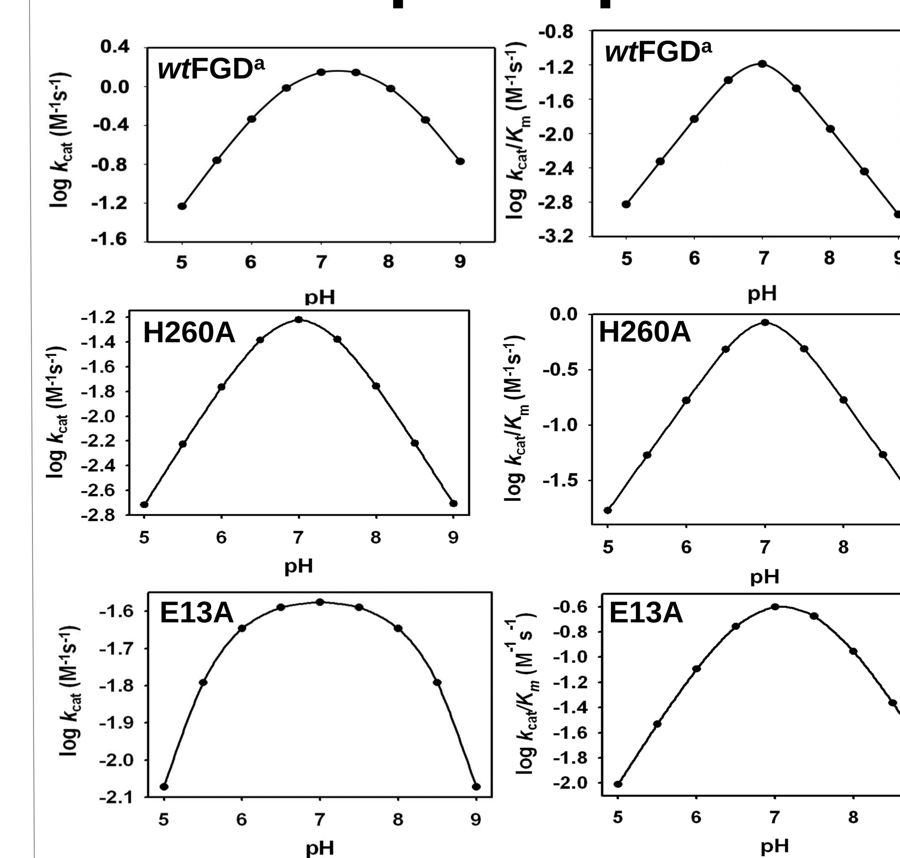


E = Enzyme
C = Oxidized Cofactor (F_{420})
S = Substrate (Glucose-6-Phosphate)
F = Conformational change within protein
I = F_{420} based Intermediate
P = Product (6-Phosphogluconolactone)
R = Reduced cofactor ($F_{420}H_2$)





pH Dependence Studies



FGD Variants	log k_{cat} vs pH		log k_{cat}/K_m vs. pH	
	pK_1	pK_2	pK_1	pK_2
[a]*wt*FGD	6.5 ± 0.3	8.0 ± 0.5	7.00 ± 0.03	7.00 ± 0.03
H260A	6.9 ± 0.3	7.1 ± 0.3	6.50 ± 0.08	7.50 ± 0.08
E13A	5.5 ± 0.3	8.6 ± 0.3	6.60 ± 0.02	7.50 ± 0.02

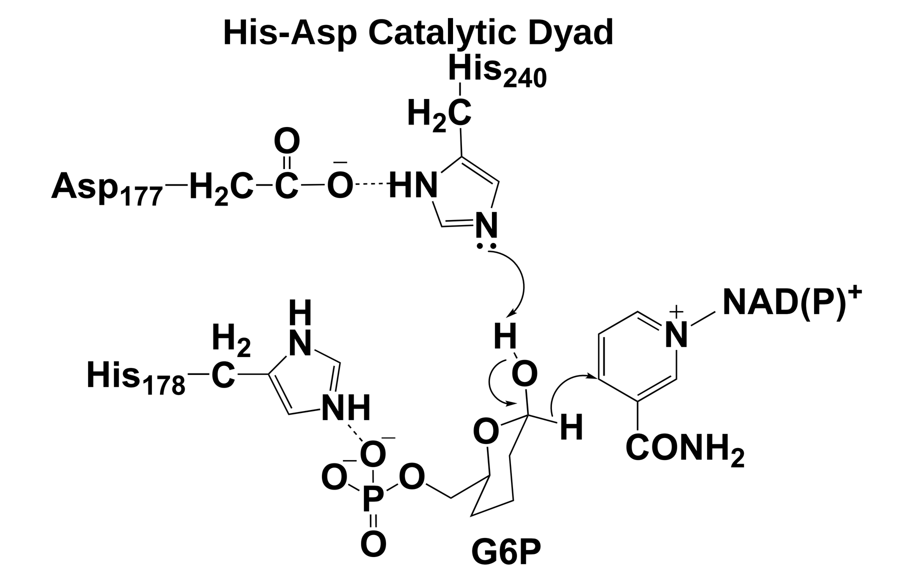
Curve fit equation (*wt*FGD)

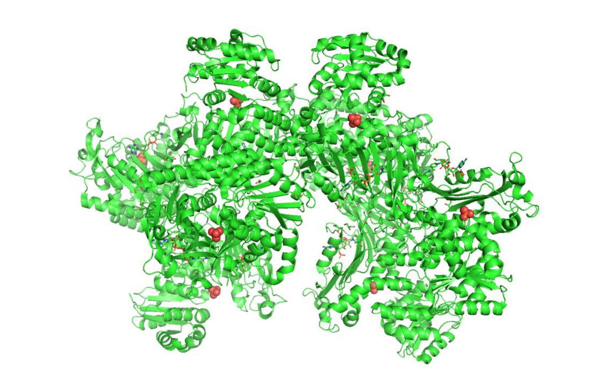
$$\log Y = \log \{Y_H / ((1+[H+] / K_1) + K_2 /[H^+])\}$$

Y = k_{cat} or k_{cat}/K_m
$\mathbf{Y_H}$ = maximum pH-indep. values
$\mathbf{K_1}$ and $\mathbf{K_2}$ is acid dissociation constants for the ascending and descending limbs respectively
[H⁺] is the proton concentration at each pH

[a]Oyugi, M. A.; Bashiri, G.; Baker, E. N.; Johnson-Winters, K., Investigating the reaction mechanism of F_{420}-dependent glucose-6-phosphate dehydrogenase from *Mycobacterium tuberculosis*: kinetic analysis of the wild-type and mutant enzymes, **2016**, *Biochemistry*, 55,5566-5577

NADP⁺ Dependent G6PD Mechanism

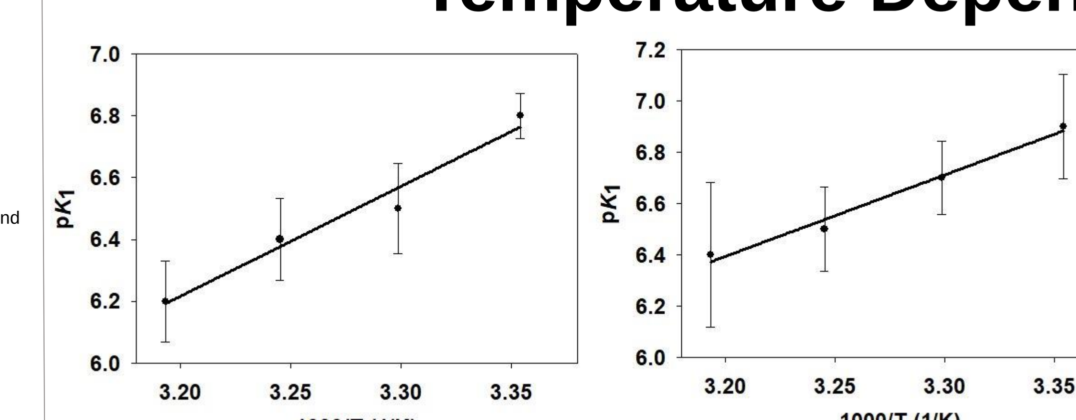




Glucose-6-phosphate dehydrogenase (G6PD)
PDB file: 6E07

Cosgrove, M.S.; Naylor, C.; Paludan, S.; Adams, M.J.; Levy, H.R. *Biochemistry* **2000** *39*, 15002-15011.

Temperature Dependence



Van't Hoff Equation
$pK_i = \Delta H_{ion}/RT - \Delta S_{ion}/R$

K_i = Acid dissociation constant for ascending limb of $\log k_{cat}$ or $\log k_{cat}/K_m$ vs pH
T= Temperature (K), 298-313K
R= Ideal Gas Constant (8.314 J•mol⁻¹•k⁻¹)

Group	pK_a[b]	ΔH_{ion} [b](kcal/mol)	Acid type
Carboxyl	3-5	±1.5	Neutral
Imidazole	5.5-7	6-7.5	Cationic
Sulfhydryl	8-9	6.5-7	Neutral
Amino	7.6-10.5	10-13	Cationic
Tyrosine	10-10.5	6	Neutral

Parameter	$\log k_{cat}$	$\log k_{cat}/K_m$
ΔH_{ion}(kcal/mol)	7.0 ± 0.9	6.3 ± 0.6
ΔS_{ion}(cal/mol•k)	10.2 ± 3.0	7.5 ± 1.9

[b]Cleland, W.W. Determining the chemical mechanisms of enzyme-catalyzed reactions by kinetic studies. **1977**, *Adv Enzymol Relat Areas Mol Bio*, 45, 273-387

Conclusions

- Amino acids H40, H260, and E13 are not involved in G6P binding. However, they do play a role in F_{420} binding
- Due to decreased activity in the steady-state experiments, we are able to conclude that all residues are involved in catalysis
- E13 and H40 act as a catalytic dyad. H40 acts as an acid donating a proton to E13 and subsequently acts as a base abstracting a proton from G6P
- Global analysis of *wt*FGD revealed the presence of intermediate and a fast chemistry/slow product release reaction

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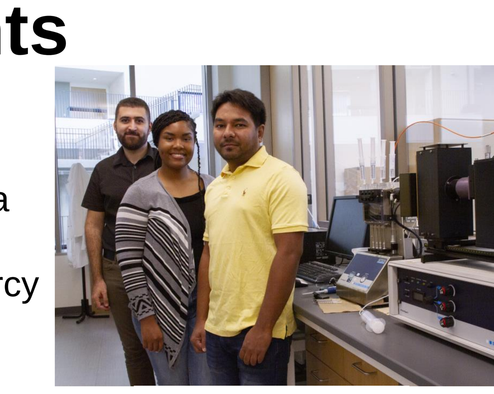
Previous lab members: Dr. Mercy Oyugi, Dr. Lindsey Davis, Ana Alvarez



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