Kinetic Analysis Reveals a Shift In Inter-subunit Communication Within $F_{420}H_2$:NADP$^+$ Oxidoreductase


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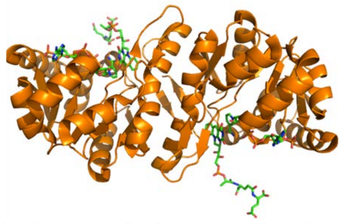
Abstract

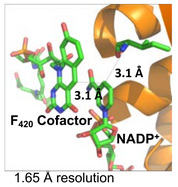
$F_{420}H_2$:NADP$^+$ Oxidoreductase (Fno) catalyzes the reversible reduction of NADP$^+$ to NADPH, using reduced F_{420} cofactor as the hydride donor. NADPH and the F_{420} cofactor, are linked to several metabolic pathways including glycolysis and methanogenesis within methanogenic and sulfate-reducing archaea. Previous steady-state kinetic data conducted on *wt*Fno displayed a Lineweaver-Burk plot, which was downward and concave in curvature, indicative of negative cooperativity. The pre steady-state kinetic studies displayed biphasic kinetics with an initial burst phase followed by a subsequent slow phase. The amplitude of the burst phase revealed 50% F_{420} cofactor reduction, indicating half-site reactivity. These studies suggest that Fno regulates NADPH production within the cell. The goal of this project is to determine which amino acids are involved in communication at the subunit interface. We have identified four amino acids which are Arg186, Thr192, Ser190, and His133. We have designed a library of Fno variants to test our hypothesis. The generated Fno variants (R186Q, R186K, R186I, T192V, T192A, H133A, H133N and S190A) were then characterized using fluorescence binding, steady-state and pre steady-state kinetic experiments. Our studies revealed that unlike *wt*Fno, the following Fno variants, R186Q, R186K, R186I, T192A, and S190A displayed no cooperativity. This suggests that R186, T192 and S190 are involved in subunit communication. The results are reported here.

Introduction

F_{420}-dependent enzymes are important in several organisms, which play vital roles in methane production, NADPH regulation, nucleic acid biosynthesis, folate biosynthesis and carbon cycling. Until our recent publications on $F_{420}H_2$:NADP$^+$ Oxidoreductase (Fno) and F_{420}-dependent glucose-6phosphate dehydrogenase (FGD), these enzymes, in general, had not been subjected to rigorous enzymological investigation. Our work has provided valuable new mechanistic and functional insights into the enzymes that use this unique cofactor. Our previous kinetic studies on Fno, which is the focus of this proposal, indicated half-site reactivity in only one of the active sites in a functional dimer. These data suggest that Fno participates in negative cooperativity kinetics and that this enzyme regulates NADPH production methanogenic organisms. Based upon our kinetic studies, we have proposed a chemically plausible mechanism and have identified several key amino acids that potentially play an important role in subunit communication within Fno.

Fno Crystal Structure





1.65 Å resolution
48 kDa homodimer
N-terminal binding site for F_{420} binding

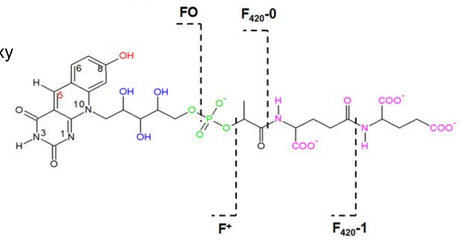
PDB File: 1jax
Warkentin, E; Mamat, B.; Sordel-Klippert, M.; Wicke, M.; Thauer R. K.; Iwata, M.; Iwata, S.; Ermler, U.; Shima S. *The EMBO Journal*, **2001**, 20, 6561-6569.

Structure of the F_{420} Cofactor

COFACTOR NAMES

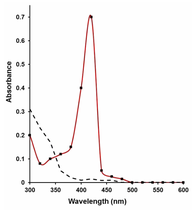
Chemical Name: 7,8-didemethyl-8-hydroxy deazariboflavin-5'-phosphorylactyl(glutamyl)$_n$glutame

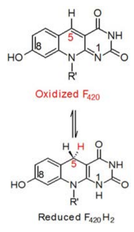
Common Names
- Factor 420
- F_{420} Cofactor

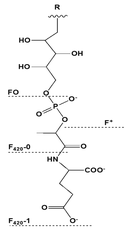


Cheeseman, P.; A. Toms-Wood; Wolfe, R. S. *J. Bacteriol.* **1972**, 112, 527–531.

F_{420} Cofactor Spectra

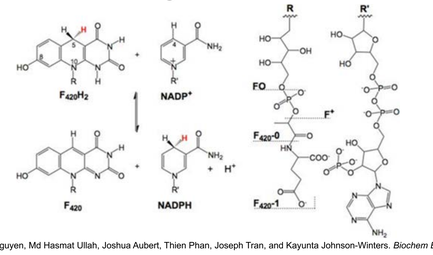






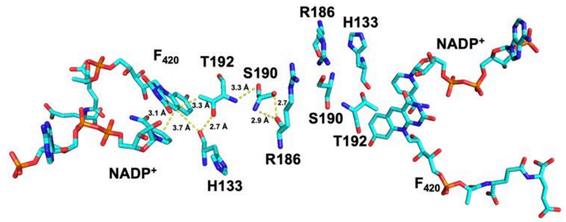
Cheesman, P., Toms-Wood, A., Wolfe, R. S. *J. Bacteriol.* **1972**, 112, 527-31.

Fno Catalyzed Reaction



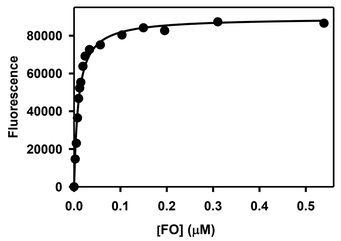
Cuong Quang Le, Mercy Oyugi, Ebenezer Joseph, Toan Nguyen, Md Hasmat Ullah, Joshua Aubert, Thien Phan, Joseph Tran, and Kayunta Johnson-Winters. *Biochem Biophys Rep.* **2016,** 9:114-120.

Subunit Inter-face Interactions



*wt*fno Binding Data

FO CONDITIONS
50 µM MES/NaOH (pH 6.50) buffer
Temp: 22 °C
NADPH was titrated into 0.2 µM of Fno
$\lambda_{excitation}$ = 290 nm and $\lambda_{emission}$ = 340 nm

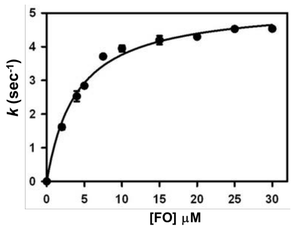


Data Fit to Hill Equation:
$\Delta F = F_{max}[L]^b/(K_d{}^b+[L]^b)$

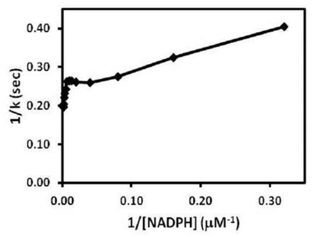
Enzyme	K_d^{FO} (nM)	K_d^{NADPH} (nM)	NADPH Hill Coefficient
***wt*Fno**[a]	3.6 ± 0.7	2.0 ± 0.3	0.61 ± 0.03
R186K	13 ± 2	64 ± 9	0.9 ± 0.1
R186Q	4.8 ± 0.5	156 ± 35	0.8 ± 0.1
R186I	12 ± 2	53 ± 5	1.0 ± 0.1
T192A	11 ± 2	101 ± 21	0.5 ± 0.1
T192V	21 ± 9	156 ± 24	0.9 ± 0.1
S190A	54 ± 15	254 ± 85	0.8 ± 0.1
T09A	7 ± 1	138 ± 35	0.8 ± 0.1
H133A	8 ± 3	176 ± 67	0.5 ± 0.1
H133N	35 ± 6	107 ± 31	0.7 ± 0.1

[a]Joseph, E.; Le, C.; Nguyen, T.; Oyugi, M.; Hossain, M.; Foss, F.; Johnson-Winters, K., *Biochemistry,* **2016**, 55, 1082-1090.

*wt*Fno Steady-State Kinetics Data



FO CONDITIONS
50 µM MES/NaOH (pH 6.50) buffer
Temp: 22 °C
FO was titrated into 0.2 µM of Fno
[NADPH]=600 µM

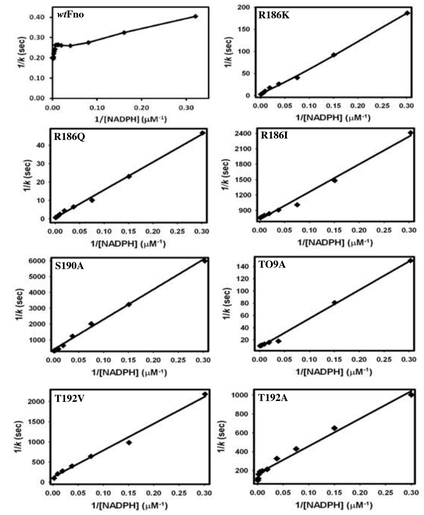


Hossain, M. S.; Le, C. Q.; Joseph, E.; Nguyen, T. Q.; Johnson-Winters, K.; Foss, F. W., Jr. *Org. Biomol. Chem,* **2015,** 13, 5082-5085.
Joseph, E.; Le, C.; Nguyen, T.; Oyugi, M.; Hossain, M.; Foss, F.; Johnson-Winters, K. *Biochemistry,* **2016**, 55, 1082-1090.

Enzyme	$^{FO}k_{cat}$ (s^{-1})	$^{FO}K_m$ (µM)	$^{FO}k_{cat}/K_m$ ($M^{-1}s^{-1}$)	$^{NADPH}k_{cat}$ (s^{-1})	$^{NADPH}K_m$ (µM)	$^{NADPH}k_{cat}/K_m$ ($M^{-1}s^{-1}$)
***wt*Fno**	5.3 ± 0.1	4.0 ± 0.4	(1.3 ± 0.3) x 10^6	5.41 ± 0.04 4.16 ± 0.07	2.3 ± 0.2 61 ± 6	(2.4 ± 0.2) x 10^{10} (6.8 ± 0.1) x 10^7
R186K	0.407 ± 0.008	1.3 ± 0.1	(3.1 ± 0.2) x 10^5	0.35 ± 0.02	279 ± 28	(1.2 ± 0.1) x 10^3
R186Q	0.0035 ± 0.0001	2.0 ± 0.3	(1.7 ± 0.2) x 10^3	0.0039 ± 0.0001	139 ± 13	(2.8 ± 0.2) x 10^1
R186I	0.0017 ± 0.0006	4.3 ± 0.8	(3.9 ± 0.2) x 10^2	0.0013 ± 0.0001	35 ± 8	(3.7 ± 0.2) x 10^1
T192V	0.0017 ± 0.0001	3.5 ± 0.4	(4.8 ± 0.6) x 10^2	0.0010 ± 0.0003	124 ± 17	0.8 ± 0.2 x 10^1
T192A	0.0025 ± 0.0007	1.4 ± 0.4	(1.7 ± 0.2) x 10^3	0.050 ± 0.001	91 ± 19	(5.4 ± 0.1) x 10^2
S190A	0.00030 ± 0.00001	0.8 ± 0.2	(3.7 ± 0.1) x 10^2	0.0039 ± 0.0004	49 ± 13	(7.9 ± 0.2) x 10^1
T09A	0.0930 ± 0.0011	0.9 ± 0.1	(1.0 ± 0.1) x 10^5	0.0976 ± 0.0012	28 ± 2	(3.4 ± 0.2) x 10^3

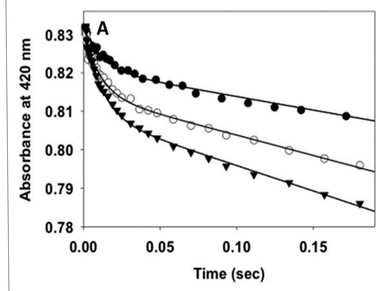
Fno Variant Double Reciprocal Plots

Fit to: $\frac{1}{k} = \frac{K_m}{k_{cat}[S]} + \frac{1}{k_{cat}}$



Joseph, E., Le, C., Nguyen, T., Oyugi, M., Hossain, M., Foss, F., Johnson-Winters, K. *Biochemistry,* **2016**, 55, 1082-1090.

Pre-Steady-State Kinetics Data



[A] Pre steady-state kinetics of hydride transfer to FO from NADPH by Fno is shown for varying Fno concentrations: 1.0 µM (solid circle), 1.5 µM (open circle), and 2.0 µM (solid triangle).

Enzyme	1 µM k_{obs} (s^{-1})	1.5 µM k_{obs} (s^{-1})	2 µM k_{obs} (s^{-1})
***wt*Fno**	47.9 ± 0.5[a] 1.99 ± 0.02		
R186K	0.45 ± 0.02	0.46 ± 0.02	0.47 ± 0.02
R186Q	0.27 ± 0.01	0.25 ± 0.01	0.23 ± 0.02
R186I	0.0040 ± 0.0006	0.0050 ± 0.00006	0.00680 ± 0.00006
T192A	0.2681 ± 0.0046	0.2748 ± 0.0057	0.2759 ± 0.0151
T192V	0.1858 ± 0.0091	0.6100 ± 0.0266	0.1372 ± 0.0007
S190A	0.046 ± 0.001	0.050 ± 0.001	0.06 ± 0.01
H133N	0.4116 ± 0.0042	0.3856 ± 0.0156	0.3518 ± 0.0083
H133A	0.0135 ± 0.0001	0.0211 ± 0.0001	0.0323 ± 0.0003
T09A	0.1709 ± 0.0022	0.1735 ± 0.0010	0.2083 ± 0.0040

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Conclusions

Binding-
- The Hill Coefficients for the Fno variants are around 1 for R186K, R186I, AND T192V, revealing no cooperativity, diverging from the behavior of *wt*fno.
- R186Q, S190A,and T09A variants all have a Hill coefficient of 0.8 ± 0.1, which is a less pronounced cooperativity.
- T192A, H133A, and H133N are similar to *wt*fno, showing negative cooperativity

Steady-state-
- The Fno variants R198K, R186Q ,R186I ,S190A, T192V and T09A did not mimic the downward and concave curvature as previously observed with *wt*Fno, but instead yielded a straight line, revealing that these Fno variants did not display cooperativity kinetics.
- T192A displayed a downward and concave curvature therefore negative cooperativity which is similar to what we see in *wt*fno.

Pre Steady-state-
- The hydride transfer step for these Fno variants was significantly slower than *wt*Fno, suggesting that they are important in catalysis.