## Supplemental Data For

# Characterization of the Flavin Monooxygenase Involved in Biosynthesis of the Antimalarial FR-900098

Kim Nguyen[1,a], Matthew A. DeSieno[2,5,a], Brian Bae[1,5,c], Tyler W. Johannes[2,b], Ryan E. Cobb[2,5], Huimin Zhao[1,2,3,4,5*], Satish K. Nair[1,3,4,5*]

[1]Department of Biochemistry, [2]Department of Chemical and Biomolecular Engineering, [3]Department of Chemistry, [4]Center for Biophysics and Computational Biology, [5]Institute for Genomic Biology
University of Illinois at Urbana-Champaign
600 South Mathews Avenue
Urbana, IL 61801, USA

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**Figure S1.** Analysis of the FrbG reaction product by HPLC. Negative control contains heat-inactivated enzyme. Each peak was also identified by MS analysis. FrbG is able to convert CMP-5'-3APn (m/z 443→322) to CMP-5'-H3APn (m/z 459→322). The enzyme was inactive on the substrate lacking nucleotide conjugation (inset). CMP-5'-3APn, CMP-5'-3-aminopropylphosphonate; CMP-5'-H3APn, CMP-5'-*N*-hydroxy-3-aminopropylphosphonate; 3APn, 3-aminopropylphosphonate.

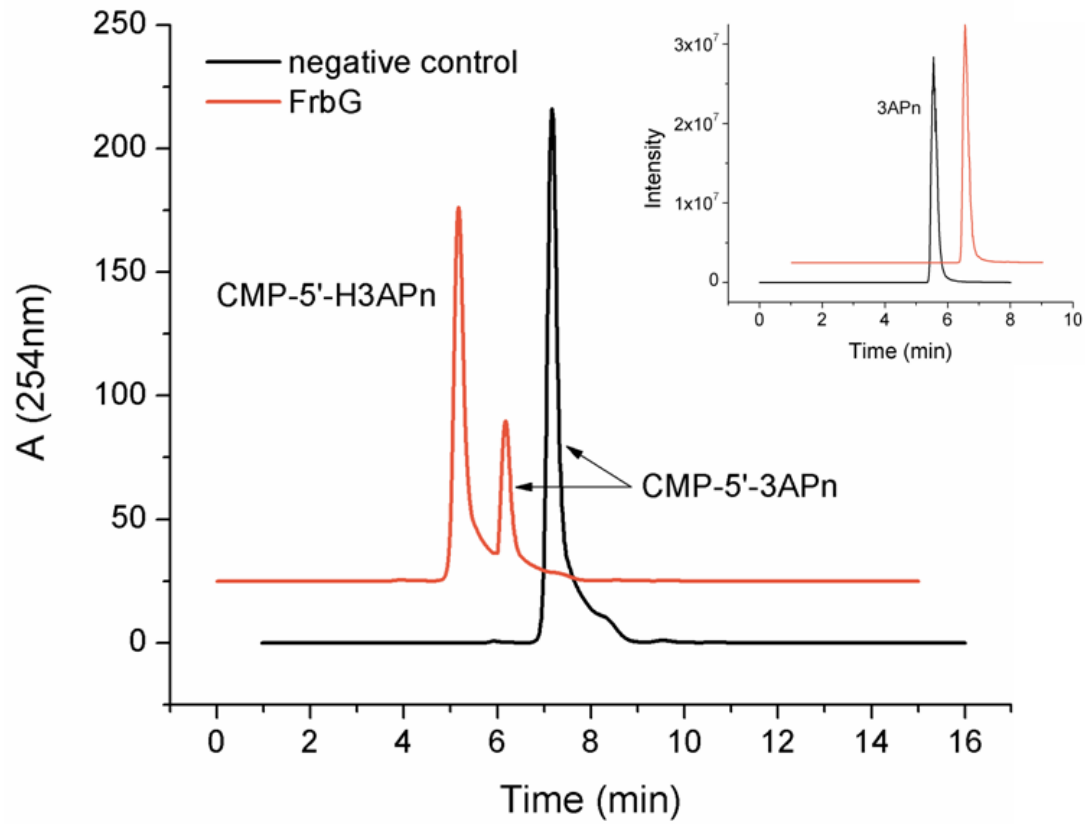
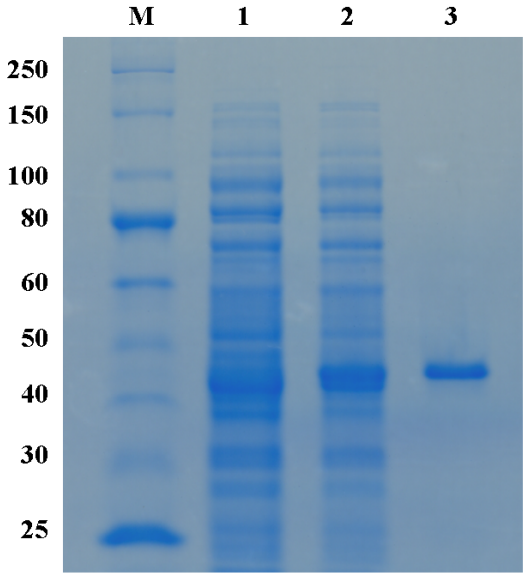


**Figure S2.** HPLC size exclusion chromatography of FrbG. (A) Overexpression and purification of recombinant FrbG in *E. coli* BL21(DE3). Lane M, protein standard (kDa); lane 1, cell-free lysate of empty pET28a; lane 2, cell-free lysate of induced FrbG culture; lane 3, purified FrbG enzyme. (B) A size exclusion standard was used to calibrate a Bio-Sil SEC-250, 300 x 7.8 mm column with a mobile phase of 0.1 M $Na_2HPO_4$, 0.15 M NaCl, 0.01 M $NaN_3$, pH 6.8 at a flow rate of 1 mL/min. The standard proteins are represented by closed squares and FrbG sample is represented by an open square.



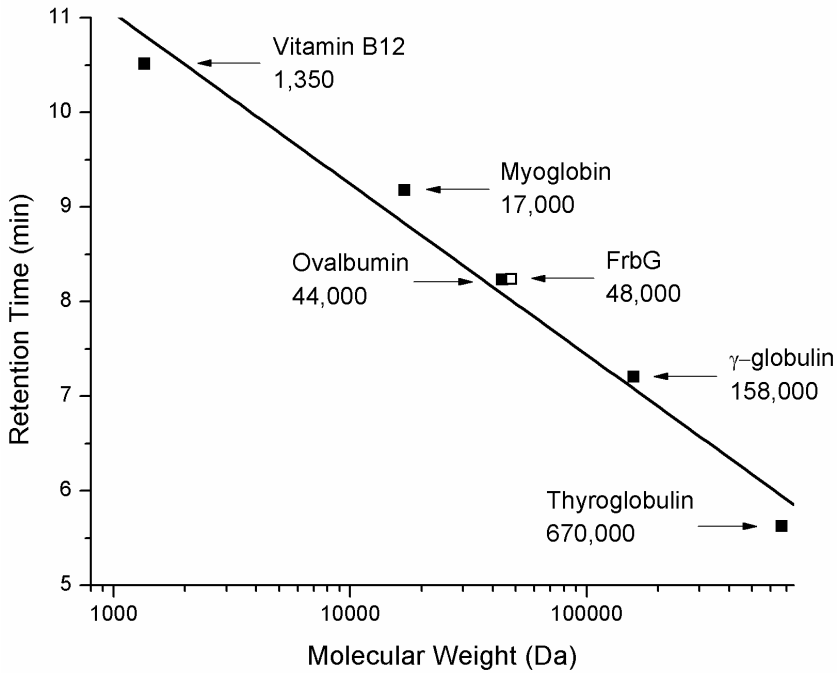



**Figure S3.** A comparison of the overall structures of (**a**) FrbG (in cyan) with (**b**) *S. pombe* FMO and (**c**) *Methylophaga* sp. SK1 FMO. The FAD and NADP(H) shown as yellow sticks. Notice that the smaller, nucleotide-binding domain of FrbG is characterized by several novel insertions that are absent from the structures of the other FMOs.

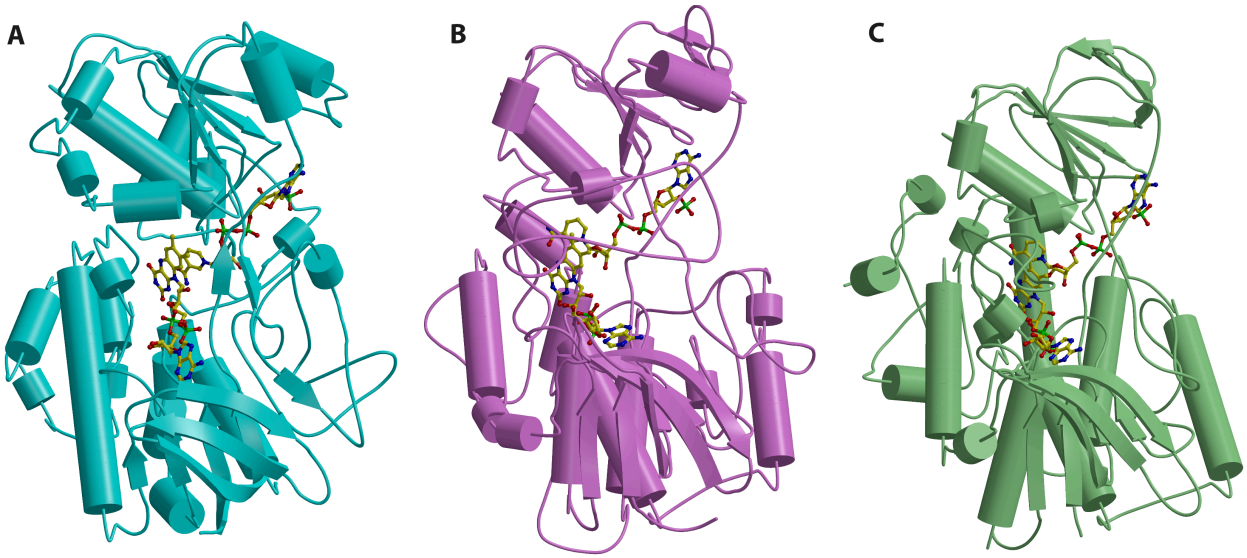


**Figure S4.** Mutational analysis of Asn-58 and His-221 in FrbG. Wild-type and mutant proteins (at a concentration of 5 μM) were tested for NADPH oxidase activity (in the absence of substrate) as well as enzymatic activity in the presence of 500 μM of CMP-5'-3APn substrate.

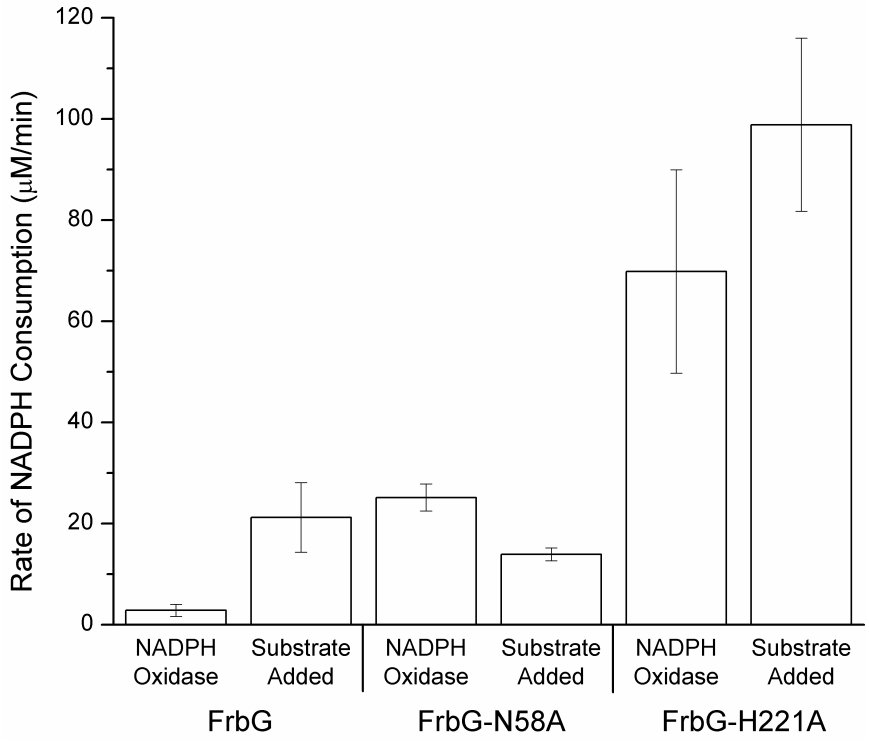


**Figure S5**. A hypothetical model for the interaction of substrate CMP-3APn with FrbG, generated by aligning the nucleotide moiety of the substrate with the position of the nucleotide of the $NADP^+$ cofactor observed in the co-crystal structure. Protein residues involved in the binding of the substrate are labeled and shown in yellow, the CMP-3APn substrate is shown in cyan and the isoalloxazine ring of FAD is shown in purple.

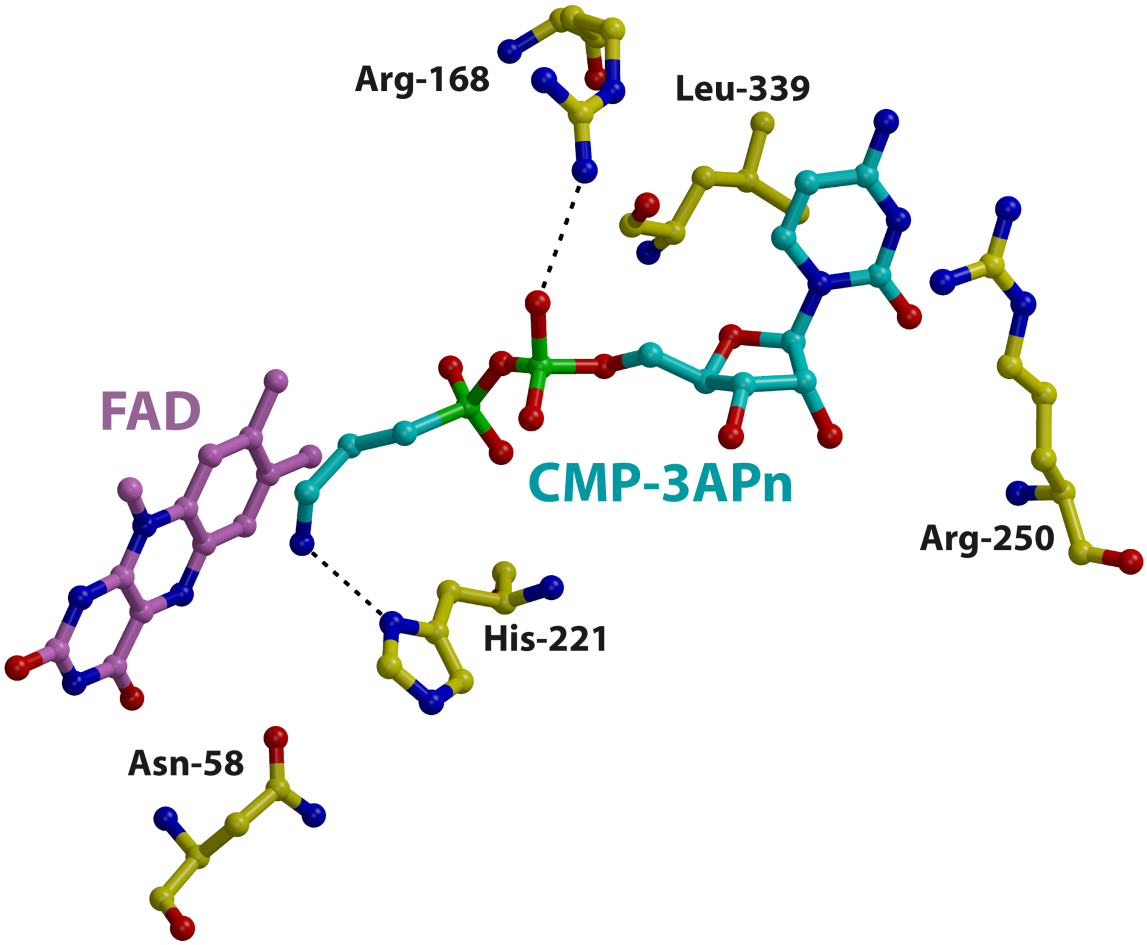


**Figure S6**. Kinetic analysis of FrbG as determined by hydroxylated product formation. Inhibition of initial velocity rates was monitored for increasing concentrations of substrate, indicating the competition with cofactor for the same binding pocket.

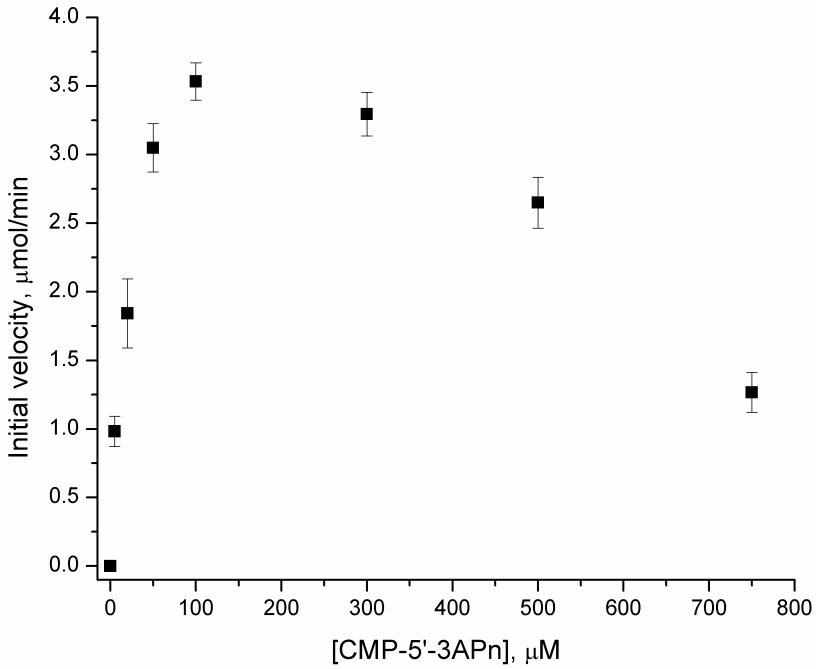


**Figure S7**. Analysis of the non-enzymatic oxidation of the hydroxylamine product to its nitroso derivative. (**a**) FrbG reaction mixtures were monitored by LC-MS following removal of the enzyme by diafiltration for loss of the hydroxylamine product (*m*/*z* = 459). (**b**) The nitroso derivative was reduced back to the hydroxylamine under anaerobic conditions using $SmI_2$, but rapidly reformed upon exposure to air. The left trace shows extracted ion chromatograms for the hydroxylamine (red curve) and the nitroso (blue curve), along with a significant CMP side product (green curve), following anaerobic reduction. The right trace shows reformation of the nitroso (blue) following 30 minutes of aerobic exposure.