Supporting Information

Identification of the NAD(P)H Binding Site of Eukaryotic UDP-Galactopyranose Mutase

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EXPERIMENTAL PROCEDURES

Crystallization. The K344A/K345A double surface mutant of AfUGM was used for all crystallization experiments. As described previously, this form of AfUGM is amenable to high resolution X-ray crystallography, whereas crystallographic studies of the wild-type enzyme are hampered by translational pseudosymmetry and weak diffraction (*1*). Residues 344 and 345 are on the surface of the enzyme, far from the active site and oligomerization interfaces, and the kinetic constants of K344A/K345A are virtually identical to those of the native enzyme (*1*). For simplicity, we will refer to K344A/K345A as AfUGM.

Hexagonal crystals of AfUGM were grown as described previously (*1*). Briefly, the crystals were grown using the sitting drop vapor diffusion method with a reservoir solution containing 1.4 M ammonium sulfate and 0.1 M sodium acetate at pH 4.5. Equal volumes of protein solution (8 mg/mL in 125 mM NaCl and 25 mM HEPES at pH 7.5) and reservoir solution were mixed, and large yellow hexagonal crystals were obtained in 3-4 days. The space group is $P6_522$ with unit cell lengths of a = 218 Å and c = 319 Å. The asymmetric unit contains one tetramer and 75 % solvent.

Synthesis of Deuterated Coenzymes. (R)NADPD was synthesized by the method of Jeong (*2*) with some modifications. 25 mg of $NADP^+$ (5.6 mM final concentration), 480 µL 2-propanol-*d*8 (1 M final concentration), and 50 units of alcohol dehydrogenase (*Thermoanaerobium brockii*) were added to 6 mL of 25 mM Tris-HCl pH 9.0. The reaction was allowed to proceed for 30 min at 40 ºC with light agitation until the A_{260}/A_{340} ratio reached a values of ~ 2.5. (S)NADPD was synthesized by the method of Viola (*3*) with some modifications. 59 mg $NADP^+$ (10 mM final concentration), 22 mg D-glucose-1-*d* (15 mM final concentration), and 120 units of glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) were added to 3.2 mL (40%

v/v) of dimethyl sulfoxide and 4.7 mL of 100 mM sodium phosphate pH 8.0. The reaction was allowed to proceed overnight for ~16 hours at 25 ºC with light agitation until the A_{260}/A_{340} ratio had plateaued at 2.5.

(R)NADD was synthesized by the method of Sucharitakul (*4*). 30 mg NAD^+ (15 mM final concentration), 8 µL formic acid-*d*2 (71 mM final concentration), and 90 units of formate dehydrogenase (*Candida boidinii*) were added to 3 mL of 50 mM sodium carbonate pH 8.5 (pH adjusted after the addition of formic acid). The reaction was allowed to proceed for 5 hours at 25ºC with light agitation until the A_{260}/A_{340} ratio plateaued at 2.3.

After all the reactions reached their desired A_{260}/A_{340} ratio; the solutions were filtered with a 5,000 molecular-weight cut-off filter (Amicon). The filtered (S)NADPD samples were then loaded onto a 5 mL DEAE column (GE Healthcare) equilibrated with 25 mM Tris-HCl pH 8.0. The bound (S)NADPD was eluted with 50 mM NaCl. This purification step was performed in order to remove dimethyl sulfoxide and phosphate as NAD(P)D has been shown previously to degrade in phosphate buffer (*2*). This chromatographic step was omitted for the (R)NAD(P)D coenzymes. Next, the samples were concentrated by rotary evaporation to ~1 mL and diluted 1:12 with ethanol, vortexed, and placed at -20 ºC for 30 minutes. The precipitated samples were separated from the supernatant by centrifugation for 20 minutes at 14,100 *g* at 4ºC. The resulting pellets were allowed to dry for 1 hour to remove any residual ethanol. The samples were then resuspended in 250 µL of 25 mM Tris-HCl pH 9.0 and stored at -20 ºC. As a control, NADH and NADPH were synthesized by the same respective methods to account for any differences from commercial samples.

Expression and Purification of *Mycobacterium tuberculosis* UGM. The gene coding for *M. tuberculosis* UGM (MtUGM) was cloned into pVP56K, and the protein expressed and purified following the procedures developed for AfUGM (*1*).

TABLE S1.
Deuterium Kinetic Isotope Effects for Hydride Transfer to AfUGM and TcUGM[a]

	AfUGM	TcUGM
[4R-4-^{2}H]-NAD(P)H		
NADPH	3.0 ± 0.1	1.85 ± 0.01
NADH	6.0 ± 0.7	3.9 ± 0.2
[4S-4-^{2}H]-NAD(P)H		
NADPH	0.93 ± 0.03	1.00 ± 0.03

[a]expressed as the ratio of k_{red} measured using saturating concentrations of [4R-4-^{2}H]-NAD(P)H or to [4S-4-^{2}H]-NADPH to k_{red} measured using NAD(P)H

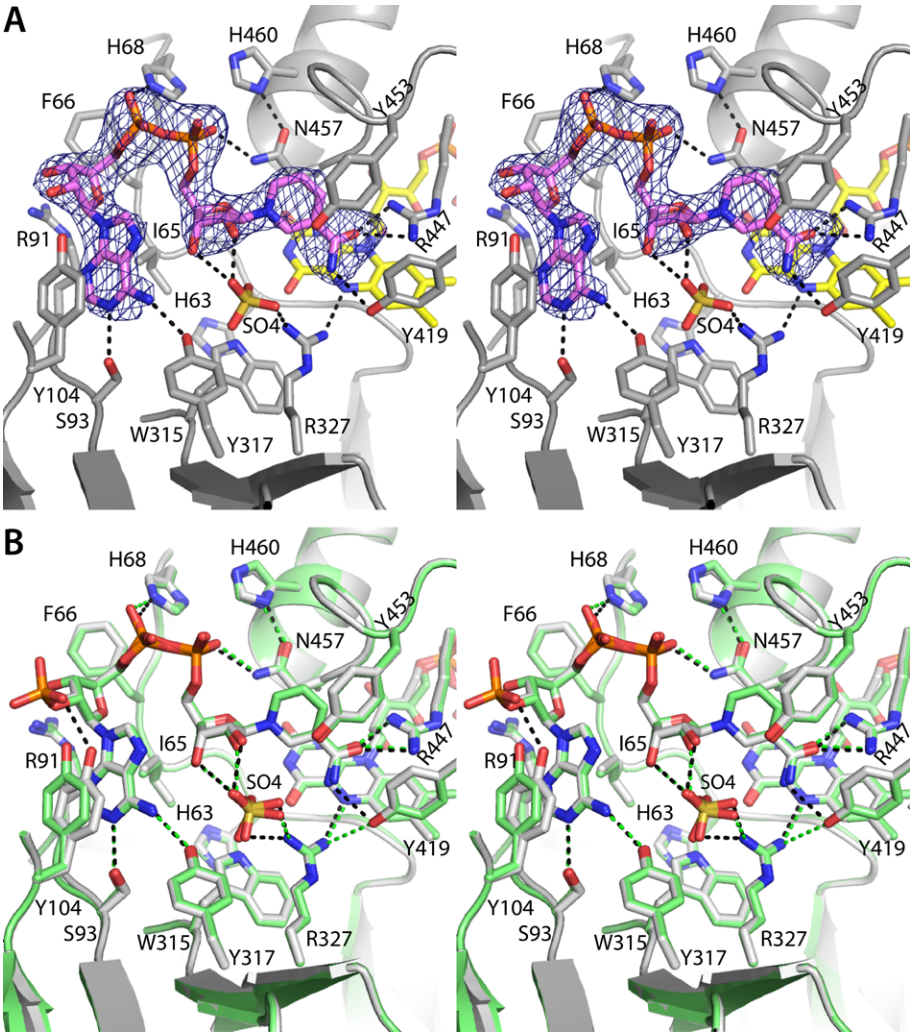

Figure S1. The active site of AfUGM$_o$-NADH (stereographic views). (A) Electron density for NADH. FAD and NADH are colored yellow and pink, respectively, and the cage represents a simulated annealing σ_A-weighted F_o - F_c omit map contoured at 3.0 σ. Prior to calculation of the map, NADH was omitted and simulated annealing refinement was performed. (B) Superposition of AfUGM$_o$-NADH (green) and AfUGM$_o$-NADPH (white).

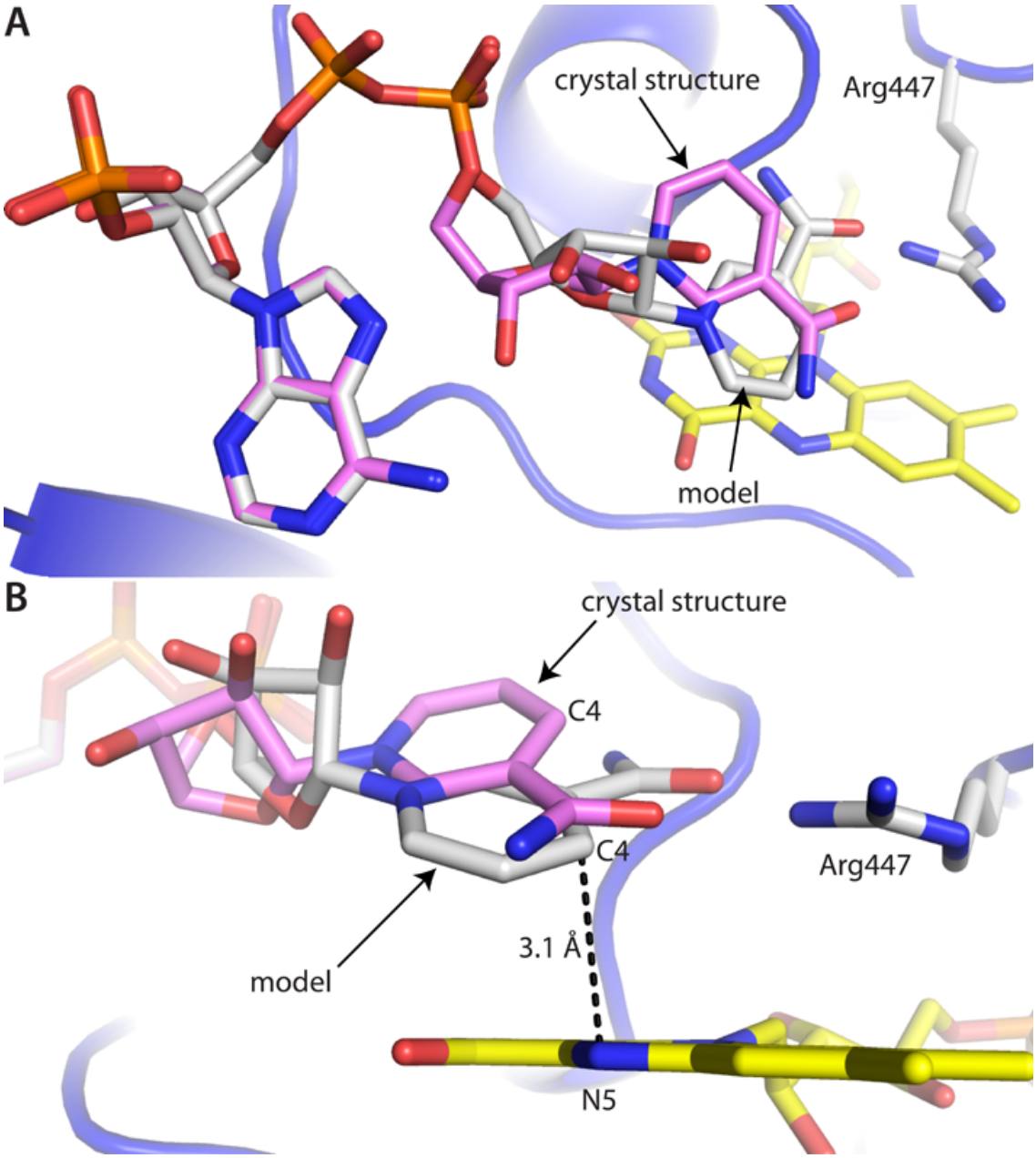

Figure S2. Two views of a model of the hydride transfer complex between NADPH and $AfUGM_o$. NADPH from the crystal structure is colored pink, and the model is shown in white. The model was created by manual torsional adjustment of the nicotinamide riboside moiety in COOT to bring the *re* face of the nicotinamide in contact with the FAD isoalloxazine, which is consistent with kinetic isotope effect data implicating pro-R stereochemistry.

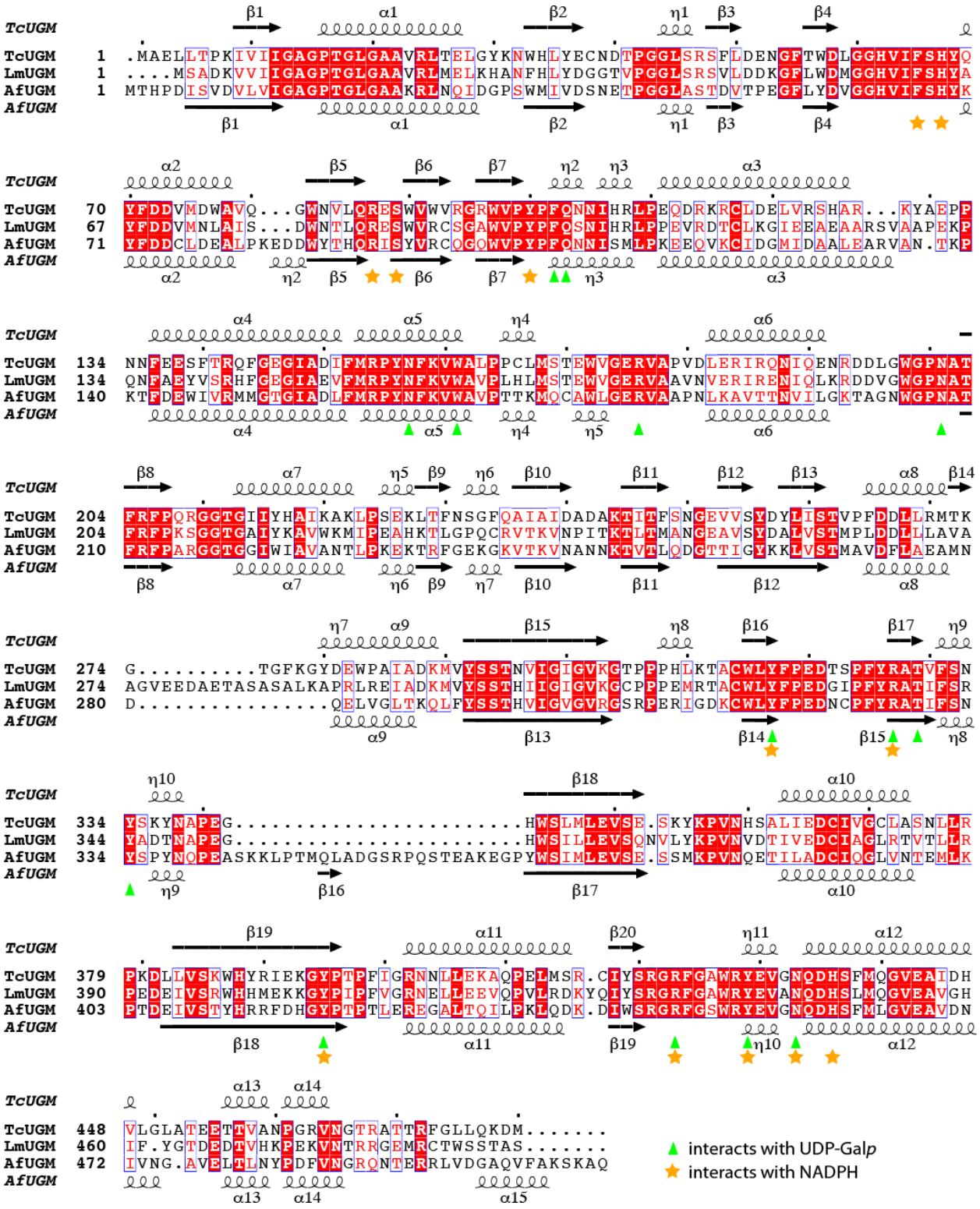

Figure S3. Sequence alignment of the UGMs from *Trypanosoma cruzi*, *Leishmania major*, and *Aspergillus fumigatus*. The secondary structure elements above and below the alignment were derived from the structures of *T. cruzi* UGM and AfUGM, respectively. This figure was created with CLUSTALW2 (*5*) and ESPript (*6*).

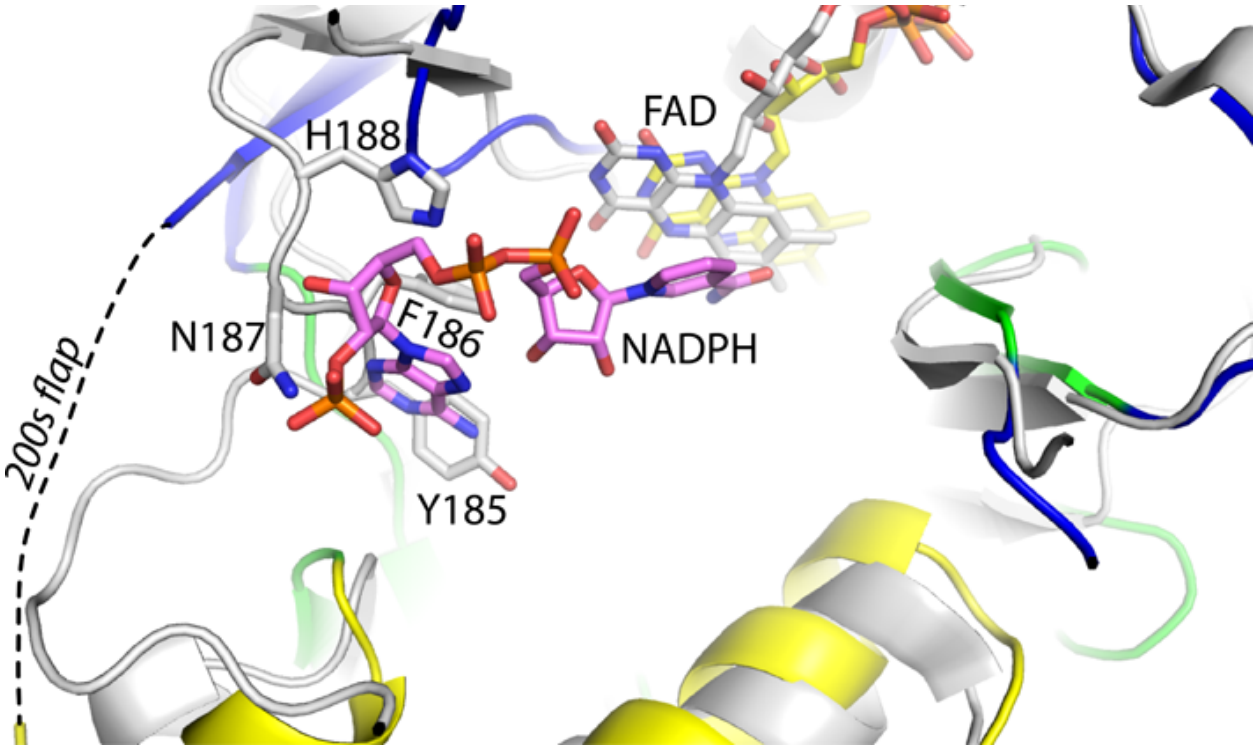

Figure S4. Superposition of the $AfUGM_o$-NADPH complex and oxidized KpUGM (PDB 2BI7). $AfUGM_o$ is shown with domains 1, 2, and 3 colored blue, yellow, and green as in Figure 2A, NADPH colored pink, and FAD in yellow. KpUGM is colored white. Selected side chains of the 180s loop of KpUGM are shown. Note that these residues occupy the space corresponding to the AMP group of NADPH, which suggests that the NADPH binding sites of KpUGM and AfUGM are different.

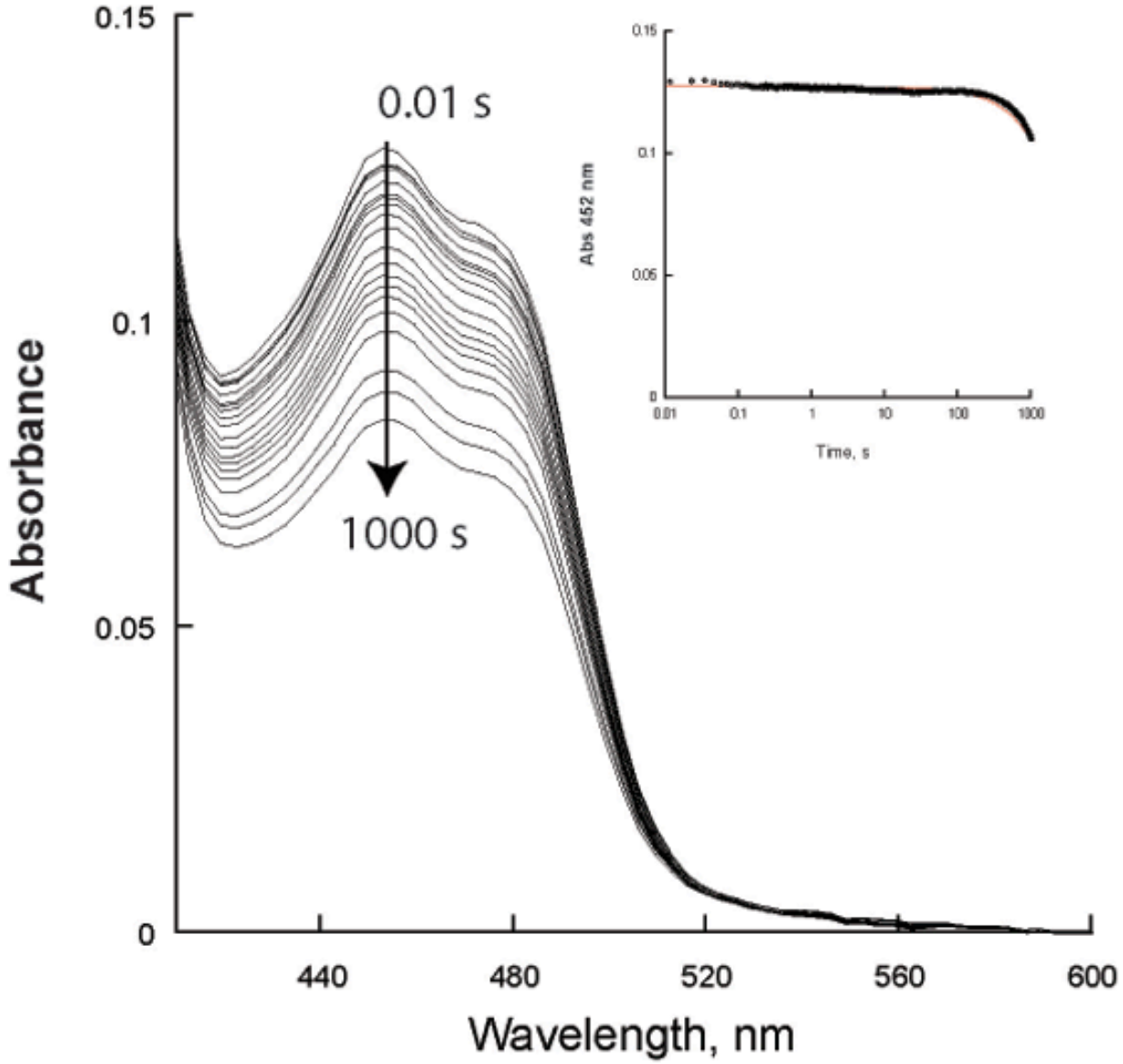

Figure S5. Reduction of *M. tuberculosis* UGM (MtUGM) by NADPH. The reaction of MtUGM (15 μM after mixing) with NADPH (2 mM after mixing) was monitored in a stopped-flow spectrophotometer under anaerobic conditions. The insert shows the absorbance at 452 nm during the first 1000 s (note the logarithmic time axis). The rate was calculated by fitting the data to a linear equation.

References

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