Supporting information

X-ray crystallography reveals a reduced substrate complex of UDP-galactopyranose mutase poised for covalent catalysis by flavin

Todd D. Gruber, William M. Westler, Laura L. Kiessling,* and Katrina T. Forest*

Materials and methods

Enzyme preparation

Hexahistidine-tagged UGM from *Klebsiella pneumoniae* was expressed and purified as previously described (*1*).

Increased trapping of covalent adduct

(A) ~5% adduct chromatogram, reproduced from (*2*). Samples (125 μL) containing 300 μM UGM, 0.5 mM UDP-Gal*p* (Calbiochem, San Diego, CA), 50 mM sodium cyanoborohydride (Fluka, Steinheim, Switzerland), and 20 mM freshly prepared sodium dithionite (Fluka, Steinheim, Switzerland) in 500 mM sodium phosphate were reacted for 15 min (ambient atmosphere) and acid-precipitated with HCl, prior to centrifugation at 9,300 x g for 8 min. Supernatant was filtered and injected (40 μL) on a Shimadzu LC-MS containing a C18 column (Supelco Discovery, 2.1 x 150 mm) equilibrated with 0.4% (v/v) formic acid. A linear gradient of acetonitrile and 0.2% (v/v) formic acid (15-85% (v/v)) over 40 min eluted the flavin species at ~10 minutes. Positive-ion mass spectral data were collected for all time points after 3 min.

(B) ~20% adduct chromatogram: similar to (A) conditions, with 1.0 mM UGM, 2 mM UDP-Gal*p*, 50 mM sodium cyanoborohydride, 30 mM dithionite, 100 mM sodium phosphate pH 7, 90 min, and anaerobic Ar atmosphere (reaction mixtures set up in an open-top container filled with Ar gas). (C) ~80% adduct chromatogram: 2.5 mM UGM, 10 mM UDP-Gal*p*, 1 M sodium cyanoborohydride, 30 min, Ar atmosphere. (D) ~90% adduct chromatogram: 1.1 mM UGM, 10 mM UDP-Gal*p*, 1 M sodium cyanoborohydride, 60 min, Ar atmosphere.

Reactions B-D were precipitated with 6 M HCl and then neutralized with 1 M sodium phosphate at pH 7, and precipitate was washed with 1 M NaCl to extract flavin adduct.

Precipitate was removed by centrifugation at 16,100 x g. Supernatant was injected onto a Varian C18 Microsorb (250 x 4.6 mm, Palo Alto, CA) column equilibrated with 95% Buffer A (H_2O with 0.4 % (v/v) formic acid) and 5% Buffer B (acetonitrile with 0.2% (v/v) formic acid). The sample was eluted with the following program: 0-5 min: isocratic (95% A, 5% B); 5-10 min: linear gradient to 85% A, 15% B; 10-30 min: linear gradient to 25% A, 75% B; 30-32 min: linear gradient to 5% A, 95% B; 32-37 min: isocratic (5% A, 95% B). Trapped adduct (peak at ~350 nm) eluted at 20 min, unreacted flavin (peaks at 380, 450 nm) at 22 min, as determined by UV/vis absorbance measured with a photo diode array detector.

^{1}H NMR of adduct

For reduced flavin NMR (Figure 1C), a solution of FAD (Sigma, St. Louis, MO) was reduced with 30 mM dithionite and placed in an anaerobic glove box (N_2/H_2 scrubbed environment, Coy Laboratory Products, Grass Lake, MI) with NMR tubes for 3 hrs to purge residual O_2 (final concentrations: 1 mM FAD, 100 mM potassium phosphate pH 6.8 in D_2O (99.9% D_2O, Cambridge Isotope Laboratories, Andover, MA), 30 mM dithionite, 33% (v/v) CD_3OD (99.8% CD_3OD, Cambridge Isotope Laboratories), 10 μM deuterated 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) (NMR standard)). Sample was added to screw-cap NMR tube (Wilmad Labglass, Buena, NJ), which was then sealed with parafilm. NMR experiments were performed at the National Magnetic Resonance Facility at Madison (NMRFAM). ^{1}H NMR (Bruker DMX 400 MHz): δ 8.42 ppm (s, 1H, adenine proton), 8.12 (s, 1H, adenine proton), 6.30 (s, 1H, isoalloxazine C9 proton), 6.16 (s, 1H, isoalloxazine C6 proton), 6.00 (d, 1H, unresolved J, ribose anomeric proton), 4.6-3.3 (multiple unresolved protons: ribose and ribityl protons), 1.96 (s, 3H, isoalloxazine methyl protons), 1.93 (s, 3H, isoalloxazine methyl protons).

For trapped covalent adduct, following generation of adduct as in condition (D) above, trapped covalent adduct was purified on a C18 Sep-pak cartridge (Waters, Milford, MA). Sample was washed with 20 mM potassium phosphate in D_2O, pH 6.8, to remove unquenched sodium cyanoborohydride and salt and eluted with 1:1 20 mM potassium phosphate pH 6.8 in D_2O:CD_3OD. Green sample (presumably partially oxidized adduct) elutes in approximately 500 μL. Sodium dithionite in D_2O was added to 25 mM to reduce the adduct (pale yellow). 10 μM DSS was added as an NMR standard. Reduced samples were placed in an anaerobic glove box with NMR tubes for 3 hrs. Final concentrations: ~1 mM adduct, 50 mM potassium phosphate buffer pH 7 in D_2O, 25 mM dithionite, ~33% CD_3OD, 10 uM DSS. Sample was added to a screw-cap NMR tube, which was then sealed with parafilm. ^1H NMR (Bruker DMX 600 MHz with Cryoprobe, with water suppression)(Adduct with small amount of unreacted FAD, Figure 1C): Adduct: δ 8.49 ppm (s, 1H, adenine proton), 8.13 (s, 1H, adenine proton), 6.68 (s, 1H, isoalloxazine C6 proton), 6.64 (s, 1H, isoalloxazine C9 proton), 6.03 (d, 1H, J = 5.3 Hz, ribose anomeric proton), 4.65-3.3 (multiple unresolved peaks: ribose, ribityl and galactose-derived protons), 2.06 (s, 1H, isoalloxazine methyl protons), 2.02 (s, 1H, isoalloxazine methyl protons); unreacted FAD: ~8.49 ppm, unresolved from adduct (adenine proton), 8.12 (s, 1H, adenine proton), 6.31 (s, 1H, isoalloxazine C9 proton), 6.17 (s, 1H, isoalloxazine C6 proton), 6.01 (d, 1H, J = 5.1 Hz, ribose anomeric proton), 1.96 (s, 3H, isoalloxazine methyl protons), 1.93 (s, 3H, isoalloxazine methyl protons).

^1H-^1H NOESY experiments (Supplementary Figure 1): Adduct generated as with condition (D), above, and purified with a C18 Sep-pak cartridge, placed in anaerobic glove box and sealed with parafilm as above. Final concentrations: ~1 mM adduct, 50 mM potassium phosphate pH 6.8, 25 mM dithionite, ~33% CD_3OD, 10 μM DSS. ^1H-^1H NOESY NMR

performed on Bruker DMX 500 with Cryoprobe. Reduced FAD: final concentration: 4 mM FAD in D_2O, 33% CD_3OD, 10 μM DSS. ^1H-^1H NOESY NMR performed on Bruker DMX 500 with Cryoprobe.

Soaking of substrate into UGM crystals

Crystals of UGM were grown in the presence of UDP-Glc as previously described (*1*). Cryoprotectant was 90 mM ammonium acetate, 45 mM sodium acetate, pH 5.6, 13.5% PEG4000, 8% glycerol (this represents 53% of Cryos Suite Condition #87 (Qiagen, Valencia, CA)) plus 15 mM L-cys and 30% methanol. Incrementally, mother liquor was removed and cryoprotectant containing 120 mM UDP-Gal*p* was added to a final UDP-Gal*p* concentration of 90 mM. Ligand exchange was allowed to occur for 24 hours. Crystals were transferred to cryoprotectant with 90 mM UDP-Gal*p* for 20 seconds and were vitrified in liquid nitrogen. For reduced UGM crystals, cryoprotectant additionally contained 100 mM freshly prepared sodium dithionite. Reduction (as determined by a color change from yellow to very pale yellow) took 15 seconds, and vitrification was carried out 165 seconds later.

X-ray diffraction analysis

X-ray diffraction data were collected at the Advanced Photon Source (LS-CAT, 21-ID-G, Argonne, IL). For both oxidized and reduced UDP-Gal*p* structures, molecular replacement was performed using Molrep (*3*) with UDP-Glc bound UGM (PDB ID 3GF4) (*1*) stripped of ligand and waters as the starting model. Refinement was performed using Refmac (v5.5.0072) (*4*). Electron density maps were analyzed using Coot (*5*).

Because of disorder, residues 129-133 of oxidized monomer B, residues 127-135 of reduced monomer B, and side chain atoms beyond Cβ for residues 164-168 of reduced monomer B were not included. Only the UDP portion of the ligand in reduced monomer A is ordered and included in the model; this ligand orientation appears to be a mixture of the oxidized orientation and reduced monomer B orientation. Omit maps showing difference density (F_O-F_C) for the ligands of reduced and oxidized monomers A and B are shown in **Supplementary Figure 2**.

Accession numbers

Coordinates and structure factors have been deposited in the PDB with accession numbers 3INR (oxidized UDP-Gal*p* bound structure) and 3INT (reduced UDP-Gal*p* bound structure).

Acknowledgments

Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor for the support of this research program (Grant 085P1000817). NMR experiments were performed at the National Magnetic Resonance Facility at Madison, supported by NIH grants P41RR02301 (BRTP/ NCRR) and P41GM66326 (NIGMS). This facility is also supported by the University of Wisconsin, the NIH (RR02781, RR08438), the NSF (DMB-8415048, OIA-9977486, BIR-9214394), and the USDA.

References

1. Gruber, T., Borrok, M., Westler, W., Forest, K., and Kiessling, L. L. (2009) *J. Mol. Biol. 391*, 327-340.
2. Soltero-Higgin, M., Carlson, E., Gruber, T. D., and Kiessling, L. L. (2004) *Nat. Struct. Mol. Biol. 11*, 539-543.
3. Vagin, A., and Teplyakov, A. (1997) *J. Appl. Cryst. 30*, 1022-1025.
4. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr. D Biol. Crystallogr. 53*, 240-255.
5. Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. D Biol. Crystallogr. 60*, 2126-2132.
6. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) *Nucleic Acids Res. 35*, W375-383.

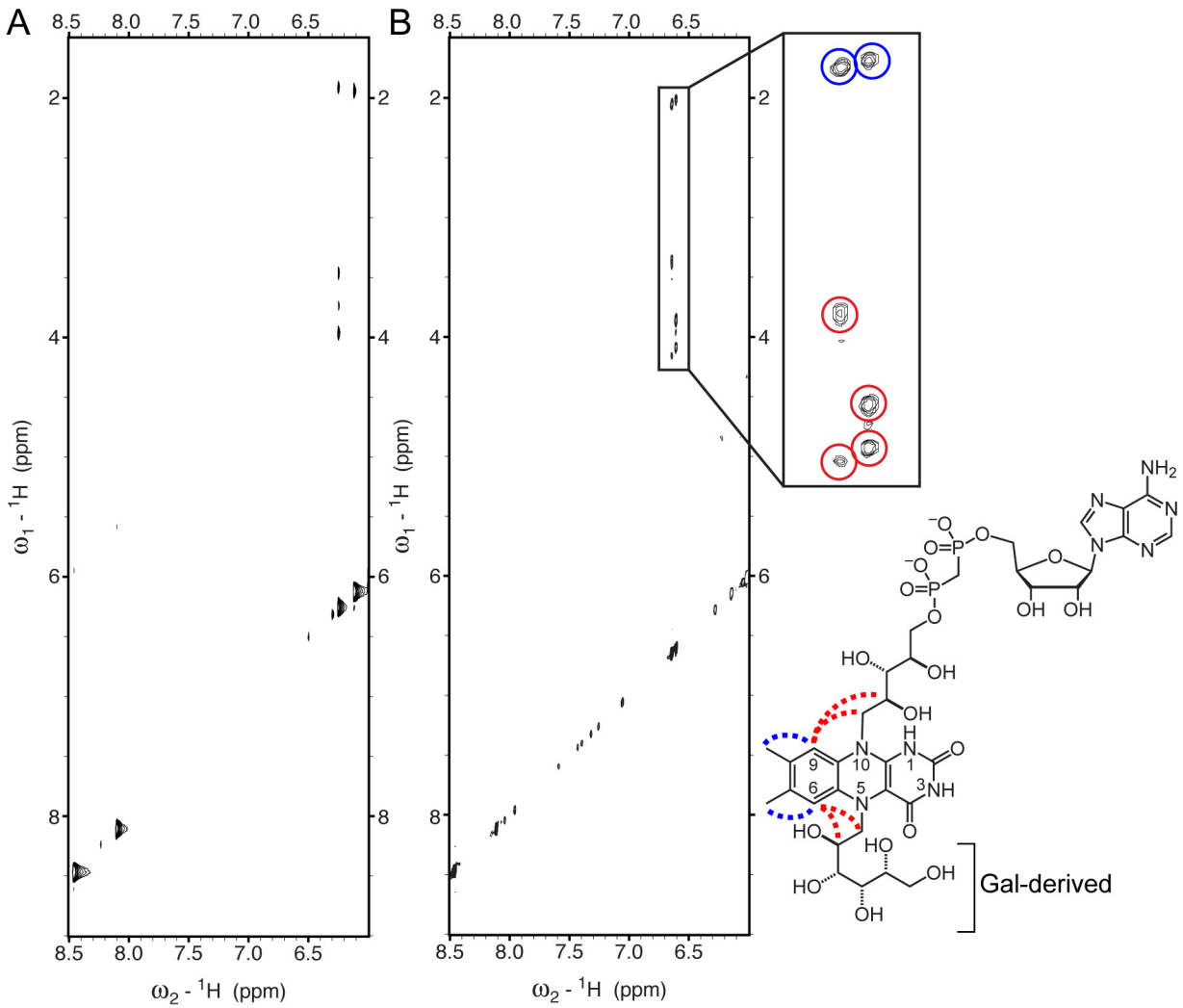
Supplementary Table 1. Data collection and refinement statistics

	Oxidized (3INR)	Reduced (3INT)
Data collection		
Resolution limits (Å) (last shell)	30.0-2.30 (2.38-2.30)	30.0-2.50 (2.59-2.50)
Unique reflections (#)	48,652 (4826)	38,510 (3,816)
Completeness (%)	98.5 (98.4)	100.0 (100.0)
R_{sym} [a] (%)	8.5 (26.6)	10.7 (32.9)
I/σ(I)	15.4 (7.1)	15.0 (6.7)
Redundancy	5.9 (5.8)	7.6 (7.5)
Wilson *B*-factor (Å^2)	39.1	56.8
Space group	P 4_1	P 4_1
Unit-cell parameters (*a*/*b*/*c*) (Å)	93.8/93.8/128.6	94.1/94.1/129.4
Refinement statistics		
Resolution range (Å)	30.0-2.30 (2.36-2.30)	30.0-2.51 (2.57-2.51)
R_{cryst} [b] (%) / R_{free} [c] (%)	19.0 (22.5) / 23.3 (30.1)	19.6 (27.7) / 24.7 (29.9)
Protein atoms	6203	6160
Water molecules	287	200
Hetero atoms	178	167
Isotropic average temperature factors (Å^2)		
Overall	33.7	43.8
Protein	33.6	43.9
Water	34.5	44.3
FAD (monomers A)	27.1	32.0
FAD (monomers B)	24.0	32.4
UDP-Gal*p* (monomer A. oxidized)	36.2	
UDP-Gal*p* (monomer B, oxidized)	59.1	
UDP (monomer A, reduced)		37.4
UDP-Gal*p* (monomer B, reduced)		64.6
Estimated coordinate error from Luzzati plot (Å)	0.21	0.27
RMSD from ideal		
Bond length (Å)	0.022	0.019
Bond angles (°)	1.8	1.8
Ramachandran plot (% favored / % allowed) (*6*)	98.4 / 100.0	96.5 / 99.9

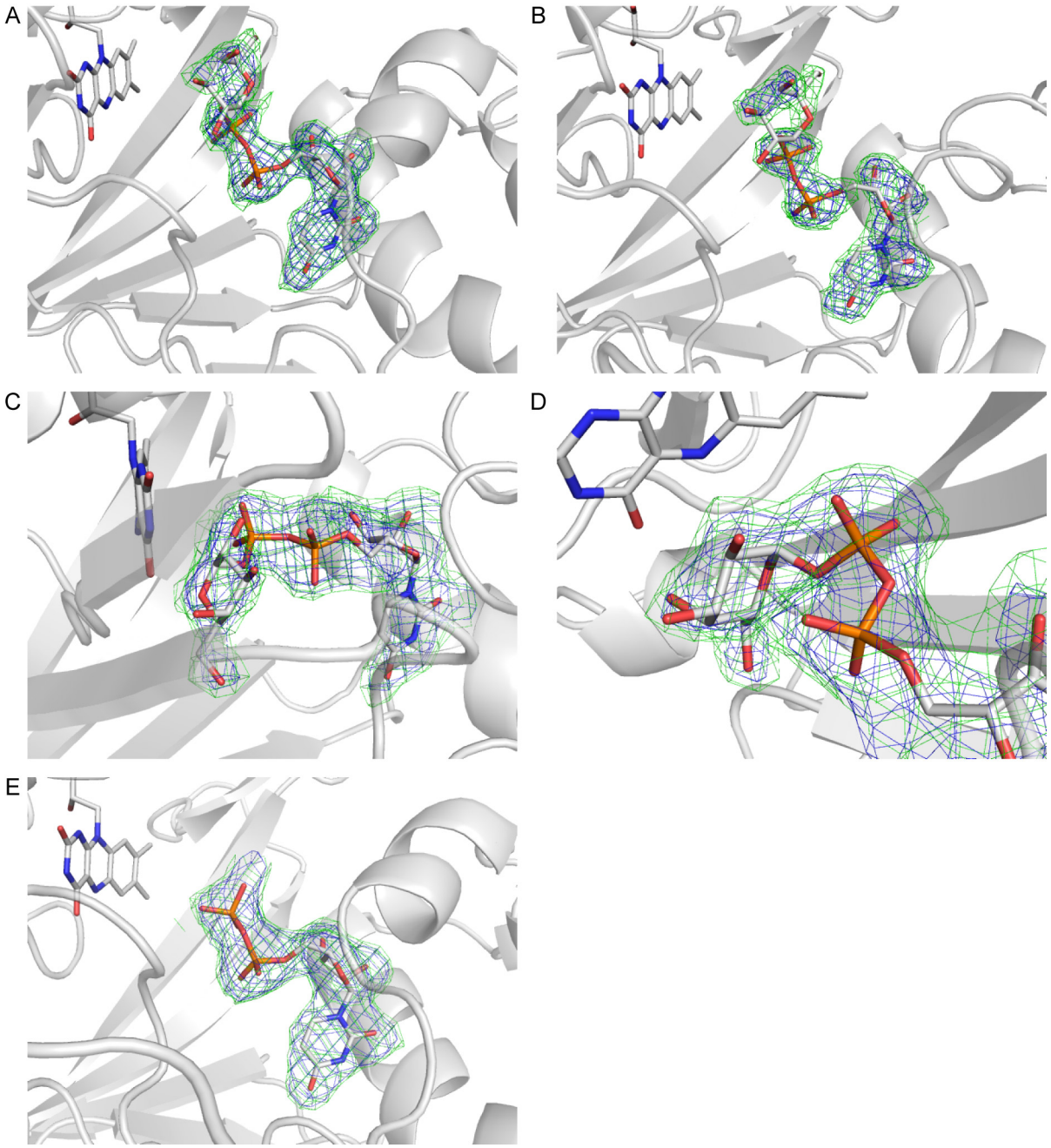
[a] $R_{sym}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl}\sum_i I_i(hkl)$ where $I(i)$ is the intensity of the ith observation of the hkl reflection and $\langle I(hkl) \rangle$ is the mean intensity from multiple measurements of the h,k,l reflection.

[b] $R_{cryst}(F) = \sum_{hkl}|F_{obs}(hkl) - F_{calc}(hkl)| / \sum_{hkl}F_{obs}(hkl)$, where $F_{obs}(hkl)$ and $F_{calc}(hkl)$ are the observed and calculated structure factor amplitudes for the h,k,l reflection.

[c] R_{free} is R_{cryst} calculated for a randomly selected test set of reflections (5%) not included in the refinement.



Supplementary Figure 1. ^{1}H-^{1}H NOESY spectra of flavin and covalent adduct 2 (aromatic region). (A) ^{1}H-^{1}H NOESY spectrum of flavin (reduced). (B) ^{1}H-^{1}H NOESY spectrum of covalent adduct 2. Covalent adduct shows peaks from the interaction of C6H and galactose-derived protons (inset).



Supplementary Figure 2. Omit maps showing F_o-F_c density when UDP-Gal*p* ligand is left out of refinement (map contoured at 3 (blue) or 2 (green) standard deviations above the mean). (A) Monomer A, oxidized, (B) monomer B, oxidized, (C) and (D) two views of monomer B, reduced, (E) monomer A, reduced (only the UDP portion of the ligand is ordered and included in this model).