Published in final edited form as:

Biochemistry. 2018 December 18; 57(50): 6848–6859. doi:10.1021/acs.biochem.8b00497.

Hysteresis and Allostery in Human UDP-Glucose Dehydrogenase Require a Flexible Protein Core.

Nathaniel R. Beattie, Brittany J. Pioso, Andrew M. Sidlo, Nicholas D. Keul, and Zachary A. Wood*

Department of Biochemistry & Molecular Biology, University of Georgia, Athens, GA 30602, USA

Abstract

Human UDP-glucose dehydrogenase (hUGDH) oxidizes UDP-glucose to UDP-glucuronic acid, an essential substrate in the phase II metabolism of drugs. The activity of hUGDH is regulated by the conformation of a buried allosteric switch (T131-loop/ α 6 helix). Substrate binding induces the allosteric switch to slowly isomerize from an inactive E* conformation to the active E state, which can be observed as enzyme hysteresis. When the feedback inhibitor UDP-xylose binds, the allosteric switch and surrounding residues in the protein core repack, converting the hexamer into an inactive, horseshoe-shaped complex (E^Q). This allosteric transition is facilitated by large cavities and declivities in the protein core that provide the space required to accommodate the alternate packing arrangements. Here, we have used the A104L substitution to fill a cavity in the E state and sterically prevent repacking of the core into the E^Q state. Steady state analysis shows that hUGDH_{A104L} binds UDP-xylose with lower affinity and that the inhibition is no longer cooperative. This means that the allosteric transition to the high UDP-xylose affinity E^Q state is blocked by the substitution. The crystal structures of hUGDH_{A104L} show that the allosteric switch still adopts the E and E* states, albeit with a more rigid protein core. However, the progress curves of hUGDH_{A104L} do not show hysteresis, which suggests that the E* and E states are now in rapid equilibrium. Our data suggests that hysteresis in native hUGDH originates from the conformational entropy of the E* state protein core.

Introduction:

UDP-glucuronic acid (UDP-GlcA) is the essential substrate in glucuronidation, a major component of phase II metabolism in mammalian cells.^{1–3} Glucuronosyltransferases catalyze the nucleophilic addition of glucuronic acid to drugs or toxins, which are then rapidly excreted from the body.⁴ Glucuronidation is exploited by some cancers as a mechanism of chemotherapeutic resistance.^{5–8} Thus, controlling glucuronidation is a potential strategy for combatting drug resistance in these cancers. Because there are 28 glucuronosyltransferase isozymes, each with broad substrate specificity, designing specific inhibitors is challenging.⁴ Alternatively, it is possible to inhibit glucuronidation by reducing the availability of the essential substrate UDP-GlcA.^{9–11} UDP-GlcA is produced by UDP-glucose dehydrogenase (UGDH), which uses two molecules of NAD⁺ to oxidize the C6

*For editorial correspondence: Zachary A. Wood, phone: 706-583-0304, FAX: 706-542-1738, zaw@uga.edu.

The atomic coordinates and structure factors have been deposited in the Protein Data Bank. (PDB entries 6C4J and 6C4K)

hydroxyl of UDP-glucose (UDP-Glc).¹² The downstream metabolite UDP-xylose (UDP-Xyl) acts as an allosteric feedback inhibitor to control the activity of hUGDH.^{13, 14} Understanding the allosteric mechanism of UGDH is an important goal in developing strategies to control glucuronidation.

Human UGDH (hUGDH) forms a hexamer, with each chain containing an NAD⁺ binding (NB), dimerization (DD) and a nucleotide-sugar binding (SB) domain (Figure 1A). The activity of hUGDH is controlled by the feedback inhibitor UDP-Xyl, which converts the enzyme into an inactive horseshoe-shaped hexamer (E^Ω) (Figure 1A).^{14, 15} This conformational change is controlled by a buried allosteric switch that links the active site to the α6 helix in the hexamer building interface (Figures 1B, C).^{15, 16} The allosteric switch (T131-loop/α6-helix) can adopt one of three distinct conformations: the active E state, the allosterically inhibited E^Ω, and an inactive E* conformation that represents a structural intermediate between the E and E^Ω states (Figure 1C).^{15–19} In the absence of ligand the enzyme favors the E* state, which resembles the E state with the exception that the NAD⁺ binding domains are rotated into an ‘open’ conformation that exposes the T131-loop and surrounding residues to solvent (Figure 1B).^{18, 20} When UDP-Glc binds, the allosteric switch and surrounding residues in the protein core adopt the active E conformation (Figures 1B, C). The activity of hUGDH is regulated by an atypical allosteric mechanism. Briefly, UDP-Xyl competes with substrate for the active site, and upon binding induces the allosteric switch to adopt the E^Ω conformation (Figures 1A, C).^{15, 18} This remarkable transition occurs because UDP-Xyl lacks the C5 hydroxymethyl of UDP-Glc, which allows the allosteric switch to move ~5 Å into the active site (Figure 1B).¹⁵ What makes this allosteric is that the binding of UDP-Xyl in the active site increases the affinity between subunits in the E^Ω hexamer.²¹ In the inhibited state, the T131-loop occludes the coenzyme binding site and reduces the affinity for UDP-Glc (Figure 1B).^{15, 18} Because the E^Ω and E states have different affinities for UDP-Glc, the allosteric transition can be observed as positive cooperativity in steady state studies of UDP-Xyl inhibition.^{15, 18}

The substrate-induced transition from the inactive E* to the active E hexamer is slow and can be observed as hysteresis in progress curves.^{18, 19, 22} It has been proposed that the source of this slow enzyme isomerization is the repacking of the allosteric switch and surrounding residues into the E state.^{18, 19, 23} This hypothesis is supported by a previous study that showed trapping the allosteric switch in the active conformation abolishes hysteresis.²³ The remarkable plasticity exhibited by the hUGDH protein core likely originates from large cavities and deep surface pockets (declivities) near the allosteric switch (Figure 1D).¹⁵ Here, we have examined how the flexibility of the hUGDH protein core contributes to both hysteresis and allostery. Using a small to large amino acid substitution (A104L) we have partially filled a cavity to stabilize the E state. Our results show that these packing defects are potential targets for regulating hUGDH activity.

Methods:

Cavity Characterization

The program Caver Analysis 1.0²⁴ was used to characterize the packing defects observed in crystal structures of hUGDH. PDB IDs 2Q3E, 4RJT and 3PTZ were used as the models for

the E, E* and E^Ω states, respectively. Each chain of the structures were converted to an individually PDB file prior to analysis which includes; 2Q3E chain A-F, 4RJT chain A-C and 3PTZ chain A-F. The volumes of cavities were quantified using an inner probe radius of 1.2 Å and outer probe radius of 1.9 Å. The inner probe radius was selected to approximate the radius of water. The outer probe radius was selected to maximize volume while preventing the cavity from opening to the surface. Declivities were defined using an inner probe radius of 1.2 Å and an outer probe radius of 2.3 Å. For the chains of 3PTZ, the outer probe radius was varied from 1.9 Å to 2.3 Å to minimize extraneous surface inclusion in the declivity volume calculation. The volumes were calculated after maximizing tessellation by using the “increase precision” option that comes as part of the Caver Analysis 1.0 program. Because the size and locations of the packing defects are nearly identical in all chains, the contributing atoms of each packing defect were identified using chain “A” of each hUGDH structure.

Rosetta VIP redesign

Chain A of hUGDH in the E state (PDB ID 2Q3E) was used as the template for RosettaVIP²⁵ core redesign. Because the initial relaxation step in the RosettaVIP protocol introduced small shifts that reduced the size of the packing defects observed in the native crystal structures, we did not include it in the redesign effort. Packing defects were detected using a probe with radius 1.2 Å. See results for residues targeted in the redesign. The protein structure was subject to iterative rounds of substitution, fixed back bone relaxation, then scoring.

Protein Expression, crystallization, and Structure Solution

Both hUGDH and hUGDH_{A104L} were recombinantly expressed in *Escherichia coli* and purified as previously described.^{15, 18–21, 23} Following purification, TEV protease was used to remove the HIS-tag and another round of purification was conducted to remove the TEV. Proteins were quantified using an Agilent 8453 UV/vis and an ε₂₈₀ of 49850 M⁻¹ cm⁻¹ for hUGDH and hUGDH_{A104L} (calculated using Protparam²⁶). The proteins were dialyzed into a storage buffer [25 mM Tris (pH 8.0) and 50 mM NaCl] and concentrated to ~20 mg/mL using a Millipore Amicon Ultra-15 10K centrifugal filter unit. Aliquots of protein were flash frozen in liquid nitrogen and stored at -80°C. All proteins were crystallized at 20°C using the hanging drop vapor diffusion method, with a 2 µL drop consisting of a 1:1 ratio of protein (10 mg/ml) and the reservoir solution. The E complex of hUGDH_{A104L} (hUGDH_{A104L}:UDP-Glc:NADH) with 3 mM UDP-Glc and 5 mM NADH in the protein solution over a reservoir of 16% polyethylene glycol 3350, 0.2 M ammonium sulfate, 0.3 M NaCl, 1% 1,2 propanediol, 0.1 M Tris (pH 8.6). Unliganded hUGDH_{A104L} (E*) was crystallized at 20°C over a reservoir containing 16% polyethylene glycol 3350, 0.5 M NaCl, and 0.1 M Tris (pH 7.6). Crystals were cryoprotected using the reservoir conditions supplemented with 15% EDG (1:1:1 ratio of ethylene glycol: dimethylsulfoxide: glycerol), then rapidly plunged into liquid nitrogen. All X-ray diffraction data was collected using the SER-CAT beamlines at the Advanced Photon Source (Argonne, IL). For the E complex (hUGDH_{A104L}:UDP-Glc:NADH), a 2.5 Å resolution data set was collected on the 22-ID beamline using a Rayonix MX300HS CCD detector. For the E* structure, a 2.65 Å resolution data set was collected on the 22-BM beamline using a MAR MX300 CCD detector. XDS²⁷ was used for data processing, and 5% of the data were set aside for cross

validation.²⁸ The E complex (hUGDH_{A104L}:UDP-Glc:NADH) was solved by molecular replacement using the PDB entry 2Q3E as a search model in Phaser-MR of the PHENIX²⁹ software package. Because the hUGDH_{A104L} E* crystal was isomorphous to the native E* structure (PDB entry 4RJT), the native R-free flags were preserved. Both structures were subjected to iterative rounds of manual rebuilding in COOT³⁰ followed by automated refinement in PHENIX²⁹. The data collection and refinement statistics are listed in Table 1.

Normalized B-factor analysis

The B-factors of the isomorphous hUGDH_{A104L} and native hUGDH (PDB entry 4RJT) E* crystal structures were set to 20 and then refined until convergence (~7 cycles) without TLS³³ or NCS restraints using PHENIX²⁹. Because the NAD domain (residues 1–212), dimerization domain (residues 213–322) and sugar binding domain (residues 323–466) of each chain behave as separate rigid bodies in the crystal, the B-factor of each C α atom was normalized relative to the other C α atoms within the same domain using the equation³⁴:

$$B_{\text{normalized}} = (B - B_{\text{mean}})/B_{\text{stdev}} \quad \text{eq (1)}$$

where B is the B-factor of the specific C α atom, B_{mean} is the average B-factor of the domain in question, and B_{stdev} is the standard deviation of the C α atom B-factors within the domain. Next, the normalized B-factors for each domain in a crystal were averaged. Because the crystals are isomorphous, the differences in the average normalized B-factors of hUGDH_{A104L} and native hUGDH will only reflect changes in flexibility due to the A104L substitution.

Sedimentation Velocity

hUGDH and hUGDH_{A104L} were dialyzed for 24 hours into 25 mM HEPES (pH 7.5) and 150 mM KCl, and then quantified as above. The samples were then diluted with the dialysis buffer to a final protein concentration of 9 μ M, loaded into 12 mm double-sector Epon centerpieces and equilibrated for 1 h at 20 °C. Sedimentation velocity data were collected at 50,000 rpm at 20 °C in an Optima XLA analytical ultracentrifuge. Data were recorded at 280 nm in radial step sizes of 0.003 cm. SEDNTERP³⁵ was used to estimate the partial specific volume of hUGDH (0.73840 mL/g) and hUGDH_{A104L} (0.73872 mL/g), and the density (1.00726 g/mL) and viscosity (0.01018) of the buffer. SEDFIT³⁶ was used to analyze the raw sedimentation data. Data were modeled as a continuous sedimentation coefficient distribution ($c(s)$), and were fit using the baseline, meniscus, frictional coefficient, and systematic time-invariant and radial-invariant noise. Fit data for both experiments had a root mean square deviations (r.m.s.d) < 0.004 AU.

Progress curve analysis of hysteresis

UDP-glucose and NAD⁺ were purchased from Sigma and UDP-xylose was purchased from Carbosource (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Reactions were monitored at 25°C using an Olis RSM 1000 Spectrophotometer equipped with a stopped-flow assembly and a 0.4 mm path length. Separate Enzyme (460 nM) and substrate (1 mM UDP-Glucose and 10 mM or 5 mM NAD⁺) solutions were made in

reaction buffer (50 mM HEPES (pH 7.5), 50 mM NaCl and 5 mM EDTA) and were equilibrated at 25°C for 5 minutes. We used the 5mM NAD⁺ concentration for hUGDH_{A104L} because the A104L substitution increased the apparent affinity for the coenzyme (Table 3). Equal volumes of enzyme and substrate solutions were then rapidly mixed using the stopped flow. Absorbance readings at 340 nm were taken every 0.1 s for a total of 200 seconds for native hUGDH and 90 seconds for hUGDH_{A104L} to monitor NADH production. Native hUGDH progress curves were fit to Frieden's equation for enzyme hysteresis³⁷:

$$P(t) = v_{ss}t - \tau(v_{ss} - v_i)(1 - e^{-t/\tau}) \quad \text{eq (2)}$$

where P is the concentration of product at time t and τ is equal to $1/k_{obs}$, where k_{obs} is the apparent rate constant for the transition between the initial rate (v_i) and the steady state rate (v_{ss}). For hysteretic enzymes, the initial velocity defined by eq 2 is the pre-steady state rate and does not follow the steady state approximation. The v_{ss} represents the initial steady state velocity and is calculated before 10% of the substrate is converted to product. The length of the lag is calculated as $e\tau$. The hUGDH_{A104L} progress curves did not display hysteresis and the initial steady state velocity was derived from the linear portion of progress curves prior to depleting 10% of the substrate. Data were fit using PRISM (GraphPad Software Inc., San Diego, CA) and all fits were analyzed using residual plots.³⁸

Steady state Kinetics

The assay conditions are as previously described^{15, 18–21, 23}, with the exception that the enzyme dilution buffer was changed to 50 mM HEPES (pH7.0), 50 mM NaCl and 5 mM EDTA. Briefly, for NAD⁺ saturation curves, the assays contained 100 nM enzyme in 50 mM HEPES (pH 7.5), 50 mM NaCl, and 5 mM EDTA with a saturating concentration of 1 mM UDP-Glucose. The same conditions were used for UDP-Glc saturation curves, except that the NAD⁺ concentration was fixed at 5 mM. Because hUGDH displays negative cooperativity with respect to NAD⁺ binding, it is difficult to saturate the low affinity sites with coenzyme (see results).¹⁹ hUGDH_{A104L} does not exhibit negative cooperativity with respect to NAD⁺ binding, and is saturated with 2.5 mM coenzyme. Prior to mixing, enzyme and substrate were incubated separately at 25 °C for 5 minutes. The reactions were initiated by rapidly mixing enzyme and substrate solutions and the progress curves were measured by continuously monitoring the change in 340nm absorbance every 0.5 s using an Agilent 8453 UV/vis spectrometer. NADH concentration was determined at 340nm using a molar absorptivity coefficient of $6220 \text{ M}^{-1} \text{ cm}^{-1}$. Because native hUGDH is hysteretic, the steady state velocities were calculated by fitting progress curves to eq.2 as described above. ^{15, 18–21, 23} hUGDH A104L does not display hysteresis the steady state velocity was determined by fitting the initial liner portion of the progress curves to a straight line. Initial steady state velocities from progress curves were fit to a sigmoidal rate equation and analyzed with residual plots³⁸:

$$v = \frac{k_{cat} [E_t][S]^h}{K_M^h + [S]^h} \quad \text{eq (3)}$$

where h is the Hill coefficient. The negative cooperativity observed in hUGDH NAD⁺ substrate saturation curves was evaluated using Kurganov's analysis as described previously.^{23, 39} Briefly, the data were fit to the equation:

$$\frac{v}{[S]} = \frac{V_{max} - v}{K_m^{eff}}, \text{ where } K_m^{eff} = K_0 + (K_{lim} - K_0)(v/V_{max}) \quad \text{eq (4)}$$

where K_0 is the approximate K_M for the high affinity binding sites and K_{lim} is the average apparent K_M for the low affinity sites.³⁹

Inhibition kinetics

The K_i for the inhibitor UDP-Xyl was determined as previously described.^{15, 18–21, 23} Briefly, UDP-Xyl competes with UDP-Glc for the active site and induces a conformational change in hUGDH to the inhibited E^Ω state. Because the E and E^Ω have different affinities for UDP-Glc and UDP-Xyl, the inhibition studies show cooperativity.¹⁸ To determine the K_i , the substrate saturation curves at different concentrations of UDP-Xyl were fit simultaneously to an equation for competitive inhibition corrected for cooperativity using global analysis in Prism:

$$v_o = \frac{k_{cat} [E_t][S]^h}{(K_M^{app})^h + [S]^h}, \text{ where } K_M^{app} = K_M(1 + \frac{[I]}{K_i}) \quad \text{eq (5)}$$

Results:

Rational design of the A104L cavity filling substitution

The cavities and declivities in the E state protein core are believed to provide space and accommodate the repacking of the allosteric switch and surrounding residues into the E^Ω conformation (Figure 1D). Our goal was to design a cavity filling amino acid substitution that would stabilize the E state and prevent the repacking of the protein core to the E^Ω state. We chose cavity C-I ($109.9 \pm 6.0 \text{ \AA}^3$) because it includes the $C\beta$ atom of A104, which is a good target for small-to-large amino acid substitutions (Figure 2A and Table S1). In fact, A104 is conserved as a phenylalanine in the non-allosteric UGDH from *Streptococcus pyogenes*, which does not have a cavity corresponding to C-I.¹⁵ The C-I cavity in hUGDH will not accommodate an A104F substitution without significant changes in the protein core. In fact, we are not able to express soluble hUGDH with the A104F substitution. This is most likely due to the low sequence identity of 23% between *Sp*UGDH and hUGDH, which has resulted in numerous changes in the protein core packing.¹⁵ As an alternative, we used

Rosetta_{VP} to predict cavity-filling amino acid substitutions that would preserve the integrity of the protein core. The following cavity C-I residues were included in the automated redesign routine of Rosetta_{VP}: T91, T93, R102, A103, A104, L106, V132, K279, D280 and N283. Cavity residues P92 and P133 were excluded because prolines reduce the configurational entropy of the peptide backbone, and are often important for protein folding and stability.⁴⁰ Additionally, P133 is conserved in non-allosteric UGDH from *Streptococcus pyogenes*, which suggests that it may be significant to the structure or function of the enzyme.¹⁵ T131 was also excluded from the redesign since it contributes to the catalytic mechanism by coordinating a nucleophilic water.⁴¹ Rosetta_{VP} identified A104L as the optimal cavity filling substitution; no other substitution was identified in multiple (<10) stochastic design simulations. The predicted A104L substitution fills approximately 61% of cavity C-I and will favor the E state by introducing steric clashes into the core of the E^Ω state (Figures 2B, C).

We also examined the A104L model for unsatisfied polar groups contributing to cavity C-I. The native cavity C-I contains three ordered water molecules that form several hydrogen bonds with polar groups in the protein core (Figure 2A). The first water (W1) forms hydrogen bonds with the carbonyl oxygens of R102 and K279, as well as the O γ of T93 and the second water molecule (W2) (Figure 2A). Water (W2) also forms hydrogen bonds with the carbonyl oxygen of T91 and the third water molecule (W3), which also accepts a hydrogen from the N δ atom of N283 (Figure 2A). The Rosetta_{VP} A104L model conserves a small cavity that can accommodate water W1 and its hydrogen bonds; however, the leucine substitution will displace W2 and W3 (Figures 2A, B). In the absence of W2 and W3, the carbonyl oxygen of T91 can still be satisfied by the N ζ of K279, and the carboxamide of N283 can be exposed to solvent by a small rotation about the χ_1 torsion angle.

The hUGDH_{A104L} crystal structure reveals a reduced cavity C-1 volume.

hUGDH with the A104L substitution (hUGDH_{A104L}) was co-crystallized with UDP-Glc and NADH to obtain the E state conformation (hUGDH_{A104L}:UDP-Glc:NADH). The structure was solved to a resolution of 2.53 Å and revealed two 32 symmetry hexamers in the asymmetric unit (Table 1). All residues are modeled with the exception of loop_{385–387} and the C-terminus (residues 467–494), which are disordered in all 12 chains. Residues 23–30, 34–54 and 69–72 in the NB domain of chain A were poorly ordered and not modeled. Each monomer contains unambiguous electron density for the bound NADH and UDP-Glc (not shown). In all chains the allosteric switch and hexamer building interface are in the E state. The largest structural difference between the chains involves the rotation of the NAD⁺ domain; chains G and J adopt the closed conformation, while the remaining ten chains have NAD⁺ domains that are rotated between 6.8° to 10.6° toward the open confirmation. In chains G and J, the allosteric switch and hexamer building interface are in the E state (Figure S1). Because the open domain conformation exposes the C-1 cavity to solvent, we will limit our analysis of the A104L induced changes in the cavity volume to the closed domain conformations in chains G and J.

The crystal structure of hUGDH_{A104L} agrees well with the Rosetta_{VP} model (Figure 2B). The A104L substitution has split cavity C-I into two, smaller cavities with volumes of

20.7±2.2 Å³ and 33.3±2.9 Å³ (Figure 2B). The smaller cavity contains weak electron density for a water corresponding to W1 in chains B, C, D, E, F, K and L. The electron density in the remaining chains is weak and did not support modeling the water. Because the absence of water W1 would leave unsatisfied polar groups, it is likely that there is a poorly ordered water in the remaining cavities (Figure 2B).^{42, 43} As expected, the L104 sidechain has displaced waters W2 and W3 (Figure 2B). As a result, the Ce and N ζ atoms of K293 are disordered, which exposes the carbonyl oxygen of T91 to solvent. To compensate for the loss of the hydrogen bond with W3, the χ_2 torsion angle of N283 has rotated from $-10.3\pm6^\circ$ to the more favorable rotamer $49.4\pm0.5^\circ$, which exposes the carboxamide to solvent. The only significant difference between the hUGDH_{A104L} structure and the Rosetta_{VIIP} model involves the χ_2 torsion angle of N283, which is rotated 180° to flip the amide and carbonyl oxygen of the carboxamide group. Because it is not possible to assign the absolute conformation of the carboxamide group in the absence of known hydrogen bond acceptors or donors at this resolution, we chose to model the χ_2 rotamer in the conformation originally reported in the 2Q3E crystal structure.¹⁶

To see if the A104L substitution destabilized the hexamer, we examined the oligomeric structure of native hUGDH and hUGDH_{A104L} using sedimentation velocity. Consistent with previous reports^{15, 18, 20, 21, 23}, the sedimentation of 9μM native hUGDH reveals a 11.4 S hexamer (78.0%), an 8.2 S tetramer (3.8%), a 5.5 S dimer (13.3%), and a 3.1 S peak that likely represents a monomer (4.9%) (Figure 2D). Similarly, the *c(s)* distribution of 9μM hUGDH_{A104L} consists of the 11.5 S hexamer (83.5%), a small amount of tetramer (<1%), 5.5 S dimer (9.6%) and the 3.0 S monomer (6.9%) (Figure 2D). The *c(s)* distribution of native hUGDH is in rapid equilibrium, with the tetramer representing a transient between the more stable hexamer and dimer states^{18, 21, 23, 44}. Because of this, it was difficult to assign an accurate S value for the tetramer in the hUGDH_{A104L} distribution.

hUGDH_{A104L} adopts the E* state.

hUGDH_{A104L} was also crystallized under conditions known to stabilize the native E* state.¹⁸ The resulting crystals diffract to a resolution of 2.65Å and are isomorphous with the native E* structure (PDB entry 4RJT) (Table 1). Briefly, the asymmetric unit contains 3 chains, with the complete hexamer being formed by crystallographic symmetry. All residues are modeled except for a surface loop (residues 385–387) and the C-terminus (residues 467–494), which are disordered in every chain. Additionally, there was incomplete main chain electron density for residue 72 in chain A as well as residues 13, 14, 49 and 154 in chain C, so they were not modeled. As in the native E* crystal structure¹⁸, the NB domains of E* hUGDH_{A104L} are rotated to the open conformation. The only significant conformational change in the hexamer involves the NB domain of hUGDH_{A104L} chain C, which is rotated ~0.9° less open than the corresponding chain in the native E* structure. The allosteric switch and hexamer-building interface of hUGDH_{A104L} are also in the E* conformation (Figure 3A). In native hUGDH, four residues in the protein core adopt χ_1 torsion angles that are unique to the E* state (Figures 3B, C). Residues S88 and E161 adopt unique χ_1 angles in the open domain E* state that would introduce steric clashes if the domain rotated to the closed conformation (Figures 3B, C). Residue E128 changes conformation with the allosteric switch and adopts a unique rotamer conformation in the E, E* and E^Ω states

(Figure 3C). Finally, S120 adopts a χ_1 rotamer angle of $67\pm 1^\circ$ which is only observed in the E* conformation. S120 is located at the C-terminus of the $\alpha 5$ helix, which is adjacent to the allosteric $\alpha 6$ helix in the hexamer building interface (Figures 3B, C). It is likely that the change in the S120 χ_1 rotamer is correlated with the movement of the $\alpha 6$ helix. In both χ_1 rotamers, the O γ of S120 caps the carboxy terminus of the $\alpha 5$ helix by contributing a hydrogen bond to the carbonyl oxygen of I116. In the E* conformation of hUGDH_{A104L}, the χ_1 rotamers of S88, E128 and E161 are conserved. However, the O γ of S120 is disordered and could not be modelled unambiguously.

The open domain conformation of E* exposes the allosteric switch and surrounding residues to solvent, which relaxes the steric packing constraints that would otherwise resist the transition between the E and E $^\Omega$ states.²⁰ A comparison of the hUGDH_{A104L} and native E* normalized B-factors shows that the A104L substitution decreases the flexibility of several residues in cavity C-1 (Figure 4A). These changes originate from the A104L substitution, which packs against the allosteric switch residues V132 and P133, and the carboxamide of N283 (Figures 4B, C). These stabilizing interactions are propagated to the surrounding second-shell residues V285, Y286 and L287, which become significantly more rigid (Figures 4A, B). In contrast, the residues that are unique to cavity C-2 and the declivities D-1 and D-2 show no significant change in flexibility (Figure 4A).

hUGDH_{A104L} does not display hysteresis or negative cooperativity.

Unlike native hUGDH, the progress curves of hUGDH_{A104L} do not exhibit hysteresis (Figures 5A, B and Table 2). In fact, we observe no lag in the progress curves of hUGDH_{A104L} even in the presence of UDP-Xyl, which is known^{18, 19} to increase the lag in native hUGDH progress curves (Table 2). Next, we examined the impact of the A104L substitution on steady state kinetics. The NAD⁺ saturation curves of hUGDH_{A104L} are hyperbolic (Hill=1), with a K_M of 48 ± 3 μ M (Figure 5C, Table 3). In contrast, the NAD⁺ saturation curves of native hUGDH are known to display negative cooperativity^{18, 19, 23} (Figure 5D Table 3). Earlier work showed that UDP-Glc binding induces an asymmetry that results in a mixture of high and low affinity NAD⁺ binding sites.¹⁹ Using Kurganov's analysis³⁹ (eq 4), the high (K_o) and low (K_{lim}) NAD⁺ binding affinities for native hUGDH can be estimated (157 ± 13 μ M and 2300 ± 500 μ M, respectively) (Figure 5E Table 3). Both native hUGDH and hUGDH_{A104L} have similar a k_{cat} with respect to NAD⁺ (0.94 ± 0.07 s⁻¹ and 0.79 ± 0.01 s⁻¹, respectively). The UDP-Glc saturation curves for both hUGDH_{A104L} and native hUGDH are hyperbolic, with similar values for K_M (9.4 ± 0.5 μ M and 7.6 ± 0.6 μ M, respectively) (Table 3). The k_{cat} values observed in the UDP-Glc and NAD⁺ saturation curves of hUGDH_{A104L} are also similar (0.82 ± 0.01 s⁻¹ and 0.79 ± 0.01 s⁻¹, respectively) (Table 3). The difference between the k_{cat} values measured in the UDP-Glc and NAD⁺ saturation curves of native hUGDH (0.59 ± 0.01 s⁻¹ and 0.94 ± 0.07 s⁻¹, respectively) is a consequence of the negative cooperativity in NAD⁺ binding, which makes it difficult to saturate hUGDH with coenzyme during the pseudo-first order steady-state analysis.¹⁵

hUGDH_{A104L} does not form the E $^\Omega$ state

Because the A104L substitution will introduce steric clashes in the E $^\Omega$ state, hUGDH_{A104L} should favor the E and E* states. Attempts to crystallize hUGDH_{A104L} in the E $^\Omega$ state using

both of the previously published conditions¹⁵ were not successful. However, the allosteric transition from E^{Ω} to the E conformation can be observed in steady state inhibition studies of native hUGDH^{18, 23}. Briefly, UDP-Xyl binding favors the E^{Ω} state, which has a low affinity for UDP-Glc. As UDP-Glc outcompetes UDP-Xyl, the allosteric transition to the high UDP-Glc affinity E state can be observed as substrate cooperativity (Figures 5F, G and Table 4). As previously shown¹⁸, the degree of cooperativity increases with inhibitor concentration (Figures 5F, G, and Table 4). Global analysis of hUGDH_{A104L} steady state inhibition curves shows that the UDP-Xyl affinity is 13-fold lower ($K_i = 2.3 \pm 0.2 \mu\text{M}$) than native hUGDH ($K_i = 0.18 \pm 0.01 \mu\text{M}$) (Table 4). In addition, none of the steady state inhibition curves for hUGDH_{A104L} show evidence of substrate cooperativity (Figure 5H). Even at saturating UDP-Xyl concentration, 15-fold higher than the K_i , the substrate saturation curves of hUGDH_{A104L} are hyperbolic (Figure 5I). This suggests that hUGDH_{A104L} does not transition to the low substrate affinity E^{Ω} state upon inhibitor binding.

The A104G substitution has no significant effect on hysteresis or allostery.

The A104L substitution reduces the volume of cavity C-I and produces a more rigid protein core (Figures 2 and 4). We also investigated the effect of increasing the cavity volume and core flexibility using the large-to-small A104G substitution. The hUGDH_{A104G} construct expresses at a much lower level relative to the native enzyme (~2.5mg/L versus ~20mg/L, respectively), which suggests that the substitution has negatively impacted folding. A decrease in stability may also explain the higher UDP-Glc K_M observed in hUGDH_{A104G} (17.2 ± 1.1 compared to 7.4 ± 0.6 in native) (Table 4). Global analysis of UDP-Xyl inhibition shows that the UDP-Xyl affinity (K_i) of hUGDH_{A104G} and substrate cooperativity are similar to that of the native enzyme (Figure 5J and Table 4). Finally, the A104G substitution does not appear to have a significant effect on enzyme hysteresis; the progress curves of hUGDH_{A104G} display lags that are similar to that observed in native hUGDH (Figure 5K and Table 2).

Discussion:

The allosteric inhibition mechanism in hUGDH involves the ligand-induced isomerization of the T131-loop from the E or E^* states to the E^{Ω} conformation (Figures 1A, B and C).^{15, 18, 20} In the absence of ligand, the NB domain is rotated open to expose the allosteric switch and surrounding core residues to solvent (Figure 6A (i)). This open conformation relaxes the core packing constraints, which allows the allosteric switch and some core residues to adopt the inactive E^* state (Figures 3 A, C). The binding of UDP-Glc and NAD^+ triggers the allosteric switch and core residues to adopt the active E state conformation, which allows the domains to rotate closed (Figure 6A (ii) and (iii)). In contrast, UDP-Xyl binding induces the allosteric switch and the protein core to repack into the E^{Ω} state (Figure 6A, (iv) and (v)). This repacking is facilitated by the remarkable plasticity of the protein core, which originates from the large cavities and declivities surrounding the T131-loop (Figures 1D, 6A)¹⁵. In previous work²³, we tested the hypothesis that the repacking of the allosteric switch is the source of hysteresis using the A136M substitution (hUGDH_{A136M}). We showed that A136M traps the allosteric switch in the active E state, and abolished ligand-induced hysteresis (Figure 6B (i $\rightarrow$ ii)). However, it was not clear if the hysteresis

was a feature of the inactive E* state, the inhibited E^Ω complex or the repacking of the protein core.

Here, we have examined the contribution of protein core flexibility to both allostery and hysteresis by filling cavity C-I with the A104L substitution (Figures 1D and 2A). This cavity is only present in the E state, and our modelling studies showed that the A104L substitution would sterically prevent the repacking of the protein core into the E^Ω conformation (Figures 2C and 6C). We tested this using steady state inhibition kinetics; the allosteric transition in native hUGDH can be observed as substrate induced cooperativity in the presence of saturating UDP-Xyl¹⁸ (Figures 5F, G and Table 4). Thus, the absence of cooperativity in UDP-Xyl inhibition studies is strong evidence that the A104L substitution prevents the formation of the E^Ω state (Figures 5H, I and Table 4). This interpretation is also consistent with the reduced UDP-Xyl affinity of hUGDH_{A104L}, which has been shown²³ to be a property of a constitutive E state hUGDH_{A136M} (Table 4).

The progress curves of hUGDH_{A104L} do not display hysteresis, despite the fact that the crystal structures show that the enzyme can adopt both the E and E* states (Figures 3A, 5A and 6C (i)). This is the first evidence that the source of hysteresis in hUGDH is not simply the movement of the allosteric switch from the E* to the E conformation.^{15, 19, 21} Our data suggests that the conformational entropy of the native hUGDH protein core is an important factor in enzyme hysteresis.¹⁹ In this model, the large packing defects allow residues in the protein core to sample nonproductive conformations as they converge to the correct E state rotamers. By filling cavity C-I with the A104L substitution, we sterically constrain the conformational freedom of the core, which should increase the rate at which the core residues converge to the E state conformation (Figure 6C(i → ii)). This interpretation is supported by our normalized B-factor analysis of the native and hUGDH_{A104L} crystal structures, which shows hUGDH_{A104L} has a more rigid protein core surrounding the substitution (Figure 4A). To see if increasing the flexibility of the protein core would increase hysteresis, we introduced the A104G substitution. hUGDH_{A104G} displays a rate of hysteresis and an inhibitor affinity that is similar to the native enzyme (Table 2 and Table 4). This suggests that the main chain flexibility of A104 does not contribute to hysteresis. It is also likely that small increase in cavity C1 volume due to the A104G substitution does not significantly increase the conformational entropy of the protein core residues. Taken together, our data shows for the first time that the packing defects in the core of hUGDH play a significant role in both the allosteric and hysteretic mechanisms of hUGDH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank Phillip G. Gross for helpful discussions. We thank the staff at the Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, which is supported in part by NIH (S10 RR25528) and S10 RR028976). We also acknowledge NIH/NIGMS for an equipment grant supporting our in-house X-ray facility (S10 OD021762).

† Funding from NIGMS (grant R01GM114298) to Z.A.W. is gratefully acknowledged.

References:

- [1]. Lin JH, and Lu AYH (1997) Role of pharmacokinetics and metabolism in drug discovery and development, *Pharmacological Reviews* 49, 403–449. [PubMed: 9443165]
- [2]. Tukey RH, and Strassburg CP (2000) Human UDP-glucuronosyltransferases: Metabolism, expression, and disease, *Annual Review of Pharmacology and Toxicology* 40, 581–616.
- [3]. Guillemette C (2003) Pharmacogenomics of human UDP-glucuronosyltransferase enzymes, *Pharmacogenomics Journal* 3, 136–158. [PubMed: 12815363]
- [4]. Ritter JK (2000) Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions, *Chem Biol Interact* 129, 171–193. [PubMed: 11154740]
- [5]. Cummings J, Boyd G, Ethell BT, Macpherson JS, Burchell B, Smyth JF, and Jodrell DI (2002) Enhanced clearance of topoisomerase I inhibitors from human colon cancer cells by glucuronidation, *Biochemical Pharmacology* 63, 607–613. [PubMed: 11992628]
- [6]. Cummings J, Ethell BT, Jardine L, Boyd G, Macpherson JS, Burchell B, Smyth JF, and Jodrell DI (2003) Glucuronidation as a mechanism of intrinsic drug resistance in human colon cancer: Reversal of resistance by food additives, *Cancer Research* 63, 8443–8450. [PubMed: 14679008]
- [7]. de Almagro MC, Selga E, Thibaut R, Porte C, Noe V, and Ciudad CJ (2011) UDP-glucuronosyltransferase 1A6 overexpression in breast cancer cells resistant to methotrexate, *Biochemical Pharmacology* 81, 60–70. [PubMed: 20854796]
- [8]. Oguri T, Takahashi T, Miyazaki M, Isobe T, Kohn N, Mackenzie PI, and Fujiwara Y (2004) UGT1A10 is responsible for SN-38 glucuronidation and its expression in human lung cancers, *Anticancer Research* 24, 2893–2896. [PubMed: 15517893]
- [9]. Kakizaki I, Kojima K, Takagaki K, Endo M, Kannagi R, Ito M, Maruo Y, Sato H, Yasuda T, Mita S, Kimata K, and Itano N (2004) A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone, *J Biol Chem* 279, 33281–33289. [PubMed: 15190064]
- [10]. Kultti A, Pasonen-Seppanen S, Jauhiainen M, Rilla KJ, Karna R, Pyoria E, Tammi RH, and Tammi MI (2009) 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3, *Exp Cell Res* 315, 1914–1923. [PubMed: 19285976]
- [11]. Wei Q, Galbenus R, Raza A, Cerny RL, and Simpson MA (2009) Androgen-stimulated UDP-glucose dehydrogenase expression limits prostate androgen availability without impacting hyaluronan levels, *Cancer Res* 69, 2332–2339. [PubMed: 19244115]
- [12]. Kalckar HM, Maxwell ES, and Strominger JL (1956) Some properties of uridine diphosphoglucose dehydrogenase, *Arch Biochem Biophys* 65, 2–10. [PubMed: 13373402]
- [13]. Bakker H, Oka T, Ashikov A, Yadav A, Berger M, Rana NA, Bai X, Jigami Y, Haltiwanger RS, Esko JD, and Gerardy-Schahn R (2009) Functional UDP-xylose transport across the endoplasmic reticulum/Golgi membrane in a Chinese hamster ovary cell mutant defective in UDP-xylose Synthase, *J Biol Chem* 284, 2576–2583. [PubMed: 19028698]
- [14]. Neufeld EF, and Hall CW (1965) Inhibition of Udp-D-Glucose Dehydrogenase by Udp-D-Xylose: A Possible Regulatory Mechanism, *Biochem Biophys Res Commun* 19, 456–461. [PubMed: 14338992]
- [15]. Kadirvelraj R, Sennett NC, Polizzi SJ, Weitzel S, and Wood ZA (2011) Role of Packing Defects in the Evolution of Allostery and Induced Fit in Human UDP-Glucose Dehydrogenase, *Biochemistry* 50, 5780–5789. [PubMed: 21595445]
- [16]. Egger S, Chaikuad A, Kavanagh KL, Oppermann U, and Nidetzky B (2010) UDP-glucose dehydrogenase: structure and function of a potential drug target, *Biochemical Society Transactions* 38, 1378–1385. [PubMed: 20863317]
- [17]. Egger S, Chaikuad A, Kavanagh KL, Oppermann U, and Nidetzky B (2011) Structure and Mechanism of Human UDP-glucose 6-Dehydrogenase, *Journal of Biological Chemistry* 286, 23877–23887. [PubMed: 21502315]
- [18]. Kadirvelraj R, Custer GS, Keul ND, Sennett NC, Sidlo AM, Walsh RM, and Wood ZA (2014) Hysteresis in Human UDP-Glucose Dehydrogenase Is Due to a Restrained Hexameric Structure That Favors Feedback Inhibition, *Biochemistry* 53, 8043–8051. [PubMed: 25478983]

- [19]. Kadirvelraj R, Sennett NC, Custer GS, Phillips RS, and Wood ZA (2013) Hysteresis and Negative Cooperativity in Human UDP-Glucose Dehydrogenase, *Biochemistry* 52, 1456–1465. [PubMed: 23363239]
- [20]. Sennett NC, Kadirvelraj R, and Wood ZA (2011) Conformational Flexibility in the Allosteric Regulation of Human UDP-alpha-D-Glucose 6-Dehydrogenase, *Biochemistry* 50, 9651–9663. [PubMed: 21961565]
- [21]. Sennett NC, Kadirvelraj R, and Wood ZA (2012) Cofactor Binding Triggers a Molecular Switch To Allosterically Activate Human UDP-alpha-D-glucose 6-Dehydrogenase, *Biochemistry* 51, 9364–9374. [PubMed: 23106432]
- [22]. Dickinson FM (1988) Studies on the Unusual Behavior of Bovine Liver UDP-Glucose Dehydrogenase in Assays at Acid and Neutral pH and on the Presence of Tightly Bound Nucleotide Material in Purified Preparations of this Enzyme, *Biochemical Journal* 255, 775–780. [PubMed: 3214424]
- [23]. Beattie NR, Keul ND, Sidlo AM, and Wood ZA (2017) Allostery and Hysteresis Are Coupled in Human UDP-Glucose Dehydrogenase, *Biochemistry* 56, 202–211. [PubMed: 27966912]
- [24]. Kozlikova B, Sebestova E, Sustr V, Brezovsky J, Strnad O, Daniel L, Bednar D, Pavelka A, Manak M, Bezdeka M, Benes P, Kotry M, Gora A, Damborsky J, and Sochor J (2014) CAVER Analyst 1.0: graphic tool for interactive visualization and analysis of tunnels and channels in protein structures, *Bioinformatics* 30, 2684–2685. [PubMed: 24876375]
- [25]. Borgo B, and Havranek JJ (2012) Automated selection of stabilizing mutations in designed and natural proteins, *Proc Natl Acad Sci U S A* 109, 1494–1499. [PubMed: 22307603]
- [26]. Gasteiger E, H. C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein Identification and Analysis Tools on the ExPASy Server, *The Proteomics Protocols Handbook*, pp. 571–607
- [27]. Kabsch W (2010) XDS, *Acta Crystallographica Section D-Biological Crystallography* 66, 125–132.
- [28]. Brunger AT (1997) Free R value: Cross-validation in crystallography, In *Macromolecular Crystallography*, Pt B (Carter CW and Sweet RM Eds.), pp 366–396.
- [29]. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, and Zwart PH (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution, *Acta Crystallographica Section D-Biological Crystallography* 66, 213–221.
- [30]. Emsley P, Lohkamp B, Scott WG, and Cowtan K (2010) Features and development of Coot, *Acta Crystallographica Section D-Biological Crystallography* 66, 486–501.
- [31]. Karplus PA, and Diederichs K (2012) Linking Crystallographic Model and Data Quality, *Science* 336, 1030–1033. [PubMed: 22628654]
- [32]. Diederichs K, and Karplus PA (1997) Improved R-factors for diffraction data analysis in macromolecular crystallography, *Nature Structural Biology* 4, 269–275. [PubMed: 9095194]
- [33]. Hayward S, and Berendsen HJC (1998) Systematic analysis of domain motions in proteins from conformational change: New results on citrate synthase and T4 lysozyme, *Proteins-Structure Function and Genetics* 30, 144–154.
- [34]. Carugo O, and Argos P (1997) Correlation between side chain mobility and conformation in protein structures, *Protein Eng* 10, 777–787. [PubMed: 9342144]
- [35]. Laue TM, Shah BD, Ridgeway TM, and Pelletier SL (1992) Analytical Ultracentrifugation in Biochemistry and Polymer Science, *R. Soc. Chem*, 90–125.
- [36]. Schuck P (2003) On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation, *Analytical Biochemistry* 320, 104–124. [PubMed: 12895474]
- [37]. Frieden C (1970) Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept, *J Biol Chem* 245, 5788–5799. [PubMed: 5472372]
- [38]. Cornish-Bowden A (2001) Detection of errors of interpretation in experiments in enzyme kinetics, *Methods* 24, 181–190. [PubMed: 11384193]
- [39]. Kurganov BI (2000) Analysis of negative cooperativity for glutamate dehydrogenase, *Biophysical Chemistry* 87, 185–199. [PubMed: 11099181]

- [40]. Matthews BW, Nicholson H, and Becktel WJ (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding, *Proc Natl Acad Sci U S A* 84, 6663–6667. [PubMed: 3477797]
- [41]. Campbell RE, Mosimann SC, van De Rijn I, Tanner ME, and Strynadka NC (2000) The first structure of UDP-glucose dehydrogenase reveals the catalytic residues necessary for the two-fold oxidation, *Biochemistry* 39, 7012–7023. [PubMed: 10841783]
- [42]. Levitt M, and Park BH (1993) Water: now you see it, now you don't, *Structure* 1, 223–226. [PubMed: 8081736]
- [43]. Matthews BW, and Liu L (2009) A review about nothing: are apolar cavities in proteins really empty?, *Protein Sci* 18, 494–502. [PubMed: 19241368]
- [44]. Zhao H, Balbo A, Brown PH, and Schuck P (2011) The boundary structure in the analysis of reversibly interacting systems by sedimentation velocity, *Methods* 54, 16–30. [PubMed: 21315155]

Figure 1:

Allostery and hysteresis in hUGDH. **(A)** The active E and allosterically inhibited E^Ω states of hUGDH. The allosteric switch (red), the NB (yellow), the dimerization domain (gray) and the SB domain (purple) are identified. The α6 of the allosteric switch helix (red) in the hexamer building interface is identified with black circles. **(B)** Cartoon depicting the rotation of the NB domain (curved arrow) about the hinge bending axis (magenta rod) from the open (grey) to the closed (cyan) state. The allosteric switch (red) and UDP-Glc (spheres) in the active site are shown. The inset box shows a close up of the active site and depicts the van der Waals clash (dots) that would occur between T131 of the allosteric switch in the E^Ω state (red) and the C5 hydroxymethyl of UDP-Glc (green). UDP-Xyl lacks the C5 hydroxymethyl. **(C)** The left hand panel depicts the E (green), E* (blue) and E^Ω (pink) conformations of the T131-loop. The right hand panel shows the corresponding conformations of the α6 helix and the F323 of the adjacent subunit in the hexamer building interface. The arrows depict the directions that the F323 in the E* state (blue) moves to adopt the E (green) or E^Ω states (pink). **(D)** Stereoviews of the allosteric switch in the E (top panel green sticks) and E^Ω (bottom panel, pink sticks) states. Cavities (blue surfaces) and declivities (yellow surface) are identified. Surfaces were calculated using a 1.2 Å probe. The residues lining each cavity are listed in Table S1

Figure 2:

Rosetta_{VIP} design of the A10L cavity filling substitution in the E state. **(A)** A stereo view of the residues that line the E state cavity C-I (cyan surface) and share hydrogen bonds (yellow dashes) with three buried waters (red spheres). A104L and the allosteric switch residues are colored purple and green, respectively. **(B)** Residues that line cavity C-I in the native hUGDH structure superimposed onto the Rosetta_{VIP} model and the hUGDH_{A104L} crystal structure. The native A104 is colored purple (native) and all other residues are grey. The A104L substitution and allosteric switch is colored slate (Rosetta_{VIP} model), orange (hUGDH_{A104L}), or green (native hUGDH). The residual surfaces (cyan) of cavity C-I in hUGDH_{A104L} and a single buried water molecule (grey sphere) are also shown. Surfaces were calculated with a 1.2Å probe. The van der Waals radii of the hUGDH_{A104L} L104 and the N283 carboxamide are depicted as yellow and pink dots, respectively. **(C)** Modeling L104 into the E protein core shows the substitution will introduce steric clashes in all

rotameric states. In the E^Q state (PDB ID 3PTZ), all preferred rotamers of L104 (purple) introduce steric clashes (red dashes). Even strained rotamers are sterically excluded (not shown). The E^Q state of the allosteric switch is shown in pink. **(D)** The $c(s)$ distribution of hUGDH_{A104L} (red) and native hUGDH (black) as determined by sedimentation velocity analysis. The M, D, T and H correspond to the predicted S value of monomer, dimer, tetramer and hexamer, respectively.

Figure 3:

The E* state of hUGDH_{A104L}. (A) The E* state of the hUGDH_{A104L} allosteric switch (yellow) superimposes onto the native E (green) and E* (blue) conformation. (B) A stereo diagram of the C α traces of the hUGDH protein core surrounding the allosteric switch in the E (green) E* (blue) and E^Q (pink) states. Residue T131 is shown as sticks, as are residues that adopt rotamers unique to the E* state. The figure was created by superimposing residues 1–124. (C) Residues that adopt rotamers unique to the E* state. The column labeled hUGDH shows representative rotamers from the E (green), E* (blue) and E^Q (pink) states as sticks with the corresponding χ 1 angles observed in all chains in the native structures represented in a Newman projection. The last two columns labeled hUGDH_{A104L} show the E (orange) and E* (yellow) conformations superimposed onto the corresponding native conformation.

Figure 4:

The A104L substitution decreases the flexibility of cavity C-1 and surrounding residues. **(A)** A histogram of the normalized B-factors (eq. 1) for the C α atoms of residues lining the packing defects (C-I, C-II, D-I, and D-II) native hUGDH (black) and hUGDH_{A104L} (red) structures. The 2nd shell identifies residues that are adjacent to the cavity C-1 which show a significant decrease in flexibility. Residues that are shared between cavity C-1 and the other packing defects are identified in the C-1 plot with green symbols (stars, circles and squares for C-II, D-I and D-II, respectively). **(B)** Stereo view of the A104L (magenta), allosteric switch (yellow), remaining cavity C-I residues (grey) and the second shell residues (blue). **(C)** The left panel depicts new van der Waals contacts between A104L (magenta) and the E* allosteric switch (yellow). The right panel is the same view with the native A104 (magenta) and the native E* allosteric switch (blue).

Figure 5:

Kinetic analysis of native and hUGDH_{A104L} (A) Stopped flow absorbance spectroscopy of an hUGDH_{A104L} progress curve with saturating substrate fit to a straight line (yellow). (B) Same as in (A) but of native hUGDH fit (yellow line) to Frieden's equation for hysteresis (eq.2) (C) An NAD⁺ substrate saturation curve with hUGDH_{A104L} fit to eq. 3. Rates are normalized by enzyme concentration to give turnover and dashed lines represent the 95% confidence interval of the fit in all panels. (D) An NAD⁺ substrate saturation curve with native hUGDH as in (c). For native hUGDH, the initial steady state velocities are obtained by fitting progress curves to Frieden's equation for hysteresis (eq.2), as described in methods. (E) An Eadie-Hofstee plot of the hUGDH NAD⁺ saturation data which is concave up (negative cooperativity), and was fit to eq. 4 to determine the K_0 and K_{lim} values in Table 4 (described in methods). (F) hUGDH UDP-glucose saturation curves with 0 μ M (black circles), 0.6 μ M (blue squares) and 1.2 μ M (red triangles) UDP-Xyl were globally fit to eq. 5. (G) hUGDH UDP-Glc saturation curves with 2.7 μ M UDP-Xyl fit to eq. 3. (H)

hUGDH_{A104L} UDP-Glc saturation curves with 0 μ M (black circles), 2.2 μ M (blue squares) and 4.3 μ M (red triangles) UDP-Xyl were globally fit to eq. 5. **(I)** hUGDH_{A104L} UDP-Glc saturation curves with 34 μ M UDP-Xyl fit to eq. 3. **(J)** hUGDH_{A104G} UDP-glucose saturation curves with 0 μ M (black circles), 0.6 μ M (blue squares) UDP-Xyl were globally fit to eq. 5. **(K)** The hUGDH_{A104G} progress curve fit to eq.2 (yellow).

Figure 6:

The proposed mechanism for allostery and hysteresis in hUGDH. **(A)** (i) A single monomer of hUGDH is represented by a grey clamshell with the allosteric switch (yellow) in the E* state and cavity C-I residues depicted as a red L-shape. The cavity C-I residues are depicted in the E state in the open domain conformation (as observed in the crystal structures) but it is likely that they can sample alternate rotamers including those of the EΩ state. (ii) Binding of UDP-Glc (green rectangle) and NAD⁺ (orange trapezium) trigger the allosteric switch to adopt the active E state and (iii) the domains rotate closed with the cavities and declivities represented as white spaces between the domains. In contrast, (iv) Binding of UDP-Xyl (blue L-shape) triggers allosteric switch the cavity C-I residues adopt the EΩ conformation and then (v) the domains rotate closed. In the EΩ conformation the allosteric switch occludes the NAD⁺ binding site. Previously, we proposed¹⁵ that the slow isomerization of E* to E (i → ii) or (i → iv) EΩ originated from the repacking of the allosteric switch and core residues. **(B)** (i) The A136M substitution (purple polygon) traps the allosteric switch in the E state. (ii) Here, binding of UDP-Glc and NAD⁺ does not induce a slow isomerization and (iii) the domains rotate closed. **(C)** (i) The A104L substitution (cyan trapezoid) limits the prevents the cavity C-I residues from sampling the EΩ state. However, the allosteric switch is free to adopt the E* state, as seen Figure 3A. (ii) Upon binding UDP-Glc and NAD⁺ the allosteric switch rapidly adopts the E state and (iii) the domains rotate closed.

Table 1.

Data collection and refinement statistics

	hUGDH _{A104L} :UDP-Glc:NADH	hUGDH _{A104L}
Protein Data Bank entry	6C4J	6C4K
Data collection		
Space Group	C121	C121
Unit cell dimensions (<i>a</i> , <i>b</i> , <i>c</i> , β)	403.5, 112.6, 184.7, 98.5°	178.5, 114.5, 96.9, 116.5°
Resolution Range	(48.63–2.53)	(48.16–2.65)
Completeness (%)	99.9 (99.9) ^a	99.7 (97.8) ^a
Redundancy	7.5 (7.5)	7.7 (7.7)
No. of reflections	2056278	389870
<i>I</i> / $\sigma(I)$	9.8 (1.0)	13.7 (1.1)
CC _{1/2} ^b	99.8 (53.3)	99.9 (52.8)
<i>R</i> _{meas} ^c (%)	14.8 (222.2)	12.5 (228.5)
Refinement		
Resolution (Å)	2.53 (2.56–2.53)	2.65 (2.71–2.65)
<i>R</i> _{work} / <i>R</i> _{free}	0.181/0.214	0.222/0.255
<i>R</i> _{free} in highest resolution shell	40.33	0.425
No. atoms Protein / Ligand / Water	43090 / 1043 / 594	10810 / 16 / 69
Wilson <i>B</i> -factor	62.3	71.5
<i>B</i> -factors (Å ²) Protein / Ligand / Water	73.2 / 72.6 / 59.1	105.3 / 77.5 / 63.5
Stereochemical Ideality		
Bond lengths (Å ²)	0.004	0.002
Bond angles (°)	0.64°	0.43°
ϕ , ψ Preferred region (%)	97.6	96.2
ϕ , ψ Additionally allowed (%)	2.4	3.8
ϕ , ψ Disallowed region (%)	0.0	0.0

^aValues in parentheses are for the highest-resolution shell (2.559–2.530 Å and 2.712–2.650 Å for 6C4J and 6C4K, respectively).^bCC_{1/2} is correlation between intensities from random half-data sets.³¹^c*R*_{meas} is the redundancy-independent merging *R* factor.³²

Table 2.

Effect of UDP-Xyl on Hysteresis

Enzyme	UDP-Xyl (μM)	τ (s^{-1})	Lag (s)
hUGDH	0	17.7 ± 1.4	48.2 ± 3.7
	1 ^a	23.9 ± 2.0	65.0 ± 5.3
hUGDH _{A104L}	0	N.D. ^b	N.D.
	11.3 ^a	N.D.	N.D.
hUGDH _{A104G}	0	16.2 ± 2.0	44.0 ± 5.3
	1 ^a	22.9 ± 2.2	62.2 ± 5.7

^aThese concentrations of UDP-Xyl are 5x K_I listed in Table 4^bN.D.= not detected

Table 3.

Steady State Parameters

Enzyme	Substrate	K_M (μM)	Hill	k_{cat} (s^{-1}) ^a	K_0 ^b	K_{lim} ^b
hUGDH	UDP-Glc	7.6 ± 0.6	1	0.59 ± 0.01 ^c		
	NAD ⁺	1160 ± 290 ^d	0.69 ± 0.05	0.94 ± 0.07	157 ± 13 ^b	2300 ± 500 ^b
hUGDH _{A104L}	UDP-Glc	9.4 ± 0.5	1	0.82 ± 0.01		
	NAD ⁺	48 ± 3	1	0.79 ± 0.01		

^aOne complete catalytic turnover produces 2 NADH.

^b K_0 and K_{lim} represent the high and low affinity sites derived from Kurganov analysis³⁹, respectively.

^cThe k_{cat} for the UDP-Glc saturation curve is underestimated due to the negative cooperativity of NAD⁺ binding (see text).

^dBecause of negative cooperativity, this value represents the apparent half-saturation of the ES complex $K_{0.5}$.

Table 4.

Global analysis of competitive inhibition

Enzyme	UDP-Xyl (μM)	UDP-Glc K_M (μM)	k_{cat} (s^{-1}) ^a	K_i UDP-Xyl (μM)	Hill
hUGDH	0	7.4 ± 0.6 ^b	0.59 ± 0.01 ^b	0.18 ± 0.01	1
	0.6				1.1 ± 0.1
	1.2				1.3 ± 0.1
	2.7 ^c	7.4 ± 0.1	0.45 ± 0.01		1.85 ± 0.14
hUGDH _{A104L}	0	10.3 ± 0.6 ^b	0.84 ± 0.01 ^b	2.3 ± 0.2	1
	2.2				1
	4.3				1
	34 ^c	8.8 ± 0.4	0.85 ± 0.01		1
hUGDH _{A104G}	0	17.2 ± 1.1	0.67 ± 0.02	0.19 ± 0.2	1
	0.6				1.2 ± 0.1

^aOne complete catalytic turnover produces 2 NADH.^bFor global analysis, this parameter was refined as a shared value.^cThis experiment was excluded from global analysis because at long lags induced at high UDP-Xyl concentrations, native hUGDH is subject to NADH inhibition before the enzyme reaches steady state velocity.