Conservation of Atypical Allostery in C. elegans UDP-Glucose Dehydrogenase

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Supporting Information

ABSTRACT: Human UDP-glucose dehydrogenase (hUGDH) oxidizes uridine diphosphate-α-D-glucose (UDP-Glc) to UDP-glucuronic acid, an essential substrate in the metabolism of drugs. The activity of hUGDH is controlled by an atypical allosteric mechanism in which the feedback inhibitor UDP-xylose competes with the substrate for the active site and triggers a buried allosteric switch to produce an inactive complex (EΩ). Previous comparisons with a nonallosteric UGDH identified six large-to-small substitutions that produce packing defects in the protein core and provide the conformational flexibility necessary for the allosteric transition. Here, we test the hypothesis that these large-to-small substitutions form a motif that can be used to identify atypical UGDHs. Caenorhabditis elegans UGDH (cUGDH) conserves this motif with the exception of an Ala-to-Pro substitution in position 109. The crystal structures of unliganded and UDP-xylose bound cUGDH show that the A109P substitution is accommodated by an Asn-to-Ser substitution at position 290. Steady-state analysis and sedimentation velocity studies show that the allosteric transition is conserved in cUGDH. The enzyme also exhibits hysteresis in progress curves and negative cooperativity with respect to NAD+ binding. Both of these phenomena are conserved in the human enzyme, which is strong evidence that these represent fundamental features of atypical allostery in UGDH. A phylogenetic analysis of UGDH shows that the atypical allostery motif is ancient and identifies a potential transition point in the evolution of the UGDH family.

INTRODUCTION

The nucleotide sugar uridine diphosphate-α-D-glucuronic acid (UDP-GlC4A) is the essential substrate of glucuronidation, a major mechanism of drug metabolism in mammals. Some cancers have been shown to exploit glucuronidation as a means of chemotherapeutic resistance. Limiting the availability of UDP-GlC4A represents a novel strategy for combatting this drug-resistance mechanism. UDP-GlC4A is produced by UDP-α-D-glucose 6-dehydrogenase (UGDH), which catalyzes the NAD+-dependent oxidation of UDP-α-D-glucose (UDP-Glc). In humans, human UDP-glucose dehydrogenase (hUGDH) is a hexamer that is allosterically regulated by the downstream metabolite UDP-α-D-xylose (UDP-Xyl) (Figure 1A). Understanding the allosteric mechanism of hUGDH is an important step toward the design of therapeutics that can reduce the cellular levels of UDP-GlC4A.

In the absence of any ligand, the three dimers of hUGDH form a weakly associated hexamer called E* (Figure 1A,B). The E* hexamer slowly isomerizes to the active E state upon binding the substrate, which produces a lag in progress curves (hysteresis). The activity of hUGDH is regulated by an atypical allosteric mechanism. What makes this atypical is that the effector (UDP-Xyl) competes with the substrate for the active site. Upon binding, the UDP-Xyl triggers the Thr131-loop/c6-helix (the allosteric switch) to change the conformation, which allosterically increases the affinity of adjacent dimers and produces an inactive horseshoe-shaped complex (EΩ) (Figure 1A,B). This allosteric transition can also be observed as positive cooperativity in inhibition studies.

The Thr131-loop of the allosteric switch is buried in the protein core and must change the conformation for the enzyme to transition between the E and EΩ states (Figure 1C). The remarkable plasticity of the protein core originates from packing defects (cavities and deep surface pockets) that provide flexibility and space for the allosteric transition (Figure 1C). Comparing hUGDH to the nonallosteric homolog from Streptococcus pyogenes (spUGDH), it was shown that the packing defects evolved from six large-to-small residue substitutions in the protein core (Figure 1D). It was hypothesized that these substitutions could be used as a sequence motif to identify other UGDHs that exhibit atypical allostery. Here, we have tested that hypothesis using Caenorhabditis elegans UGDH (cUGDH), which shares 61% sequence identity with hUGDH but has a single substitution in one of the allosteric motif residues (A109P) (Figures 1D and 2). In the human enzyme, we have previously shown that the A109L substitution in this motif abolishes allostery. Here, we...
that show that cUGDH conserves the allosteric transition. The crystal structure shows that the A109P substitution is accommodated by a second N290S mutation. In addition, we observe both hysteresis and negative cooperativity with respect to NAD+ binding (which has also been reported in the human enzyme).13,14,16,18 We suggest that the conservation of both hysteresis and negative cooperativity is good evidence that these phenomena are defining characteristics of atypical allostery in UGDH. Prior to this work, atypical allostery had only identified in the human enzyme. By combining structural and kinetic studies with a phylogenetic analysis, we shed light on the evolution of atypical allostery in UGDH.

**RESULTS**

**cUGDH Forms the EΩ State.** The defining feature of the allosteric transition is the formation of the horseshoe-shaped EΩ complex.17 The crystal structure of cUGDH in complex with UDP-Xyl lacks the CS′CH2OH, which triggers the allosteric switch to produce the EΩ state. (D) The atypical allostery motif residues (orange boxes) previously hypothesized to facilitate allostery in UGDH.

![Figure 1](image1.png)

**Figure 1.** Allosteric transition in hUGDH. (A) Hexameric hUGDH forms from the association of three dimers. The allosteric switch (magenta), NAD+ binding (NB) domain (cyan), dimerization domain (green), and nucleotide–sugar binding (SB) domain (red) are identified. (B) In the inactive E* state, the NB and SB domains adopt an "open" conformation (curved arrows). The binding of the substrate (green spheres) induces the formation of the active, E state, while UDP-Xyl (red spheres) produces the inactive, horseshoe-shaped EΩ complex. (C) The E and EΩ conformations of the allosteric switch (magenta). The packing defects in the protein core are colored cyan. In the E state, a water molecule (red sphere) forms hydrogen bonds with the CS′CH2OH of UDP-Glc and Thr131 of the allosteric switch. UDP-Xyl lacks the C5′CH2OH, which triggers the allosteric switch to produce the EΩ state. (D) The atypical allostery motif residues (orange boxes) previously hypothesized to facilitate allostery in UGDH.
Figure 3. Inhibited EΩ State of cUGDH (A) The NB domain (cyan), dimerization domain (green), SB domain (red), and allosteric switch (magenta) are shown in a single chain of cUGDH in the complex with UDP-Xyl (gray spheres) (B) The cUGDH horseshoe-shaped hexameric complex (EΩ), depicted with rigid body rotation axes (blue rods) in the hexamer interfaces. The active sites with UDP-Xyl (red spheres) and allosteric switches (magenta) are also identified. (C) A stereodiagram of the Fobs – Fcalc density map for UDP-Xyl contoured at 3σ and calculated by omitting UDP-Xyl subjecting the model to simulated annealing. (D) Hydrogen bonds (dashed lines) and van der Waals (red feathers) interactions with UDP-Xyl (gray highlighting). (cUGDH/UDP-Xyl) was solved and refined to a resolution of 2.45 Å (Figure 3A,B and Table 3). The asymmetric unit contains 12 chains that are structurally equivalent, with a small number of disordered residues at the N- and C-termini (≤7 and ≤11 residues, respectively). Each chain of cUGDH also contains a well-ordered molecule of UDP-Xyl in the active site, which conserves all of the same ligand interactions previously described for the human enzyme (Figure 3C,D). In addition, there is a second molecule of UDP-Xyl bound in the NAD⁺ binding site that is most likely an artefact of the high UDP-Xyl concentrations (1 mM) in the crystallization buffer (not shown); at high concentrations, UDP-Xyl is known to bind promiscuously to the NAD⁺ site in hUGDH.18

The cUGDH structure superimposes 453 corresponding Ca atoms onto hUGDH (PDB entry 3PTZ) with an rmsd of 0.7 Å and sequence identity of 61%. The domain structure is assigned based on homology to the human enzyme: NAD⁺ binding domain (NB; res. 1-219), dimerization domain (DD; res. 220-329), and sugar-binding domain (SB; res. 330-481) (Figure 2). The application of crystallographic symmetry shows that all 12 chains of cUGDH assemble into horseshoe-shaped hexamers, similar to the UDP-Xyl bound EΩ state observed in the human enzyme (Figure 3B). The largest difference between the cUGDH and hUGDH EΩ hexamers involves a 5.6° larger rigid body rotation of adjacent dimers, which produces a wider opening in the cUGDH ‘horseshoe’ complex.

UDP-Xyl Binding Induces Formation of the EΩ Hexamer.

The UDP-Xyl binding allosterically stabilizes the UGDH hexamer in the solution.17,19,20 Sedimentation velocity analysis of 9 μM unliganded cUGDH reveals a c(s) distribution consisting of an 11.2 S (68.5%), 8.3 S (8.7%), 5.6 S (21.6%), and 3.3 S (1.2%) species (Figure 4A). This is consistent with the distribution of the hUGDH unliganded EΩ
open domain conformation resembles the E* state of hUGDH, which represents an intermediate in the allosteric transition from E to E2 (Figure 1B).13−15 The Thr136-Loop/α6-helix (allosteric switch) in cUGDH was identified based on homology with the human enzyme (Figure 2). A comparison of the unliganded cUGDH structure with the human enzyme confirms that the allosteric switch is in the E* conformation (Figure 4E). This analysis also shows that cUGDH and hUGDH undergo a similar conformational change in the transition from the E* and E2 states (Figure 4E).17,19 The only significant difference in the Thr136-Loop portion of the allosteric switch involves the conserved residue Val139 (cUGDH numbering) (Figure 5A). In cUGDH, the side chain of Val139 is weakly ordered and is modeled in the gauche+, gauche−, and trans rotamers in the different peptides of the crystal structure, while in hUGDH, the side chain is in the trans conformation (−170°). The rotational disorder of the valine appears to be in response to the bulky pyrrolidine ring of Pro109, which displaces the Val139 Ca atom by 1.4 Å relative to the human enzyme. This displacement also induces the conserved residue Leu228 to change rotamers (Figure 5A). The largest conformational difference between the enzymes involves the α6-helix; in the allosteric transition from the E* hexamer to the E2 complex, the cUGDH helix rotates by 14 ± 3° but only 6 ± 2° in the human enzyme (Figure 5B). The difference in rotation originates from several changes in the packing interactions between the α6-helix and the groove formed between the α5-helix and the β6-strand. First, the Cβ atom of Ala141 and the Cγ atom of Val114 are ∼4.7 Å apart, which creates a small void that weakens the packing of the α6-helix in cUGDH (Figure 5C). In hUGDH, Val114 is replaced with an isoleucine, which fills the void with a Cα atom and packs tightly against the alanine. Another significant change involves an alanine-to-serine substitution at position 118 in cUGDH, which is buried in a hydrophobic pocket where it weakens a hydrogen bond in the α5-helix by competing for the main-chain carbonyl oxygen of Val114 (Figure 5D). The most significant change in the α6-helix involves Ala152 in cUGDH, which packs into a buried hydrophobic cluster formed by Ala122, Leu149, and Phe161 (Figure 5E). In the human enzyme, the hydrophobic cluster is maintained with conservative substitutions, but Ala152 is replaced by an asparagine (Asn147 in hUGDH, Figure 5E). As a result, the C-terminus of the α6-helix in cUGDH has locally unfolded to expose the Asn to the solvent. There is also a two-residue insertion in the loop following the α6-helix in cUGDH, but it does not appear to contribute directly to the change in the helix rotation (Figures 2 and 5E).

The hexamer-building interface was examined to determine the origin of the increased rotation of the α6-helix in cUGDH (Figure 5F and Supporting Information Figure 1). The most significant differences involve the conserved residues Phe330 and Pro440, both of which pack directly against the α6-helix. In cUGDH, Phe330 adopts a η1 torsion angle of −69° and packs against the α6-helix residue Cys147 (Figure 5F). In contrast, Cys147 is replaced with an arginine in the human enzyme, which forces the corresponding Phe into a strained, weakly ordered rotamer of −102°. The conserved residues Glu143 and Ser144 change conformations to produce a complementary packing surface for Phe330 in each enzyme. The conserved residue Pro440 is also displaced by ∼1.9 Å relative to the human enzyme (Figure 5F). This shift is a
consequence of the altered α6-helix conformation and the replacement of alanine by Glu151 in cUGDH.

**Atypical Allostery Motif in cUGDH.** With the exception of the alanine-to-proline substitution at position 109, hUGDH and cUGDH conserve the atypical allostery motif and most of the associated packing defects in the EΩ hexamer (cavity C-1Ω and deep surface pockets D-1Ω and D-2Ω) (Figures 2 and 6A–C). In hUGDH, the main-chain amide of the alanine forms a hydrogen bond with the carboxamide of an asparagine (Asn283, human numbering) (Figure 6D). The proline substitution in cUGDH removes the amide, which would break the hydrogen bond (Figure 6E). As a result, the selective pressure on the asparagine is relaxed, and it has been replaced with Ser290, which forms a hydrogen bond with an ordered water molecule in the deep surface pocket D-2Ω. The pyrrolidine ring of Pro109 also alters the structure of the deep surface pocket D-1Ω by introducing a steric clash that induces the rotameric changes in Val139 and Leu228 that were described in the previous section (Figure 5A). The only other change in D-1Ω involves Val114, which replaces an isoleucine in the human enzyme.

**cUGDH Conserves Hysteresis and Cooperativity.** The atypical allosteric hUGDH undergoes a slow, substrate-induced isomerization from the inactive E* to the active E state, which can be observed as a lag in progress curves. The cUGDH progress curves also display a similar hysteresis, with a lag that is ~30% shorter than that observed in hUGDH (Figure 7A,B). The kinetic parameters for cUGDH were determined using the steady-state velocities derived from Frieden’s equation for the enzyme hysteresis. The NAD+ saturation curve exhibits negative cooperativity (Hill coefficient = 0.82), with a $K_{0.5}$ and $k_{cat}$ of 333 μM and 1.7 s$^{-1}$, respectively (Figure 7C and Table 1). This is comparable to the NAD+ negative cooperativity in saturation curves that has previously been reported for the human enzyme (Figure 7D and Table 1). In contrast, the UDP-Glc saturation curve is hyperbolic, with a $K_M$ and $k_{cat}$ of 55 μM and 1.2 s$^{-1}$, respectively (Figure 7E and Table 1). The lower $k_{cat}$ is a result of the negative cooperativity associated with NAD+ binding, which makes it difficult to saturate cUGDH with co-enzyme to achieve pseudo-first-order conditions. Substrate cooperativity in the presence of saturating UDP-Xyl has been observed in hUGDH and is strong evidence of the allosteric transition from a low UDP-Glc affinity EΩ complex to a higher UDP-Glc affinity E hexamer. Steady-state analysis of UDP-Xyl inhibition yields a $K_i$ of 6.9 μM, which corresponds to a ~16-fold weaker affinity for the inhibitor than that seen in hUGDH (Figure 7E,F and Table 2). The cUGDH UDP-Glc saturation curves also exhibit positive

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**Figure 5.** Structural divergence in the cUGDH allosteric switch. (A) The Pro109 substitution in cUGDH displaces the Val139 (magenta), which causes Leu228 to change rotamer relative to hUGDH (gray). (B) The α6-helix in the EΩ conformation of cUGDH (magenta, cyan) is rotated more than that observed in hUGDH. (C) Val114 (cyan) replaces an isoleucine in hUGDH (gray). VDWs distances (dots) and the α6-helix (magenta) are identified. (D) Ser118 of cUGDH (cyan) replaces an alanine in hUGDH (gray). (E) Stereoview depicting significant changes in the packing of the α6-helix of cUGDH (magenta) against the α5-helix and β6-strand (cyan) compared to the hUGDH (gray). cUGDH contains an insertion that lengthens the α6-helix (orange). (F) Stereoview of changes in the packing interactions that form the hexamer-building interface of cUGDH (colored by domain; NB: cyan, allosteric switch: magenta, dimerization domain: green, and SB: red). hUGDH is colored gray.
Figure 6. Packing defect residues (PD residues) are conserved in cUGDH. (A) A cutaway of the cUGDH E\(\Omega\) structure protein core depicting cavity C-1\(\Omega\). (B, C) Same as in (A), depicting deep surface pockets D-1\(\Omega\) and D-2\(\Omega\), respectively. (D) In hUGDH, Asn283 in D-2\(\Omega\) forms a hydrogen bond with the backbone amide of Ala104 (hUGDH numbering). (E) In cUGDH, the alanine and asparagine are replaced with a Pro109 and Ser290, respectively. Ser290 forms a hydrogen bond with a water molecule (red sphere).

Figure 7. Kinetic analyses of cUGDH and hUGDH. (A) Representative cUGDH progress curve (black line) from stopped-flow absorbance spectroscopy, which is fit to eq 1 (yellow line) (eq 1). (B) The length of the progress curve lag under the same conditions for cUGDH and hUGDH. (C) NAD\(^+\) saturation curve with cUGDH fit to eq 3 (thin line). Rates (black dots) have been normalized by the enzyme concentration to give turnover, and dashed lines represent 95% confidence intervals. (D) NAD\(^+\) saturation curve with hUGDH. (E) cUGDH substrate saturation curves with 0 \(\mu\)M (black), 4 \(\mu\)M (blue), and 15 \(\mu\)M UDP-Xyl (red), which were globally fit to eq 4. (F) hUGDH substrate saturation curves with 0 \(\mu\)M (black) and 1 \(\mu\)M (blue) UDP-Xyl, which were globally fit to eq 4. Kinetic parameters derived from all fits can be found in Tables 1 and 2.

Table 1. Kinetic Parameters for cUGDH and hUGDH

| enzyme | ligand   | \(K_M\) (\(\mu\)M) | \(k_{cat}\) \(^a\) (s\(^{-1}\)) | \(K_M\) (\(\mu\)M) | \(k_{cat}\) \(^a\) (s\(^{-1}\)) | Hill (h) |
|--------|----------|----------------|----------------|----------------|----------------|----------|
| cUGDH  | UDP-Glc  | 55 \(\pm\) 3   | 1.23 \(\pm\) 0.03 | 333 \(\pm\) 18 | 1.7 \(\pm\) 0.1 | 0.8 \(\pm\) 0.1 |
|         | NAD\(^+\) |                 |                 |                 |                  | 1.0      |
| hUGDH  | UDP-Glc  | 11.0 \(\pm\) 0.8| 0.71 \(\pm\) 0.02| 1094 \(\pm\) 244| 1.1 \(\pm\) 0.1 | 0.6 \(\pm\) 0.1 |
|         | NAD\(^+\) |                 |                 |                 |                  | 1.0      |

\(^a\)One complete catalytic turnover produces two molecules of NADH.
Table 2. Global Analysis of UDP-Xylose Inhibition

| enzyme | UDP-Xyl (μM) | $K_{iA}$ (μM)$^{a}$ | $K_{iD}$ (s$^{-1}$)$^{a}$ | $K_i$ (μM)$^{a}$ | Hill (h) |
|--------|--------------|---------------------|-----------------------------|-------------------|---------|
| cUGDH  | 0            | 54 ± 3              | 1.23 ± 0.03                 | 6.9 ± 0.6         | 1.0     |
|        | 4            |                     |                             |                   | 1.0 ± 0.1|
|        | 15           |                     |                             |                   | 1.4 ± 0.1|
| hUGDH  | 0            | 11.0 ± 0.8          | 0.70 ± 0.01                 | 0.42 ± 0.04       | 1.0     |
|        | 1            |                     |                             |                   | 1.3 ± 0.1|

$^{a}$Shared parameter in the global fitting.

cooperativity in the presence of 15 μM UDP-Xyl (Hill coefficient of 1.4) (Figure 7E and Table 2).

**Evolutionary Analysis of Atypical Allostery in UGDH.** The evolution of atypical allostery in UGDH was examined using over 70,000 sequences. A phylogenetic tree of 31 representative UGDH sequences from diverse eukaryotic and prokaryotic phyla is shown in Figure 8. Based on overall sequence similarity and taxonomic representation, these 31 sequences can be broadly classified into 8 clades. Since the dendrogram shown in Figure 8 is a gene tree and not a species tree, taxonomically diverse species such as bacteria and protists often fall in different clades. Here, our definition of clades is based on the overall similarity of full-length UGDH sequences. The nature of amino acids present in the atypical allosteric motif (including the newly defined S/N290 position) was compared across phyla (Figure 8). Although both the atypical allosteric motif residues and the packing defect residues are generally conserved across the eukaryotic and prokaryotic phyla (clades 1–7), they are strikingly different in the nonallosteric S. pyogenes clade (clade 8), which was used as an outgroup (see methods) (Figures 8 and 9). If a clade contains a single, conservative variation, then it is identified as likely allosteric, but if there are significant changes, then it is assumed to be nonallosteric (Figure 9). Sequences in clades 1–2 strictly conserve the allosteric motif residues, while notable variations are observed in clades 3–7. In clade 3, the Capsaspora phylum absolutely conserves the motif, while the Platyhelminthes substitute a serine for the alanine at position 141. The impact of the serine is likely to be negligible since the $O\gamma$ would point into the deep surface pocket D-285, which is large enough to accommodate a water molecule for hydrogen bonding. In clades 5–8, we observe the persistent conservation of a Thr substitution in position 141. This is most pronounced in the Stramenopiles, Bacillariophyta, and Phaeophyceae phyla of clade 5, where the Thr141 substitution represents the only significant variation in either the atypical motif or packing defect residues. In contrast, the remaining phyla of clade 5 also contain a tryptophan substitution in the packing defect residue position 114, which will likely introduce significant clashes in the inhibited state of the enzyme. In clade 6, the protist Parasabalia clusters with bacterial phyla Lentisphaerae, Cyanobacteria, and Verrucomicrobia. The grouping of protists and bacteria in gene dendrograms has previously been observed in other gene families and is often attributed to lateral gene transfer between protists and bacteria.26 The Ascomycota (clade 7) contain a leucine substitution at position 109, which is interesting because the equivalent substitution in the human enzyme has been shown to eliminate alloster by introducing steric clashes in the deep surface pocket D-285. In general, the overall conservation of the motif residues in fungal and cyanobacterial clades (clades 6,7) suggests that the atypical allosteric mechanism is likely ancient and evolved progressively in higher eukaryotes (clades 1,2) (Figure 9). Because of the specificity for UDP-Xyl, it is unlikely that the atypical allosteric inhibition mechanism would be present in the UGDHs of organisms that do not produce this nucleotide–sugar. Unfortunately, data concerning the distribution of UDP-Xyl throughout various phyla is poorly characterized and incomplete.

**DISCUSSION**

What makes allostery in UGDH atypical is that the feedback inhibitor competes with the substrate for the active site to trigger a transition to the E$\Omega$ state (Figure 1B).15,17 Key to this remarkable transition is the atypical allosteric motif, which identifies a series of large-to-small amino acid substitutions that produce the packing defects in the protein core that are necessary to accommodate both the E and E$\Omega$ conformations of the buried allosteric switch (Figures 4E and 6A–C).14,17 This motif is conserved in cUGDH with the exception of an alanine to proline substitution in position 109 (Figure 2). In an early study with the human enzyme, it was shown that a leucine substitution at the equivalent position abolished allostery.14 Here, we show that despite the A109P substitution, the crystal structure of cUGDH/UDP-Xyl still forms the
allosterically inhibited EΩ hexamer (Figure 3B). The structure also suggests a reason for why Ser290 is replaced with an asparagine in hUGDH (Figure 6D,E). There, the asparagine forms a hydrogen bond with the main-chain amide of the alanine in position 109 of the atypical motif (Ala104 in hUGDH) (Figure 6D,E). The Pro109 substitution in cUGDH removes the amide, which relaxes the selective pressure on the asparagine and allows the Ser290 substitution.

A comparison of the E* and EΩ cUGDH structures shows that the allosteric switch undergoes a conformational change similar to that observed in the human enzyme but with a larger rotation of α6-helix (Figures 4E and 5B). It has been shown that changes in the conformation of the α6-helix can affect the stability of the hUGDH hexamer and the affinity for UDP-Xyl.13,14,17 Here, we have shown that the EΩ state of cUGDH is a weakly associated hexamer, while the allosterically inhibited enzyme forms a stable EΩ complex, similar to the human enzyme (Figure 4A,B).15,17 However, the UDP-Xyl affinity of cUGDH is ~16-fold weaker than that of the human enzyme, despite the fact that both enzymes conserve the same interactions in the binding site (Table 2 and Figure 3D). Since UDP-Xyl is coupled with the hexamer-building interface through the allosteric switch, it is likely that the changes in the rotation of the α6-helix are responsible for the reduced affinity (Figure 4B).

Hysteresis and allosteroy have been shown to be coupled in both hUGDH.13−15,17 Our observation of hysteresis in cUGDH suggests that this is a defining characteristic of the atypical allosteric mechanism (Figure 7A,B). The allosteric transition can be observed as positive cooperativity in steady-state inhibition studies (Figure 7E,F and Table 2). Briefly, the EΩ conformation has low affinity for the substrate UDP-Glc (Figure 1D).15,17 As UDP-Glc outcompetes the inhibitor, the enzyme allosterically switches to the high substrate affinity E* state, resulting in a sigmoidal substrate saturation curve. Both hUGDH and cUGDH also display negative cooperativity with respect to NAD+ binding (Figure 7C,D and Table 1). The negative cooperativity in hUGDH is poorly understood at a structural level, but it is known to originate from a substrate-induced asymmetry in the enzyme. Negative cooperativity is believed to be linked to the allosteric mechanism, because amino acid substitutions that disrupt allostery also abolish negative cooperativity.13,14 The conservation of negative cooperativity in cUGDH strongly suggests that this phenomenon is also a defining characteristic of the atypical allosteric mechanism.

To obtain new insights into the evolution of allosteroy in UGDH, we have examined the patterns of conservation and variation in the atypical allosteric motif residues across diverse species (Figures 8 and 9). Phyla that conserve Ala109 also conserve Asn290, supporting our hypothesis that these residues are conserved to maintain the allosteric mechanism.
residues may have co-evolved. Because the dendrogram shown in Figure 8 is a gene tree and not a species tree, taxonomically diverse species such as bacteria and protists often fall in different clades, which are based only on the overall similarity of full-length UGDH sequences.\(^24,25\) UGDHs in higher eukaryotes (clades 1–3) generally conserve the motif residues and are likely to be allosteric, while clade 8, which is predominantly bacterial, is unlikely to be nonallosteric as it displays striking divergence in motif residues (Figures 8 and 9). Specifically, phyla in clade 8 show no conservation of the amino acids corresponding to the motif residues A108, A141, A142, and N290. Notably, some bacterial sequences (Cyanobacteria, Lentisphaerae, and Verrucomicrobia) fall in a different clade (clade 6) and are more similar to eukaryotic UGDHs (49–60% similarity to sequences in clades 1–3) than they are to other bacterial sequences (23–37% sequence similarity to sequences in clade 8). Interestingly, clade 6 bacterial sequences also share some of the allosteric motif residues (A108, A142, N290) suggesting an ancient origin for the proposed allosteric mechanism. We also note the diversity of protist UGDH sequences as they also fall into distinct clades in the phylogenetic tree. For example, Capsaspora, a protist most closely related to animals, clusters with UGDH sequences in clades 1 and 2 and conserves nearly all of the allosteric motif residues, whereas other ciliated protozoa such as Intermacronucleata lack the allosteric motif residues and group with bacterial sequences in clade 8. It is also interesting that some protists such as Parabasalia group with bacterial sequences in clade 6 and share some of the allosteric motif residues (A108, N290). Grouping of protists and bacteria in gene dendrograms has previously been observed in other gene families and is often attributed to lateral gene transfer between protists and bacteria.\(^26\) Although we have focused our analysis on the allosteric motif residues, it is likely that variations beyond the allosteric motifs contribute to the placement of protist and bacterial sequences in different clades. These variations, along with those in the remaining clades (clades 4–7), will require additional analysis to fully map the allosteric diversity of the UGDH superfamily. In particular, we are intrigued by the selective conservation of threonine at position 141 in clades 4–7 (Figures 9). If that substitution prevents the formation of the E\(^6\) complex, then it may be that we have identified a key step in the evolution of atypical allosterity in UGDH. Future work will focus on mapping these evolutionary steps through the experimental characterization of UGDHs from the Stramenopiles, Bacillariophyta, and Phaeophyceae phyla in clade 5. Because these sequences contain the Thr141 substitution and conserve most of the packing defect residues, they appear to be important transition points between nonallosteric bacterial UGDH and allosteric human UGDH.

**MATERIALS AND METHODS**

Protein Expression and Purification. The enzymes cUGDH (UniProt ID: Q19905-1) and hUGDH (UniProt ID: O60701-1) were recombinantly expressed in *Escherichia coli* and purified, as previously described.\(^13–20\) Briefly, cUGDH is cloned into modified pET-15b vectors (Norclone) with N-terminal hexahistidine affinity tags adjacent to a tobacco etch virus (TEV) cleavage site. Recombinant proteins were purified using a Talon-immobilized metal affinity column, and the affinity tags were removed with 5 μM TEV protease for ∼16 h at 20 °C. Proteins were dialyzed into a storage buffer (25 mM TRIS pH 8.0 and 50 mM NaCl) and then concentrated to 20 mg mL\(^{-1}\) using an Ultra-15 10k centrifugal filter. Proteins were quantified from dilution replicates (N ≥ 6) using the molar extinction coefficients of 53 806 and 49 850 M\(^{-1}\) cm\(^{-1}\) for cUGDH and hUGDH, respectively (calculated with the program PROTPARAM). Proteins were aliquoted and flash-frozen in liquid nitrogen and then stored at −80 °C.

Protein Crystallization and Structure Determination. The cUGDH protein with bound UDP-Xyl was crystallized at 20 °C using the hanging drop vapor diffusion method, with a 2 μL drop that consisted of a 1:1 mixture of protein (10 mg mL\(^{-1}\)) and reservoir solution. Optimized crystals were obtained using a reservoir solution that consisted of citric acid buffer pH 5.0, 200 mM LiCl, 1 mM UDP-Xyl, and 4% PEG 8000. These crystals were soaked in a cryoprotectant mixture composed of the same reservoir solution supplemented with 15% v/v of the cryoprotectant solution (1:1:1 ethylene glycol/dimethyl sulfoxide/glycerol ratio) and then rapidly plunged into liquid nitrogen. Diffraction data were collected on the 22-ID beamline (SERCAT) at Argonne National Laboratory (Argonne, IL) using a MAR 300 mm CCD detector. The 2.45 Å data set was processed with XDS in the space group P2\(_2\)\(_2\)\(_1\)\(_2\), setting aside 5% of the data for cross-validation.\(^27,28\) The structure was solved by molecular replacement using PHENIX and a single dimer of the unliganded cUGDH crystal structure (PDB: 2O3J; unpublished) as a search model. During refinement, the structure was subjected to iterative cycles of manual model rebuilding using COOT and the automated refinement procedure that is implemented within the PHENIX software suite.\(^29–31\) The R\(_\text{free}\) test set was corrupted during refinement, so a posterior R-free was calculated by selecting 7% of the reflections for a new cross-validation data set and subjecting the final model to Cartesian dynamics at 4000° to decouple the test set. Data collection and refinement statistics can be found in Table 3.

Structural Analyses. The sequence alignment for cUGDH, hUGDH, and spUGDH was generated using Sequoia.\(^34\) The ligand interactions in the cUGDH/UDP-xylose structure were determined using LigPlot\(^35\). The program DynDom\(^36,37\) was used to identify the hinge-bending axes for the domain rotation between the open and closed states and the rigid body rotation axis that relates the 32 symmetry hexamer to the horseshoe-shaped conformation. PISA\(^38\) and DIMPLOT\(^35\) were used to identify interacting residues at the hexamer-building interfaces in the cUGDH and hUGDH structures. The α6-helix rotation angle was calculated using PyMOL (https://pymolwiki.org/index.php/AngleBetweenHelices). Briefly, the monomers from the unliganded and UDP-Xyl bound structures were superimposed via least-squares-refinement using the CA residues 1-132, and the rotation of the α6-helix was measured by using the CA residues 141-151. Both PyMOL\(^39\) and UCSF Chimera 1.13\(^40\) were used to generate figures.

Sedimentation Velocity. Sedimentation velocity experiments were conducted, as previously described.\(^15–17,19–20\) Briefly, 9 μM cUGDH was dialyzed >12 h at 4 °C into a buffer with or without 80 μM of UDP-Xyl containing 25 mM HEPES pH 7.5 and 150 mM KCl. Samples were loaded into 12 mm double-sector Epon centerpieces equipped with quartz window cells. Cells were then loaded into an An60 Ti rotor and equilibrated to 20 °C for 1.5 h. Sedimentation velocity data were collected using an Optima XLA ultracentrifuge at 50 000 rpm for 8–10 h. Absorbance data were recorded at
Steady-State and Transient-State Kinetics. All steady-state kinetic assays were performed, as previously described.13–20 Briefly, assays contained 100 nM enzyme and either saturating amounts of UDP-Glc or NAD+ in the reaction buffer (50 mM HEPES pH 7.5, 50 mM NaCl, and 5 mM EDTA). Solutions containing enzyme and substrate/cofactor were incubated separately at 25 °C for 5 min, and then the reaction was initiated by rapidly mixing the two solutions. The progress of the reaction monitored NADH production (E340 nm = 6220 M−1·cm−1) on a Agilent 8453 UV/vis spectrophotometer at 25 °C, with absorbance readings collected every 0.5 s. Because the progress curves for both cUGDH and hUGDH display hysteresis, the initial velocity (v0) represents a transient that violates the steady-state approximation.13,15,16,18 Thus, the initial steady-state velocities (vss) were derived from fitting progress curves to Frieden’s equation for hysteresis before the depletion of 10% substrate,13–15,18,23

\[
P(t) = v_{ss} - v_0(1 - e^{-t/\tau})
\]

where P is the product produced at time t and \( \tau \) is the relaxation time of the lag. The length of the lag is equal to \( \tau \). All data were fit using the nonlinear regression analysis in PRISM (GraphPad Software). UDP-Glucose substrate saturation curves were fit to eq 2

\[
v_0 = \frac{k_{cat}[E_i][S]}{K_M + [S]}
\]

where \( v_0 \) is the initial steady-state velocity (in eq 1), \( [E_i] \) is the enzyme concentration (100 nM), and \( [S] \) is the variable substrate concentration. Both enzymes display negative cooperativity in NAD+ saturation curves and were fit to the sigmoidal rate eq 3

\[
v_0 = \frac{k_{cat}[E_i][S]^h}{(K_{0.5})^h + [S]^h}
\]

where \( K_{0.5} \) is the half saturation point and \( h \) is the Hill coefficient. The affinity (\( K_i \)) for UDP-Xyl was determined by globally fitting substrate saturation curves with and without inhibitor to a competitive inhibition model with cooperativity (eq 4) using PRISM:

\[
v_0 = \frac{k_{cat}[E_i][S]^h}{(K_{0.5})^h + [S]^h}
\]

where \( K_{0.5} \) is the half saturation point and \( h \) is the Hill coefficient. The affinity (\( K_i \)) for UDP-Xyl was determined by globally fitting substrate saturation curves with and without inhibitor to a competitive inhibition model with cooperativity.
Accession Codes
Homo sapiens UGDH (UniProt ID: O60701-1) C. elegans UGDH (UniProt ID: Q19905-1) The atomic coordinates and structure factors for UDP-Xyl bound cUGDH have been deposited in the Protein Data Bank (PDB entry: 6OM8).

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The authors declare no competing financial interest.

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ABBREVIATIONS
UGDH, UDP-glucose dehydrogenase; hUGDH, Homo sapiens UGDH; cUGDH, Caenorhabditis elegans UGDH; spUGDH, Streptococcus pyogenes UGDH

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