Supplemental information

MATERIAL AND METHODS

Recombinant production and purification of hUGDH

The coding gene for hUGDH was amplified from I.M.A.G.E. Consortium Clone ID IMAGE: 3916854. It was cloned in a truncated version that produces a 27 amino acid-long deletion from the C-terminus of the protein. A pBEN-derived plasmid vector was used. The final construct encoded the short-form hUGDH (residues 1 – 467) fused to N-terminal SET1 and SBP tags, followed by a tobacco etch virus (TEV) protease cleavage site. If not mentioned otherwise, truncated enzyme was used and is referred to as hUGDH. The final plasmid constructs were expressed in the phage-resistant *E. coli* strain BL21(DE3)-R3 harboring the pRARE2 plasmid for rare codon expression. A Luria-Bertani or Terrific Broth medium was used that was supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. The starter culture was grown overnight at 37°C and used to inoculate the main culture to an initial OD_{600} of 0.01. Cultures were further grown at 37°C to an OD_{600} of ~0.8. High-level soluble protein production was performed overnight at 18°C at an isopropyl-β-D-thiogalactopyranoside concentration of 0.2 mM. Cells were harvested by centrifugation at 5000 rpm for 20 minutes (4 °C), and the cell pellet was resuspended in 150 mL Streptavidin binding buffer (100 mM TRIS, 150 mM NaCl, pH 8) supplemented with Complete Protease Inhibitors (Complete, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics Ltd.) and stored at 20 °C. Cells were lysed using an EmulsiFlex-C5 high pressure homogenizer (Avestin) at 4 °C. Affinity purification with a Strep-Tactin affinity column was performed according to a protocol supplied by the manufacturer (IBA GmbH). Buffer exchange to 50 mM HEPES (pH 7.5) containing 300 mM NaCl and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP) was carried out using a NAP-10 (GE Healthcare) desalting column. SET1 and SBP tags were cleaved off by treatment with TEV protease. Note: It was shown that the tags did not affect the activity of hUGDH so it would be possible to work with the tagged preparation. Further purification was done by size-exclusion chromatography (Superdex 200 16/60 HiLoad, GE/Amersham Biosciences) and anion-exchange chromatography (HiTrap-Q HP), which used a linear gradient between no and 1 M NaCl in 50 mM HEPES (pH 7.5). Fractions containing hUGDH were combined, desalted, concentrated by ultrafiltration (Amicon Ultra MWCO 30 kDa, Millipore) to about 15-20 mg/mL and stored in 50 mM HEPES (pH 7.5) until further use.

The enzyme preparations obtained by the procedures described above appeared homogeneous by the criterion of a single protein band in SDS PAGE. ESI-TOF-MS analysis was also used to verify the expected mass 52019 g/mol of the protein subunit. Using analytical gel filtration and dynamic light scattering, it was shown that hUGDH as isolated had a molecular mass of ~ 320 kDa, consistent with a homohexameric assembly of protein subunits. Molar protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of 48360 M^{-1} cm^{-1} that is based on amino acid

analysis. Alternatively, the BCA protein assay (ThermoScientific) was applied. It was confirmed that both methods of protein determination agreed within less than 10%.

A control experiment was performed in which the gene encoding full-length hUGDH was placed into a pLIC-SGC1 expression vector. The final construct encoded hUGDH fused to an N-terminal His_6-tag further equipped with a TEV cleavage site. Protein production in *E. coli* was done as described above. Purification was carried out on a Ni Sepharose column according to instructions of the supplier (GE Healthcare) using a stepwise elution with imidazole. Eluted fractions containing hUGDH were gel filtered to 50 mM HEPES (pH 7.5) containing 300 mM NaCl and 0.5 mM TCEP by using a NAP-10 column (GE Healthcare). Subsequent steps were carried out as described before and yielded purified protein whose ESI-TOF-MS mass of 55112 ± 1 g/mol was in excellent agreement with expectation from the amino acid sequence (55111 g/mol) lacking the His_6-tag. Molar protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of 49850 M^{-1} cm^{-1} that is based on amino acid analysis. Table 2 (main text) shows that kinetic parameters of full-length and short-form hUGDH were identical within limits of error. Binding of UDP-Glc measured by ITC was also identical in both enzyme preparations.

Mutagenesis

Site-directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the protocol supplied by the manufacturer. The oligonucleotides used are listed in 5´- 3´ direction and mutated codons are underlined.

C276A: Fw: GTTGGGTTTGGTGGGAGCGCTTTCCAAAAGGATGTTCTG,
Rev: TCAGAACATCCTTTTGGAAAGCGCTCCCACCAAACCC.

C276S: Fw: GTTGGGTTTGGTG GGAGCTCTTTCCAAAAGGATGTTCTG,
Rev: TCAGAACATCCTTTTGGAAAGAGCTCCCA CCAAACCC**.**

T131A: Fw: GTGACTGAGAAAAGCGCAGTTCCAGTGCGGG,
Rev: CCCGCACTGGAACTGCGCTT TTCTCAGTCAC.

Plasmid miniprep DNA of all mutated genes was fully sequenced to verify introduction of the desired mutations and to confirm that no misincorporations of nucleotides had occurred. Mutants of hUGDH were isolated and characterized as described above for the wild-type enzyme.

Isothermal titration calorimetry (ITC)

ITC experiments were carried out with a VP-ITC titration microcalorimeter from MicroCal, equipped with a ThermoVac module. The used cell had volume of 1.4189 mL and a 250 µL microsyringe was applied. All experiments were carried out at 10°C while stirring at 295 rpm. A 10 mM HEPES buffer (pH 7.5) containing 150 mM NaCl was used. Solutions were degassed, by gentle swirling under vacuum,

before loading them to the microcalorimeter. The microsyringe was filled with a solution of the ligand (1 mM UDP-glucose; or 0.5 mM NAD^+) in 10 mM HEPES (pH 7.5; 150 mM NaCl) and carefully inserted into the calorimetric cell, which was filled with the protein sample (2.1 mL; 20 – 50 µM) dissolved in the above HEPES buffer. The system was first allowed to equilibrate until the cell temperature reached 10°C and an additional delay of 120 sec was applied. All titrations were conducted using an initial control injection of 2 µL followed by 29 identical injections of 8 µL with a duration of 16 sec (per injection) and a spacing of 200 sec between injections. The titration experiments were designed in such a fashion to ensure complete saturation of the enzymes before the final injection. The heat of dilution for the ligands was independent of their concentration and corresponded to the heat observed from the last injection, following the saturation of the enzyme, thus facilitating the estimation of the baseline of each titration from the last injection. The collected data were corrected for ligand heats of dilution (measured on separate experiments by titrating the ligands into 10 mM HEPES, pH 7.5, 150 mM NaCl) and deconvoluted using the MicroCal™ Origin software supplied with the instrument to yield enthalpies of binding (ΔH) and binding constants (K_d) as described elsewhere (1). Thermodynamic parameters were calculated using the fundamental thermodynamic relationship, $\Delta G = \Delta H - T\Delta S = -RT\ln K_d$, where R is gas constant, T is temperature, and ΔG, ΔH, and ΔS are the changes in free energy, enthalpy and entropy of binding respectively. A single binding site model was employed, supplied with the MicroCal™ Origin software package. Detailed background information is described elsewhere (1,2).

Analytical gel filtration

Size-exclusion chromatography (Superdex 200 16/60 HiLoad, GE/Amersham Biosciences) using 50 mM phosphate buffer containing 150 mM NaCl at pH 7.5 was applied. 0.5 mL of 15 mg/mL wild-type hUGDH was loaded at a flow rate of 0.5 mL/min. Analysis and correlation of elution profile to gel filtration standard (Biorad) yields the mass and thus oligomeric state of the native protein according to the protocol supplied by the manufacturer.

Dynamic light scattering (DLS)

Samples of protein preparation (from 0.1 to 1.0 mg/mL) were centrifuged (Ultrafree-MC Centrifugal Filter Units, Millipore) at 12,000g, 3 min, and 4 °C to remove potential large impurities (particles >1 µm). DLS measurements were carried out at ambient temperature at a scattering angle of 90° (Spectroscatter 201, RiNA). A volume of 20 µL of each sample was used to fill a 1.5 × 1.5 mm cuvette (Hellma). Autocorrelations over 20 s were collected, and the instrument software deduced the distribution of particles according to their hydrodynamic radius, N(Rh), from Laplace inversion.

References

1. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem.* **179**(1), 131-137
2. Jelesarov, I., and Bosshard, H. R. (1999) *J. Mol. Recognit.* **12**(1), 3-18

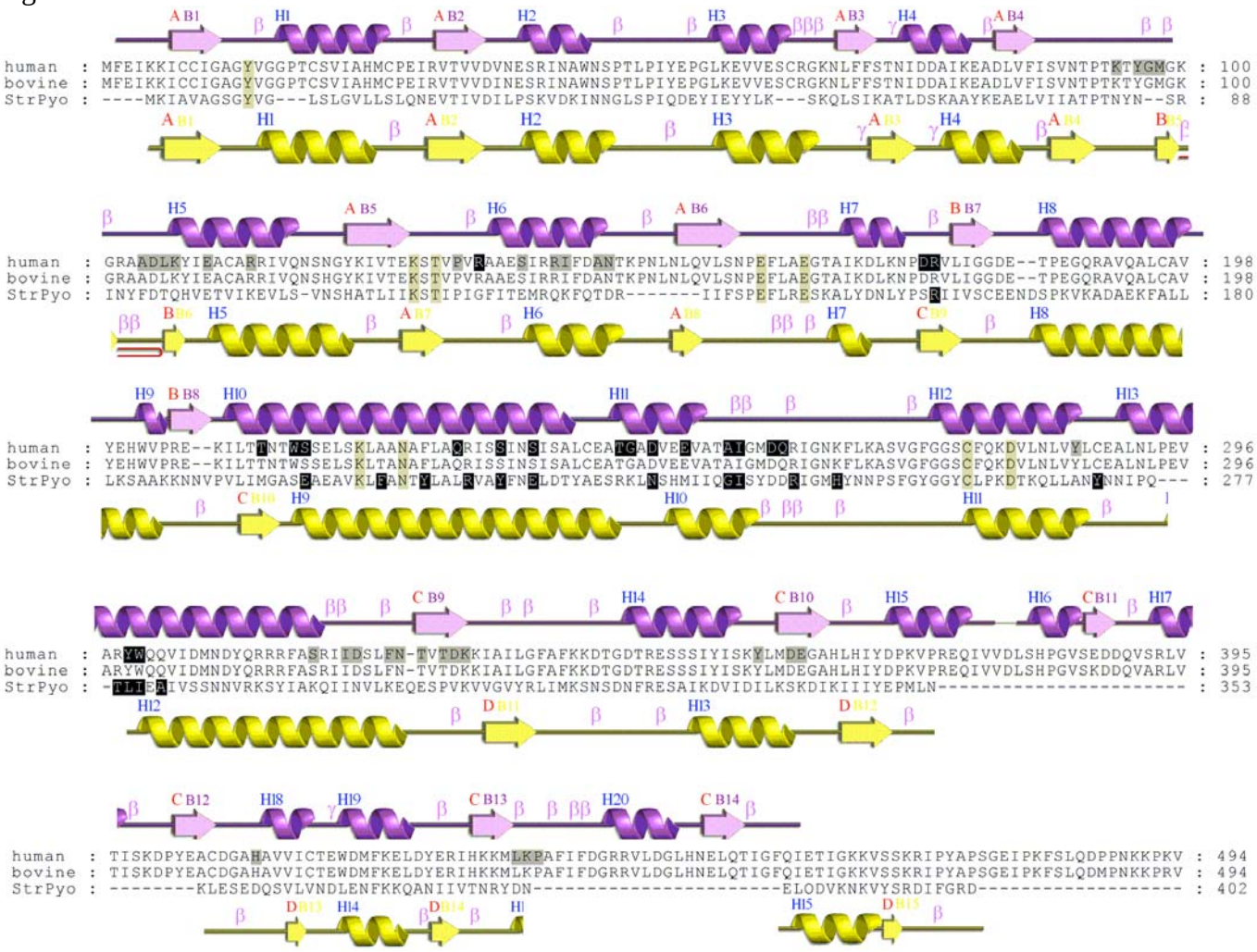
SUPPLEMENTAL DATA

Figure legends

Figure S1. Multiple alignment of selected UGDH sequences. Human (O60701/1-494 *Homo sapiens*), bovine (P12378/1-494 *Bos taurus*), and StrPyo (P0C0F4/1-402 *Streptococcus pyogenes*) are shown. Conserved catalytic residues are indicated as light grey. Residues forming hydrogen bonds responsible for dimerization are shown as white text and black background. Amino acids involved in higher oligomerization of hUGDH are highlighted in grey. Secondary structure elements for hUGDH (PDB: 2Q3E) are shown on top of the alignment, those for *Sp*UGDH (PDB: 1DLI) are shown underneath. Helices are numbered H1-H15/20 and strands B1-B14/15 and by their sheets A-C/D. Motifs are indicated as beta turn (β), gamma turn (γ) and beta hairpin (⟹).

Figure S2. Representative mass spectrum obtained from deconvoluted ESI-MS data recorded from a reaction of the C276S mutant. The mutant (20 μM subunits) was incubated in the presence of 15 mM NAD^+ and 1 mM UDP-Glc. A sample taken after 200 min was analyzed. Peak 1 (on the left) corresponds to “native” (i.e. non-derivatized) enzyme. Peak 2 (on the right) shows a mass increase of 562 ± 2 g/mol, consistent with formation of a covalent adduct, presumably on Ser^{276}, that was derived from oxidation of the UDP-Glc substrate. Measurement of the appearance of NADH confirmed that reaction had occurred under the conditions used. Note: For the experiment described, a preparation of C276S was used that had not undergone treatment with TEV protease and therefore, still contained the tags employed for expression and purification. The presence of these tags did not interfere with activity.

Figure S1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bovine : YEHWVPRE--KILTTNTWSSELSKLTANAFLAQRISSINSISALCEATGADVEEVATAIGMDQRIGNKFLKASVGFGGSCFQKDVLNLVYLCEALNLPEV : 296
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human : ARYWQQVIDMNDYQRRRFASRIIDSLFN-TVTDKKIAILGFAFKKDTGDTRESSSIYISKYLMDEGAHLHIYDPKVPREQIVVDLSHPGVSEDDQVSRLV : 395
bovine : ARYWQQVIDMNDYQRRRFASRIIDSLFN-TVTDKKIAILGFAFKKDTGDTRESSSIYISKYLMDEGAHLHIYDPKVPREQIVVDLSHPGVSKDDQVARLV : 395
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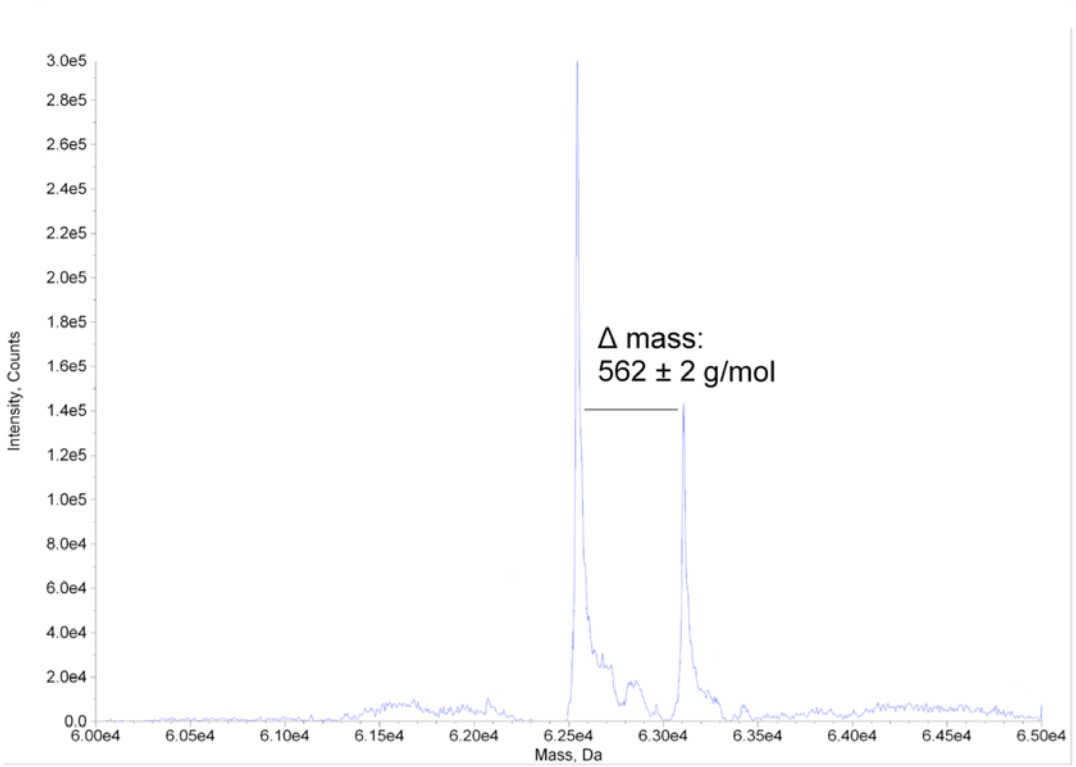
Figure S2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562 ± 2 g/mol
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