The Structural Basis of Substrate Promiscuity in Glucose Dehydrogenase from the Hyperthermophilic Archaeon *

Sulfolobus solfataricus

Christine C. Milburn, Henry J. Lamble, Alex Theodossis, Steven D. Bull, David W. Hough, Michael J. Danson, and Garry L. Taylor

From the Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, Scotland, United Kingdom and the Department of Chemistry or Centre for Extremophile Research, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

The hyperthermophilic archaeon Sulfolobus solfataricus grows optimally above 80 °C and utilizes an unusual, promiscuous, non-phosphorylative Entner-Doudoroff pathway to metabolize both glucose and galactose. The first enzyme in this pathway, glucose dehydrogenase, catalyzes the oxidation of glucose to gluconate, but has been shown to have activity with a broad range of sugar substrates, including glucose, galactose, xylose, and L-arabinose, with a requirement for the glucose stereo configuration at the C2 and C3 positions. Here we report the crystal structure of the apo form of glucose dehydrogenase to a resolution of 1.8 Å and a complex with its required cofactor, NADP⁺, to a resolution of 2.3 Å. A T41A mutation was engineered to enable the trapping of substrate in the crystal. Complexes of the enzyme with d-glucose and d-xylose are presented to resolutions of 1.6 and 1.5 Å, respectively, that provide evidence of selectivity for the β-anomeric, pyranose form of the substrate, and indicate that this is the productive substrate form. The nature of the promiscuity of glucose dehydrogenase is elucidated, and a physiological role for this enzyme in xylose metabolism is suggested. Finally, the structure suggests that the mechanism of sugar oxidation by this enzyme may be similar to that described for human sorbitol dehydrogenase.

The hyperthermophilic archaean Sulfolobus solfataricus grows optimally at 80–85 °C and pH 2–4, utilizing a wide range of carbon and energy sources, and has been used as a model organism of archaeal sugar metabolism, being subject to extensive and comprehensive investigations (1, 2). Central metabolism in S. solfataricus involves a variant of the Entner-Doudoroff pathway (3). Typically this pathway has been described as non-phosphorylative (3), proceeding with no net ATP production, and with analogous pathways being described for the thermophilic archaean Sulfolobus acidocaldarius (4), Thermoplasma acidophilum (5), and Thermoproteus tenax (6), as well as certain strains of Aspergillus fungi (7, 8). In this pathway, glucose dehydrogenase and gluconate dehydrogenase catalyze the oxidation of glucose to gluconate and the subsequent dehydration of gluconate to 2-keto-3-deoxygluconate (KDG). KDG aldolase then catalyzes the cleavage of KDG to glyceraldehyde and pyruvate. The glyceraldehyde is phosphorylated by glyceraldehyde kinase to give 2-phosphoglycerate. A second molecule of pyruvate is then produced from this by the actions of enolase and pyruvate kinase. Glucose dehydrogenase and KDG aldolase from S. solfataricus have been reported to have high activity with galactose and 2-keto-3-deoxygalactonate, respectively (9), with recent reports demonstrating the activity of gluconate dehydratase from this organism with galactonate (10, 11). Consequently, it was proposed that the entire central metabolic pathway in this organism is promiscuous for the metabolism of glucose and galactose. This situation is in contrast with other microorganisms, where separate enzymes and pathways are present for the metabolism of the two sugars. A parallel part-phosphorylative Entner-Doudoroff pathway has also been described as an alternative route for glucose metabolism, and it has been demonstrated that this pathway is equally promiscuous for the metabolism of both glucose and galactose in S. solfataricus (12, 13). However, the pathways intersect at the KDG aldolase level, and thus the role and substrate specificity of glucose dehydrogenase is unchanged in both pathways.

Glucose dehydrogenase from S. solfataricus (SsGDH) has previously been assigned to the medium-chain alcohol/polyol dehydrogenase/reductase (MDR) branch of the superfamily of pyridine-nucleotide-dependent alcohol/polyol/sugar dehydrogenases (14). These enzymes are characterized by a chain length of 350–375 residues and conserved structural zinc-binding and nucleotide-binding sites. SsGDH has dual-cofactor specificity for NAD/NADP⁺ (9) and contains a GXGXXG motif (residues 188–193) characteristic of nucleotide-binding folds (15, 16). SsGDH has four conserved cysteine residues equivalent to the residues involved in the binding of a structural zinc ion in T. acidophilum glucose dehydrogenase (TaGDH) (17). SsGDH also possesses catalytic zinc coordinating residues, including Cys39 and His66, which align with equivalent residues present throughout the alcohol dehydrogenase family (18), with Gln150 being predicted to replace what is typically a second conserved cysteine as a zinc ligand. On this basis it has been predicted that SsGDH and TaGDH will oxidize their sugar substrates via a mechanism similar to that of other, characterized, MDR family members (17). Previous structural and mechanistic studies of MDR family members have centered on liver alcohol dehydrogenase (LADH) (19–21) and sorbitol dehydrogenase (SDH) (22, 23), whereas the structure of TaGDH has only been described in an apo form (17). Thus there are no currently available structures of an MDR family member in complex

8 This work was supported by a Biotechnology and Biological Sciences Research Council grant (to M. J. D., D. W. H., S. D. B., and G. L. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2cd9, 2cda, 2cdb, and 2cdc) have been deposited in the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ [http://www.rcsb.org/]).

1 To whom correspondence should be addressed. Tel.: 44-1334-467301; Fax: 44-1334-462595; E-mail: glt2@st-andrews.ac.uk.

2 The abbreviations used are: KDG, 2-keto-3-deoxygluconate; LADH, liver alcohol dehydrogenase; SDH, sorbitol dehydrogenase; MDR, medium-chain alcohol/polyol dehydrogenase/reductase; GDH, glucose dehydrogenase; TaGDH, T. acidophilum GDH; SsGDH, S. solfataricus GDH; MES, 4-morpholineethanesulfonic acid.
with a ring form sugar substrate. As such, current predictions on the structural basis of the promiscuity of this enzyme have been open to wide interpretation.

Finally, it is known that *Sulfolobus* species and a number of halophilic archaea are capable of utilizing pentoses as a carbon/energy source, although to date there has been little investigation into the route they catabolize xylose. In *Haloarcula marismortui*, xylose dehydrogenase is induced in the halophilic archaeon during growth on D-xylose (24). It was proposed that the oxidation of xylose by this enzyme, to produce xylonate, is the first step of xylose catabolism in this organism. This situation contrasts to the situation in bacteria, where the pentose phosphate pathway is the major route for xylose catabolism in this organism. This situation contrasts to the situation in bacteria, where the pentose phosphate pathway is the major route for xylose catabolism in this organism. This situation contrasts to the situation in bacteria, where the pentose phosphate pathway is the major route for xylose catabolism in this organism.

**EXPERIMENTAL PROCEDURES**

**Cloning of SsGDH and the Generation of T41A and T41V Mutant Proteins**—The cloning of wild-type SsGDH (gi: 3786221) into the pREC7 plasmid and the transformation of JM109 cells for expression has previously been described (9). Mutagenesis was performed on the SsGDH gene cloned into the NdeI and BamHI sites of expression vector pET-3a (Novagen). QuikChange site-directed mutagenesis (Stratagene) was employed with primer sequences 5'-GGTATTTGTGCT-GATAGAGGATAAGTTATGG-GCT-GTTGAGATGATGTTATGG-G3' (T41A) and 5'-GGTATTTGTTTTGGT-TGGTGTGAGATGATGTTATGG-G3' (T41V), following the manufacturer's protocol (mutations are indicated by bold italics). Sequenced clones containing the required mutation were transformed into BL21(DE3) cells.

**Expression and Purification of Recombinant SsGDH**—Expression and purification of wt-SsGDH has been described previously (26). Briefly, SsGDH was expressed in JM109 cells from the pREC7 plasmid at 37 °C without induction. After expression the protein was purified in four steps: heat treatment at 60 and 80 °C, followed by gel filtration on Superdex 75 26/60, anion exchange chromatography, and, finally, affinity chromatography using Reactive Red-500 dye affinity medium and an NaCl gradient of 0 to 1.5 M. Purified SsGDH samples were dialyzed into a final buffer of 50 mM Tris-base (pH 7.5), 20 mM MgCl₂ and concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the protein being expressed from the pET3A plasmid in BL21(DE3) cells at 37 °C without induction. After expression the protein was purified in four steps: heat treatment at 60 and 80 °C, followed by gel filtration on Superdex 75 26/60, anion exchange chromatography, and, finally, affinity chromatography using Reactive Red-500 dye affinity medium and an NaCl gradient of 0 to 1.5 M. Purified SsGDH samples were dialyzed into a final buffer of 50 mM Tris-base (pH 7.5), 20 mM MgCl₂ and concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience), with the concentration being verified at 3.5 mg/ml with a spin concentrator (VivaScience).

**Assay of Enzyme Activity**—Glucose dehydrogenase activity was determined spectrophotometrically by following the increase in absorbance at 340 nm, corresponding to the reduction of NAD(P)⁺ by over 3 min at...
Glucose Dehydrogenase Promiscuity

70 °C. Assays were performed in 100 mM HEPES buffer (pH 7.5 at 70 °C) containing 20 mM MgCl₂, 10 mM NAD⁺ or 1 mM NADP⁺, and 0–200 mM D-glucose, D-galactose, or D-xylose. Kinetic parameters were determined by the direct linear method of Eisenthal and Cornish-Bowden (27, 28).

Crystalization—The hanging drop, vapor-diffusion method was used for producing crystals. Hanging drops were formed by mixing 1 μL of protein solution with 1 μL of a mother liquor solution. The apo enzyme was crystallized using a mother liquor of 12% (v/v) polyethylene glycol 8000, 0.1 mM Tris-base (pH 8.0), and 4.5% (v/v) propan-2-ol. Orthorhombic crystals appeared after 2 days and grew to 0.3 × 0.1 × 0.1 mm in size. Crystals were frozen in a nitrogen gas stream after being soaked in 10%, followed by 20% (v/v) glycerol in mother liquor for 10–15 s each. Crystals of SsGDH (wt and T41A/T41V) in complex with NADP⁺ were formed by incubating the purified protein with 1 mM NADP⁺ for ~12 h at room temperature before crystallization with a mother liquor of 8% (v/v) polyethylene glycol 8000, 0.1 mM Tris-base (pH 8.0), and 4.5% (v/v) propan-2-ol. Orthorhombic crystals appeared after 3 days and grew to an average size of 0.2 × 0.15 mm in size. wt-NADP⁺-complexed crystals were frozen using the same conditions as the apo crystals. T41A/V mutant proteins in complex with glucose/xylose were obtained by stepwise equilibration of T41A/V-NADP⁺-co-crystals with 40 mM (2 min), 130 mM (2 min), 500 mM (10 min), and 800 mM (30 s) glucose/xylose dissolved in mother liquor, followed by cryoprotection with 20% (v/v) ethylene glycol in mother liquor with 800 mM glucose/xylose for 15 s before being frozen in the nitrogen gas stream.

Data Collection—Data were collected at the European Synchrotron Radiation Facility at a temperature of 100 K. Data were processed using MOSFLM (29) and scaled using Scala from the CCP4 version 5.0.2 Suite (30). The apo, NADP⁺-bound, and T41V-NADP⁺glucose/xylose structures were of space group P2₁2₁2₁ with a dimer in the asymmetric unit, whereas the structures of the T41A-NADP⁺glucose/xylose complexes were of space group P2₁ with a tetramer in the asymmetric unit. Data processing statistics are shown in Table 1.

Structure Solution and Refinement—The apo structure of SsGDH was solved to a resolution of 1.8 Å by molecular replacement using CNS (31), with a monomer of TaGDH (17) as the search model. The model phases were input to warpNtrace (32), which was able to build 683 out of 732 residues. The final refined model has R = 0.192 (Rfree = 0.229). The structure of SsGDH in complex with NADP⁺ was solved to a resolution of 2.3 Å by molecular replacement, using AMoRe (30, 33) with the apo-SsGDH structure as a search model. The final model has R = 0.195 (Rfree = 0.246). The structures of the T41A mutant protein in complex with NADP⁺ and glucose/xylose were solved by molecular replacement using AMoRe (30, 33). The final models have R = 0.194/0.204 (Rfree = 0.222/0.242) for the glucose/xylose-bound structures, to resolutions of 1.6 and 1.5 Å, respectively. In all cases, iterative model building in O (34), together with refinement in REFMAC (30), was carried out. All structures revealed varying levels of disorder in residues 51–58. Refinement statistics for all structures are shown in Table 1. Structures of the T41V mutant protein with NADP⁺ and complexed with glucose/xylose were also obtained; however, statistics for these structures are not reported, because refinement did not progress past initial rounds due to the higher quality of the T41A data.

The atomic coordinates and structure factors for the apo, NADP⁺, glucose, and xylose complex structures have been deposited in the RCSB Protein Data Bank (www.rcsb.org/pdb) under accession codes 2cd9, 2cdα, 2cdβ, and 2cdc, respectively.

RESULTS

Overall Structure—The crystal structure of SsGDH confirms the enzyme as a 160-kDa homo-tetramer (9, 17, 35) (Fig. 1A). The SsGDH monomer comprises 366 amino acids and possesses a secondary/tertiary structure closely resembling that of TaGDH, with which it shares a 34% sequence identity (17), with a root mean square deviation of 1.34 Å based on Ca positions. It is composed of two domains, a nucleotide-binding domain (residues 190–308) and a catalytic domain (residues 1–189 and 309–366) (Fig. 1B).

The catalytic domain is highly conserved between SsGDH and TaGDH, being formed by interactions between N-terminal and C-terminal segments. Residues 94–109, which lie between strands β9 and β10, coil to form a conserved structural zinc-binding lobe, with cysteine residues 93, 96, 99, and 107 coordinating to a well ordered zinc. Conserved catalytic zinc coordinating residues Cys⁶⁹ and His⁵⁶, along with Glu⁷⁵ within the β7 strand of the catalytic domain, lie within the cleft between this domain and the nucleotide-binding domain and were found to coordinate a zinc (occupancy of ~0.8) (Fig. 2A). A well ordered water molecule is found coordinating to this zinc and capable of forming additional interactions with residues Glu⁵⁴, His⁶⁶, and Glu⁷⁷. The locations of the zinc atom and water molecule were confirmed by calculating an anomalous difference Fourier map (data not shown), and suggest that the catalytic zinc could occupy the site of the coordinated water molecule, and vice versa, within the apo structure. A partially-occupied catalytic zinc site (0.6–0.7 occupancy) was also reported for sorbitol dehydrogenase (36). The positioning of the zinc atom within the SsGDH catalytic site, like that of TaGDH, more closely resembles the pattern of interacting residues seen in the apo human SDH structure (22), than the structures of LADH (21, 37).

The N-terminal segment of the catalytic domain is linked to the nucleotide-binding domain by a large central helix (α2), and the α3-helix connects the end of the nucleotide-binding domain to the C-terminal segment of the catalytic domain (Fig. 1B). The nucleotide-binding domain consists of a Rossmann fold containing six parallel strands and, as with the previously reported structures (17, 21, 22, 38), this sheet can be characterized by a left-handed twist of ~100° and forms the core of the Rossman fold. The GXXGXXG nucleotide-binding motif is located within the center of a deep cleft between the catalytic and nucleotide-binding domains, ~10 Å from the catalytic zinc ion (Figs. 1B and 2A). SsGDH assembles into a tetramer with His⁹⁷ from one monomer contributing to the active site of an adjacent monomer, leading to the narrowing of one end of the binding cleft. His⁹⁷ thus has the potential to interact with bound substrate. From comparison with the TaGDH structure, it would appear that the role of the structural zinc is also to maintain the tetrameric nature of GDH (17).

NADP⁺-bound Structure—Binding of NADP leads to a conformational change within the βD–αE loop (residues 252–260). As with SDH and LADH (21, 22), the NADP⁺ molecule adopts a linear shape closely matching the backbone trace of the nucleotide-binding motif. The NAD⁺ molecule makes several specific interactions with the nucleotide binding pocket of GDH, one being a stacking interaction between the nicotinamide ring and Phe²⁷⁷, whereas Phe²⁷⁷ moves to stack the other side of the nicotinamide ring (Fig. 2, A and B). This movement may in part result from interactions between the C3-hydroxyl of the cofactor ribose adjacent to the nicotinamide ring and the backbone nitrogen of Phe²⁷⁷. Three further interactions are made between the nicotinamide amide group and the backbone of Leu²⁰⁵, Phe²⁷⁷, and Asn²⁶⁷. Two hydrogen bonding interactions are made from the two bridging phosphate groups, with the phosphate closest to the nicotinamide ring being within 3.2 Å of...
the backbone nitrogen of Ile^{192} (part of the GXGXXG motif) and the phosphate closest to the adenosine ring interacting with Lys^{64} from the catalytic domain, as a result of a 2.3-Å shift in position of this side chain between the apo and NADP^{+}-bound states. Within the adenosine portion of the cofactor, the nucleotide hydroxyl forms two interactions, one to the backbone oxygen of Thr^{189} (which constitutes the first X of the GXGXXG motif) and the other to the side chain of Asn^{211}, which moves 2.5 Å from its apo position to form this interaction. As predicted by comparison with other NADP^{+}-preferring enzymes, Arg^{213} makes two hydrogen bonds to the 3'-phosphate of this nucleotide, whereas the oxygen linking the phosphate to the nucleotide is capable of forming two hydrogen bonds to Asn^{211}. Arg^{212} also moves from its apo position to avoid steric clashes and stacks against the adenosine ring. As predicted, the previously described movement of the side chain of Asn^{211} leads to both its O61 and N82 atoms being within ~3.3 Å of the adenosine 2-hydroxyl, thus stabilizing the 2'-phosphate and acting as a determinant for NADP^{+} specificity (14, 17, 21, 39).

In contrast to predictions based on LADH (20, 21, 40), but in agreement with human SDH (22), we do not observe any deprotection of the catalytic zinc, with a zinc-coordinated water molecule being observed at the same position as in the apo structure, and the coordination of the zinc remaining the same between the apo and cofactor-bound structures. This suggests that the water molecule in SsGDH may have a catalytic function equivalent to the proposed function for the water molecule in SDH (22). The anomalous signal for the NADP^{+}-bound SsGDH structure indicates a lesser movement of the catalytic zinc between the zinc binding position and the site that is occupied by the zinc-coordinated water molecule than was seen in the apo structure.

**Common Features of the Glucose/Xylose Complexes**—Because attempts to capture substrate or product within the SsGDH active site were unsuccessful, mutations that would decrease or abolish activity were designed based on the proposed LADH mechanism (19). This suggested hydride extraction from the substrate to the N4 position of the nicotinamide ring, with simultaneous proton abstraction to a conserved Thr/Ser (equivalent to Thr^{41} of SsGDH). Thus T41A and T41V mutations were made, and kinetic analysis showed that these mutations resulted in decreased, but not abolished, activity (Table 2), which lends support to the theory that this residue acts by optimizing the pK_a of the interactions as opposed to proton abstraction (17, 21). Both the T41A and
T41V mutations were successful in enabling the trapping of glucose and xylose in the active site of the enzymes when 800 mM soaks of co-crystallized SsGDH/H18528 crystals were carried out at room temperature, with the resulting sugar ring occupancies being 0.9. Attempts were made to trap galactose, but it was found to be less soluble in the crystallization buffers. Due to inferior data quality and resolution, the data from the T41V mutant protein are not included here beyond the kinetic explanations offered by the T41A structure for the T41V kinetics, although the T41A and T41V structures of SsGDH in complex with both NADP/glucose and NADP/xylose were nearly identical in both side-chain, main-chain, and sugar positioning.

In both the T41A-NADP-glucose and T41A-NADP-xylose structures, the NADP+ molecules in all subunits are positioned/configured in a near identical fashion to the NADP+ molecule from the wild-type NADP+ complex structure, and in addition, the βD–αE loop is in the cofactor-bound conformation as previously described. The anomalous Fourier transform maps indicate only one position for the catalytic zinc within the carbohydrate complex structures, suggesting that the binding of the sugar may further enforce one position for the catalytic zinc.

Glucose Complex—Examination of the T41A-NADP+ glucose structure revealed a well defined ring of density close to the catalytic zinc and the nicotinamide ring of the NADP+ cofactor (Fig. 3A). The pyranose ring is found in the “chair” configuration and is angled such that it stacks with the nicotinamide ring. The trapped glucose is in the βD-form, with the C1-hydroxyl in the equatorial configuration; this brings the C1 of the glucose ring to within 3.7 Å of the reactive C4 position of the nicotinamide ring, which is ideal for hydride transfer. Closer examination of the nicotinamide ring indicated a slight degree of puckering to the ring, perhaps implying partial reduction to NADPH (41, 42). In the glucose complex, the C1-hydroxyl is able to form direct interactions with the zinc-coordinated water molecule (3.4 Å) and is 3.7 Å from the catalytic zinc and 3.9 Å from the S atom of Cys39, which is coordinated to the zinc. It also hydrogen-bonds to an additional water molecule that in turn interacts with a hydroxyl group of the nicotinamide ribose.

FIGURE 2. A, nucleotide-binding groove of SsGDH (apo structure). The nucleotide-binding domain is shown in red, with the position of the GXXGXXG motif shown in yellow. The catalytic domain is shown in blue, with the zinc ion shown as a magenta sphere and the green sphere representing the zinc-coordinated water molecule. Residues lining the nucleotide-binding groove are shown with gray carbons, while the C-monomer is shown in wheat color, with His39 highlighted by black carbons. B, nucleotide-binding groove of SsGDH (NADP+–bound structure). Coloring is as for A, but with the NADP+ molecule shown with purple carbons, red oxygens, blue nitrogens, and yellow phosphates. Unbiased Fc − Fc difference electron density is shown as a green mesh (contoured at 2.25σ). Hydrogen bonds between the protein and NADP+ are shown as black broken lines.
These interactions are unlikely to have been effected by the mutation. Had Thr⁴¹ not been mutated to alanine, an additional hydrogen bond would have been available to the nicotinamide ring and interacts with just two water molecules. Thus fewer hydrogen bonds can be made to α-xylose than β-xylose, and models suggest that in α-glucose/α-xylose the C1-hydroxyl would block hydride extraction. In addition, the C1-hydroxyl of α-xylose would not be capable of interacting with any of the residues predicted to carry out the proton abstraction.

The xylene complexed structure also contains an additional four xylene molecules, almost exclusively in the α-form, far from the active site at the monomer interfaces and sitting within a small “pocket” lined mainly with uncharged polar/hydrophobic residues. All interactions other than a hydrogen-bond between the C1-hydroxyl of the xylene and the backbone of Lys⁵³⁷ are either with water molecules or are water-mediated. It is unlikely that there is a physiological relevance to the presence of these additional sugar molecules.

**DISCUSSION**

Although the substrate promiscuity of SsGDH has been recently described (9), the majority of structural studies on members of the MDR family have focused on the oxidation of straight-chain substrates, and the structure of TaGDH was reported in the apo state (17). Crystallization details of apo, binary, and ternary complexes of GDH from *Haloflexa mediterranei* have been reported, but there are currently no published molecular details (43, 44). Hence the structures described in this report represent the first examples of ring form substrates in complex with an MDR family member. By mutating a residue, which from previous MDR family member studies was suggested to play an important role in the oxidation process, by way of accepting the proton from the substrate (19), we have been able to observe SsGDH in complex with both D-glucose and D-xylose. These structures enable prediction of the features contributing to the promiscuity of this enzyme, similar to the studies carried out on the 2-keto-3-deoxygluconate aldolase from *S. sulfataricus* (45). The structures also provide evidence that the enzyme would be active with only one anomer of the sugars, while suggesting that it is able to select for this anomer preferentially. In addition, the observations provide support to the proposal that D-xylose is a natural substrate of this enzyme. Finally, it has been possible to use the substrate-complexed structures to propose a mechanism of oxidation for SsGDH and homologous glucose dehydrogenase enzymes, by comparison with the mechanisms proposed for horse LADH and human SDH.

**α:β Selection and Kinetics**—The crystal structures of the SsGDH T41A mutant protein in complex with NADP⁺ and D-glucose/D-xylose suggest a role for Thr⁴¹ in either selection for binding the β (equatorial) form of the sugars, or in aiding the conversion from the α- to the β-form by providing favorable interactions for this form. In the equatorial position, the C1-hydroxyl is liable to form productive interactions, by way of hydrogen bonds with Thr⁴¹, the catalytic zinc-coordinated water, and a further water molecule; however, it is positioned 3.7 Å from the C4 position of the nicotinamide ring, with no intervening hydroxyl group to prevent the hydride transfer. The α-anomer, as seen in the structure of SsGDH in complex with NADP⁺ and D-xylose and from modeling α-D-glucose into the active site, is unable to make these hydrogen bonding interactions and, as such, would be expected to bind with lower affinity than the β-D-forms of the sugars. In addition, in the α-form, the C1-hydroxyl would come between the C1 position and the reactive position of the NADP⁺ nicotinamide ring, thus preventing hydride transfer. Finally, an axial C1-hydroxyl would be unable to interact with the residues predicted to accept the proton (19, 22). As such it would appear that GDH would only be active against one C1-hydroxyl anomer, and that the "productive" form of the substrate is the β-form, for which selection is optimized.

**TABLE 2**

**Kinetic parameters of SsGDH at 70 °C**

Reactions were carried out at pH 7.5 with 10 mM NAD+ or 1 mM NADP+ in 100 mM HEPES buffer containing 20 mM MgCl₂. 1 unit corresponds to the formation of 1 μmol of NAD(P)H per minute.

| Substrate  | Cofactor | Kₐ for sugar mₒ | Vₘₐₓ units/mg | s⁻¹ | kₐ | kₑₐ/Kₐ |
|-----------|---------|-----------------|---------------|-----|----|--------|
| Glucose   | NAD⁺    | 1.50 (±0.05)    | 110 (±5)      | 75  | 50 |        |
|           | NADP⁺   | 1.30 (±0.05)    | 70 (±2)       | 48  | 57 |        |
| Galactose | NAD⁺    | 0.57 (±0.01)    | 90 (±1)       | 61  | 108|        |
|           | NADP⁺   | 0.44 (±0.01)    | 55 (±1)       | 37  | 85 |        |
| Xylose    | NAD⁺    | 0.25 (±0.01)    | 90 (±5)       | 61  | 245|        |
|           | NADP⁺   | 0.18 (±0.01)    | 65 (±3)       | 44  | 246|        |

| T41A      |        |                |               |     |    |        |
|-----------|--------|----------------|---------------|-----|----|--------|
| Glucose   | NAD⁺   | 24.8 (±0.8)    | 57 (±0.4)     | 39  | 1.6|        |
|           | NADP⁺  | 33.3 (±0.5)    | 20 (±1)       | 14  | 0.4|        |
| Galactose | NAD⁺   | 118 (±1)       | 82 (±0.8)     | 56  | 0.5|        |
|           | NADP⁺  | 109 (±4)       | 33 (±0.7)     | 22  | 0.2|        |
| Xylose    | NAD⁺   | 29.2 (±0.6)    | 120 (±1)      | 81  | 2.8|        |
|           | NADP⁺  | 20.4 (±0.4)    | 33 (±0.2)     | 22  | 1.1|        |

| T41V      |        |                |               |     |    |        |
|-----------|--------|----------------|---------------|-----|----|--------|
| Glucose   | NAD⁺   | 72.5 (±1.1)    | 9 (±0.06)     | 6   | 0.09|        |
|           | NADP⁺  | 59.0 (±1.6)    | 6 (±0.10)     | 4   | 0.07|        |
| Galactose | NAD⁺   | 204 (±6)       | 12 (±0.2)     | 8   | 0.04|        |
|           | NADP⁺  | 175 (±6)       | 11 (±0.2)     | 7   | 0.04|        |
| Xylose    | NAD⁺   | 76.3 (±1.6)    | 17 (±0.1)     | 12  | 0.15|        |
|           | NADP⁺  | 65.8 (±2.4)    | 11 (±0.2)     | 7   | 0.11|        |

These values were reported previously (9).
Glucose Dehydrogenase Promiscuity

FIGURE 3. A, glucose bound to the SsGDH active site in the A-monomer. Coloring is as in Fig. 2B with the mutation T41A highlighted by orange carbons and the glucose molecule shown with purple carbons and red oxygens, with both C6-hydroxyl conformations. Unbiased F$_{o}$ − F$_{c}$ electron density for the substrate is shown as green mesh (contoured at 2.25 σ). Hydrogen bonds between the protein and glucose are shown as broken black lines, and gray broken lines indicate interactions of 3.5–3.7 Å that are possible hydrogen bonds at the moment of catalysis. Asp$^{154}$ sits below the sugar ring interacting with the C2- and C3-hydroxyls. B, xylose bound to the SsGDH active site of monomer A. Coloring is as in A, but the glucose molecule is shown in the equatorial β-form with purple carbons and red oxygens, and in the axial (α-form) with wheat-colored carbons. Unbiased F$_{o}$ − F$_{c}$ electron density for the substrate is shown as green mesh (contoured at 2.25 σ). Hydrogen bonds between the protein and β-xylose are shown as broken black lines, and gray broken lines indicate interactions of ~3.5–3.7 Å that are possible hydrogen bonds at the moment of catalysis. Hydrogen bonds to the α-form are not shown, because most, with the exception of the C1-OH interactions, are maintained and no new hydrogen bonds are formed in the α-form. C, superposition of glucose (green) and xylose (blue) in the active site of the A-monomer. The two positions for O6 of glucose are displayed, as are the two positions of O1 of xylose. Glu$^{114}$ undergoes a conformational change between the glucose and xylose complex structures; the alternative position for this residue in the xylose structure is depicted in wheat-colored carbons.
TABLE 3
Details of key interactions between SsGDH and glucose or xylose

| Sugar atom | Glucose | Xylose |
|------------|---------|--------|
| C1         | Nicotinamide C4 | 3.7 | 4.1 |
| O1, β-anomer | Zinc-H2O O5 | 2.4 | 3.2 |
| O2         | Zinc-H2O       | 2.6 | 2.6 |
| O3         | Asp154 O62     | 2.6 | 2.6 |
| O4         | Glu114 O6e     | 3.1 | 3.0 |
| O6         | Glu114 Oe1     | 3.2 | 3.2 |
| O6 (alternate position) | His1100 O6e2 | 3.3 | 3.3 |

Zinc-H2O refers to the zinc-coordinated water molecule. Distances are in Å.

In part the structures account for the observed alterations in kinetic parameters when Thr41 is mutated (Table 2). The increase in $K_m$ for the T41A mutant protein is likely to be as a result of the loss of an important hydrogen bond to the C1-hydroxyl, coupled to a decreased ability to select the productive $\beta$ (equatorial)-form of the sugars (leading to inhibition by the $\alpha$-form). Decreases in $K_m$ and $K_{cat}/K_m$ may be caused by the loss of the proposed ability of this interaction to “optimize” the pK$\alpha$ of the adjacent substrate atoms for proton/hydride extract. The further increase in $K_m$ for the sugars observed when Thr41 is mutated to a valine is possibly due to steric hindrance of the methyl groups of valine with the C1-hydroxyl of the $\beta$-sugar, which is not observed in the wild-type or T41A mutant proteins. It is also possible that the T41A mutation results in a lesser degree of selection for the active anomer, in turn decreasing the catalytic rate due to inhibition from the non-productive form. In the crystal structures of the T41V mutant protein (data not shown), the active sites exhibit the sugar molecules in approximately the same $\alpha:\beta$ ratio as seen in the T41A mutant protein structures, thus any decrease in selectivity is too weak to be detected at the temperatures at which these soaks were performed. It is possible that the reason for the successful trapping of soaked sugars in the T41A mutant protein as opposed to the wild-type enzyme is not only due to a decrease in turnover of the substrate but is also influenced by a higher effective soaking concentration due to the enzyme being more readily able to bind both anomers. Clearly the enzyme still favors the $\beta$-form, because this is the only form seen in the glucose-bound structures, and is the major form seen in the active sites of the xylose structures, although the relative $\alpha:\beta$ equilibrium in the soaking solutions may also be relevant.

Comparisons of the xylose-complexed structure, where there are mixed $\alpha:\beta$ forms of the sugar present, with the glucose-complexed structure, where the glucose is present almost entirely as $\beta$-d-glucose, have important implications for the studies on the relative rates of SsGDH with differing substrates. There is no greater selection pressure for the $\beta$-form in the glucose-complexed structure than there is the xylose-complexed structure, and it could be that the differences observed may be due to differing $\alpha:\beta$ ratios within the complex sugar solutions used in the crystal soaking.

Promiscuity—The substrate profile of SsGDH has been extensively described (9), with the enzyme being able to oxidize a range of five and six carbon sugars. The consensus was that this enzyme has a requirement for substrates to match the stereo configuration of glucose at the C2 and C3 positions, while the C4-hydroxyl may be in the glucose or galactose epimer positions, the C5/C6 position has no specific stereo requirements, and the C1-hydroxyl position (axial/equatorial, i.e. $\alpha$ or $\beta$ sugar forms) was unknown. From the structures described in this study, it can be seen that there is a common orientation/position for both the five and six carbon sugars and that the majority of interactions between enzyme and either substrate are indeed made between the C1-, C2-, and C3-hydroxyls. From modeling experiments it can be seen that, should the C2- or C3-hydroxyls be present in alternate epimers from those seen in d-glucose, then the majority of hydrogen bonds (in total approximately nine) would be lost, leading to a greatly decreased affinity of the enzyme for these sugars.

Alternate epimers of the C2-hydroxyl would be unlikely to form any described interactions, including those with the zinc-coordinated water molecule, with only the potential to form a hydrogen bond to His66, thus explaining the specificity for this configuration at the C2 position. These observations account for the very low level of activity (<1% of the activity of the enzyme with glucose) with 2-deoxy-d-glucose and NAPD$^+$ as the cofactor (9), as critical interactions to the zinc-coordinated water, Glu150 and Asp154, would be lost. In further studies we detected no decrease in activity of the enzyme with glucose when in the presence of 2-deoxy-d-glucose, indicating a lack of competition of 2-deoxy-d-glucose with glucose for the binding site (data not shown) and, hence, a lack of binding that accounts for the low activity.

An alternate configuration for the C3-hydroxyl would lead to the loss of all interactions other than those to Asp154, although it could potentially bring the hydroxyl within 3 Å of the oxygen of the nicotinamide amide group of NAPD$^+$. The large number of interactions from the C2/C3 positions also provides an explanation for the lack of activity against l-glucose and l-xylose.

Modeling experiments of the C4 epimer of glucose (galactose) show that, in the galactose configuration, the C4-hydroxyl would still be able to form one hydrogen bond to Glu114, although it would be unable to hydrogen bond to Asn307. However, the model shows that, in the galactose configuration, a second hydrogen bond to Glu114 would be likely, and thus there are no structural reasons for the difference in affinity or catalytic rate between glucose and galactose.

GDH binds both five- and six-carbon sugars in a similar manner, and it appears that the difference in $K_m$ between the C5 and C6 sugars, or between a C6 sugar and its 6-deoxy equivalent, is due to the loss of one hydrogen bonding interaction. From the d-glucose complex structure it can be seen that there are two potential interactions within the C6-hydroxyl region, which are positioned such that the hydroxyl can only interact with one or other at a given time. It is possible that glucuronic acid, with a carboxyl group at the 6 position, would bind with higher affinity than is seen for glucose. As with galactose, it is not possible to explain from the structure why there is such a large difference in the activity of the enzyme with xylose when compared with d-glucose and 6-deoxy-d-glucose (9), because both d-xylose and 6-deoxy-d-glucose are predicted from our structures to result in the loss of just one hydrogen bond. It is also not possible from the structures presented here to determine why SsGDH shows greater activity for some substrates (d-glucose and l-arabinose) with NAPD$^+$ as a cofactor but with other substrates NAD$^+$ has a far greater activity (l-xylose and l-fucose) (9).

Mechanistic Implications—Oxidation of the carbohydrate in GDH involves transfer of a hydride ion from the C1 position of the substrate to NADP$^+$, coupled to proton abstraction from the hydroxyl group at C1. In contrast to the LADH structures and the 3.0-Å structure of rat SDH (23), but in agreement with the crystal structures of human SDH (22), the structures presented here indicate that the primary coordination sphere of the catalytic zinc includes a water molecule, Glu67, His66, and Cys39 (SsGDH numbering) and that the zinc is not deprotected by cofactor binding (46), suggesting a potential role for the coordinated water. In addition, the arrangement of residues interacting with the catalytic zinc, along with the mutagenesis data presented here, rules out Thr41 as being the proton accep-
Glucose Dehydrogenase Promiscuity

tor in this enzyme, as proposed for LADH, instead suggesting that it utilizes a similar mechanism to that of human SDH. However, unlike human SDH we do not see movement of the catalytic zinc upon substrate binding (22, 46), and the catalytic zinc remains tetra-coordinated. In addition, in StGDH and a number of other glucose dehydrogenase enzymes (S. acidocaldarius and Picrophilus torridus), Glu150 of human SDH is replaced by a glutamine (Gln150 in StGDH), which would be unable to perform the role of proton acceptor suggested for the equivalent glutamate. Gln150 has been confirmed by mass spectrometric methods to not be deamidated at high temperatures, and thus it is unable to accept the proton (data not shown). In StGDH, the substrate C1-hydroxyl lies 3.7 Å from the catalytic zinc, and 3.4 Å from the zinc-coordinated water, and so interactions between the catalytic zinc and the substrate could occur via the coordinated water. It is, however, conceivable that these structures do not capture the moment of proton abstraction/hydride transfer and that during the oxidation process the substrate C1-hydroxyl approaches the inner sphere of zinc coordination, making it penta-coordinated, and enabling the mechanism to proceed as described for human SDH (22). In these scenarios, the zinc-coordinated water would, as a hydroxide, accept the proton and would need to be stabilized by Glu150, a conserved residue that also interacts with the zinc, or would pass the proton to the solvent once the product had left the active site.

Acknowledgments—We thank the staff at the European Synchrotron Radiation Facility and the European Union for funds to access the facility, the St. Andrews Biomolecular Sciences mass spectrometry and proteomics facility for mass spectrometry verification of the identity of the purified/crystallized protein, and James Naismith for useful discussions.

REFERENCES
1. Verhees, C. H., Kengen, S. W., Tuininga, J. E., Schut, G. J., Adams, M. W., De Vos, W. M., and Van Der Oost, J. (2003) Biochem. J. 375, 231–246
2. Grogan, D. W. (1989) J. Bacteriol. 171, 6710–6719
3. DeRosa, M., Gambaccorta, A., Nicolaus, B., Giardina, P., Poerio, E., and Buonocore, V. (1984) Biochem. J. 224, 407–414
4. Selig, M., Xavier, K. B., Santos, H., and Schonheit, P. (1997) Arch. Microbiol. 167, 217–232
5. Budgen, N., and Danson, M. J. (1986) FEBS Lett. 196, 207–210
6. Siebers, B., and Hensel, R. (1993) FEMS Microbiol. Lett. 111, 1–8
7. Elshafei, A. M. (1989) Acta Biotechnol. 9, 485–489
8. Elzainy, T. A., Hassan, M. M., and Allam, A. M. (1973) J. Bacteriol. 114, 457–459
9. Lambie, H. J., Hough, D. W., and Danson, M. J. (2003) J. Biol. Chem. 278, 34066–34072
10. Lambie, H. J., Milburn, C. C., Taylor, G. L., Hough, D. W., and Danson, M. J. (2004) FEBS Lett. 576, 133–136
11. Kim, S., and Lee, S. B. (2005) Biochem. J. 387, 271–280
12. Lambie, H. J., Theodossis, A., Milburn, C. C., Taylor, G. L., Hough, D. W., and Danson, M. J. (2005) FEBS Lett. 579, 6865–6869
13. Ahmed, H., Ettema, T. J., Tjaden, B., Greirling, A. C., van der Oost, J., and Siebers, B. (2005) Biochem. J. 390, 529–540
14. Edwards, K. J., Barton, J. D., Rossjohn, J., Thorn, J. M., Taylor, G. L., and Ollis, D. L. (1996) Arch. Biochem. Biophys. 328, 173–183
15. Rao, S. T., and Rossmann, M. G. (1973) J. Mol. Biol. 76, 241–256
16. Wierenga, R. K., De Maeyer, E. C. H., and Hol, W. G. J. (1985) Biochemistry 24, 1346–1357
17. John, J., Creennell, S. J., Hough, D. W., Danson, M. J., and Taylor, G. L. (1994) Structure 2, 385–393
18. Johnsen, U., and Schonheit, P. (2004) J. Biol. Chem. 279, 6198–6207
19. Soderberg, T. (2005) Archacta 1, 347–352
20. Theodossis, A., Milburn, C. C., Heyer, N. I., Lamble, H. J., Hough, D. W., Danson, M. J., and Taylor, G. L. (2004) Acta Crystallogr. F61, 112–115
21. Cornish-Bowden, A., and Edelhoch, R. (1974) Biochem. J. 139, 712–730
22. Edelhoch, R., and Cornish-Bowden, A. (1974) Biochem. J. 139, 715–720
23. Novaza, J. (1994) Acta Crystallogr. Sect. A 50, 157–163
24. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
25. Giardina, P., de Biasi, M. G., de Rosa, M., Gambaccorta, A., and Buonocore, V. (1986) Biochem. J. 239, 517–522
26. Jeffery, J., Chesters, J., Mills, C., Sadler, P. J., and Jovin, H. (1984) EMBO J. 3, 357–360
27. Ryder, U. (1995) Protein Sci. 4, 1124–1132
28. Rossman, M. G., Doras, D. O., and Olsen, K. W. (1974) Nature 249, 194–199
29. Scrutton, N. S., Berry, A., and Perham, R. N. (1990) Nature 348, 38–43
30. Eklund, H., Horjales, E., Jovin, H., Branden, C. L., and Jeffery, J. (1985) Biochemistry 24, 8005–8012
31. Reis, K., Allard, S. T., Hegeman, A. D., Murshudov, G., Philp, D., and Naismith, J. H. (2003) J. Am. Chem. Soc. 125, 11872–11878
32. Meijers, R., Morris, R. J., Adolph, H. W., Merli, A., Lamzin, V. S., and Cedergren-Zeppezauer, E. S. (2001) J. Biol. Chem. 276, 9516–9521
33. Ferrer, J., Fisher, M., Burke, J., Sedelnikova, E. E., Baker, P. J., Gilmore, D. J., Bonete, M. J., Pire, C., Esclapez, J., and Rice, D. W. (2003) Acta Crystallogr. D. Biol. Crystallogr. 59, 1877–1882
34. Esclapez, J., Britton, K. L., Baker, P. J., Fisher, M., Pire, C., Ferrer, J., Bonete, M. J., and Rice, D. W. (2005) Acta Crystallogr. Struct. F. Struct. Biol. Commun. 61, 743–746
35. Theodossis, A., Walden, H., Westwick, E. J., Connaris, H., Lamble, H. J., Hough, D. W., Danson, M. J., and Taylor, G. L. (2004) J. Biol. Chem. 279, 43886–43892
36. Li, H., Hallows, W. H., Punzi, J. S., Pankiewicz, K. C., Watanabe, K. A., and Goldstein, B. M. (1994) Biochemistry 33, 11734–11744