Catalytic mechanism of Zn²⁺-dependent polyol dehydrogenases: kinetic comparison of sheep liver sorbitol dehydrogenase with wild-type and Glu¹⁵⁴ → Cys forms of yeast xylitol dehydrogenase

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Co-ordination of catalytic Zn²⁺ in sorbitol/xylitol dehydrogenases of the medium-chain dehydrogenase/reductase superfamily involves direct or water-mediated interactions from a glutamic acid residue, which substitutes a homologous cysteine ligand in alcohol dehydrogenases of the yeast and liver type. Glu¹⁵⁴ of xylitol dehydrogenase from the yeast *Galactocandida mastotermitis* (termed GmXDH) was mutated to a cysteine residue (E154C) to revert this replacement. In spite of their variable Zn²⁺ content (0.10–0.40 atom/subunit), purified preparations of E154C exhibited a constant catalytic Zn²⁺ centre activity (k_{cat}) of $1.19 \pm 0.03 \text{ s}^{-1}$ and did not require exogenous Zn²⁺ for activity or stability. E154C retained $0.019 \pm 0.003 \%$ and $0.74 \pm 0.03 \%$ of wild-type catalytic efficiency ($k_{\text{cat}}/K_{\text{sorbitol}} = 7800 \pm 700 \text{ M}^{-1} \cdot \text{s}^{-1}$) and $k_{\text{cat}} (=161 \pm 4 \text{ s}^{-1})$ for NAD⁺-dependent oxidation of sorbitol at 25 °C respectively. The pH profile of $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C decreased below an apparent pK of 9.1 ± 0.3 , reflecting a shift in pK by about +1.7–1.9 pH units compared with the corresponding pH profiles for GmXDH and sheep liver sorbitol dehydrogenase (termed sSDH). The difference in pK for profiles determined in

¹H₂O and ²H₂O solvent was similar and unusually small for all three enzymes ($\approx +0.2 \log$ units), suggesting that the observed pK in the binary enzyme–NAD⁺ complexes could be due to Zn²⁺-bound water. Under conditions eliminating their different pH-dependences, wild-type and mutant GmXDH displayed similar primary and solvent deuterium kinetic isotope effects of 1.7 ± 0.2 (E154C, 1.7 ± 0.1) and 1.9 ± 0.3 (E154C, 2.4 ± 0.2) on $k_{\text{cat}}/K_{\text{sorbitol}}$ respectively. Transient kinetic studies of NAD⁺ reduction and proton release during sorbitol oxidation by sSDH at pH 8.2 show that two protons are lost with a rate constant of $687 \pm 12 \text{ s}^{-1}$ in the pre-steady state, which features a turnover of 0.9 ± 0.1 enzyme equivalents as NADH was produced with a rate constant of $409 \pm 3 \text{ s}^{-1}$. The results support an auxiliary participation of Glu¹⁵⁴ in catalysis, and possible mechanisms of proton transfer in sorbitol/xylitol dehydrogenases are discussed.

Key words: alcohol dehydrogenase, catalysis, medium-chain dehydrogenases/reductases, proton relay, zinc ligand.

INTRODUCTION

The MDRs (medium-chain dehydrogenases/reductases) constitute a large superfamily of basically two protein types, with and without Zn²⁺. They are comprised of different Zn²⁺-dependent ADHs (alcohol dehydrogenases), historically exemplified by enzymes of the yeast and liver type [1,2]. Among these ADHs, the PDHs (polyol dehydrogenases) and related forms establish a distinct enzyme family and include as their main activities, SDH (sorbitol dehydrogenase; EC 1.1.1.14) and XDH (xylitol dehydrogenase; EC 1.1.1.9) [1,2].

PDHs are functional homotetramers in which each subunit contains a single Zn²⁺ that is bound in the active site and is required for catalysis [3]. The ligand field of Zn²⁺ in PDHs differs from that of horse liver [4] and yeast ADH (PDB code: 2HCY) where a conserved triad of two cysteine residues and one histidine residue constitutes, together with a water molecule, the primary co-ordination sphere. Residue Cys¹⁷⁴ (using the horse liver enzyme numbering) is replaced by a glutamic acid residue (position 155 in human liver SDH) which is conserved among the PDHs [3,4]. X-ray structures of human liver SDH [5] and the related NADP(H)-dependent whitefly ketose reductase [6] [which, like the NAD(H)-dependent SDHs, catalyses the interconversion of sorbitol and fructose, however, with a preference for keto group reduction] reveal that this glutamic acid residue is not a ligand

to the Zn²⁺, but co-ordinates the metal ion via a water molecule (Figure 1). In contrast, a 3.0 Å (1 Å = 0.1 nm) structure of rat liver SDH determined the glutamic acid residue to be linked directly [7], consistent with suggestions from earlier mutagenesis data for the same enzyme [8] and electronic absorption studies of human and sheep liver SDHs [9,10]. Another glutamic acid residue (position 70 in human SDH) is a Zn²⁺ ligand in all three PDH structures, whereas the corresponding Glu⁶⁸ of horse liver ADH is not co-ordinating in resting enzyme complexes [11]. However, computational studies suggest that Glu⁶⁸ could co-ordinate transiently during catalysis, allowing product release to take place efficiently [12]. The structure of human liver SDH bound with NADH and an inhibitor that is competitive against fructose shows that the inhibitor chelates the Zn²⁺ through an oxygen and a nitrogen atom and that Glu⁷⁰ is dissociated from the now pentacoordinated metal ion [5].

What is the exact role of the positional exchange Cys → Glu in the Zn²⁺ ligand field of PDHs compared with that of ADHs? As proposed originally by Eklund et al. [4], a strong hydrogen bond between the negatively charged glutamic acid side chain and the primary hydroxy group adjacent to the secondary hydroxy group undergoing reaction could be an essential element of PDH-specific substrate-binding recognition and catalysis. The X-ray structure of the inhibitor complex of human liver SDH [5] (Figure 1) supports this notion by showing a very short distance

Abbreviations used: ADH, alcohol dehydrogenase; KIE, kinetic isotope effect; MDR, medium-chain dehydrogenase/reductase; PDH, polyol dehydrogenases; SDH, sorbitol dehydrogenase (D-sorbitol:NAD⁺ 2-oxidoreductase, EC 1.1.1.14); sSDH, sheep liver SDH; SEC, size-exclusion chromatography; XDH, xylitol dehydrogenase (xylitol:NAD⁺ 2-oxidoreductase, EC 1.1.1.9); GmXDH, XDH from *Galactocandida mastotermitis*.

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Figure 1 Participation of Glu¹⁵⁵ in Zn²⁺ co-ordination by human liver SDH

The figure was derived from the 2.0 Å crystal structure of enzyme bound with NADH and an inhibitor (PDB: 1PL6) [5]. Residues within a 4 Å distance to Zn²⁺ are depicted. Black and grey dashed lines indicate co-ordination to Zn²⁺ and hydrogen bonding respectively.

(2.4 Å) between the side chain of Glu¹⁵⁵ and the primary hydroxy group on the inhibitor which is also in co-ordination distance to the Zn²⁺ (2.4 Å). Kinetic evidence for a Glu → Cys variant of rat SDH, reported by Karlson and Höög [8], is also consistent with a major function of glutamic acid in substrate binding because the site-directed replacement caused a large (500-fold) decrease in sorbitol binding affinity (K_{sorbitol}), whereas the catalytic centre activity (k_{cat}) of the mutant was even ≈ 4 times higher than that of the wild-type. In another study of sLSDH (sheep liver SDH), analogues of sorbitol in which the C1 hydroxy group was replaced with a hydrogen, amino or thiol group were converted with an efficiency of between 0.36% to 4.8% of $k_{\text{cat}}/K_{\text{sorbitol}}$ at pH 7.4 [13]. The effect of substrate modification on decreasing k_{cat}/K was mainly due to an increase in apparent K , also suggesting that the hydroxy group of sorbitol is required for binding. A second possibility is that the replacement of the thiol group ligand in ADH by a carboxy [7] or a H₂O/hydroxy group ligand [5] in PDH alters the electrophilic character of Zn²⁺ and therefore, changes the propensity of the metal ion to bind alcohol and stabilize the proposed alkoxide intermediate state of the reaction through inner-sphere co-ordination. Relevant information could come from kinetic studies, including KIEs (kinetic isotope effects) and pH dependencies for the enzymatic reactions, as has been shown in earlier studies on ADH [14].

In the present study we report on the functional consequences of replacing Glu¹⁵⁴ of GmXDH (XDH from the yeast *Galactocandida mastotermitis*) with a cysteine residue. GmXDH is a representative microbial member of MDR-type PDHs and catalyses the NAD⁺-dependent oxidation of xylitol into D-xylulose, this reaction representing the second step of the catabolic pathway for xylose in the yeast [15]. The Glu¹⁵⁴ of GmXDH is homologous in the sequence to Glu¹⁵⁵ of human SDH (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/404/bj4040421add.htm>). We also describe a detailed steady-state kinetic comparison, using results of pH and KIE studies, of wild-type and E154C forms of GmXDH with the well-characterized sLSDH [3,16,17]. The evidence obtained supports a role for Glu¹⁵⁴ as a modulator of, but not its direct participation in, general base catalysis to

enzymatic alcohol oxidation. Zn²⁺-bound water, through which Glu¹⁵⁴ is possibly linked with the catalytic metal, would be a likely candidate to fulfil the function of the catalytic base. The glutamic acid residue is additionally important, but not essential, for substrate binding. Analysis of transient reactions catalysed by sLSDH suggests a kinetic scenario for the proton transfer involved in the catalytic mechanism, and possible routes of proton exchange between enzyme and bulk solvent are discussed.

EXPERIMENTAL

Materials

Unless mentioned otherwise, all materials and chemicals have been described elsewhere [18]. ²H₂O solvent (99.9% ²H) and D-sorbitol were from Sigma–Aldrich. [2-²H]D-sorbitol was prepared by enzymatic reduction of D-fructose according to a protocol reported previously [18]. Unlabelled reference material was synthesized in exactly the same way. [¹H] and [²H]sorbitol contained a small amount of unreacted D-fructose ($\approx 8\%$), as shown by HPLC analysis with a Bio-Rad HPX-87C column operated at 80 °C using water as eluent, and refractive index detection. Deuterium labelling at position 2 of sorbitol was estimated by NMR to be $\geq 99\%$. sLSDH was obtained from Sigma–Aldrich (# S-3764) and used without further purification.

Mutagenesis and enzyme production

E154C is a GmXDH mutant in which Glu¹⁵⁴ is replaced by a cysteine residue. Numbering for GmXDH starts with 1 for the initiator methionine. The site-directed mutation Glu¹⁵⁴ → Cys was introduced with the PCR-based overlap extension method [19] using the plasmid vector pBTac1 [20] containing the gene encoding wild-type GmXDH as the template. Briefly, two fragments of the target gene sequence were amplified with Synergy DNA polymerase (Genecraft) in separate PCR reactions. Each reaction used one flanking primer carrying the appropriate restriction site (indicated in bold) that hybridized at one end of the target sequence: primer 1, 5'-CCGAAACAGA**AATTC**ATGTCTA-3'; primer 2, 5'-GTTCGAC**GGATC**CTTACTC-3'; and one internal primer that hybridized at the site of the mutation and contained the mismatched bases (indicated by underlining): primer 1, 5'-CTTAAAGGACAA**ACT**AAAGC-3'; primer 2, 5'-GCTTTAG-TTTGTCCTTTAAAG-3'. After extension and amplification of the overlapping fragments, the resulting construct was digested with EcoRI and BamHI, and the desired band was gel-purified and subcloned into the appropriately digested expression vector. Plasmid mini-prep DNA was sequenced to verify that the desired mutation had been introduced and that no misincorporation of nucleotides had occurred as a result of DNA polymerase errors. Recombinant wild-type and mutant proteins were produced in *Escherichia coli* JM 109 transformed with the respective expression plasmid. Wild-type GmXDH was purified as described elsewhere [18]. E154C was isolated using a modified two-step procedure consisting of dye-ligand affinity chromatography followed by preparative gel filtration. To avoid contamination of E154C by wild-type enzyme, all purifications of the mutant were carried out with Procion Red HE3B (Red 120) freshly immobilized on Sepharose 4B-CL. Fractions of eluted protein containing XDH activity were brought to 50 mM potassium phosphate (pH 7.0), and concentrated by ultrafiltration. Aliquots of 200 μ l of a ~ 10 mg/ml solution of E154C were loaded on a Superdex 200 SEC (size-exclusion chromatography) column (GE Healthcare) equilibrated with 50 mM potassium phosphate buffer

(pH 7.0). Both the applied sample and the elution buffer contained 2 mM NAD⁺ to stabilize the enzyme during SEC.

Steady-state kinetic studies

Initial rates of NAD⁺-dependent sorbitol oxidation were determined by measuring the increase in absorbance at 340 nm with a Beckman DU-650 spectrophotometer at 25 °C. The enzyme was diluted in the assay such that a plot of absorbance against reaction time was linear for at least 1 min. Reactions were always started by adding the enzyme. If not stated otherwise, a 100 mM Tris/HCl buffer (pH 9.0) was used. Apparent kinetic parameters were determined at a constant saturating concentrations of sorbitol (300 mM for GmXDH, 25 mM for sLSDH and 2 M for E154C) or NAD⁺ (10 mM for all enzymes), and various concentrations of NAD⁺ or sorbitol. In the presence of a saturating NAD⁺ level, wild-type GmXDH and sLSDH showed partial substrate inhibition at sorbitol concentrations of > 300 mM and > 24 mM respectively. The reported kinetic parameters of GmXDH and sLSDH are from measurements at non-inhibiting substrate concentrations, covering the range 0.1–15 times and 0.1–8 times the apparent K_m for sorbitol respectively.

pH and kinetic isotope effects

pL-dependences (where L is ¹H or ²H) of kinetic parameters for sorbitol oxidation by wild-type enzymes and the E154C mutant were determined in ¹H₂O or ²H₂O solvent. If not stated otherwise, a solution containing the dry buffer components indicated below was prepared and titrated to the desired pL, using NaOL or LCl. The p²H was determined using the relation p²H = pH meter reading + 0.4 [21]. Alternatively, deuterated buffer was prepared by ¹H → ²H exchange whereby protonic buffer mixture alone (100 mM Mes and 100 mM Tris) or containing 10 mM NAD⁺ was taken to dryness by freeze-drying, and salts were redissolved in ²H₂O immediately prior to use. Thus the p²H values of prepared deuterated buffers differed from those of the corresponding protonic buffers that were treated in exactly the same way by 0.6 pL units.

The pL-dependence studies for sLSDH and GmXDH were performed in the pL range 5.2–9.0 using buffer mixtures of 100 mM Mes and 100 mM Tris. Variation in ionic strength across the chosen pL range was small ($I \approx 0.10$ M), and control experiments in which 25 mM NaCl was added to the assays revealed the absence of significant ionic strength effects under the conditions used. The pL-dependence of E154C was analysed in the pL ranges 7.1–9.0 and 9.0–10.0 using 100 mM Tris and 100 mM glycine respectively. Ionic strength effects were not controlled. However, rate measurements in the presence of 25 mM NaCl and the agreement between kinetic parameters determined in Tris and glycine buffers (at pL 9.0), suggest that the observable pH-dependence was not strongly affected by relevant changes in ionic strength. All initial rates were recorded at various concentrations of sorbitol in the presence of 10 mM NAD⁺. The reactions were started by adding 10 µl of enzyme (2% of total volume of assay) from a concentrated protein stock solution in 20 mM Tris/HCl buffer (pL 7.5). For use in ²H₂O, the enzymes were gel-filtered employing deuterated 20 mM Tris/HCl buffer and allowed to equilibrate with solvent for 0.5 h before the initial-rate measurement. Although the completeness of ¹H → ²H exchange at protonic sites on the enzymes was not controlled, it was shown that extension of the equilibration time in deuterated solvent from 0.5 to 10 h did not affect the activity and therefore solvent KIEs (see below). Because of concerns regarding enzyme stability, exchange of protein protons for deuterons via freeze-drying was

not considered. Michaelis–Menten parameters were calculated from initial rates measured at each value of pL, and data obtained in ¹H₂O and ²H₂O were treated separately. Solvent KIEs on kinetic parameters were obtained from different sets of experiments.

For determination of KIEs, initial rates of sorbitol oxidation with unlabelled and deuterium-labelled sorbitol substrate or solvent were compared under exactly identical conditions. Because the presence of ≈ 8% fructose in the enzymatically prepared [¹H]sorbitol did not affect kinetic parameters of wild-type enzymes, in comparison with results obtained with commercial sorbitol, the level of contamination in unlabelled and deuterium-labelled sorbitol was assumed to be compatible with the determination of primary deuterium KIEs. Solvent KIEs were determined under conditions in which the enzymatic rate (v) was independent of pL where $dv/dpL \approx 0$. Calculation of KIEs on k_{cat} and $k_{cat}/K_{sorbitol}$ was performed according to the method of Cook and Cleland [22].

Stopped-flow measurements

Rapid mixing stopped-flow experiments were carried out at 25 °C using an Applied Photophysics SX.18MV Stopped-Flow Reaction Analyser equipped with a 20 µl flow cell giving a path-length of 1 cm. The enzyme was gel-filtered twice to a reaction buffer (pH 8.0) composed of 0.5 mM Tris/HCl, 3.2 µM Phenol Red, 10 mM NAD⁺ and 59 mM NaCl. The ionic strength of this buffer equalled that of 100 mM Tris/HCl (pH 8.0). The substrate solution contained 60 mM sorbitol dissolved in reaction buffer lacking enzyme. Transient reactions were started by mixing 100 µl of solution containing the pre-formed enzyme–NAD⁺ complex (6–16 µM sLSDH) with 100 µl of substrate solution, and the time course of formation of NADH or proton release was monitored at 340 nm or 556 nm respectively. Data acquisition was performed with Applied Photophysics software. Under the conditions used, the instrument had a dead time of approximately 1.5 ms. Appropriate controls lacking either sLSDH or sorbitol were recorded under exactly the same conditions. Calibration of the Phenol Red pH indicator was performed by titrating the reaction buffer with HCl. A plot of absorbance change at 556 nm against the proton concentration was linear, within a 95% confidence interval, in the range of 1–100 µM protons with a slope value of 430 M⁻¹ · cm⁻¹.

Data processing

Parameter values were calculated by fitting the appropriate equation to the data, using non-linear least squares regression analysis with the SigmaPlot 2001 program version 7.0. Eqn (1) describes a mechanism with one ionizable group where both protonated and unprotonated forms can react. Y is a kinetic parameter, k_L and k_H are limiting values at low and high pH respectively and $k_H > k_L$, K is an apparent acid dissociation constant and is the point where Y has the average value of k_L and k_H , and $[H^+]$ is the proton concentration. Eqn (2) describes a simple mechanism in which one ionizable group has to be unprotonated to be active and the highest activity is attained at k_H [23].

$$\log Y = \log\{(k_H + k_L[H^+]/K)/(1 + [H^+]/K)\} \quad (1)$$

$$\log Y = \log\{k_H/(1 + [H^+]/K)\} \quad (2)$$

$$v = k_{cat}[E][S]/\{K_{sorbitol}(1 + [I]/K_i) + [S]\} \quad (3)$$

$$[NADH]_t \text{ or } [H^+]_t = Y_0 + \Pi(1 - e^{-k_{obs}t}) + k_{ss}t \quad (4)$$

Eqn (3) describes linear competitive inhibition where $[I]$ is the inhibitor concentration and K_i is the apparent dissociation constant of I . $[E]$ was calculated from a molecular mass of the enzyme subunit of 37.8 kDa and was based on measurements of protein concentration (sLSDH) or the concentration of Zn^{2+} associated with the protein preparation (GmXDH; E154C). Eqn (4) was used to fit the pre-steady state and steady-state phases of multitransformation time traces recorded in stopped-flow experiments [24]. Data recorded at 340 nm and 556 nm were converted into $[NADH]$ and $[H^+]$ using molar extinction coefficients of $6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $4307 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively. k_{obs} and Π represent the transient rate constant and the product burst respectively; k_{ss} corresponds to the steady-state rate constant, t is the reaction time and Y_0 is the starting point of reaction.

Other methods

Metal analysis of protein samples was performed with inductively coupled plasma MS, as described previously [15]. Protein concentrations were determined using the Bio-Rad dye-binding assay.

RESULTS

Characterization of E154C

Purification of E154C from the *E. coli* cell extract required a protocol different from that utilized for isolation of the wild-type [18]. Results are summarized in Supplementary Table S1 (at <http://www.BiochemJ.org/bj/404/bj4040421add.htm>), and elution profiles of E154C during dye-ligand chromatography and the subsequent SEC are shown in Supplementary Figures S2 and S3 (at <http://www.BiochemJ.org/bj/404/bj4040421add.htm>) respectively. E154C was obtained with an overall yield of $\approx 62\%$. The presence of 2 mM NAD^+ was essential to prevent enzyme inactivation during SEC. Supplementary Figure S4 (at <http://www.BiochemJ.org/bj/404/bj4040421add.htm>) shows SDS/PAGE analysis of purified E154C. The apparent molecular mass of the subunit of E154C was 38.7 kDa, which is in agreement with a molecular mass of 37.4 kDa calculated from the amino acid sequence.

Purified E154C eluted from an analytical gel-filtration column as a single protein peak with an apparent molecular mass of 160 kDa that contained all of the applied protein and enzyme activity. In a similar manner to the wild-type, the mutant seemed to be a functional homotetramer. Different preparations of E154C ($n = 4$) that appeared homogeneous in SDS/PAGE (see Supplementary Figure S4 at <http://www.BiochemJ.org/bj/404/bj4040421add.htm>) contained between 0.1 and 0.4 atom Zn^{2+} /protein subunit. Although the source of this variable Zn^{2+} content remained unknown, the activity of E154C solutions based on the molarity of Zn^{2+} -containing active sites was constant across the different enzyme preparations. Exogenous Zn^{2+} (added as $ZnSO_4$) in the concentration range 1–100 μM was a strong irreversible inhibitor of wild-type and the mutant, the IC_{50} value of about $5 \pm 1 \mu\text{M}$ being identical for both enzymes. Note that E154C was stable during the initial rate assays described below, suggesting that inactivation due to release of the active-site Zn^{2+} did not occur in the time-span of the kinetic experiments.

Kinetic parameters for sorbitol oxidation by GmXDH and E154C (corrected for the gram equivalent of bound Zn^{2+}) are summarized in Table 1 along with the corresponding parameters for sLSDH. Values of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C were $0.74 \pm 0.03\%$ and $0.019 \pm 0.003\%$ of the corresponding wild-type values. However, the Michaelis–Menten constant for NAD^+ was

Table 1 Kinetic parameters of sLSDH, GmXDH and E154C mutant for NAD^+ -dependent oxidation of sorbitol determined at pH 9.0 and $25.0 \pm 0.1^\circ\text{C}$

*Calculation of kinetic parameters for GmXDH and E154C is based on the molarity of Zn^{2+} -bound protein subunits.

Parameter	E154C*	GmXDH*	sLSDH
k_{cat} (s^{-1})	1.19 ± 0.03	161 ± 4	28 ± 1
K_{sorbitol} (mM)	785 ± 82	21 ± 2	3.0 ± 0.2
$k_{\text{cat}}/K_{\text{sorbitol}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	1.5 ± 0.2	7800 ± 700	9500 ± 700
K_{NAD^+} (μM)	290 ± 35	430 ± 40	500 ± 99
$k_{\text{cat}}/K_{\text{NAD}^+}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	4100 ± 510	$3.7 \times 10^5 \pm 4 \times 10^4$	$5.7 \times 10^4 \pm 1.1 \times 10^4$

the same, within limits of experimental error, in wild-type and E154C forms of GmXDH. sLSDH differed from GmXDH mainly in the value of K_{sorbitol} which was about one order of magnitude higher in the yeast enzyme.

pL-dependence studies

pL profiles of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ for sLSDH and GmXDH are shown in Figures 2(A)–2(C). With the exception of the pL profile for k_{cat} of GmXDH (results not shown) which exhibited a complex pL-dependence that was not further pursued, all other pL profiles were sigmoidal, showing a decrease in rate from a constant value at high pH to a smaller constant value at low pH. These values were fitted with Eqn (1), and the results are presented in Table 2. The pL profiles of $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ also displayed a decrease in rate at low pL but, unlike wild-type GmXDH and sLSDH, the activity of the mutant was lost completely below pK (Figure 2D). The data were fitted with Eqn (2), and results are presented in Table 2. The pL profiles were obtained with an experimental design in which determination of kinetic parameters was performed at several pL values that were, however, always the same in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. The solvent isotope effect on buffer pK implies that the buffer ratio was different in the two solvents at each pL of measurement [25]. To clearly demonstrate that these pL profiles were valid, we recorded kinetic parameters of GmXDH at different buffer ratios that were constant in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. Results are shown in Figure 2(C), superimposed on the data obtained using constant-pL measurements. Also note that because full pL-rate profiles were recorded with each enzyme, the solvent isotope effect on buffer pK will not interfere with determination of pK values for the enzymatic reactions in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$.

KIE studies

Table 3 summarizes primary deuterium KIEs on k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ for reactions catalysed by sLSDH as well as wild-type and E154C forms of GmXDH at pH 9.0. KIEs on kinetic parameters of GmXDH at pH 7.5 are also shown in Table 3. Solvent KIEs were measured at pL 9.0 (sLSDH, wild-type GmXDH) or pL 10.0 (E154C) where pL profiles of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ approach plateau values and thus differences in pL profiles of the respective kinetic parameter in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ are eliminated (wild-type GmXDH; sLSDH) or minimized (E154C). Instability of E154C precluded initial rate measurements at pL > 10.0. The solvent KIE on the apparent dissociation constant of the binary complex of sLSDH and NADH (K_{NADH}) was determined from initial rate studies in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ where NADH was used as product inhibitor of the NAD^+ -dependent oxidation of sorbitol. Under conditions

Figure 2 pH profiles of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ for sISDH and of $k_{\text{cat}}/K_{\text{sorbitol}}$ for wild-type and E154C mutant forms of GmXDH

● and ○ indicate measurements in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ solvent respectively. Error bars give the S.D. of the respective kinetic parameter, and lines are non-linear fits of the data with the appropriate equation (see Table 2). In (C), △ indicates measurements with deuterated buffers that had been prepared through $^1\text{H} \rightarrow ^2\text{H}$ exchange via freeze-drying. ▲ Shows corresponding measurements in $^1\text{H}_2\text{O}$. Insets show double-log plots of the data to illustrate differences in the shape of the pH profiles at low pH, (C), sigmoidal; (D), non-sigmoidal.

in which $[\text{NAD}^+]$ was varied and [sorbitol] was constant and saturating, NADH was a linear competitive inhibitor against NAD^+ . The value of K_{iNADH} in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$, obtained from fits of Eqn (3) to the data, was $20 \pm 4 \mu\text{M}$ and $10 \pm 2 \mu\text{M}$ respectively, yielding a solvent KIE on K_{iNADH} of $2.0 \pm 0.6 (= 20/10)$.

Transient kinetics

Results of KIE studies suggested that sISDH was the most practical enzyme with which to perform transient kinetic experiments. Because $^{\text{D}}k_{\text{cat}}$ and $^{\text{D}}k_{\text{cat}}/K_{\text{sorbitol}}$ (where $^{\text{D}}$ denotes the primary deuterium KIE on the respective parameter) were similar at pH 9.0, the dissociation constant of sorbitol from sISDH– NAD^+ cannot be much higher than $K_{\text{sorbitol}} (= 3.0 \text{ mM})$. Rapid mixing of a concentrated sorbitol solution ($\geq 100 \text{ mM}$) with a solution of the enzyme in reaction buffer lacking NAD^+ produced high absorbance noise at 340 nm during the first 4 ms, probably

because of differences in viscosity of the two solutions. Using sISDH and a sorbitol concentration of 60 mM, we could achieve full saturation of the enzyme in the steady state and, at the same time, avoid the long effective dead times. This would have not been possible with wild-type and E154C forms of GmXDH.

Figure 3 shows typical stopped-flow progress curves of NADH production and proton release, recorded by the increase of $A_{340\text{nm}}$ and the decrease in $A_{556\text{nm}}$ respectively, obtained under conditions of limiting concentration of sISDH ($8 \mu\text{M}$) that allowed multiple turnover of the enzyme present. Pre-formed enzyme– NAD^+ was used, and the concentrations of NAD^+ and sorbitol were saturating. Both time courses exhibited burst kinetics where an initial exponential increase in product formation during the first 6–10 ms was followed by a steady-state rate of product appearance, which in the case of NADH production was in excellent agreement with expectations from results of initial rate studies. The proton release rate in the steady state was identical with that of coenzyme reduction, as required by the enzymatic

Table 2 pK values from pL profiles for sISDH, GmXDH and E154C mutant

pL profiles of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ for sISDH and GmXDH were fitted with Eqn (1). pL profiles of $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C were fitted with Eqn (2). n.d., not determined. *Superscripts indicate measurements in $^1\text{H}_2\text{O}$ ($^1\text{H}_2\text{O}$) and $^2\text{H}_2\text{O}$ ($^2\text{H}_2\text{O}$); †correlation coefficients from non-linear regression analysis are given in parenthesis.

log (Parameter)*	pK	Limiting values (s^{-1} ; $\text{M}^{-1} \cdot \text{s}^{-1}$)
sISDH		
$k_{\text{cat}}^{^1\text{H}_2\text{O}}$	7.10 ± 0.15 (0.99)†	$k_{\text{L}} = 3.5 \pm 0.3$ $k_{\text{H}} = 26 \pm 2$
$k_{\text{cat}}^{^2\text{H}_2\text{O}}$	7.3 ± 0.3 (0.96)	$k_{\text{L}} = 3.1 \pm 0.4$ $k_{\text{H}} = 13 \pm 1$
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^1\text{H}_2\text{O}}$	7.15 ± 0.05 (0.999)	$k_{\text{L}} = 75 \pm 11$ $k_{\text{H}} = 8980 \pm 245$
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^2\text{H}_2\text{O}}$	7.3 ± 0.1 (0.99)	$k_{\text{L}} = 69 \pm 21$ $k_{\text{H}} = 6813 \pm 533$
GmXDH		
$k_{\text{cat}}^{^1\text{H}_2\text{O}}$	n.d.	-
$k_{\text{cat}}^{^2\text{H}_2\text{O}}$	n.d.	-
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^1\text{H}_2\text{O}}$	7.4 ± 0.1 (0.99)	$k_{\text{L}} = 17 \pm 8$ $k_{\text{H}} = 6484 \pm 351$
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^2\text{H}_2\text{O}}$	7.6 ± 0.1 (0.99)	$k_{\text{L}} = 11 \pm 4$ $k_{\text{H}} = 4275 \pm 406$
E154C		
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^1\text{H}_2\text{O}}$	9.1 ± 0.3 (0.995)	$k = 4.1 \pm 0.5$
$(k_{\text{cat}}/K_{\text{sorbitol}})^{^2\text{H}_2\text{O}}$	9.3 ± 0.2 (0.99)	$k = 2.6 \pm 0.2$

Table 3 Primary ^2H and solvent KIEs on kinetic parameters for sorbitol oxidation by sISDH, GmXDH and E154C at pL 9.0 and $25.0 \pm 0.1^\circ\text{C}$

*Superscripts D and D_2O indicate primary and solvent ^2H KIEs respectively. n.a., not analysed. ‡primary deuterium KIEs determined at pH 7.5; §determined at pL 10.0.

Parameter*	E154C	GmXDH	sISDH
$^2\text{H} k_{\text{cat}}$	n.a.	1.16 ± 0.03 (1.1 ± 0.1)‡	1.13 ± 0.08
$^2\text{H} k_{\text{cat}}/K_{\text{sorbitol}}$	2.4 ± 0.2	1.20 ± 0.07 (1.9 ± 0.3)‡	1.25 ± 0.14
$^2\text{H} k_{\text{cat}}/K_{\text{NAD}^+}$	n.a.	1.2 ± 0.1	0.9 ± 0.2
$^2\text{H}_2\text{O} k_{\text{cat}}$	n.a.	1.49 ± 0.05	2.1 ± 0.1
$^2\text{H}_2\text{O} k_{\text{cat}}/K_{\text{sorbitol}}$	1.7 ± 0.2 §	1.7 ± 0.1	1.5 ± 0.3
$^2\text{H}_2\text{O} k_{\text{cat}}/K_{\text{NAD}^+}$	n.a.	1.1 ± 0.1	1.0 ± 0.2

reaction studied, sorbitol + $\text{NAD}^+ \rightarrow$ fructose + $\text{NADH} + \text{H}^+$. The multiple turnover progress curves were fitted with Eqn (4), yielding apparent first-order rate constants of $409 \pm 3 \text{ s}^{-1}$ and $687 \pm 12 \text{ s}^{-1}$ for NADH production and proton loss respectively. In the burst phase, 0.9 ± 0.1 enzyme equivalents of NADH were produced. The enzyme equivalent of protons released in the pre-steady state was 1.8 ± 0.2 , hence twice that of NADH. Note that the reported values are averages from seven independent stopped-flow experiments.

DISCUSSION

The Zn^{2+} centre of human liver SDH differs from that of horse liver ADH mainly in the substitution of the Cys¹⁷⁴ ligand [4] by a water molecule linked through the secondary sphere Glu¹⁵⁵ [5]. (In the discussion that follows we do not consider a role for the glutamic acid residue in directly co-ordinating the Zn^{2+} , as suggested by the 3.0 Å structure [7] and mutagenesis data of the closely related SDH from rat [8].) Little is known about how the primary co-ordination sphere of the active-site Zn^{2+} influences the

Figure 3 Stopped-flow progress curves of formation of NADH and release of proton during NAD^+ -dependent oxidation of sorbitol catalysed by sISDH

Traces with the higher signal-to-noise ratio are proton release measurements. Non-linear fits of the individual progress curves with Eqn (4) are shown as grey lines. Controls in which sorbitol was lacking and no reaction took place are also shown.

various steps in the catalytic mechanism of these two, and related, MDR enzymes. We have therefore substituted the relevant Glu¹⁵⁴ of GmXDH with cysteine residues to mimic in the sequence the Zn^{2+} ligation pattern of ADH and carried out a detailed kinetic comparison of wild-type and E154C mutant enzymes using pH and deuterium KIE studies. To establish relationships between microbial XDH and mammalian SDH with regard to reaction mechanism, we examined sISDH in a parallel series of kinetic experiments. Purified E154C was shown to have retained a functional Zn^{2+} centre that displayed fractional saturation with Zn^{2+} of ≤ 0.4 . The catalytic efficiency of the mutant for NAD^+ -dependent oxidation of sorbitol at pH 9.0 was decreased $10^{3.7}$ -fold in comparison with the corresponding wild-type value, suggesting that Glu¹⁵⁴ is relatively more important for catalytic function than Zn^{2+} binding.

pL-dependences of wild-type enzymes and E154C

k_{cat} for sISDH and $k_{\text{cat}}/K_{\text{sorbitol}}$ for GmXDH and sISDH in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ displayed sigmoidal pH-dependences with level asymptotes at high and low pH. There are several mechanisms that fit the observed profiles, but the simplest is one in which a single ionizable group is present on enzyme- NAD^+ and both unprotonated and protonated forms can react. (The sorbitol substrate does not ionize in the pL range studied.)

Results of our pH profile analysis for sISDH in $^1\text{H}_2\text{O}$ (Table 2) agree with earlier findings of Lindstad and McKinley-McKee [17]. It was shown by these authors that the k_{cat} of sorbitol oxidation by sISDH was limited by the dissociation of NADH over the pH range 5.2–9.9 [17]. The pH profile for the rate constant of NADH release was sigmoidal with an apparent pK of 7.45, which we calculate from the two pK values of 7.1 and 7.7, given in [17], as $\text{pK} = (\text{pK}_1 + \text{pK}_2)/2$. From fits of the pH profile of k_{cat} with Eqn (1), we obtained a nearly identical pK of 7.10 ± 0.15 . The previously reported pH profile for $k_{\text{cat}}/K_{\text{sorbitol}}$ displayed a pK of 7.1, also consistent with a pK value of 7.15 ± 0.05 found in the present study. In spite of the similar pK values observed in the pH

profiles of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$, there was a much larger decrease in $k_{\text{cat}}/K_{\text{sorbitol}}$ (120-fold) than k_{cat} (7.5-fold) on going from the high-pH plateau to the one at low pH.

The p*K* values for the fall of k_{cat} and $k_{\text{cat}}/K_{\text{sorbitol}}$ at low pH were both elevated by $\approx +0.2$ pH units in ²H₂O solvent. The observed solvent isotope effect on the kinetically determined p*K* was lower than expected ($\Delta\text{p}K \approx +0.48$) if the observed pH-dependence of the enzyme was due to the ionization of a group such as imidazole, carboxy or amino [26]. The comparison of p*L* profiles in ¹H₂O and ²H₂O may thus assist in the assignment of the apparent p*K* values to groups, as follows.

For sLSDH, the H₂O/hydroxy ligand to Zn²⁺ and a histidine residue were considered to be candidate groups whose ionization could determine the pH-rate profile of the enzyme [17]. The present results support the Zn²⁺-bound water because the $\Delta\text{p}K$ for it in ²H₂O is supposedly smaller than +0.48. Quinn and Sutton [26,27] report that certain hydrated metal ions such as Fe³⁺, Gd³⁺ and La³⁺ have $\Delta\text{p}K$ values of $\leq +0.3$. Likewise, the $\Delta\text{p}K$ of Co²⁺-water in carbonic anhydrase appears to be also small [28]. (However, the kinetically determined $\Delta\text{p}K$ for carbonic anhydrase was $\approx +0.5$ [29]). With regard to Zn²⁺-water, Welsh et al. [30] reported that p*L*-dependencies of k_{cat} for NAD⁺-dependent oxidation of *p*-methoxybenzyl alcohol by yeast ADH displayed a $\Delta\text{p}K$ ($= 8.35 - 8.14 = +0.21$) in ²H₂O. They suggested that the small $\Delta\text{p}K$ could be due to the hydrated metal ion. Note that because catalysis is rate-limiting in the reaction of the yeast enzyme, a comparison of p*L* profiles of k_{cat} for yeast ADH and $k_{\text{cat}}/K_{\text{sorbitol}}$ for sLSDH is meaningful. Northrop and Choi [31] showed that the deuterium fractionation factor, Φ , of Zn²⁺-H₂O for enzyme-NAD⁺ of yeast ADH is approximately 0.56. (Φ is an isotope exchange equilibrium constant [26]). Welsh et al. [30] described that the effect of solvent deuteration on the p*K* of a protonic species BH can be expressed by the relation $\Delta\text{p}K = \log [\Phi(\text{BH})/\Phi(\text{H}_3\text{O}^+)]$. Because $\Phi(\text{H}_3\text{O}^+) = 0.33$ [26], we can estimate that $\Delta\text{p}K$ for Zn²⁺-OH₂ is $+0.23$ ($= \log 0.56/0.33$). However, in spite of the seemingly excellent agreement between the calculated $\Delta\text{p}K$ of Zn²⁺-OH₂ and the kinetically determined $\Delta\text{p}K$ for sLSDH-NAD⁺ in ²H₂O, it must be emphasized that the possible kinetic complexity of $k_{\text{cat}}/K_{\text{sorbitol}}$ and the hydrogen-bonded system through which Zn²⁺-water is linked with protein residues (see Figure 1) make the assignment of apparent p*K* to group uncertain. Solvent isotope effect studies of horse liver ADH provide a note of caution because unlike the yeast enzyme, $\Delta\text{p}K$ values for the decrease in rate in p*L* profiles of k_{cat} and k_{cat}/K for NAD⁺-dependent oxidation of cyclohexanol at high p*L* were $+1.39 \pm 0.21$ [32].

pH profiles of GmXDH were similar to those of sLSDH. The apparent p*K* describing the decrease of $k_{\text{cat}}/K_{\text{sorbitol}}$ in ¹H₂O and ²H₂O solvent was 7.4 ± 0.1 and 7.6 ± 0.1 respectively. Asymptote levels of $k_{\text{cat}}/K_{\text{sorbitol}}$ at high and low pH differed by a factor of 372. The pH-dependence of $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C was clearly distinguished from that for the wild-type, showing an upshift in p*K* by $+1.7$ pH units to a value of 9.1 ± 0.3 . In ²H₂O, the p*K* was 9.3 ± 0.2 , hence again a $\Delta\text{p}K$ ($= +0.2$) that was significantly smaller than +0.48. The pH profile of the mutant did not decrease to an asymptote level at low pH, suggesting that unlike the wild-type, the protonated form of E154C does not react. Considering the $\Delta\text{p}K$ of $+0.2$ in ²H₂O solvent, the observable p*K* values of 7.4 ± 0.1 and 9.1 ± 0.3 in the NAD⁺ complexes of wild-type and E154C forms of GmXDH respectively are tentatively assigned to a Zn²⁺-bound water. Mutation of Glu¹⁵⁴ did not abolish the pH-dependence because Zn²⁺-water is still expected to be present in E154C. However, the carboxylate side chain of the glutamic acid residue could facilitate deprotonation of Zn²⁺-water through a proton relay or hydrogen bonding, whereas Cys¹⁵⁴, due to the

smaller size and the higher p*K* of its side chain, would not be able to fulfil the analogous function.

Interpretation of primary deuterium and solvent KIEs

At the optimum pH of 9.0, primary deuterium KIEs on kinetic parameters for NAD⁺-dependent oxidation of sorbitol by GmXDH and sLSDH were small or not significantly different from unity. This result implies that with both enzymes, hydride transfer was not rate-limiting for the overall reaction ($^Dk_{\text{cat}}$) as well as for the catalytic cascade ($^Dk_{\text{cat}}/K_{\text{sorbitol}}$) which includes all steps from sorbitol binding to enzyme-NAD⁺ up to the dissociation of fructose from enzyme-NADH. Release of NADH was probably the slowest step in both enzymatic reactions under k_{cat} conditions, consistent with previous suggestions for the kinetic mechanism of sLSDH [16] and GmXDH [15,18].

The KIE on $k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C at pH 9.0 was 2.4 ± 0.2 , indicating that the chemical step of hydride transfer had become partly rate-limiting for the relevant sequence of reaction steps as a consequence of the site-directed replacement. However, the differences in $^Dk_{\text{cat}}/K_{\text{sorbitol}}$ for E154C and wild-type at pH 9.0 appear to reflect solely the altered pH-dependence in the mutant. ($^Dk_{\text{cat}}/K_{\text{sorbitol}}$) values are similar at the p*K* of the wild-type enzyme-catalysed reaction (pH 7.5) and at the p*K* of the E154C-catalysed reaction (pH 9.0).

At p*L* 9.0, values of $^{D2O}k_{\text{cat}}$ (where D2O indicates the solvent KIE) for sLSDH and wild-type GmXDH were significantly greater than unity. K_{inADH} for sLSDH determined under the same p*L* conditions also showed a sizable solvent KIE, and $^{D2O}K_{\text{inADH}} \approx ^{D2O}k_{\text{cat}}$. The net rate of NADH release (k'_{NADH}) is thought to control k_{cat} of sLSDH ([17] and the present study) and contributes to the value of K_{inADH} . The results therefore suggest that k'_{NADH} is sensitive to solvent deuteration. For sLSDH and wild-type GmXDH at p*L* 9.0, there was a much larger solvent KIE on $k_{\text{cat}}/K_{\text{sorbitol}}$ than on $k_{\text{cat}}/K_{\text{NAD}^+}$, indicating that proton transfers within the steps of $k_{\text{cat}}/K_{\text{sorbitol}}$ could be partly rate-limiting. Under conditions where $k_{\text{cat}}/K_{\text{sorbitol}}$ did not show a p*L*-dependence, values of $^{D2O}k_{\text{cat}}/K_{\text{sorbitol}}$ for wild-type and E154C forms of GmXDH were almost identical, suggesting that Glu¹⁵⁴ is not directly involved in catalytic proton transfer. If it were, a Glu → Cys substitution would arguably make the proton transfer more rate-limiting, detectable as a significant increase in $^{D2O}k_{\text{cat}}/K_{\text{sorbitol}}$ for E154C in comparison with wild-type. We note that the thiol group of cysteine has a fractionation factor in ²H₂O of ≈ 0.5 [21], but involvement of the side chain of Cys¹⁵⁴ as a protonic site in the active centre of E154C at p*L* 10.0 is not probable. The results of the KIE studies suggest that Glu¹⁵⁴ participates in, but is not essential for hydrogen transfer in the catalytic reaction of GmXDH.

Hydrogen transfer in the catalytic mechanism, and proposed participation of Glu¹⁵⁴

Multiple turnover stopped-flow kinetic experiments for sorbitol oxidation by sLSDH detected a pre-steady state burst of formation of NADH which corresponded to $\approx 90\%$ of the enzyme present. This observation requires that there be a slow step after hydride transfer to NAD⁺ and is fully consistent with evidence from steady-state kinetics reported in the present study and by others [17]. Analysis of the kinetic transient also showed that proton transfers during the reduction of enzyme-bound NAD⁺ are not rapid equilibrium processes. Their rate is comparable with that of hydride transfer to NAD⁺, and relative estimation of the corresponding k_{obs} values reveals that proton loss appears to be slightly faster than coenzyme reduction, suggesting that proton dissociation from Zn²⁺-bound sorbitol could precede the transfer

Figure 4 Alternative proton shuttle pathways for SDH/XDH

The structure of human liver SDH is shown (PDB: 1PL6) [5]. Hydrogen-bonded networks connecting the active site with bulk water are indicated with arrows. Black and grey dashed lines indicate co-ordination to Zn^{2+} and hydrogen bonding respectively.

of hydride ion from alcohol to NAD^+ . The proposed timing of hydrogen transfer steps involved in the chemical oxidation of alcohol substrate by sSDH may thus be very similar to that of liver ADH [33].

Analysis of the stopped-flow progress curves in Figure 3 also revealed that two protons are lost per NADH produced in the pre-steady state. Various steps of the mechanism of sSDH are known to be pH-dependent [17], and a reasonable explanation for the observed proton stoichiometry, consistent with proton release kinetics and in agreement with recent results for liver ADH [34,35], is that formation of the reactive ternary complex is kinetically coupled with deprotonation of the sSDH- NAD^+ complex. The weak pH-dependence of k_{cat} for sSDH and GmXDH is in agreement with this notion.

Rapid proton release may be facilitated by a catalytic base acting through a proton-relay system. Pauly et al. [5] have proposed a mechanism of human SDH in which Zn^{2+} -bound water functions as a base, abstracting a proton from the reactive 2'-hydroxy group of sorbitol. Results of our pL-dependence studies support a catalytic base function of Zn^{2+} -water. The side chains of Glu⁷⁰ and Glu¹⁵⁴ which were both hydrogen-bonded with Zn^{2+} -water in a model of the ternary complex [5] could participate in a novel type of proton-relay system that connects Glu⁷⁰ with

aqueous solvent, as shown in Figure 4 and discussed below. The suggested role of Glu¹⁵⁴ (Glu¹⁵⁵ in human SDH) in the catalytic mechanism of PDH enzymes is summarized in Scheme 1.

At the optimum pH for sorbitol oxidation, Zn^{2+} -water is deprotonated in the enzyme- NAD^+ complex and hydrogen bonds with the negatively charged side chain of Glu¹⁵⁴ (Scheme 1A). When sorbitol binds, the carboxylate of Glu¹⁵⁴ forms another hydrogen bond with the substrate C1 hydroxy group, which probably helps in positioning the reactive C2 alcohol of the substrate and the catalytic groups on the enzyme, in particular Zn^{2+} and the hydroxy group co-ordinated with it, for a reaction to take place. Substrate binding displaces Glu⁶⁹ as a Zn^{2+} ligand such that the side chain of it can now substitute the side chain of Glu¹⁵⁴ in forming a hydrogen bond with Zn^{2+} -OH (Scheme 1B). Significantly, interactions provided by the side chain of Glu⁶⁹ establish a proton-relay through which the hydroxy group of Zn^{2+} -bound alcohol is associated with bulk water (compare with Figure 4; Glu⁷⁰ in human SDH). Below the pK of about 7.2–7.4 in enzyme- NAD^+ , Zn^{2+} -water is protonated, causing a large decrease in activity in both wild-type and mutant enzymes. However, loss of activity is only partial in wild-type, whereas it is complete in the E154C mutant. We would like to suggest that the side chain of Glu¹⁵⁴ serves in partially restoring the catalytic base function of Zn^{2+} -OH (together with Glu⁶⁹) by partly taking up a proton from Zn^{2+} -OH₂, as indicated in Scheme 1(C). Note that partial negative charge remaining on the H₂O/hydroxy ligand co-ordinating to Zn^{2+} might also confer electrostatic stabilization to the positively charged nicotinamide ring of NAD^+ in a position suitable for hydride transfer. Now, if Glu¹⁵⁴ becomes partly protonated (Scheme 1C), its ability to form a charged hydrogen bond with the C1 hydroxy group of sorbitol will be lost and, consequently, apparent binding of sorbitol will be weakened substantially, as is observed at low pH. Due to its smaller size, the side chain of Cys¹⁵⁴ is probably too far away from Zn^{2+} -OH₂ to function in an analogous proton relay. In addition, even if it were brought through a protein conformational change into a position suitable for proton uptake from Zn^{2+} -OH₂, it would probably be present in its protonated form and thus inactive below the observed pK of enzyme- NAD^+ .

Scheme 1 is subject to the caveat that residues of the hydrogen-bonded system in liver ADH, Ser⁴⁸ and His⁵¹, through which the hydroxy group of Zn^{2+} -bound alcohol is connected via the 2'-hydroxy group of the nicotinamide ribose with bulk water, are conserved in the sequence of human SDH at positions 46 and 49 (Figure 4), and at homologous positions in sSDH and GmXDH sequences. However, a recent study of MDR-type glucose dehydrogenase (from *Sulfolobus solfataricus*) supports a role of Glu⁶⁷ in facilitating proton abstraction from substrate to Zn^{2+} -OH and passing on the proton to solvent [36].

Scheme 1 Proposed participation of Glu¹⁵⁴ in the catalytic mechanism of SDH/XDH

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