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ABBREVIATIONS

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); slSDH; sheep liver SDH; XDH, xylitol dehydrogenase (xylitol:NAD⁺ 2-oxidoreductase, EC 1.1.1.9); GmXDH, XDH from *Galactocandida mastotermitis*. E154C is a GmXDH mutant in which Glu-154 was replaced by a cysteine. Numbering for GmXDH starts with 1 for the initiator methionine.

SYNOPSIS

Coordination of catalytic Zn²⁺ in sorbitol/xylitol dehydrogenases of the medium-chain dehydrogenase/reductase superfamily involves direct or water-mediated interactions from a Glu residue, which substitutes a homologous Cys ligand in alcohol dehydrogenases of the yeast and liver type. Glu-154 of xylitol dehydrogenase from the yeast Galactocandida mastotermitis (GmXDH) was mutated into Cys (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 Zn^{2+} centre activity (k_{cat}) of 1.19±0.03 s⁻¹ and did not require exogenous Zn^{2+} for activity or stability. E154C retained 0.019±0.003 % and 0.74±0.03 % of wild-type catalytic efficiency $(k_{cat}/K_{sorbitol})$; = 7800±700 M⁻¹s⁻¹) and k_{cat} (= 161±4 s⁻¹) for NAD⁺-dependent oxidation of sorbitol at 25 °C, respectively. The pH profile of $k_{cat}/K_{sorbitol}$ for E154C decreased below an apparent pK of 9.1 \pm 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 (slSDH). The difference in pK for profiles determined in ${}^{1}H_{2}O$ and ${}^{2}H_{2}O$ solvent was similar and unusually small for all three enzymes ($\approx +0.2 \log \text{ units}$), 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 $k_{cat}/K_{sorbitol}$ respectively. Transient kinetic studies of NAD⁺ reduction and proton release during sorbitol oxidation by sISDH at pH 8.2 show that two protons are lost with a rate constant of $687\pm12 \text{ s}^{-1}$ in the pre-steady state, which features a turnover of 0.9 ± 0.1 enzyme equivalents as NADH produced with a rate constant of 409 ± 3 s⁻¹. The results support an auxiliary participation of Glu-154 in catalysis, and possible mechanisms of proton transfer in sorbitol/xylitol dehydrogenases are discussed.

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

INTRODUCTION

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

PDHs are functional homotetramers in which each subunit contains a single Zn^{2+} that is bound in the active site and required for catalysis [3]. The ligand field of Zn^{2+} in PDHs differs from that of horse liver [4] and yeast ADH (PDB code: 2 HCY) where a conserved triad of two cysteines and one histidine constitutes, together with a water molecule, the primary coordination sphere. Residue Cys-174 (numbering of the horse liver enzyme) is replaced by a glutamic acid (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 catalyzes the interconversion of sorbitol and fructose, however, with a preference for keto group reduction) reveal that this Glu is not a ligand to the Zn^{2+} but coordinates the metal ion via a water molecule (Figure 1). A 3.0 Å structure of rat liver SDH, by contrast, determined the Glu 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 Glu (position 70 in human SDH) is a Zn^{2+} ligand in all three PDH structures whereas the corresponding Glu-68 of horse liver ADH is not coordinating in resting enzyme complexes [11]. Computational studies suggest, however, that Glu-68 could coordinate 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 Zn^{2+} through an oxygen and a nitrogen atom and that Glu-70 is dissociated from the now pentacoordinated metal ion [5].

What is the exact role of the positional exchange Cys \rightarrow Glu in the Zn²⁺ ligand field of PDHs compared with that of ADHs? As proposed originally by Eklund and colleagues [4], a strong hydrogen bond between the negatively charged Glu side chain and the primary hydroxyl adjacent to the secondary hydroxyl undergoing reaction could be an essential element of PDHspecific 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 (2.4 Å) between the side chain of Glu-155 and the primary hydroxyl on the inhibitor which is also in coordination distance to the Zn^{2+} (2.4 Å). Kinetic evidence for a Glu \rightarrow Cys variant of rat SDH, reported by Höög and co-workers [8], is also consistent with major function of Glu 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 hydroxyl was replaced by a hydrogen, amino, or sulfhydryl were converted with an efficiency of between 0.36% to 4.8% of $k_{cat}/K_{sorbitol}$ 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 1-OH of sorbitol is required for binding. A second possibility is that the replacement of the $-S^{-1}$ ligand in ADH by a $-CO_{2}^{-1}$ [7] or a H₂O/OH⁻¹ ligand [5] in PDH alters the electrophilic character of Zn^{2+} and therefore, changes the propensity of the metal ion to bind alcohol and stabilize the proposed alkoxide intermediate state of the reaction through innersphere coordination. 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 works of ADH [14].

In this paper we report on the functional consequences of replacing Glu-154 of GmXDH (XDH from the yeast *Galactocandida mastotermitis*) by a Cys residue. GmXDH is a representative microbial member of MDR-type PDHs and catalyzes 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]. Its Glu-154 is homologous in the sequence to Glu-155 of human SDH (see Supplementary Figure S1). 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 of Glu-154 as a modulator of, but not its direct participation in general base catalysis to enzymatic alcohol oxidation. Zn^{2+} -bound water through which Glu-154 is possibly linked with the catalytic metal would be a likely candidate to fulfill the function of the catalytic base. The glutamate is additionally important, but not essential for substrate binding. Analysis of transient reactions catalyzed by slSDH suggests a kinetic scenario for the proton transfers 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]. 2 H₂O solvent (99.9% 2 H) and D-sorbitol was from Sigma-Aldrich. 2-[2 H]-D-sorbitol was prepared by enzymatic reduction of D-fructose according to a reported protocol [18]. Unlabelled reference

material was synthesised in exactly the same way. *Protio-* and *deuterio-*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

The site-directed mutation Glu-154 \rightarrow 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 PCRs. Each reaction used one flanking primer carrying the appropriate restriction site (bold) that hybridised at one end of the target sequence

1: 5'-CCG AAA CAG AAT TCA TGT CTA-3'; 2: 5'-GTC GAC GGA TCC TTA CTC-3'

and one internal primer that hybridised at the site of the mutation and contained the mismatched bases (underlined)

1': 5'-CTT AAA GGA CAA ACT AAA GC-3'; 2': 5'-GCT TTA GTT TGT CCT TTA AG-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 appropriate digested expression vector. Plasmid mini-prep DNA was sequenced to verify that the desired mutation had been introduced and that no misencorporation of nucleotides had occurred as a result of DNA polymerase errors. Recombinant wild-type and mutant proteins were produced in *E. coli* JM 109 transformed with the respective expression plasmid. Wild-type GmXDH was purified as described elsewhere [18]. E154C was isolated by 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) immobilised freshly 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. Twohundred μ L aliquots of a ~ 10 mg/mL solution of E154C were loaded on a Superdex 200 size exclusion chromatography (SEC) 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 appropriately 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 mentioned 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 varied 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 (where L is ¹H or ²H) dependences of kinetic parameters for sorbitol oxidation by wild-type enzymes and E154C mutant were determined in ¹H₂O or ²H₂O solvent. If not mentioned 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 metre reading + 0.4 [21]. Alternatively, deuterated buffer was prepared by ${}^{1}\text{H} \rightarrow {}^{2}\text{H}$ exchange whereby protonic buffer mixture alone (100 mM MES, 100 mM Tris) and containing NAD⁺ (10 mM) was taken to dryness by lyophilisation, and salts were redissolved in ${}^{2}\text{H}_{2}\text{O}$ immediately prior to use. The p ${}^{2}\text{H}$ values of thus 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 sISDH and GmXDH were carried out in the pL range 5.2 – 9.0 using buffer mixtures of MES (100 mM) and Tris (100 mM). 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 good agreement between kinetic parameters determined in Tris and glycine buffers, pL 9.0, suggests that the observable pH dependence was not strongly affected by relevant changes in ionic strength. All initial rates were recorded at varied concentrations of sorbitol in the presence of 10 mM NAD⁺. The reactions were started by adding the enzyme (10 µL; 2 % of total volume of assay) from a concentrated protein stock solution in 20 mM Tris buffer, pL 7.5. For use in ²H₂O, the enzymes were gelfiltered employing deuterated 20 mM Tris buffer and allowed to equilibrate with solvent for 0.5 h before the initial-rate measurement. Although the completeness of ${}^{1}H \rightarrow {}^{2}H$ 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 later). Because of concerns regarding enzyme stability, exchange of protein protons for deuterons via lyophilisation was not considered. Michaelis-Menten parameters were calculated

from initial rates measured at each value of pL, and data obtained in ${}^{1}\text{H}_{2}\text{O}$ and ${}^{2}\text{H}_{2}\text{O}$ 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 $\approx 8\%$ fructose in the enzymatically prepared *protio*-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 done according to Cook and Cleland [23].

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 μ L flow cell giving a pathlength of 1 cm. The enzyme was gelfiltered twice to a reaction buffer (pH 8.0) composed of 0.5 mM Tris/HCl, 3.2 μ M Phenol Red, 10 mM NAD⁺, and 59 mM NaCl. The ionic strength of this buffer equals 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 μ L solution containing the pre-formed enzyme-NAD⁺ complex (6 – 16 μ M slSDH) with 100 μ L 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 $1 - 100 \,\mu\text{M}$ protons with a slope value of $430 \,\text{M}^{-1}\text{cm}^{-1}$.

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 programme version 7.0. Equation (1) describes a mechanism with one ionisable 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. Equation (2) describes a simple mechanism in which one ionisable group has to be unprotonated to be active and highest activity is attained at k_H [22].

$$\log Y = \log((k_{\rm H} + k_{\rm L} [{\rm H}^+]/K)/(1 + [{\rm H}^+]/K))$$
(1)

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

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

$$[NADH]_{t} \text{ or } [H^{+}]_{t} = Y_{0} + \Pi(1 - e^{-k_{obs}t}) + k_{ss}t$$
(4)

Equation (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 Zn²⁺ associated with the protein preparation (GmXDH; E154C). Equation (4) was used to fit the pre-steady state and steady-state phases of multiturnover 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 M⁻¹cm⁻¹ and 430 M⁻¹cm⁻¹, respectively. k_{obs} and Π represents the transient rate constant and the product burst, respectively; k_{ss} corresponds to the steady state rate constant, t is the reaction time and Y₀ is the starting point of reaction.

Other methods

Metal analysis of protein samples was performed with inductively coupled plasma MS, as described elsewhere [15]. Protein was determined with 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 utilised for isolation of the wild-type [18]. Results are summarised in Supplementary Table S1, and elution profiles of E154C during dye ligand chromatography and the subsequent SEC are shown in Supplementary Figures S2 and S3 respectively. E154C was obtained in an overall yield of $\approx 62\%$. The presence of 2 mM NAD⁺ was essential to prevent enzyme inactivation during SEC. Supplementary Figure S4 shows an SDS polyacrylamide gel of purified E154C. The apparent molecular mass of the subunit of E154C is 38.7 kDa, which is in good agreement with 37.4 kDa calculated from the amino acid sequence.

Purified E154C eluted from an analytical gel filtration column as a single protein peak of apparent molecular mass of 160 kDa that contained all of the applied protein and enzyme activity. Like the wild-type, the mutant thus seems to be a functional homotetramer. Different preparations of E154C (N = 4) that appeared homogeneous in SDS PAGE (see Supplementary Figure S4) contained between 0.1 to 0.4 atom Zn²⁺/protein subunit. While the source of this variable Zn²⁺ content remained unknown, the activity of E154C solutions based on the molarity of Zn²⁺-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 mutant, the IC₅₀ value of about 5±1 µM being identical for both enzymes. Note that E154C was stable during the initial rate assays to be described below, suggesting that inactivation due to release of the active-site Zn^{2+} does 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 summarised in Table 1 along with the corresponding parameters for slSDH. Values of k_{cat} and $k_{cat}/K_{sorbitol}$ for E154C are 0.74±0.03 % and 0.019±0.003 % of the corresponding wild-type values. However, the Michaelis-Menten constant for NAD⁺ is the same, within limits of experimental error, in wild-type and E154C forms of GmXDH. slSDH differs from GmXDH mainly in the value of $K_{sorbitol}$ which is about one order of magnitude higher in the yeast enzyme.

pL dependence studies

pL profiles of k_{cat} and $k_{cat}/K_{sorbitol}$ for slSDH and GmXDH are shown in Figures 2 (panels A – C). With the exception of the pL profile for k_{cat} of GmXDH (data 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. They were fitted with eqn (1), and results are collected in Table 2. The pL profiles of $k_{cat}/K_{sorbitol}$ for E154C in ¹H₂O and ²H₂O 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 2, panel D). The data were fitted with eqn (2), and results are given in Table 2. The pL profiles were was performed at several pL values that were, however, always the same in ¹H₂O and ²H₂O. The solvent isotope effect on buffer pK implies that the buffer ratio was different in the two solvents

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at each pL of measurement [25]. To clearly demonstrate that these pL profiles are 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 (panel 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 p*K* will not interfere with determination of p*K* 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 $k_{cat}/K_{sorbitol}$ for reactions catalyzed 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 $k_{cat}/K_{sorbitol}$ approach plateau values and thus differences in pL profiles of the respective kinetic parameter in ¹H₂O and ²H₂O are eliminated (wild-type GmXDH; slSDH) or minimised (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_{iNADH}) was determined from initial rate studies in ¹H₂O and ²H₂O where NADH was used as product inhibitor of the NAD⁺-dependent oxidation of sorbitol. Under conditions in which [NAD⁺] was varied and [sorbitol] was constant and saturating, NADH was a linear competitive inhibitor against NAD⁺. The value of K_{iNADH} in ¹H₂O and ²H₂O, obtained from fits of eqn (3) to the data, was 20±4 µM and 10±2 µM respectively, yielding a solvent KIE on K_{iNADH} of 2.0±0.6 (= 20/10).

Transient kinetics

Results of KIE studies suggested that slSDH was the most practical enzyme with which to perform transient kinetic experiments. Because ${}^{D}k_{cat}$ and ${}^{D}k_{cat}/K_{sorbitol}$ (where superscript D denotes the primary deuterium KIE on the respective parameter) were similar at pH 9.0, the

dissociation constant of sorbitol from slSDH-NAD⁺ cannot be much higher than K_{sorbitol} (= 3.0 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 slSDH 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 4 shows typical stopped-flow progress curves of NADH production and proton release, recorded by the increase of A_{340nm} and the decrease in A_{556nm} respectively, obtained under conditions of limiting concentration of slSDH (8 µM) that allowed multiple turnovers 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 to that of coenzyme reduction, as required by the enzymatic reaction studied, sorbitol + NAD⁺ \rightarrow fructose + NADH + H⁺. The multiple turnover progress curves were fitted with eqn (4), yielding apparent first-order rate constants of 409±3 s⁻¹ and 687±12 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 stoppedflow experiments.

DISCUSSION

The Zn^{2+} centre of human liver SDH differs from that of horse liver ADH mainly in the substitution of the Cys-174 ligand [4] by a water molecule linked through the secondary sphere Glu-155 [5]. (In the discussion that follows we do not consider a role of the Glu in directly coordinating 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 coordination sphere of the active-site Zn^{2+} influences the various steps in the catalytic mechanism of these two and related MDR enzymes. We have therefore substituted the relevant Glu-154 of GmXDH by Cys 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 slSDH 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 Glu-154 is relatively more important for catalytic function than Zn^{2+} binding.

pL dependences of wild-type enzymes and E154C

 k_{cat} for sISDH and $k_{cat}/K_{sorbitol}$ for GmXDH and sISDH in ¹H₂O and ²H₂O 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 where a single ionisable group is present on enzyme-NAD⁺ and both unprotonated and protonated forms can react. (The sorbitol substrate does not ionise in the pL range studied.)

Results of our pH profile analysis for slSDH in 1 H₂O (Table 2) agree well with earlier findings of Lindstad and McKinley-McKee [17]. It was shown by these authors that the k_{cat} of sorbitol oxidation by slSDH 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 ref. [17], as pK = $(pK_1 + pK_2)/2$. From fits of the pH profile of k_{cat} with eqn (1), we obtain a nearly identical pK of 7.10±0.15. The previously reported pH profile for $k_{cat}/K_{sorbitol}$ displayed a pK of 7.1, also consistent with a pK value of 7.15±0.05 found in this work. In spite of the similar pK values seen in the pH profiles of k_{cat} and $k_{cat}/K_{sorbitol}$, there was a much larger decrease in $k_{cat}/K_{sorbitol}$ (120fold) than k_{cat} (7.5-fold) upon going from the high-pH plateau to the one at low pH.

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

For slSDH, the H₂O/OH⁻ ligand to Zn²⁺ and a histidine residue were considered to be candidate groups whose ionisation could determine the pH-rate profile of the enzyme [17]. The present results support the Zn²⁺-bound water because the ΔpK 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 ΔpK values of \leq +0.3. Likewise, the ΔpK of Co²⁺-water in carbonic anhydrase appears to be also small [28]. (However, the kinetically determined ΔpK for carbonic anhydrase was \approx +0.5 [29]). With regard to Zn²⁺-water, Klinman and coworkers [30] reported that pL dependencies of k_{cat} for NAD⁺-dependent oxidation of *p*-methoxy-benzylalcohol by yeast ADH displayed a ΔpK (= 8.35 – 8.14 = +0.21) in ²H₂O. They suggested that the small ΔpK 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 pL profiles of k_{cat} for yeast ADH and $k_{cat}/K_{sorbitol}$ 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 pK of a protonic species BH can be expressed by the relation $\Delta pK = \log [\Phi(BH)/\Phi(H_3O^+)]$. Because is $\Phi(H_3O^+) = 0.33$ [26], we can estimate that ΔpK for Zn²⁺-OH₂ is +0.23 (= log 0.56/0.33). However, in spite of the seemingly excellent agreement between the calculated ΔpK of Zn²⁺-OH₂ and the kinetically determined ΔpK for slSDH-NAD⁺ in ²H₂O, it must be emphasised that the possible kinetic complexity of $k_{cat}/K_{sorbitol}$ and the hydrogen-bonded system through which Zn²⁺-water is linked with protein residues (see Figure 1) make the assignment of apparent pK to group uncertain. Solvent isotope effect studies of horse liver ADH provide a note of caution because unlike the yeast enzyme, ΔpK values for the decrease in rate in pL profiles of k_{cat} and k_{cat}/K for NAD⁺-dependent oxidation of cyclohexanol at high pL were +1.39±0.21 [32].

pH profiles of GmXDH were similar to those of slSDH. The apparent pK describing the decrease of $k_{cat}/K_{sorbitol}$ in ¹H₂O and ²H₂O solvent was 7.4±0.1 and 7.6±0.1 respectively. Asymptote levels of $k_{cat}/K_{sorbitol}$ at high and low pH differed by a factor of 372. The pH dependence of $k_{cat}/K_{sorbitol}$ for E154C was clearly distinguished from that for the wild-type, showing an upshift in pK by +1.7 pH units to a value of 9.1±0.3. In ²H₂O, the pK was 9.3±0.2, hence again a ΔpK (= +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 ΔpK 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 Zn^{2+} -bound water. Mutation of Glu-154 did not abolish the pH dependence because Zn^{2+} -water is expected to be still present in E154C. However, the carboxylate side chain of the Glu could facilitate deprotonation of Zn^{2+} -water through a proton relay or hydrogen bonding whereas Cys-154 due to the smaller size and the higher p*K* of its side chain would not be able to fulfill 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 (${}^{D}k_{cat}$) as well as for the catalytic cascade (${}^{D}k_{cat}/K_{sorbitol}$) 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 $k_{cat}/K_{sorbitol}$ 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 ${}^{D}k_{cat}/K_{sorbitol}$ for E154C and wild-type at pH 9.0 appear to reflect solely the altered pH dependence in the mutant. ${}^{D}(k_{cat}/K_{sorbitol})$ values are similar at the pK of the wild type enzyme-catalyzed reaction (pH 7.5) and at the pK of the E154C-catalyzed reaction (pH 9.0).

At pL 9.0, values of ${}^{D_2O}k_{cat}$ (where superscript D₂O indicates the solvent KIE) for slSDH and wild-type GmXDH were significantly greater than unity. K_{iNADH} for slSDH determined under the same pL conditions also showed a sizable solvent KIE, and ${}^{D_2O}K_{iNADH} \approx {}^{D_2O}k_{cat}$. The net rate of NADH release (k'_{NADH}) is thought to control k_{cat} of slSDH ([17]; this work) 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 pL 9.0, there was a much larger solvent KIE on $k_{cat}/K_{sorbitol}$ than on k_{cat}/K_{NAD^+} , indicating that proton transfers within the steps of $k_{cat}/K_{sorbitol}$ could be partly rate limiting. Under conditions where $k_{cat}/K_{sorbitol}$ did not show a pL dependence, values of $D_2O_{k_{cat}}/K_{sorbitol}$ for wild-type and E154C forms of GmXDH were almost identical, suggesting that Glu-154 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 $D_2O_{k_{cat}}/K_{sorbitol}$ for E154C in comparison with wild-type. We note that the thiol group of Cys has a fractionation factor in ²H₂O of ≈ 0.5 [21] but involvement of the side chain of Cys-154 as a protonic site in the active centre of E154C at pL 10.0 is not probable. The results of the KIE studies suggest that Glu-154 participates in, but is not essential for hydrogen transfers in the catalytic reaction of GmXDH.

Hydrogen transfers in the catalytic mechanism, and proposed participation of Glu-154

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 here 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 to 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 Zn^{2+} -bound sorbitol could precede the transfer of hydride ion from alcohol to

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

Analysis of the stopped-flow progress curves in Figure 4 also revealed that two protons are lost per each NADH produced in the pre-steady state. Various steps of the mechanism of slSDH 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 slSDH-NAD⁺ complex. The weak pH dependence of k_{cat} for slSDH and GmXDH is in good 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-OH group of sorbitol. Results of our pL dependence studies support a catalytic base function of Zn^{2+} -water. The side chains of Glu-70 and Glu-155 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 Glu-70 with aqueous solvent, as shown in Figure 5 and discussed below. The suggested role of Glu-154 (Glu-155 in human SDH) in the catalytic mechanism of PDH enzymes is summarised 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 Glu-154 (Scheme 1, panel A). When sorbitol binds, the carboxylate of Glu-154 forms another hydrogen bond with the substrate C1 hydroxyl, which probably helps in positioning the reactive C2 alcohol of the substrate and the catalytic group on the enzyme, in particular Zn^{2+} and the hydroxyl coordinated with it, for reaction to take place. Substrate binding displaces Glu-69 as a Zn^{2+} ligand such that the side chain of it can now substitute the side chain of Glu-154 in forming a hydrogen bond with Zn^{2+} -OH (panel B). Significantly, interactions provided by the side chain of Glu-69 establish a proton relay through which the hydroxyl group of Zn^{2+} -bound alcohol is associated with bulk water (cf. Figure 4; Glu-70 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, activity loss is only partial in wild-types whereas it is complete in E154C mutant. We would like to suggest that the side chain of Glu-154 serves in partially restoring the catalytic base function of Zn^{2+} -OH (together with Glu-69) by partly taking up a proton from Zn^{2+} -OH₂, as indicated in panel C of Scheme 1. Note that partial negative charge remaining on the H_2O/OH^- ligand coordinating 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 Glu-154 becomes partly protonated (Scheme 1, panel C), 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 Cys-154 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, Ser-48 and His-51, through which the hydroxyl of Zn^{2+} -bound alcohol is connected via the 2'-hydroxyl 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 slSDH and GmXDH sequences. However, a recent study of MDR-type glucose dehydrogenase (from *Sulfolobus solfataricus*) supports a role of Glu-67 in facilitating proton abstraction from substrate to Zn^{2+} -OH and passing on the proton to solvent [36].

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REFERENCES

- 1 Riveros-Rosas, H., Julian-Sanchez, A., Villalobos-Molina, R., Pardo, J. P. and Pina, E. (2003) Diversity, taxonomy and evolution of medium-chain dehydrogenase/reductase superfamily. Eur. J. Biochem. **270**, 3309-3334
- 2 Persson, B., Zigler, J. S., Jr. and Jörnvall, H. (1994) A super-family of medium-chain dehydrogenases/reductases (MDR). Sub-lines including zeta-crystallin, alcohol and polyol dehydrogenases, quinone oxidoreductase enoyl reductases, VAT-1 and other proteins. Eur. J. Biochem. **226**, 15-22
- Jeffery, J. and Jörnvall, H. (1988) Sorbitol dehydrogenase. Adv. Enzymol. Relat. Areas Mol. Biol. **61**, 47-106
- 4 Eklund, H., Horjales, E., Jörnvall, H., Bränden, C. I. and Jeffery, J. (1985) Molecular aspects of functional differences between alcohol and sorbitol dehydrogenases. Biochemistry **24**, 8005-8012
- 5 Pauly, T. A., Ekstrom, J. L., Beebe, D. A., Chrunyk, B., Cunningham, D., Griffor, M., Kamath, A., Lee, S. E., Madura, R., McGuire, D., Subashi, T., Wasilko, D., Watts, P., Mylari, B. L., Oates, P. J., Adams, P. D. and Rath, V. L. (2003) X-ray crystallographic and kinetic studies of human sorbitol dehydrogenase. Structure **11**, 1071-1085
- Banfield, M. J., Salvucci, M. E., Baker, E. N. and Smith, C. A. (2001) Crystal structure of the NADP(H)-dependent ketose reductase from *Bemisia argentifolii* at 2.3 Å resolution. J. Mol. Biol. **306**, 239-250
- Johansson, K., El-Ahmad, M., Kaiser, C., Jörnvall, H., Eklund, H., Höög, J. and Ramaswamy, S. (2001) Crystal structure of sorbitol dehydrogenase. Chem. Biol. Interact. 130-132, 351-358
- 8 Karlsson, C. and Höög, J. O. (1993) Zinc coordination in mammalian sorbitol dehydrogenase. Replacement of putative zinc ligands by site-directed mutagenesis. Eur. J. Biochem. **216**, 103-107
- 9 Feiters, M. C. and Jeffery, J. (1989) Zinc environment in sheep liver sorbitol dehydrogenase. Biochemistry **28**, 7257-7262
- 10 Maret, W. (1989) Cobalt(II)-substituted class III alcohol and sorbitol dehydrogenases from human liver. Biochemistry **28**, 9944-9949
- 11 Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B. O., Tapia, O., Bränden, C. I. and Akeson, A. (1976) Three-dimensional structure of horse liver alcohol dehydrogenase at 2-4 Å resolution. J. Mol. Biol. **102**, 27-59

- 12 Ryde, U. (1995) On the role of Glu-68 in alcohol dehydrogenase. Protein Sci. **4**, 1124-1132
- 13 Lindstad, R. I., Koll, P. and McKinley-McKee, J. S. (1998) Substrate specificity of sheep liver sorbitol dehydrogenase. Biochem. J. **330** 479-487
- 14 LeBrun, L. A., Park, D. H., Ramaswamy, S. and Plapp, B. V. (2004) Participation of histidine-51 in catalysis by horse liver alcohol dehydrogenase. Biochemistry **43**, 3014-3026
- 15 Lunzer, R., Mamnun, Y., Haltrich, D., Kulbe, K. D. and Nidetzky, B. (1998) Structural and functional properties of a yeast xylitol dehydrogenase, a Zn²⁺-containing metalloenzyme similar to medium-chain sorbitol dehydrogenases. Biochem. J. **336** 91-99
- 16 Lindstad, R. I., Hermansen, L. F. and McKinley-McKee, J. S. (1992) The kinetic mechanism of sheep liver sorbitol dehydrogenase. Eur. J. Biochem. **210**, 641-647
- 17 Lindstad, R. I. and McKinley-McKee, J. S. (1995) Effect of pH on sheep liver sorbitol dehydrogenase steady-state kinetics. Eur. J. Biochem. **233**, 891-898
- 18 Nidetzky, B., Helmer, H., Klimacek, M., Lunzer, R. and Mayer, G. (2003) Characterization of recombinant xylitol dehydrogenase from *Galactocandida mastotermitis* expressed in *Escherichia coli*. Chem. Biol. Interact. **143-144**, 533-542
- 19 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**, 51-59
- 20 Habenicht, A., Motejadded, H., Kiess, M., Wegerer, A. and Mattes, R. (1999) Xylose utilisation: cloning and characterisation of the xylitol dehydrogenase from *Galactocandida mastotermitis*. Biol. Chem. **380**, 1405-1411
- 21 Schowen, K. B. and Schowen, R. L. (1982) Solvent isotope effects of enzyme systems. Methods Enzymol. **87**, 551-606
- 22 Cleland, W. W. (1982) The use of pH studies to determine chemical mechanisms of enzyme-catalyzed reactions. Methods Enzymol. **87**, 390-405
- 23 Cook, P. F. and Cleland, W. W. (1981) Mechanistic deductions from isotope effects in multireactant enzyme mechanisms. Biochemistry **20**, 1790-1796.
- 24 Fersht, A. (1999) Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding. W. H. Freeman and Company
- 25 Schowen, K. B. J. (1978) Solvent isotope effects. In Transition states of biochemical processes (Gandour, R. D. and Schowen, R. L., eds.), pp. 225-283, Plenum Press, New York
- 26 Quinn, D. M. and Sutton, L. D. (1991) Theoretical basis and mechanistic utility of solvent isotope effects. In Enzyme mechanism from isotope effects (Cook, P. F., ed.), pp. 73-126, CRC Press, Boca Raton
- 27 Quinn, D. M. (2006) Theory and practice of solvent isotope effects. In Isotope effects in chemistry and biology (Kohen, A. and Limbach, H.-H., eds.), pp. 995-1018, CRC Press, Boca Raton
- 28 Kassebaum, J. W. and Silverman, D. N. (1989) Hydrogen/deuterium fractionation factors of the aqueous ligand of cobalt in $\text{Co}(\text{H}_2\text{O})_6^{2+}$ and Co(II)-substituted carbonic anhydrase. J. Am. Chem. Soc. **111**, 2691-2696
- 29 Pocker, Y. and Bjorkquist, D. W. (1977) Comparative studies of bovine carbonic anhydrase in H_2O and D_2O . Stopped-flow studies of the kinetics of interconversion of CO_2 and HCO_3^- . Biochemistry **16**, 5698-5707
- 30 Welsh, K. M., Creighton, D. J. and Klinman, J. P. (1980) Transition-state structure in the yeast alcohol dehydrogenase reaction: the magnitude of solvent and α -secondary hydrogen isotope effects. Biochemistry **19**, 2005-2016

- 31 Northrop, D. B. and Cho, Y. K. (2000) Effects of high pressure on solvent isotope effects of yeast alcohol dehydrogenase. Biophys. J. **79**, 1621-1628
- 32 Taylor, K. B. (1983) Solvent isotope effects on the reaction catalyzed by alcohol dehydrogenase from equine liver. Biochemistry **22**, 1040-1045
- 33 Sekhar, V. C. and Plapp, B. V. (1990) Rate constants for a mechanism including intermediates in the interconversion of ternary complexes by horse liver alcohol dehydrogenase. Biochemistry **29**, 4289-4295
- 34 Kovaleva, E. G. and Plapp, B. V. (2005) Deprotonation of the horse liver alcohol dehydrogenase-NAD⁺ complex controls formation of the ternary complexes. Biochemistry **44**, 12797-12808
- 35 Plapp, B. V. (2006) Catalysis by alcohol dehydrogenases. In Isotope effects in chemistry and biology (Kohen, A. and Limbach, H.-H., eds.), pp. 811-835, CRC Press, Boca Raton

TABLES

Table 1. Kinetic parameters of slSDH, GmXDH and E154C mutant for NAD⁺-dependent oxidation of sorbitol determined at pH 9.0 and $25.0\pm0.1^{\circ}$ C.

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

| parameter | $E154C^*$ | GmXDH^* | slSDH |
|--|----------------|--------------------------|-----------------------|
| $k_{\text{cat}} [\text{s}^{-1}]$ | 1.19 ± 0.03 | 161 ± 4 | 28 ± 1 |
| $K_{\rm sorbitol} [{\rm mM}]$ | 785 ± 82 | 21 ± 2 | 3.0 ± 0.2 |
| $k_{\text{cat}}/K_{\text{sorbitol}} [\text{M}^{-1}\text{s}^{-1}]$ | 1.5 ± 0.2 | 7800 ± 700 | 9500 ± 700 |
| $K_{\rm NAD^+}$ [μ M] | 290 ± 35 | 430 ± 40 | 500 ± 99 |
| $L / V = [M^{-1} e^{-1}]$ | 4100 + 510 | $3.7 \times 10^5 \pm$ | $5.7 \times 10^4 \pm$ |
| $\kappa_{\text{cat}}/\Lambda_{\text{NAD}}+[\mathbf{W} \mathbf{S}]$ | 4100 ± 310 | 4×10^4 | 1.1×10^4 |

Table 2. pK values from pL profiles for slSDH, GmXDH andE154C mutant.

pL profiles of k_{cat} and $k_{cat}/K_{sorbitol}$ for slSDH and GmXDH were fitted with eqn (1). pL profiles of $k_{cat}/K_{sorbitol}$ for E154C were fitted with eqn (2). *Superscripts indicate measurements in ¹H₂O and ²H₂O; [†]correlation coefficients from non-linear regression analysis are given in parenthesis; [‡] not determined

| $\log(n_{0}n_{1}n_{1}n_{2}n_{2}n_{1}n_{2}n_{1}n_{2}n_{2}n_{1}n_{2}n_{2}n_{2}n_{2}n_{2}n_{2}n_{2}n_{2$ | nK | limiting values | |
|---|--------------------|----------------------------|--|
| log (parameter) | pre | $[s^{-1}]; [M^{-1}s^{-1}]$ | |
| | slSDH | | |
| - 1H2O | 7.10 ± 0.15 | $k_{\rm L} = 3.5 \pm 0.3$ | |
| $\kappa_{\rm cat}$ | $(0.99)^{\dagger}$ | $k_{\rm H} = 26 \pm 2$ | |
| L 2H2O | 7.3 ± 0.3 | $k_{\rm L} = 3.1 \pm 0.4$ | |
| <i>k</i> _{cat} | (0.96) | $k_{\rm H} = 13 \pm 1$ | |
| $(l_{\rm L}/l_{\rm L})^{1\rm H_2O}$ | 7.15 ± 0.05 | $k_{\rm L} = 75 \pm 11$ | |
| $(\kappa_{cat}/\Lambda_{sorbitol})$ | (0.999) | $k_{\rm H} = 8980 \pm 245$ | |
| $(l_{\mathbf{V}})^{2H_2O}$ | 7.3 ± 0.1 | $k_{\rm L} = 69 \pm 21$ | |
| $(\kappa_{cat}/\Lambda_{sorbitol})$ | (0.99) | $k_{\rm H} = 6813 \pm 533$ | |
| | GmXDH | | |
| $k_{\rm cat}^{1\rm H_2O}$ | n.d. [‡] | - | |
| $k_{\rm cat}^{\rm 2H_2O}$ | n.d. | - | |
| $(k \mathbf{K} \dots)^{1\text{H}_2\text{O}}$ | 7.4 ± 0.1 | $k_{\rm L} = 17 \pm 8$ | |
| (K _{cat} /K _{sorbitol}) | (0.99) | $k_{\rm H} = 6484 \pm 351$ | |
| $(k \mathbf{K} \dots)^{2H_2O}$ | 7.6 ± 0.1 | $k_{\rm L} = 11 \pm 4$ | |
| (K _{cat} /K _{sorbitol}) | (0.99) | $k_{\rm H} = 4275 \pm 406$ | |
| | E | 154C | |
| $(k / K)^{1H_2O}$ | 9.1 ± 0.3 | $k = 4.1 \pm 0.5$ | |
| (Vcat ^{/11} sorbitol) | (0.995) | $n = 7.1 \pm 0.5$ | |
| $(k_{cat}/K_{sorbital})^{2H_2O}$ | 9.3 ± 0.2 | $k = 2.6 \pm 0.2$ | |
| ('Car - * 50101101) | (0.99) | | |

Table 3. Primary deuterium and solvent KIEs on kinetic parameters for sorbitol oxidation by slSDH, GmXDH and E154C at pL 9.0 and 25.0±0.1°C.

^{*}Superscripts D and D₂O indicate primary and solvent deuterium KIEs respectively. [†]not analysed; [‡]primary deuterium KIEs determined at pH 7.5; [§]determined at pL 10.0.

| Parameter* | E154C | GmXDH | slSDH |
|--|--------------------|----------------------------|-----------------|
| $D_{k_{cat}}$ | n.a. [†] | 1.16 ± 0.03 | 1.13 ± 0.08 |
| | | $(1.1 \pm 0.1)^{\ddagger}$ | |
| $^{\rm D}k_{\rm cat}/K_{\rm sorbitol}$ | 24+02 | 1.20 ± 0.07 | 1 25 + 0 14 |
| | 2.4 ± 0.2 | $(1.9 \pm 0.3)^{\ddagger}$ | 1.23 ± 0.14 |
| ${}^{\mathrm{D}}k_{\mathrm{cat}}/K_{\mathrm{NAD}^{+}}$ | n.a. | 1.2 ± 0.1 | 0.9 ± 0.2 |
| $^{D_2O}k_{cat}$ | n.a. | 1.49 ± 0.05 | 2.1 ± 0.1 |
| $^{\rm D_2O}k_{\rm cat}/K_{\rm sorbitol}$ | $1.7 \pm 0.2^{\$}$ | 1.7 ± 0.1 | 1.5 ± 0.3 |
| $^{\mathrm{D}_{2}\mathrm{O}}k_{\mathrm{cat}}/K_{\mathrm{NAD}^{+}}$ | n.a. | 1.1 ± 0.1 | 1.0 ± 0.2 |

FIGURE LEGENDS

Figure 1. Participation of Glu-155 in Zn^{2+} coordination 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 Zn^{2+} are depicted. Yellow and black dashed lines indicate coordination to Zn^{2+} and hydrogen bonding respectively.

Figures 2. pL profiles of k_{cat} and $k_{cat}/K_{sorbitol}$ for slSDH and of $k_{cat}/K_{sorbitol}$ for wild-type and E154C mutant forms of GmXDH. Open and filled circles indicate measurements in ¹H₂O and ²H₂O 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 panel C, filled triangles indicate measurements with deuterated buffers that had been prepared through ¹H \rightarrow ²H exchange via lyophilisation. Open triangles show corresponding measurements in ¹H₂O. Insets show double-log plots of the data to illustrate differences in shape of the pL profiles at low pH (panel C: sigmoidal; panel D: non-sigmoidal).

Figure 3. Stopped-flow progress curves of formation of NADH and release of proton during NAD⁺-dependent oxidation of sorbitol catalyzed by slSDH. 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.

Figure 4. Alternative proton shuttle pathways for sorbitol/xylitol dehydrogenases. The structure of human liver SDH is shown (PDB: 1PL6) [5]. Hydrogen-bonded networks connecting the active site with bulk water are indicated by arrows.

Scheme 1. Proposed participation of Glu-154 in the catalytic mechanism of sorbitol/xylitol dehydrogenases.

FIGURES



Figure 1



Figure 2, panel A



Figure 2, panel B



Figure 2, panel C



Figure 2, panel D



Figure 3



Figure 4

Scheme 1

