Structural and functional properties of a yeast xylitol dehydrogenase, a Zn²⁺-containing metalloenzyme similar to medium-chain sorbitol dehydrogenases

Regina LUNZER, Yasmine MAMNUN, Dietmar HALTRICH, Klaus D. KULBE and Bernd NIDETZKY¹

Division of Biochemical Engineering, Institute of Food Technology, Universität für Bodenkultur (BOKU), Muthgasse 18, A-1190 Wien, Austria

The NAD⁺-dependent xylitol dehydrogenase from the xylose-assimilating yeast *Galactocandida mastotermitis* has been purified in high yield (80 %) and characterized. Xylitol dehydrogenase is a heteronuclear multimetal protein that forms homotetramers and contains 1 mol of Zn²⁺ ions and 6 mol of Mg²⁺ ions per mol of 37.4 kDa protomer. Treatment with chelating agents such as EDTA results in the removal of the Zn²⁺ ions with a concomitant loss of enzyme activity. The Mg²⁺ ions are not essential for activity and are removed by chelation or extensive dialysis without affecting the stability of the enzyme. Results of initial velocity studies at steady state for D-sorbitol oxidation and D-

fructose reduction together with the characteristic patterns of product inhibition point to a compulsorily ordered Theorell–Chance mechanism of xylitol dehydrogenase in which coenzyme binds first and leaves last. At pH 7.5, the binding of NADH ($K_i \approx 10 \mu\text{M}$) is approx. 80-fold tighter than that of NAD⁺. Polyhydroxyalcohols require at least five carbon atoms to be good substrates of xylitol dehydrogenase, and the C-2 (S), C-3 (R) and C-4 (R) configuration is preferred. Therefore xylitol dehydrogenase shares structural and functional properties with medium-chain sorbitol dehydrogenases.

INTRODUCTION

The initial pathway of D-xylose catabolism in yeast comprises the NAD(P)H-dependent reduction of D-xylose to xylitol followed by the NAD⁺-dependent oxidation of xylitol to D-xylulose [1]. The first step of this sequential redox reaction is catalysed by aldose reductase (alditol:NAD(P)⁺-1-oxidoreductase; EC 1.1.1.21) and the second step by xylitol dehydrogenase (XDH; xylitol:NAD⁺-2-oxidoreductase; EC 1.1.1.9). The yeast aldose reductases, often termed xylose reductases, are members of the aldo/oxo reductase superfamily. As a result of structural and mechanistic studies with aldose reductases from mammalian sources [2–4], the structure–function relationships in aldose reductase enzymes are at present quite well understood. An overall structural similarity in mammalian and yeast aldose reductases is determined by a sequence identity of approx. 40 % [5,6] that includes a strict conservation of the catalytic residues [4] and thus, most probably, the mechanism of action. In contrast with aldose reductase, however, the structural and functional properties of yeast XDH have not been investigated in comparable detail.

A structural characterization from the corresponding genes has been performed with two forms of XDH that are found in the D-xylose-fermenting yeast *Pichia stipitis* [7]. One XDH, the *XYL2* gene product, belongs to the medium-chain alcohol dehydrogenase (ADH) family that comprises mammalian sorbitol dehydrogenases, prokaryotic threonine dehydrogenases, Zn²⁺-containing ADHs and ζ -crystallin [7,8]. A close relationship of XDH to sorbitol dehydrogenase (SDH; L-iditol:NAD⁺-2-oxidoreductase; EC 1.1.1.14) has been proposed, essentially on the basis of three facts. First, one Zn²⁺-binding site, corre-

sponding to the catalytic Zn²⁺ at the active site of SDH [8–11], is found in XDH. A second structural Zn²⁺ site, as present in many ADHs [8,9,12,13], has not been detected by sequence comparison. The putative ligands to the Zn²⁺ ion in XDH are cysteine (Cys-41), histidine (His-66) and glutamate (Glu-159) [7]. This coordination of the metal ion is considered typical of sorbitol dehydrogenases [8,9,14,15] rather than ADHs, in which glutamate is replaced by a second cysteine residue [9,16]. Secondly, XDH will not oxidize primary alcohols and exhibits a substrate spectrum similar to that of SDH [7,8]. Thirdly, relative to ADH, various loop structures that are thought to determine the subunit association state of the respective member of the family are missing from both XDH and SDH [7,8,14]. Mammalian SDH is a tetramer composed of four identical subunits [8,10]. In addition to the *XYL2* gene product, another XDH has been found in *P. stipitis*. This second XDH belongs to the family of short-chain dehydrogenases and contains those two active-site residues, a tyrosine and a lysine, that are thought to be functionally important in all members of this family [17]. The enzyme involved in the conversion of D-xylose by *P. stipitis* is the *XYL2* gene product. The physiological function of the second XDH, however, is not completely clear.

At present, there are only sparse biochemical data describing the properties of yeast XDHs in relation to the members of the medium-chain ADH family and that would support the conclusions that were derived from a comparison of the primary structures of XDH and SDH [7]. For example, a role of metal ions in XDH-mediated catalysis has, to the best of our knowledge, not been investigated. The XDHs are thought to be dimers [18,19] or eventually tetramers ([20], and unpublished work in [7]) composed of protomers of molecular masses 30–40 kDa and

Abbreviations used: ADH, alcohol dehydrogenase (alcohol:NAD⁺-1-oxidoreductase; EC 1.1.1.1); ANS, 8-anilino-1-naphthalene sulphonate; MALDI-MS, matrix-assisted laser desorption/ionization MS; SDH, sorbitol dehydrogenase (L-iditol:NAD⁺-2-oxidoreductase; EC 1.1.1.14); XDH, xylitol dehydrogenase (xylitol:NAD⁺-2-oxidoreductase; EC 1.1.1.9).

¹ To whom correspondence should be addressed (e-mail nide@mail.boku.ac.at).

exhibit a polyol substrate preference in the order xylitol > ribitol $\approx$ D-sorbitol [18,21]. The XDH from *P. stipitis* was shown to follow an ordered kinetic mechanism [22].

In the present study we have isolated in high yields the XDH from a recently identified [23] and classified D-xylose-assimilating yeast strain, *Galactocandida mastotermitis* and performed a detailed characterization of the enzyme. We show that XDH is a heteronuclear multimetal enzyme containing one catalytic Zn²⁺ ion and six Mg²⁺ ions that are present in the native XDH but are removed by extensive dialysis. The results reveal a basic structural and functional similarity of XDH to members of the medium-chain ADHs, especially SDH. However, some differences pertaining to metal content and kinetic properties have been found.

EXPERIMENTAL

Materials

Unless mentioned otherwise, all chemicals were of the highest purity available from Sigma (Deisenhofen, Germany). Materials for protein chromatography and electrophoresis were from Pharmacia (Uppsala, Sweden). D-Xylosone (D-threo-2-pentosulose) and D-glucosone (D-arabino-hexosulose) were prepared enzymically from D-xylose and D-glucose respectively by using pyranose 2-oxidase [24] from *Trametes multicolor*.

Organism and growth conditions

The yeast strain used for the production of XDH, *Galactocandida* (gen. nov.) *mastotermitis* (spec. nov.), was a gift from Dr. H. Prillinger (Institute of Applied Microbiology, BOKU, Vienna, Austria). It has been isolated from the termite *Mastotermis darwiniensis* [23]. The organism was grown in a 20 litre bioreactor at constant pH (5.0), dissolved oxygen concentration (25% saturation) and temperature (25 °C). The growth medium consisted of yeast extract (4 g/l), enzymically digested peptone from soybean meal (6 g/l), D-xylose (20 g/l) and 50 mM KH₂PO₄. At the end of the logarithmic growth phase, typically 24 h of cultivation, the cells (approx. 9 g dry cell mass/l of medium) were harvested by centrifugation at 4 °C and 13000 g for 25 min, washed twice and suspended in 20 mM potassium phosphate buffer, pH 7.0 (1:2, w/w). Cell disruption was performed in a continuously operated ball mill (Dynamill, KDL Bachofen AG, Basel, Switzerland) with glass beads 0.25–0.5 mm in diameter. The average time of disruption was 7 min, and the temperature was kept below 10 °C. Cell debris was removed at 113000 g and 4 °C (25 min), and the recovered supernatant was stored at –70 °C.

Assays

All measurements were performed with a Beckman DU-650 spectrophotometer. XDH activity was assayed routinely in the direction of polyol oxidation by measuring the reduction of NAD⁺ at 340 nm (1–5 min, rate of 0.05–0.1 ΔA /min) at 25 °C. The standard assay contained 220 mM xylitol (or D-sorbitol) and 2.44 mM NAD⁺ in 100 mM 2-amino-2-methyl-1-propanol buffer, pH 10.0. In the direction of ketose reduction, 440 mM D-fructose and 0.244 mM NADH in 50 mM Tris buffer, pH 7.0, were used. All reactions were started by the addition of coenzyme (20 μ l); the final reaction volume was 1 ml. One unit of enzyme activity refers to 1 μ mol of NADH produced or consumed/min. All rates were corrected for the appropriate blank readings with controls lacking either the enzyme or the polyol/ketose. Protein was assayed with the Bio-Rad reagent with BSA as standard.

Protein purification

Dye-ligand chromatography

The triazine dye Procion Red HE3B (colour index Red 120; ICI, Manchester, U.K.) was immobilized on Sepharose 4B-CL [25]. The cell extract was loaded on a 5 cm $\times$ 5 cm column of the dye matrix (10 mg of protein/ml of gel) previously equilibrated in 50 mM phosphate buffer, pH 7.0. Elution was performed with a step gradient of 0–1 M KCl at a flow rate of 8 ml/min. Under these conditions XDH was eluted at 0.2 M salt concentration.

Hydrophobic interaction chromatography

The XDH preparation was brought to 1 M (NH₄)₂SO₄, and 17 mg of protein was applied to a 0.9 cm $\times$ 3 cm column of phenyl-Sepharose 4B-CL high sub, fast flow, equilibrated with 20 mM Tris, pH 8.0. Elution was performed at 2 ml/min with a step gradient of (NH₄)₂SO₄; XDH was eluted at 0% salt.

Affinity purification

After gel filtration on a Sephadex G25c column (5 cm $\times$ 15 cm; 20 mM phosphate buffer, pH 7.0), XDH was applied to a Red 120-Sepharose 4B-CL column (0.9 cm $\times$ 3 cm). The elution of bound XDH was accomplished with 2 mM NADH in phosphate buffer, pH 7.0. Before further use XDH was dialysed for 24 h or at least doubly gel-filtered to remove bound coenzyme. Fluorometric analysis (see below) indicated the absence of bound nucleotide.

Purity and structural characterization

The purity of isolated XDH was controlled by SDS/PAGE, non-denaturing anionic PAGE (with PhastGel Gradient 8–25 in both cases) and isoelectric focusing (with PhastGel IEF 4–6.5) performed on a Pharmacia PhastSystem unit in accordance with the instructions of the supplier with silver staining of protein bands. Staining for XDH activity was performed by assaying the polyol-oxidizing activity of XDH. The gel was incubated at pH 10.0 with 250 mM xylitol as substrate, 1 mM NAD⁺ as coenzyme employing 5-methyl phenazine methosulphate (2 mg/ml) and Nitro Blue Tetrazolium chloride (6 mg/ml) as electron acceptors. The molecular mass of XDH subunits was determined by SDS/PAGE; that of the native enzyme was determined by gel filtration on a Superose 12 column (1.0 cm $\times$ 30 cm).

Kinetics

Unless indicated otherwise, the experiments were performed in 50 mM potassium phosphate buffer, pH 7.5. Kinetic analyses for D-fructose reduction and D-sorbitol oxidation were performed at 25 °C by measuring initial velocities at several fixed concentrations of the coenzyme (NADH, NAD⁺) when D-fructose or D-sorbitol was the varied substrate and at several fixed concentrations of D-fructose or D-sorbitol when coenzyme (NADH, NAD⁺) was varied. The kinetic parameters were calculated as follows:

$$V(A,B) = k_{\text{cat}} EAB / (K_{\text{ia}} K_{\text{b}} + K_{\text{b}} A + K_{\text{a}} B + AB) \quad (1)$$

where V is the reaction rate, A and B are the substrate concentrations, k_{cat} is the catalytic constant, E is the total concentration of XDH, K_{a} and K_{b} are the Michaelis constants for

substrates A and B, and K_{ia} is the apparent dissociation constant of the binary enzyme-substrate complex, assuming a compulsory order of substrate binding [26]. The kinetic parameters were calculated with an iterative non-linear fitting procedure without weighting (SigmaPlot 5.0). The discrimination between different plausible kinetic mechanisms was performed by product inhibition studies [26]. By using different concentrations of D-fructose, D-xylulose or NADH as inhibitors, a series of kinetic measurements was performed at saturating and non-saturating constant concentrations of D-sorbitol or NAD⁺ with the other substrate, NAD⁺ or D-sorbitol, being varied. Identification of the type of inhibition was by double-reciprocal analysis. The corresponding inhibition constants were derived from a non-linear fitting procedure (SigmaPlot 5.0). The apparent kinetic parameters for different polyol and ketose substrates were determined in 50 mM Tris/HCl buffer, pH 7.5, at constant concentrations of NAD⁺ and NADH respectively. The initial velocity and substrate concentration pairs were fitted to the single-substrate Michaelis-Menten equation.

Modifications of XDH

Reactive thiols in native and denatured XDH were determined at pH 7.0 and 25 °C with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) [27]. Denaturation was accomplished by incubating a solution of 0.64 μ M XDH at 25 °C in the presence of 5.7 g/l SDS for 45 min. Treatment of XDH with the metal-chelating EDTA was performed at 25 °C in 50 mM Tris/HCl buffer or 50 mM phosphate buffer, pH 7.5–9.0. To 500 μ l of a solution containing 5–10 μ M XDH tetramer was added 50 μ l of a 5–20 mM solution of EDTA in buffer. The mixture was incubated for 15 min; the residual XDH activity was then assayed. Protection studies were performed in the same way except that XDH was preincubated with 250 mM xylitol, 50 mM D-xylulose, 2 mM NAD⁺ or 0.22 mM NADH, i.e. five times the apparent K_m value for the corresponding component, for 15 min before the chelator was added. To remove the chelating compound, 500 μ l of reaction mixture was doubly gel-filtered with NAP5 columns (Pharmacia) equilibrated in Tris or phosphate buffer.

Metal analyses

Metal analyses were performed by high-resolution inductively coupled plasma MS [28,29] on an instrument developed by Finnigan MAT (Bremen, Germany). A broad metal standard was used as reference, and the elements ¹¹B, ²⁴Mg, ²⁷Al, ⁵¹V, ⁵²Cr, ⁵⁵Mn, ⁵⁸Ni, ⁵⁹Co, ⁶³Cu, ⁶⁴Zn, ⁷⁵As, ⁸²Se, ⁸⁶Sr, ⁹⁸Mo, ¹⁰⁷Ag and ¹¹⁴Cd were quantified [29]. The protein sample was gel-filtered on NAP5 columns (Pharmacia) with ultrapure MilliQ-grade water (Millipore) before analysis, and all tubes were exhaustively rinsed with this water to avoid contamination. The blanks contained less than 1 % of the metals detected in the protein sample. For the high-resolution inductively coupled plasma MS measurements, the sample was diluted appropriately; the metal concentration in the sample was approx. 500 μ g/l. The S.D. for the measurements was no more than 4 %.

Matrix-assisted laser desorption/ionization MS (MALDI-MS)

XDH was analysed by MALDI-MS with a DYNAMO linear time-of-flight spectrometer (Thermo BioAnalysis, Hemel Hempstead, Herts., U.K.). The instrument was operated with delayed extraction off and deflector on. XDH was mixed with an equal volume of matrix solution (20 g/l sinapinic acid in a 7:3 mixture of acetonitrile and 1.0 g/l trifluoroacetic acid in water). A 1 μ l sample of the protein mixture, containing approx. 0.1 pmol of

XDH, was dried on sample plates with wells and analysed. The mass axis was externally calibrated with BSA (66431 Da).

Fluorescence spectroscopy

Fluorescence measurements were performed on a Hitachi Spectrofluorometer F 2000 (Hitachi, Tokyo, Japan) at 25 ± 1 °C in cells of 1 cm optical path with bandwidths of 5 nm for the excitation and emission wavelengths. Binding of coenzyme to XDH was determined by the stepwise addition of 1.5 μ l of 2 mM NADH to 340 μ l of a 1–2 μ M enzyme solution. The dilution by the addition of NADH was kept to a maximum of 3.5 %. Controls were obtained by the same procedure without enzyme. The excitation wavelength was set to 360 nm, and the emission was measured between 400 and 600 nm. Binding of the hydrophobic probe 8-anilino-1-naphthalene sulphonic acid (ANS) was determined at an emission wavelength of 460 nm after excitation at 390 nm. To 250 μ l of a solution of XDH (approx. 0.75 μ M) in 50 mM Tris/HCl buffer, pH 7.5, were added 40 μ l of ANS (0.1 mM); the mixture was allowed to equilibrate for 5 min before the fluorescence was recorded. Each fluorescence measurement was recorded in triplicate to decrease errors; the results shown represent mean values of these independent readings. Correction for the appropriate blank values was made in all cases.

RESULTS

Enzyme purification, structure and properties

XDH was obtained in homogeneous form by means of an efficient three-step purification summarized in Table 1. As in many protocols for the purification of NAD⁺-dependent dehydrogenases, we made use of dye ligand chromatography. The ligand, Red HE3B, seems to mimic the coenzyme, NADH, in its interaction with XDH. Thus affinity elution with nucleotide was possible, yielding an XDH preparation in good overall recoveries of more than 80 % that was judged to be more than 99 % pure by SDS/PAGE and non-denaturing anionic PAGE (Figures 1A and 1B). Staining for NAD⁺-dependent XDH activity in non-denaturing gels allowed an unequivocal identification of the protein as XDH (results not shown). No evidence was obtained during the purification that other proteins displaying XDH activity are present in the cell extract. To remove coenzyme bound to the active site of XDH, the enzyme was either doubly gel-filtered on NAP5 columns or dialysed against water for 24 h at 4 °C so that the protein showed no fluorescence emission at 460 nm after excitation at 340–360 nm.

Analysis by denaturing SDS/PAGE indicated that XDH is composed of one or more identical subunits of molecular mass approx. 40 kDa. The molecular mass of the XDH subunit determined by MALDI-MS was 37.4 kDa. Gel-permeation chromatography revealed a molecular mass for the native protein of 160 kDa (results not shown). Hence XDH is a tetramer. The isoelectric point of XDH is 6.0–6.1, as determined by chromatofocusing, isoelectric focusing and pH titration. The activity of XDH is strictly NAD⁺/NADH-dependent, and NADP⁺ and NADPH are not accepted as coenzymes. Other electron acceptors such as 2,6-dichlorophenol-indophenol or potassium ferricyanide cannot be used by XDH. When employed in concentrations of up to 2 mM, the components of the anabolic reduction charge, NADP⁺ and NADPH, and the energy charge, AMP, ADP and ATP, had no significant effect on the NAD⁺-dependent oxidation of D-sorbitol. Various metabolic intermediates of the pentose phosphate pathway and the citric acid cycle, tested at 10 mM, did not affect XDH activity significantly. Potent inhibitors of

Table 1 Purification of XDH

The material processed was approx. 75 g of wet yeast biomass. The purification steps were as follows: Red 120, dye-ligand chromatography with Red 120–Sepharose 4B-CL; HIC, hydrophobic interaction chromatography with phenyl-Sepharose 4B-CL high-sub, fast flow.

Purification step	Total units	Protein concentration (mg/ml)	Volumetric activity (units/ml)	Volume (ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	1750	6.48	10.9	160	1.69	1	100
Red 120 (salt elution)	1702	0.42	21.7	78	51.7	31	97
HIC	1499	0.32	28.3	53	89.1	53	86
Red 120 (affinity elution)	1449	0.79	120.8	12	152.0	90	83

Figure 1 Purity and properties of XDH

(A) SDS/PAGE. Left lane, purified protein; right lane, molecular mass standards (indicated at the right in kDa). The standards were, from bottom to top, lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin and BSA. (B) Non-denaturing PAGE. Left lane, molecular mass standards (indicated at the left in kDa); middle lane, purified protein; right lane, purified protein after treatment with 1 mM EDTA for 30 min at pH 7.5.

XDH are Co^{2+} , Mn^{2+} and Zn^{2+} ions that, when employed at a concentration of 5 mM, caused 65%, 64% and 100% inhibition of enzyme activity respectively. For XDH, the pH optima are 7.0–7.5 and 10.0–10.5 for substrate reduction and oxidation respectively. At 25 °C, XDH is most stable in the pH range 8.0–9.0 and, its stability strongly depends on the buffer component. The half-life in 50 mM Tris buffer, pH 7.5, was approx. 300 h, whereas that in 20–50 mM potassium phosphate, pH 7.5, was only 24 h. No thiol compounds are necessary to protect XDH against inactivation. The temperature optima for enzyme activity in polyol oxidation at pH 9.0 and ketose reduction at pH 7.0 are both 45 °C, and a similar 2.5-fold increase in activity is observed for the temperature range 25–45 °C (results not shown). Thus the corresponding activation energy is estimated to be approx. 39 kJ/mol at both pH values. The inactivation of XDH at temperatures no lower than 45 °C is faster at pH 7.0 than at pH 9.0, in agreement with the results for long-term stability of the enzyme at 25 °C.

Metal content and its role in catalytic activity and structural stability

XDH contains 1.10 ± 0.10 Zn^{2+} ions per protomer. In addition, each XDH subunit was found to contain 5.90 ± 0.35 Mg^{2+} ions

Table 2 Removal of metal ions and inactivation of XDH by EDTA

Enzyme preparation	XDH activity (%)	Metal content (mol/mol XDH subunit)
XDH, doubly gel-filtered	100	1.1 Zn^{2+} ; 5.9 Mg^{2+}
XDH, dialysed	50 ± 10	0.6 Zn^{2+} ; 0 Mg^{2+}
EDTA-treated XDH	$5 \pm 2^*$	0.15 Zn^{2+} ; 0 $\text{Mg}^{2+} \dagger$
EDTA-treated XDH, xylitol (250 mM) protection	$80 \pm 10^*$	0.8 Zn^{2+} ; 0 $\text{Mg}^{2+} \dagger$

* The residual XDH activity after 15 min incubation at 25 °C in the presence of 1 mM EDTA is given (50 mM potassium phosphate, pH 7.5).

† The metal content of EDTA-treated XDH was determined after double gel-filtration on a NAP5 column.

(Table 2). The Mg^{2+} content was verified in three independent enzyme preparations including production in *G. mastotermis* and purification (as in Table 1). After extensive dialysis (24 h) against water, Mg^{2+} ions were removed completely. Approx. 50% of Zn^{2+} ions were removed during dialysis, correlating with a decrease in the specific activity of the resulting enzyme preparation to one-half. When XDH was incubated with chelating agents such as EDTA in concentrations of 1.0 mM for 15 min in either phosphate or Tris buffer, pH 7.5–9.0, enzyme activity was lost almost completely. The extent of inactivation was, however, dependent on the EDTA concentration and increased with increasing EDTA. The treatment with 1.0 mM EDTA at pH 8.0 and subsequent double gel-filtration resulted in the removal of 100% and 85% of enzyme-bound Mg^{2+} and Zn^{2+} respectively (Table 2). When XDH was preincubated with 250 mM xylitol for 15 min at pH 8, and then 1.0 mM EDTA was added, the inactivation could be prevented efficiently (Table 2). D-Xylulose (50 mM) as well as NADH (0.5 mM) and NAD^+ (2 mM) afforded similar protection to that by xylitol (250 mM). The inactivation by EDTA was pH-dependent, and the stability of XDH increased with increasing pH values. It is interesting that the protection with xylitol selectively prevented the removal of Zn^{2+} ions (Table 2). The Mg^{2+} -depleted XDH (by EDTA treatment) retained full enzyme activity and was stable at 4 and 25 °C. (For the analysis EDTA had to be removed with caution by at least double gel-filtration. Traces of remaining chelator were found to cause the rapid inactivation of XDH, thus feigning a decreased stability of the Mg^{2+} -depleted XDH.) When, after treatment with 1.0 mM EDTA for 15 min without protection with xylitol, the inactive XDH was diluted 10-fold into buffer or subjected to gel filtration, approx. 20% of the original XDH activity was recovered. This recoverable enzyme activity is in agreement with the Zn^{2+} content of the EDTA-treated XDH (Table 2). It decreased, however, with increasing time of incubation in the presence of EDTA (15–60 min), thus probably

Figure 2 Kinetic analysis of XDH at steady state for the NAD^+ -dependent oxidation of D-sorbitol

(A) Double-reciprocal plot from initial velocity measurements, with the concentration of NAD^+ varied and the concentration of D-sorbitol held constant. (B) Double-reciprocal plot from initial velocity measurements, with the concentration of D-sorbitol varied and the NAD^+ concentration held constant.

indicating the progressive removal of the Zn^{2+} ion with time. No XDH activity was recovered when an up to 3-fold molar excess of Zn^{2+} was added to the gel-filtered apoenzyme. For XDH it seems that chelating agents form a stable complex with the Zn^{2+} ion in the holoenzyme first and then only slowly remove the metal from the protein. Judging from the analysis by non-denaturing anionic PAGE (Figure 1B), the EDTA-treated inactive enzyme retains the tetrameric structure of the native XDH. The exposure of hydrophobic groups at the protein surface, measured fluorometrically by the binding of the hydrophobic dye ANS, was significantly (30%) lower in the native enzyme, relative to XDH inactivated by treatment with chelator.

Inhibition and activation of XDH

Many thiol compounds are potent inhibitors of ADHs and SDHs [30–32]. They are competitive inhibitors with respect to the alcohol, bind to the binary enzyme–nucleotide complex and

display inhibition constants (K_i) in the micromolar to low millimolar range. In marked contrast, the XDH-mediated oxidation of D-sorbitol (at pH 10) was not inhibited by 1 mM dithiothreitol, for which a K_i of 2.4 μM for the SDH from sheep liver has been determined. Titration of reactive cysteine residues in XDH gave a value of one thiol per native protomer and approximately four per denatured protomer. The difference in enzyme activities of native and modified XDH was less than 25%. Therefore the cysteine residue derivatized with 5,5'-dithiobis-(2-nitrobenzoic acid) in the native protomer is not essential for catalysis. As found with SDH [31], detergents such as Tween 20 (1 g/l) had a significant activating effect on the maximum initial velocity of D-sorbitol oxidation (35%).

Initial velocity studies

In D-sorbitol oxidation, initial velocities obtained with D-sorbitol as the varied substrate when NAD^+ was constant, and initial velocities obtained with NAD^+ as the varied substrate when D-sorbitol was constant, yielded straight converging lines that intersected to the left of the ordinate in the double-reciprocal plot of initial velocity against varied reactant concentration (Figures 2A and 2B). The secondary plots of the resulting slopes and intercepts against the reciprocals of the second fixed substrate or coenzyme, were linear (results not shown). In D-fructose reduction, when D-fructose was varied and NADH was constant and when NADH was varied and D-fructose was constant, linear intersecting primary Lineweaver–Burk plots were obtained (Figures 3A and 3B; note that not all results are shown for reasons of clarity), and the replots of slopes and intercepts against the reciprocal of the second fixed substrate were linear (results not shown). The results indicate that the kinetic mechanism is sequential, which implies that substrate and coenzyme must add to the enzyme before any product is released [26]. No substrate inhibition was observed in polyol oxidation by D-sorbitol (1 M or less) and ketose reduction by D-fructose (2 M or less).

Product inhibition

To determine the steady-state kinetic mechanism of XDH in better detail, product inhibition studies in the direction of NAD^+ -dependent D-sorbitol oxidation were carried out. When NAD^+ was the variable substrate and D-sorbitol concentration was held constant, NADH gave a competitive inhibition pattern at both non-saturating and saturating concentrations of D-sorbitol. The replot of the slope against NADH was also linear. When D-sorbitol was the variable substrate and NAD^+ was held constant, D-fructose gave a competitive inhibition pattern both at saturating and non-saturating concentrations of NAD^+ . The competence of D-fructose to inhibit the NAD^+ -dependent D-sorbitol oxidation was low; the corresponding inhibition constant was approx. 200 mM. With NAD^+ as the variable substrate, no inhibition by D-fructose was observed at saturating concentrations of D-sorbitol. To confirm the existence of a competitive ketose/polyol reactant pair in the steady-state kinetic mechanism of XDH, further inhibition experiments were performed with D-xylulose. For XDH there is a large difference in the apparent Michaelis constants for D-xylulose (10 mM) and D-fructose (approx. 1 M). Therefore D-xylulose is expected to be a better inhibitor than D-fructose of the NAD^+ -dependent oxidation of D-sorbitol. D-Xylulose was a strong competitive inhibitor with respect to D-sorbitol both at saturating and non-saturating constant concentrations of NAD^+ (Table 3). (It was proved that in the back reaction, i.e. ketose reduction, D-sorbitol was a

Figure 3 Kinetic analysis of XDH at steady state for the NADH-dependent reduction of D-fructose

(A) Double-reciprocal plot from initial velocity measurements, with the concentration of NADH varied and the concentration of D-fructose held constant. (B) Double-reciprocal plot from initial velocity measurements, with the concentration of D-fructose varied and the NADH concentration held constant.

competitive inhibitor with respect to D-xylulose at high and low concentrations of NADH.) Furthermore D-xylulose was a mixed-type inhibitor with respect to NAD⁺ at non-saturating concen-

trations of D-sorbitol. High concentrations of D-sorbitol could, however, overcome this inhibition. When D-sorbitol was the variable substrate and NAD⁺ concentration was held constant, NADH gave a mixed non-competitive inhibition pattern at high concentrations of NAD⁺ (5 mM). The inhibition constants increased with increasing NAD⁺ concentration (1–5 mM), indicating that saturation with NAD⁺ can probably overcome the inhibition. Full saturation with NAD⁺ was probably difficult to achieve in the experiments ($100K_m^{NAD} \approx 20$ mM). The inhibition patterns are summarized in Table 3.

Coenzyme binding to free XDH

When NADH (1–40 μ M) was added to a solution of XDH (2 μ M), a slight increase in coenzyme fluorescence (at 460 nm) was observed relative to a blank that contained coenzyme but not XDH, indicative of the binding of NADH to the free enzyme. However, the fluorescence enhancement was too weak (a maximum of approx. 15%) for a determination of the dissociation constant and the stoichiometry of NADH binding.

Kinetic constants

The initial velocity patterns together with the patterns of product inhibition and coenzyme binding are consistent with a Theorell–Chance mechanism in which coenzyme binds first and leaves last. This kinetic mechanism is also found with the structurally and functionally related SDH and ADH [8,33]. The results for D-sorbitol oxidation and D-fructose reduction were fitted to eqn. (1). The resulting kinetic constants are summarized in Table 4. The competitive inhibition constant of NADH with respect to NAD⁺ ($K_i = 12 \pm 2$ μ M at 0.1 M D-sorbitol) matches with the value for K_i^{NADH} determined from kinetic measurements (Table 4). The internal consistency of the kinetic parameters in Table 4 was checked with the kinetic Haldane relationship for a Theorell–Chance mechanism:

$$K_{eq}(\text{app}) = k_{cat,ox} K_m^{RO} K_i^{NADH} / (k_{cat,red} K_m^{ROH} K_i^{NAD}) \quad (2)$$

where $K_{eq}(\text{app})$ is the apparent equilibrium constant, $k_{cat,ox}$ and $k_{cat,red}$ are the catalytic constants for alcohol oxidation and carbonyl reduction, K_i^{NADH} and K_i^{NAD} are apparent dissociation constants of the binary XDH–nucleotide complexes, and K_m^{RO} and K_m^{ROH} are Michaelis constants for D-fructose and D-sorbitol respectively. The kinetic constants in Table 4 give a value of

Table 3 Analysis of product inhibition for the NAD⁺-dependent oxidation of D-sorbitol catalysed by XDH

The inhibition constants were determined by non-linear regression. See the Kinetics section for details. Abbreviations: n.d., not determined; c, competitive; uc, uncompetitive.

Inhibitor	Variable substrate	Concentration of fixed substrate (M)	Inhibition type	Inhibition constant (M)
NADH	NAD ⁺	Saturating (1.0)	Competitive	5×10^{-5}
NADH	NAD ⁺	Non-saturating (0.10)	Competitive	1.2×10^{-5}
NADH	D-Sorbitol	Saturating (5×10^{-3})*	Mixed-type	8×10^{-5} (c); 4×10^{-4} (uc)
D-Fructose	D-Sorbitol	Saturating (5×10^{-3})*	Competitive	0.20
D-Fructose	D-Sorbitol	Non-saturating (5×10^{-4})	Competitive	n.d.
D-Xylulose	D-Sorbitol	Saturating (5×10^{-3})*	Competitive	10^{-3}
D-Fructose	NAD ⁺	Saturating (1.0)	No inhibition	—
D-Xylulose	NAD ⁺	Non-saturating (0.1)	Mixed-type	n.d.

* Complete saturation with NAD⁺ probably requires nucleotide concentrations higher than 20 mM.

Table 4 Kinetic parameters for XDH for NAD⁺-dependent D-sorbitol oxidation and NADH-dependent D-fructose reduction

The values were determined by non-linear regression. ROH and RO are D-sorbitol and D-fructose; K_i is the apparent dissociation constant, $k_{cat,ox}$ is the catalytic constant for the reaction of NAD⁺ and D-sorbitol, $k_{cat,red}$ is the catalytic constant for the reverse reaction. The values for $k_{cat,ox}$ and $k_{cat,red}$ are based on the XDH tetramer. Values are means $\pm$ S.D.

D-Sorbitol oxidation		D-Fructose reduction	
Parameter	Value	Parameter	Value
$k_{cat,ox}$ (s ⁻¹)	203 $\pm$ 62	$k_{cat,red}$ (s ⁻¹)	858 $\pm$ 124
K_i^{NAD} (μ M)	810 $\pm$ 260	K_i^{NADH} (μ M)	10 $\pm$ 8
K_m^{ROH} (mM)	32 $\pm$ 6	K_m^{RO} (mM)	1270 $\pm$ 441
K_m^{NAD} (μ M)	211 $\pm$ 35	K_m^{NADH} (μ M)	39 $\pm$ 20

Table 5 Individual rate constants for XDH

Rate constants were calculated by the method of Segel [26] from the steady-state kinetic parameters for XDH in Table 4 for a Theorell–Chance kinetic mechanism with six velocity constants.

Rate constant	Value	Rate constant	Value
k_1 (mM ⁻¹ ·s ⁻¹)	962	k_{-1} (s ⁻¹)	779
k_2 (mM ⁻¹ ·s ⁻¹)	6.34	k_{-2} (mM ⁻¹ ·s ⁻¹)	0.68
k_3 (s ⁻¹)	220	k_{-3} (mM ⁻¹ ·s ⁻¹)	22 000

0.116 for this expression at pH 7.5, or 3.66 nM for the pH-independent value of K_{eq} . This latter value is in good agreement with the experimentally observed value of K_{eq} of 3.7 nM for the expression [D-fructose][NADH][H⁺]/[D-sorbitol][NAD⁺] [33]. By using the relation of the kinetic parameters to the six individual rate constants for the Theorell–Chance mechanism [26], the rate constants given in Table 5 were calculated. The good agreement of the first-order rate constants k_3 (reflecting the dissociation of XDH–NADH) and k_{-1} (reflecting the dissociation of XDH–NAD⁺) with $k_{cat,ox}$ and $k_{cat,red}$ respectively suggests that XDH–coenzyme dissociation might be rate-limiting both in

D-sorbitol oxidation and D-fructose reduction, which is a requirement for the Theorell–Chance mechanism.

Substrate spectrum

The substrate spectrum in the direction of polyol oxidation and ketose reduction is summarized in Table 6. Like mammalian SDH [8] and XDH from *P. stipitis* [18], XDH from *G. masto-termitis* does not oxidize primary alcohols (methanol, ethanol or butanol). The three-carbon polyol glycerol seems to be the smallest substrate recognized by XDH. For a series of polyols composed of four to six carbons, the absolute configuration of xylitol or D-sorbitol, C-2 (S), C-3 (R), C-4 (R), is preferred, and at least five carbon atoms are required for full catalytic competence; compare, for example, D,L-threitol with xylitol. Correct interactions between XDH and the substrate both at the 2 and 4 position of the polyol are critical for catalysis. Relative to D-sorbitol, D-mannitol, C-2 (R), C-4 (R), and galactitol, C-2 (S), C-4 (R), are very poor substrates of XDH, as are D-arabinitol, C-2 (R), C-4 (R), and L-arabinitol, C-2 (S), C-4 (S), relative to xylitol. In contrast with sheep liver SDH [33], the configuration at C-5, (R) in D-sorbitol and (S) in L-iditol, seems to be important for the XDH-mediated catalysis. The significant contribution to catalytic competence of interactions at the C-3 position is clearly inferred from a comparison of the apparent rates and catalytic efficiencies observed with xylitol, C-3 (R), and ribitol, C-3 (S). The binding energy derived from non-covalent interactions of XDH with other polyol substrates was calculated relative to xylitol by the equation $\Delta\Delta G = -RT\ln[(k_{cat}/K_m)_{polyol}/(k_{cat}/K_m)_{xylitol}]$. Compared with xylitol a loss of binding energy was found with all polyol substrates: D-sorbitol (1.3 kJ/mol), L-iditol (8.0 kJ/mol), D-mannitol (approx. 12 kJ/mol), ribitol (6.8 kJ/mol), D,L-threitol (approx. 11 kJ/mol) and glycerol (approx. 15 kJ/mol). In the direction of ketose reduction, the catalytic efficiencies determine a preference of XDH in the order D-xylulose > D-ribulose > L-erythrulose > D-fructose > L-sorbose. Dicarbonyl compounds such as D-xylosone and D-glucosone (containing an aldehyde group at C-1 and an oxo group at C-2) were not accepted as substrates by XDH, suggesting that the hydroxy group at C-1 is important for substrate recognition. Considering that the amino groups of Tris might react with (1) the catalytic Zn²⁺ of XDH [34] or (2) carbonyl functions of the ketose [34], we determined the kinetic parameters for some polyol and ketose substrates in 50 mM phosphate buffer, pH 7.5. The kinetic

Table 6 Substrate spectrum of XDH

Reactions for polyol oxidation and carbonyl reduction were performed at 25 °C in 50 mM Tris/HCl buffer, pH 7.5. The constant concentrations of NAD⁺ and NADH were 2.44 mM and 244 μ M respectively. Abbreviation: n.d., not determined. When saturation with substrate was not achieved, k_{cat}/K_m was determined from the linear dependence of the initial velocity on the substrate concentration. Note, this allows the determination of k_{cat}/K_m even though each kinetic constant cannot be determined separately.

Polyol	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)	Ketose	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
Glycerol	5.5	746	0.01	Dihydroxyacetone	n.d.	n.d.	n.d.
D,L-Threitol	n.d.	n.d.	0.04	L-Erythrulose	n.d.	n.d.	4
Xylitol	142	39	3.6	D-Xylulose	2763	10	276
L-Arabinitol	n.d.	n.d.	< 0.01				
D-Arabinitol	n.d.	n.d.	< 0.01				
Ribitol	48	209	0.23	D-Ribulose	n.d.	n.d.	55
D-Sorbitol	114	55	2.1	D-Fructose	1300	1370	1.0
L-Iditol	81	557	0.14	L-Sorbose	235	1468	0.2
D-Mannitol	n.d.	n.d.	0.03				
Galactitol	n.d.	n.d.	< 0.01				

parameters for one particular substrate were not exactly identical in Tris and phosphate buffers. However, the overall conclusions pertaining to the substrate spectrum of XDH remain unaffected by the choice of the buffer.

DISCUSSION

The biochemical characterization of yeast XDH described in this work revealed structural and functional properties of XDH that have not hitherto been pointed out. The XDH from *G. mastotermitis*, similarly to XDHs from several other yeast species [35,36], is inducible by D-xylose, and its principal physiological role is the oxidation of xylitol to D-xylulose in the catabolic pathway of D-xylose. In contrast with *P. stipitis* [7], evidence of multiple NAD⁺-dependent or NADP⁺-dependent forms of XDH has not been found in *G. mastotermitis*. The isolated XDH seems similar to the *XYL2* gene product from *P. stipitis* [7,18] and mammalian SDHs [8]. A close relationship between XDH and SDH was found for the following characteristics of structure and function: (1) the presence of one, most probably catalytic, but not a second structural Zn²⁺ ion per protein subunit, (2) the tetrameric quaternary structure, and (3) the spectrum of substrate structures that can be oxidized or reduced and the kinetic mechanism. These results corroborate the conclusions of others [7], based on a detailed examination of the primary structure of XDH from *P. stipitis* in relation to structures of known members of the medium-chain ADH family [8]. In contrast with our observations, the XDHs isolated from *Candida shehatae* [19] and *P. stipitis* [18] were reported to be dimers (rather than tetramers) of approx. 41 kDa and approx. 32 kDa protomers respectively. In spite of the proposed overall relationship, the XDH described here differs in several respects from mammalian SDHs, as discussed below.

Kinetic mechanism

The kinetic mechanism of XDH for D-sorbitol oxidation and D-fructose reduction is sequential and compulsorily ordered. Like mammalian SDHs [33,37], the central ternary complexes, XDH–NAD⁺–sorbitol and XDH–NADH–ketose, seem not to be kinetically significant; in agreement with the characteristic patterns of product inhibition a Theorell–Chance mechanism is proposed for XDH. The kinetic mechanism probably involves rate-limiting enzyme–coenzyme dissociation in the direction of D-sorbitol oxidation and D-fructose reduction. Unlike SDH [33], abortive ternary complexes (e.g. SDH–NADH–sorbitol) seem not to be formed during catalysis by XDH; this is manifested by the absence of substrate inhibition in the direction of polyol oxidation and ketose reduction.

Substrate spectrum

The relative catalytic efficiencies in a series of polyol substrates are similar for XDH and SDH: xylitol > D-sorbitol > ribitol > L-iditol ≫ D,L-threitol. The lower limit to the substrate size and the specific steric requirements in particular at the 2 and 4 positions of the polyol are comparable for both enzymes. The probable major difference between the two enzymes is that the apparent maximum rate for XDH is dependent on the substrate structure (the present study), whereas that for SDH is not [33,37]. In other words, XDH, but not SDH, relies on the use of binding energy derived from non-covalent interactions with the polyol substrate to decrease the activation energy for the rate-limiting step. The common rate-limiting step in the catalytic mechanism of SDH is believed to be the dissociation of the binary SDH–NADH complex, and therefore a similar if not identical value for

the maximum rate is observed for a range of different polyols [33]. In terms of catalytic efficiency, SDH distinguishes between substrate stereoisomers because of widely varying apparent binding constants rather than catalytic-centre activities [33]. With XDH, both kinetic parameters seem to contribute to its specificity, evident from a comparison of the data in Table 6. Considering the Theorell–Chance mechanism of XDH for D-sorbitol oxidation (with rate-limiting enzyme–coenzyme product dissociation), this result seems surprising. However, the formation of ternary enzyme–coenzyme–substrate complexes whose interconversion is rate-limiting and substrate-specific seems probable for XDH and has been found with sheep liver SDH for a class of poor C-3 substrates such as glycerol or propanediol [38] as well as other substrates [39]. The reason for the increase in k_{cat} for xylitol oxidation (relative to D-sorbitol) and D-xylulose reduction (relative to D-fructose) is unknown, but could indicate a substrate-specific enhancement of the off-rate of the nucleotide, which has been found for example for ADH with the cyclohexanol/cyclohexanone substrate pair [40].

For D-sorbitol oxidation, the apparent kinetic parameters of XDH and SDH are different. Relative to sheep liver SDH [33], the catalytic constant and the K_m for D-sorbitol are respectively 11-fold and 9-fold higher for XDH. The apparent dissociation constants of the binary XDH–nucleotide complexes are similar to those for SDHs from sheep liver [33] and bovine lens [37]. It is interesting to notice how these differences in kinetic parameters seem to reflect the different physiological functions of XDH and SDH. In contrast with SDH, which does not catalyse a high-flux metabolic route in mammalian tissues where it might even have a role as a detoxification catalyst [8,39], the XDH-mediated oxidation of xylitol is part of the mainstream pathway for D-xylose utilization in yeast. Thus, compared with SDH, the maximization of the reaction rate under physiological conditions is more important for XDH, to guarantee a high flux of carbon. According to the generally accepted tenets of enzyme evolution for fluxional efficiency [41], XDH should bind the polyol substrate weakly and concomitantly increase the value for k_{cat} , which was observed.

Metal content and its relation to enzyme activity and stability

The complete inactivation of XDH after the dissociation of Zn²⁺ by complexation with chelating compounds indicates that this metal ion is essential for enzyme activity in XDH. The protection by NAD(H) or substrate against the EDTA-induced removal of Zn²⁺ and concomitant inactivation in line with the known role of Zn²⁺ in the related medium-chain SDH [8] seems to suggest that the Zn²⁺ ion of XDH is located in the active site of the enzyme. However, the results of protection experiments have to be interpreted with care because non-specific effects exerted especially by polyhydroxylated compounds such as xylitol cannot be ruled out completely. The dissociation of Zn²⁺ was not accompanied by changes in quaternary structure of XDH (which seems to make a purely structural role of the Zn²⁺ ion unlikely), but at least in our hands no enzyme activity could be recovered anew by supplementing apoXDH with Zn²⁺. Mg²⁺ ions have not, to the best of our knowledge, been described in relation to SDHs and ADHs. Six Mg²⁺ ions per XDH subunit were detectable and could be removed by dialysis or treatment with EDTA. They were found neither to participate in catalysis nor to have a role in the conformational stability of the enzyme. Whether the XDH-bound magnesiums could have other important functions, for example *in vivo*, is unknown, and a characterization in more detail of the Mg²⁺-depleted XDH could therefore be interesting.

We thank Dr. Thomas Prohaska and Professor Fritz Altmann (Institute of Chemistry, BOKU, Vienna, Austria) for support and help with metal analyses and MALDI-MS analyses respectively, and Christian Leitner (Institute of Food Technology, BOKU, Vienna, Austria) for providing the D-xylulose and the D-glucosone substrates. The work was supported by an F.W.F. (Austrian Science Foundation) grant, P-12569-MOB to B.N.

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