NAD(P)H-dependent aldose reductase from the xylose-assimilating yeast *Candida tenuis*

Isolation, characterization and biochemical properties of the enzyme

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During growth on D-xylose the yeast *Candida tenuis* produces one aldose reductase that is active with both NADPH and NADH as coenzyme. This enzyme has been isolated by dye ligand and anion-exchange chromatography in yields of 76%. Aldose reductase consists of a single 43 kDa polypeptide with an isoelectric point of 4.70. Initial velocity, product inhibition and binding studies are consistent with a compulsory-ordered, ternary-complex mechanism with coenzyme binding first and leaving last. The catalytic efficiency (k_{cat}/K_m) in D-xylose reduction at pH 7 is more than 60-fold higher than that in xylitol oxidation and reflects significant differences in the corresponding catalytic centre activities as well as apparent substrate-binding

constants. The enzyme prefers NADP(H) approx. 2-fold to NAD(H), which is largely due to better apparent binding of the phosphorylated form of the coenzyme. NADP⁺ is a potent competitive inhibitor of the NADH-linked aldehyde reduction (K_i 1.5 μ M), whereas NAD⁺ is not. Unlike mammalian aldose reductase, the enzyme from *C. tenuis* is not subject to oxidation-induced activation. Evidence of an essential lysine residue located in or near the coenzyme binding site has been obtained from chemical modification of aldose reductase with pyridoxal 5'-phosphate. The results are discussed in the context of a comparison of the enzymic properties of yeast and mammalian aldose reductase.

INTRODUCTION

Aldehyde reductases are cytosolic enzymes that catalyse the NADPH-dependent reduction of an extremely wide variety of carbonyl compounds to the corresponding alcohols. The occurrence of aldehyde reductases is widespread and has been reported in man and animals, and also in yeasts and filamentous fungi. In agreement with their extraordinarily broad substrate specificity, aldehyde reductases are thought to accomplish versatile physiological functions. These functions include the detoxification of reactive carbonyl-containing metabolites in mammalian tissues [1,2], the formation of intracellular polyol as a mechanism to counteract detrimental osmotic effects [3] and the reductive assimilation of D-xylose in yeast [4]. A classification of aldehyde reductases into an enzyme family was originally based on similar chemical and physical properties as well as substrate specificity [5]. Recently it has received a sound structural basis as more primary protein structures have become available from gene-sequencing data [6].

Aldose reductase (alditol:NAD(P)⁺ 1-oxidoreductase, EC 1.1.1.21; ALR) belongs to this family and has a substrate spectrum that ranges from aldoses and alduronic acids to aromatic and aliphatic aldehydes and even steroids [7–10]. Hence ALR can catalyse the reduction of glucose to sorbitol. Owing to the excessive formation of the polyol at hyperglycaemic concentrations of glucose in human tissues, ALR has been implicated in the onset of diabetic complications [1,11,12]. Probably as a consequence of this fact, numerous studies have been published that deal with the isolation and characterization of ALR from mammalian tissues and provide the basic information for the development of therapeutic ALR inhibitors [1,5]. Important details about structure–function relationships in ALR have

recently become available from crystallographic and mechanistic studies on human and bovine ALR [13–16].

In yeast, ALR has an entirely different role as it promotes the first step in D-xylose metabolism, an NADPH-dependent or, in some cases, NADH-dependent reduction of D-xylose to xylitol. In the successive polyol dehydrogenase-catalysed, predominantly NAD⁺-dependent, reaction xylitol is oxidized to D-xylulose. The interest in this sequential redox reaction and the enzymes involved is linked to the process of ethanol production from pentose sugars contained in hydrolysates of hemicellulose (reviewed in [4]). It has recently been pointed out that the conversion of D-xylose to ethanol by yeasts requires an NADH-dependent ALR activity [17,18]; otherwise, serious imbalances in the cellular redox system are expected to occur during anoxic or oxygen-limited growth, with NADH being accumulated at the expense of NADPH. Interestingly, D-xylose-fermenting *Pichia stipitis* and *Candida shehatae* were shown to produce one single ALR that is active both with NADPH and NADH [9,19]. Other yeasts such as *Pachysolen tannophilus* synthesize multiple forms of ALR with different coenzyme specificities [20–22]. The significant dual coenzyme specificity distinguishes the *P. stipitis* and the *C. shehatae* enzymes from most other ALRs so far isolated from mammalian or microbial sources. We have recently become interested in the yeast *Candida tenuis* CBS 4435 because this organism produces comparable NADH- and NADPH-linked aldehyde-reducing activities during growth on D-xylose [23].

In the present study we have isolated the NADH-dependent ALR activity from *C. tenuis* and characterized the responsible enzyme biochemically in some detail. The results are used for a comparison of the enzymic properties of ALRs from yeasts and mammals, including the issues of coenzyme and substrate specificity in ALR. We felt that such a comparison was important

Abbreviations used: ALR; aldose reductase (alditol:NAD(P)⁺ 1-oxidoreductase; EC 1.1.1.21); Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); PLP, pyridoxal 5'-phosphate; TNPS, 2,4,6-trinitrophenylsulphonic acid.

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because the various elegant studies performed with the related ALRs from mammals and yeast have so far proceeded on separate paths.

EXPERIMENTAL

Materials

Material for gel chromatography and electrophoresis was from Pharmacia (Uppsala, Sweden). The dyes Procion Red H-8B (Reactive Red 31) and Procion Green H-E4BD (Reactive Green 19) were obtained from ICI (Manchester, U.K.). Pyridoxal 5'-phosphate (PLP), 2,4,6-trinitrophenylsulphonic acid (TNPS), Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid); Nbs₂] and all coenzymes and enzyme substrates were of the highest purity available and purchased from Sigma (Deisenhofen, Germany). Aldosones (D-glucosone, D-arabino-hexos-2-ulose; D-xylosone, D-threo-pentos-2-ulose) were obtained by complete conversion of the respective aldose sugar (D-glucose, D-xylose) with the enzyme pyranose oxidase [24]. As judged from TLC, the aldoses were more than 95 % pure (C. Leitner and D. Haltrich, unpublished work). NAD⁺-dependent formate dehydrogenase was from Boehringer (Mannheim, Germany) and NADP⁺-dependent glucose-6-phosphate dehydrogenase from Sigma.

Micro-organism and preparation of the cell-free crude extract

The organism used throughout this study was *C. tenuis* CBS 4435 obtained from the Centraalbureau voor Schimmelcultures (Yeast Division, Delft, The Netherlands). It was cultivated at 25 °C in shaken flasks at 110 rev./min with YPX medium containing 20 g/l D-xylose, 4 g/l yeast extract and 4 g/l casein peptone. After approximately 24 h of growth, cells (typically 25–30 g of wet biomass/litre) were harvested by centrifugation, washed and suspended in doubly distilled water (1:2, w/v). Cell disruption was performed in a continuously operated ballmill (Dynamill; Bachofen AG, Basel, Switzerland) with glass beads of 0.25–0.5 mm diameter. The average (residence) time during continuous disruption was 7 min, and the temperature was kept below 10 °C. Cell debris was removed by centrifugation at 9000 g and 4 °C (25 min), and the recovered supernatant (crude cell extract) was stored at –70 °C. Any turbidity present after thawing was removed by ultracentrifugation at 110000 g and 4 °C for 40 min.

Assays

All measurements were performed with a Beckman DU-650 spectrophotometer. ALR activity was assayed at 25 °C by monitoring the oxidation of NAD(P)H and reduction of NAD(P)⁺ at 340 nm (1–5 min, rate of 0.05–0.1 ΔA/min) in the forward and reverse directions of reaction respectively. One unit of enzyme activity refers to 1 μmol of NAD(P)H consumed or produced/min. All rates were corrected for the appropriate blank readings accounting for the non-specific oxidation of NAD(P)H such as autooxidation mediated by aldose sugars [25]. The standard assay mixture for the forward direction of reaction was 300 mM phosphate buffer (pH 6.0)/707 mM D(+) -xylose/220 μM NAD(P)H. Assays in the reverse direction were performed in 50 mM phosphate buffer (pH 7.0)/950 mM xylitol/2.5 mM NAD(P)⁺. All reactions were started by the addition of coenzyme. Unless stated otherwise, all ALR activities reported refer to the NAD(H)-linked activity of the enzyme. Alcohol dehydrogenase [0.93 % (v/v) ethanol/1.9 mM NAD⁺ (pH 9.0)], glucose-6-phosphate dehydrogenase [3.14 mM D-glucose 6-phosphate/0.38 mM NADP⁺ (pH 7.5)], glutamate de-

hydrogenase [6.5 mM α-oxoglutarate/0.2 mM NADH (pH 8.0)], glutathione reductase [60.4 mM GSSG/0.24 mM NADPH (pH 7.4)], isocitrate dehydrogenase [66.6 mM isocitrate/0.17 mM NAD(P)⁺ (pH 7.5)], lactate dehydrogenase [5.18 mM L-lactate/6.5 mM NAD⁺ (pH 9.3)], malate dehydrogenase [100 mM malate/1.5 mM NAD⁺ (pH 9.2)], proteinase [1.85 % (w/v) casein; pH 7.5], transaldolase [2.59 mM D-fructose 6-phosphate/0.17 mM D-erythrose 4-phosphate (pH 7.6)] and transketolase [0.17 mM D-xylulose 5-phosphate/0.17 mM D-erythrose 4-phosphate (pH 7.6)] were measured as described by Bergmeyer [26]. Activities are expressed as units/mg of protein. Protein was determined spectrophotometrically [27] at 595 nm by using the Bio-Rad protein assay, with BSA as standard (0.1–1.0 mg/ml).

Protein purification

Dye-ligand affinity chromatography

The matrix for the preparation of the affinity gel was Sepharose 4B-CL. For dye-matrix coupling, 1 ml of gel was suspended in 1 ml of dye solution (10 mg of dye/ml of doubly distilled water). The mixture was shaken gently at pH 11.0 in the presence of 2 % NaCl for at least 48 h at 30 °C. Then the gel was filtered and washed with water, 1 M NaOH and 1 M NaCl until the eluate was colourless. All affinity gels were stored in 20 % (v/v) ethanol until use. Screening of a suitable dye for the purification of ALR was performed in accordance with published procedures [28,29]. Binding and elution of ALR activity and total protein in small-scale batch experiments were monitored.

After the preselection of two triazine dyes, purification of ALR was performed by column chromatography (GradiFrac system; Pharmacia). The centrifuged cell extract with a conductivity of 2.7 mS/cm was applied to the dye-ligand column (2.5 cm × 5.0 cm or 5.0 cm × 5.5 cm; approx. 10–15 mg of protein/ml of gel) equilibrated in 50 mM potassium phosphate buffer, pH 7.0. Adsorbed protein was eluted at a flow rate of 2.5 cm/h with a step gradient of 1 M and 2 M NaCl, with absorbance detection at 280 nm. Fractions of 20 ml were collected; those containing ALR activity were pooled.

Concentration and gel filtration

Concentration of ALR was achieved by (NH₄)₂SO₄ precipitation at 70 % saturation and subsequent dissolution of the centrifuged pellet (25000 g, 4 °C, 60 min) in a small volume of 20 mM Tris/HCl, pH 7.0. Desalted preparations of ALR were obtained by gel filtration on Sephadex G25 coarse, previously equilibrated with 20 mM Tris/HCl buffer, pH 7.0 (flow rate 3.5 cm/h).

Ion-exchange chromatography

Anion-exchange chromatography was performed on a Pharmacia FPLC system with a Mono-Q HR 5/5 column equilibrated with 20 mM Tris/HCl buffer, pH 7.0, that contained 0.1 % Tween-20 to stabilize ALR. Less than 10 mg of protein was applied to this column. Elution was accomplished with 1 M NaCl in buffer in a linear gradient from 0 to 100 mM NaCl in 20 column vol. The flow rate was 1 ml/min. Fractions of 1 ml were collected and those containing ALR were pooled and stored at –70 °C.

Electrophoresis

The efficiency of each purification step was controlled by SDS/PAGE, non-denaturing anionic PAGE (with PhastGel Gradient 8–25 in both cases) and isoelectric focusing PAGE

(with PhastGel IEF 3–9) performed on a Pharmacia PhastSystem unit in accordance with the instructions of the supplier. Staining for activity was performed by assaying the polyol-oxidizing activity of ALR. The gel was incubated at pH 8.9 with 950 mM xylitol as substrate, 2.5 mM NADP⁺ as coenzyme employing 5-methyl phenazine methosulphate (2 mg/ml) and Nitro Blue Tetrazolium (6 mg/ml) as electron acceptors.

Quaternary structure and molecular mass

The molecular mass of the ALR subunits was determined by SDS/PAGE (see the Electrophoresis section); that of the native enzyme was determined by gel filtration on Superose 12 (1.5 cm × 30 cm). The Superose 12 column was equilibrated in 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl, and protein elution was performed at a constant flow rate of 1.5 ml/min. For calibration, cytochrome *c* (12.4 kDa), myoglobin (17.6 kDa), β -lactoglobulin (35 kDa), egg albumin (43 kDa) and BSA (67 kDa) were used as standards.

Determination of the isoelectric point

The pI of ALR was determined by chromatofocusing on a Mono-P HR 5/20 column. An appropriate amount of enzyme was applied to the column, previously equilibrated with 25 mM piperazine/HCl, pH 5.5. ALR was eluted with Polybuffer 74 (Pharmacia), fractions of 1 ml were collected, and the pH of the fraction that contained the enzyme was measured. In addition, the pI was determined by isoelectric focusing and electrophoretic titration curve analysis with Phastgel IEF 3–9. An isoelectric calibration kit (Pharmacia) was used as standard and silver staining was employed for revealing protein bands.

N-terminal sequencing and amino acid analysis

N-terminal sequencing of 100 pmol of ALR was performed by automatic Edman degradation in a Model 477 A gas-phase protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.). On-line analysis of the phenylthiohydantoin derivatives under standard operating conditions of the sequencer was used. For total amino analysis, ALR (1.0 mg/ml) was dried under nitrogen and subjected to hydrolysis *in vacuo* with 6 M HCl. The hydrolysis was performed at 110 °C for 24 and 48 h. The dried hydrolysate was dissolved in phosphate buffer and then subjected to analysis by reverse-phase HPLC with pre-column derivatization of the amino acids with *o*-phthalaldehyde [30]. Fluorescence detection (excitation at 340 nm; emission at 450 nm) was employed, and norleucin was used as internal standard.

Kinetics

The buffer used in these experiments was 50 mM sodium phosphate, pH 7.0. The apparent kinetic constants were determined at 25 °C in the forward [NAD(P)H-dependent D-xylose reduction] and reverse [NAD(P)⁺-dependent xylitol oxidation] directions of reaction. One substrate was varied over a concentration range of approx. 5–10 K_m . The other substrate was employed at a constant, saturating concentration. All kinetic constants were calculated by non-linear regression methods, fitting the observed rate data to a rectangular hyperbola (BMDP statistical software, Los Angeles, CA, U.S.A.). An extension of the hyperbola accounting for substrate inhibition was included as required:

$$V(A) = k_{cat}[E][A]/\{K_m(A) + [A] + [A]^2/K_i(A)\} \quad (1)$$

where $V(A)$ represents the reaction rate, k_{cat} the catalytic constant, $[E]$ the total concentration of ALR, $[A]$ the con-

centration of the varied substrate, $K_m(A)$ the apparent Michaelis constant for A, and $K_i(A)$ the substrate inhibition constant for A.

Detailed kinetic analyses for D-xylose reduction and xylitol oxidation were performed by measuring initial velocities at several fixed concentrations of coenzyme (NADH, NAD⁺) when D-xylose (or xylitol) was the varied substrate and at several fixed concentrations of D-xylose (or xylitol) when coenzyme (NADH, NAD⁺) was varied. Kinetic parameters were calculated by fitting the after equation to the experimental data:

$$V = k_{cat}[E][A][B]/\{K_i(A)K_m(B) + K_m(B)[A] + K_m(A)[B] + [A][B]\} \quad (2)$$

where V is the reaction rate, $[A]$ and $[B]$ are substrate concentrations and $K_i(A)$ is the dissociation constant of the binary enzyme–substrate complex, assuming a compulsory order of substrate binding [31]. Discrimination between different plausible kinetic mechanisms was performed by product inhibition studies [31]. By using different concentrations of xylitol or NADP⁺ (or ATP) as inhibitors, a series of kinetic measurements were performed at saturating and non-saturating constant concentrations of D-xylose or NADH, with the other substrate, NADH or D-xylose, being varied. When Dixon analysis of inhibitor data did not allow an unequivocal identification of the type of inhibition, the statistical significance of the corresponding inhibition constants (S.D., sum of squares) derived from a non-linear fitting procedure was considered to prevent ambiguities.

Chemical modifications of ALR

Free thiols in native and fully denatured [4% (w/v) SDS treatment] ALR were determined at pH 7.27 and 25 °C with a 10–20-fold molar excess of Ellman's reagent, Nbs₂. A molar absorption coefficient of $13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 410 nm for the corresponding Nbs₂[−] anion was assumed [32]. Titration of accessible amino groups in native ALR was determined colorimetrically [33]. A 1000-fold molar excess of TNPS over protein was used, and the formation of the 2,4,6-trinitrophenylamino sulphite complexes was measured at 420 nm (ϵ 19 200 $\text{M}^{-1} \cdot \text{cm}^{-1}$).

Modification of ALR (0.37 μM) with PLP at 25 °C was performed in the dark in 50 mM sodium phosphate buffer, pH 7.0, with various molar ratios of reagent to protein (20:1 to 550:1). The extent of modification was determined by measuring the enzyme activity of aliquots withdrawn at different time intervals from the incubation mixture. The enzyme activity during the time course of inactivation was expressed as the percentage of the control or as the remaining fraction of the control. Protection experiments were performed by preincubating enzyme with NADH (0.1–0.2 mM), 2'-AMP (0.1 mM), ATP (0.1 mM) or D-xylose (200 mM) for 5 min before adding PLP. Before and after modification with PLP, free amino groups in ALR were quantified by titration with TNPS. To prepare a stable PLP-modified enzyme, the ALR–PLP complex was reduced by adding a 5-fold molar excess of NaBH₄ over PLP concentration. After two steps of gel filtration on a NAP-5 column (Pharmacia), estimates of the stoichiometry of incorporation of PLP were based on a molar absorption coefficient of $10\,700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 325 nm for the N⁶-phosphopyridoxyl-lysine [34]. The reversion of the modification reaction was studied as follows: with unreacted PLP removed by gel filtration, the ALR–PLP complex was incubated in 50 mM sodium phosphate buffer; the recovery of enzyme activity was monitored over a period of 1 h.

RESULTS

Metabolic enzyme activities in *C. tenuis*

The cell extract of *C. tenuis* grown on 20 g/l D-xylose for 24 h contained ALR with a specific activity of 1.50 units/mg. We were interested to see how this value compared with other key metabolic enzyme activities in the cell. The results are summarized in Table 1 and demonstrate that ALR and xylitol dehydrogenase, which catalyse the sequential two-step conversion of D-xylose into D-xylulose, represent the highest enzyme activities among those tested. It is noteworthy that transaldolase and transketolase, responsible for the metabolic flux of D-xylulose through the pentose phosphate pathway, are comparably low in *C. tenuis*. The ALR activity in crude cell extracts was stable for several days at 4 °C, which is probably because of the absence of detectable proteinase activities in *C. tenuis*.

Purification of ALR

ALR was purified to apparent electrophoretic and chromatographic homogeneity by a three-step procedure, summarized in Table 2. The isolated enzyme had a specific NADH-linked activity of 20.6 ± 1.0 units/mg and was recovered in an overall yield of 76%. The ratio of the NADPH to NADH-dependent D-xylose-reducing activity remained constant throughout purification. Hence these different activities belong to the same enzyme. The pure enzyme preparation was stable at 4 and –20 °C for more than 4 months.

Dye-ligand chromatography

The successful application of triazine dyes, bound to an appropriate matrix, for the isolation of various oxidoreductases including ALR has been well documented in the literature. In an initial screening [28,29] the dyes Procion Red H-8B (Reactive Red 31) and Procion Green H-E4BD (Reactive Green 19) were selected for the purification of ALR from *C. tenuis*. Column chromatography of the cell extract on Green 19–Sepharose 4B-CL yielded a 7-fold purified ALR protein, and the recovery of enzyme activity was quantitative. Use of the dye Red 31 as a ligand gave a higher purification factor (8.6) with a yield of 85%. When the efficiency of the subsequent step in the purification sequence was taken into account, Red 31 proved to be the better choice for dye-ligand chromatography than Green 19.

Table 1 Enzyme activities in the crude cell extract of D-xylose-grown *C. tenuis*

The organism was cultivated on 20 g/l D-xylose for 24 h. All measurements were made at 25 °C. For other details see the Experimental section.

Enzyme	Activity (units/mg)
ALR	1.50
Alcohol dehydrogenase	0.29
Lactate dehydrogenase	0.32
Malate dehydrogenase	1.13
Isocitrate dehydrogenase (NADP ⁺ -dependent)	0.02
Isocitrate dehydrogenase (NAD ⁺ -dependent)	0.06
Glucose-6-phosphate dehydrogenase	1.36
Glutamate dehydrogenase	0.11
Xylitol dehydrogenase (NAD ⁺ -dependent)	4.35
Transaldolase	0.59
Transketolase	0.42
Glutathione reductase	0.50
Proteinase	0.00

Table 2 Purification of ALR from *C. tenuis*

Purification step	Yield of enzyme (%)	Volumetric activity (units/ml)	Specific activity (units/mg)	Purification (fold)
Crude extract	100	11.85	1.50	1
Red 31	84.8	11.80	12.9	8.6
Gel filtration	76.3	16.0	14.3	9.5
Mono-Q	76.3	16.2	20.64	13.8

Figure 1 Enzyme purification documented by SDS/PAGE

Lanes 1–5 (left to right): lane 1, protein standards; lane 2, crude cell extract; lane 3, fraction after dye-ligand chromatography; lanes 4 and 5, purified protein. The standards were, from top to bottom, lactalbumin (14.1 kDa), trypsin inhibitor (20 kDa), carboanhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase b (94 kDa). All samples were reduced and then alkylated by iodoacetamide. Silver staining was used for revealing protein bands.

Ion-exchange chromatography

In initial experiments we observed that ALR was inactivated during chromatography on a Mono-Q or Q-Sepharose column. Finding that Tween-20 had a positive effect on ALR activity and stability, the detergent (0.1%, w/v) was added to the equilibration and elution buffers. The effect was a more than 5-fold improved recovery of ALR activity after elution from the Mono-Q column. After dye-ligand chromatography, the method yielded pure ALR in yields of approx. 80%.

Electrophoretic purity and identification of ALR

The purified enzyme preparation migrated as a single protein band in SDS/PAGE (Figure 1) and non-denaturing PAGE (Figure 2). The staining for activity shown in Figure 2 allowed an unequivocal identification of the protein band as ALR. The enzyme was revealed by its reverse reaction, that is xylitol oxidation, with NADP⁺ as coenzyme. This strategy allows a clear-cut differentiation between ALR and NAD⁺-dependent xylitol dehydrogenase activities. Additional proof of purity came from isoelectric focusing and electrophoretic pH titration analysis, where a single protein band or migration curve was seen respectively (results not shown).

Physicochemical properties of ALR

The results of SDS/PAGE revealed a subunit molecular mass of the protein of 43 kDa. The molecular mass of native ALR

Figure 2 Anionic gel electrophoresis of purified ALR and identification of the enzyme

Lanes 1–4 (left to right): lane 1, standard proteins; lane 2, pure enzyme stained by Coomassie Brilliant Blue; lanes 3 and 4, pure enzyme stained for enzyme activity (for details see the Experimental section). The standards were, from top to bottom, BSA (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobin (669 kDa).

Figure 3 The effect of pH on ALR activity in the forward and reverse directions of reaction

Symbols: $\diamond$, D-xylose reduction with 250 μ M NADH; $\square$, xylitol oxidation with 500 μ M NAD⁺. Other reaction conditions were as in the standard assay described in the Experimental section.

determined by gel filtration was 48 kDa. Hence ALR is a monomeric protein. The isoelectric point of the protein was determined by isoelectric focusing, pH titration analysis and chromatofocusing on a Mono P column: it is 4.70 ± 0.15 , and the results obtained by the three methods were in good agreement. The temperature optimum of ALR was found to be 50 °C; the stability of the enzyme (48 h incubation period) started to decrease at 30–35 °C. The enzyme activity increased linearly from 25 to 50 °C, resulting in a 4.5-fold activation of ALR. The pH optima in the forward and reverse directions of reaction were 6.0 and 8.9, and both pH–activity curves were nearly symmetrical (Figure 3). Because even at its optimal pH the rate of xylitol oxidation never exceeded 10% of that of xylose reduction at pH 6, the enzyme is a ‘reductase’.

Total amino acid composition of ALR

The N-terminus of ALR is blocked, so automated Edman degradation of the protein did not proceed. The total amino acid

Table 3 Amino acid composition of ALR from *C. tenuis*

The results represent the mean values of two independent determinations including hydrolysis. For methodology see the Experimental section. Cysteine was measured by titration with Ellman's reagent (Nbs₂) of reactive thiols in ALR denatured in SDS (4%, w/v); Trp and Pro were not determined.

Amino acid	Content (mol.%)	Amino acid	Content (mol.%)
Asparagine or aspartic acid	13.5	Tyrosine	3.6
Glutamine or glutamic acid	10.5	Valine	6.8
Serine	4.6	Methionine	0.4
Histidine	2.1	Isoleucine	6.1
Glycine	8.4	Phenylalanine	6.0
Threonine	4.9	Leucine	9.8
Arginine	4.0	Lysine	6.4
Alanine	11.2	Cysteine	1.1

composition of ALR is shown in Table 3, and the results were used for a comparison with ALRs from other microbial and mammalian sources. We used the program PropSearch [35] to search for functional ‘homologues’ based on total amino acid composition in the Swissprot, SwissNew and PIR protein databases. The additional information of the pI and the molecular mass of ALR was included and given weight factors from 1 to 10. No other ALR was identified as significantly similar. However, the amino acid composition of the *C. tenuis* enzyme clearly resembles that of the ALR from *C. shehatae* [19], which has not been deposited in any of the databases queried.

Kinetics

Apparent kinetic constants

The ALR from *C. tenuis* is active with NADP(H) and NAD(H) as coenzyme. This was proved not only in initial velocity experiments but also by allowing the reaction to proceed to a conversion of more than 10% of the respective coenzyme form. Furthermore, in D-xylose reduction (100 mM substrate), complete conversion into xylitol was achieved when 50 μ M coenzyme (NADPH or NADH) and a second enzymic system capable of regenerating the phosphorylated or non-phosphorylated reduced coenzyme respectively was employed: D-glucose 6-phosphate (100 mM)/D-glucose-6-phosphate dehydrogenase for NADPH and formate (100 mM)/formate dehydrogenase for NADH. The apparent kinetic constants of ALR were determined in the direction of D-xylose reduction and xylitol oxidation by using the phosphorylated and non-phosphorylated forms of the coenzyme (Table 4). The kinetic constants (k_{cat} and K_m) differ widely when D-xylose and xylitol are compared as the substrate of the ALR-catalysed reaction. The resulting catalytic efficiencies confirm the previous conclusion that the enzyme is a reductase. The apparent binding of NADP(H) was 5.3- and 2.2-fold greater than that of NAD(H) in the reduction and oxidation direction of reaction respectively. The catalytic constant observed with NADH in the forward reaction was approx. 20% lower than that with NADPH. In the reverse reaction, k_{cat} values with NADP⁺ and NAD⁺ were identical within statistical significance.

Initial velocity studies

In D-xylose reduction, initial velocities obtained with D-xylose as the varied substrate when NADH was constant, and initial velocities obtained with NADH as the varied substrate when D-xylose was constant, yielded straight converging lines that

Table 4 Apparent kinetic constants of ALR from *C. tenuis* in D-xylose reduction and xylitol oxidation

Reactions were performed at 25 °C in 50 mM phosphate buffer, pH 7.0. The calculation was by non-linear regression analysis. The constants are k_{cat} , the catalytic constant, and K_m , the Michaelis constant, for the respective substrate or coenzyme.

Varied substrate	Constant substrate	k_{cat} (app) (s^{-1})	K_m (app) (μM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
NADH	D-Xylose (300 mM)	18.1	25.4 (μM)	7.13×10^5
NADPH	D-Xylose (300 mM)	21.9	4.8 (μM)	4.61×10^6
D-Xylose	NADH (0.3 mM)	18.2	87 (mM)	2.10×10^2
D-Xylose	NADPH (0.3 mM)	21.5	72 (mM)	2.96×10^2
NAD ⁺	Xylitol (500 mM)	0.89	58.7 (μM)	1.52×10^4
NADP ⁺	Xylitol (500 mM)	0.82	26.6 (μM)	3.09×10^4
Xylitol	NAD ⁺ (1 mM)	0.87	257 (mM)	3.39

intersected to the left of the ordinate for the double-reciprocal plot of initial velocity against varied substrate concentration (Figure 4). The results indicate that the apparent Michaelis constant for one substrate is dependent on the concentration of the other substrate. The secondary plots of the resulting slopes and intercepts against the reciprocals of the second fixed substrate or coenzyme were also linear. At higher substrate concentrations, however, uncompetitive substrate inhibition by D-xylose was observed (Figure 4, inset), and a substrate inhibition constant, $K_i(S)$, of 1.5 ± 0.3 M was determined at saturating concentrations of NADH. Competitive substrate inhibition because of the formation of a dead-end enzyme–D-xylose–D-xylose complex was ruled out because saturation with NADH could not overcome the inhibition. In xylitol oxidation, when [xylitol] was varied and [NAD⁺] was constant and when [NAD⁺] was varied and [xylitol] was constant, linear intersecting primary Lineweaver–Burk plots were obtained (Figure 5), and the replots of slopes and intercepts against the reciprocal of the second fixed substrate were linear (Figure 5, inset). No substrate inhibition by xylitol was observed. The kinetic mechanism is thus sequential, which implies that substrate and coenzyme must add to the enzyme, thus forming a ternary enzyme–substrate–coenzyme complex, before any product is released [31].

Product inhibition

To determine the order of substrate binding in the kinetic mechanism of *C. tenuis* ALR, product inhibition studies for the forward reaction, D-xylose reduction, were performed. When NADH was the variable substrate and [D-xylose] was held constant, NADP⁺ gave a linear competitive inhibition pattern at both saturating and non-saturating concentrations of D-xylose. The replot of the slope against [NADP⁺] was also linear. Similar results were obtained with the coenzyme analogue ATP. The inhibitory pattern of NAD⁺ was competitive, but the competence to inhibit the NADH-dependent D-xylose reduction was low [$K_i(Q) > 150 \mu M$]. The inhibition pattern is described by:

$$V = k_{cat}[E][A]/(K_m(A)\{1 + [Q]/K_i(Q)\} + [A]) \quad (3)$$

where [A] is the concentration of NADH, $K_m(A)$ is the Michaelis constant of NADH, [Q] is the concentration of NADP⁺ (or ATP or NAD⁺) and $K_i(Q)$ is the dissociation constant of NADP⁺ (or ATP). With NADH as the variable substrate, xylitol was a linear uncompetitive inhibitor at saturating concentrations of D-xylose. When D-xylose was the variable substrate and [NADH] was held constant, both xylitol and NADP⁺ gave a linear mixed non-competitive inhibition pattern at non-saturating concentrations

Figure 4 Initial-rate analysis of ALR from *C. tenuis* with NADH as the variable substrate

Double-reciprocal plot from the initial velocity measurements of the reduction of D-xylose by NADH catalysed by ALR. The D-xylose concentration was held constant at 50 ($\diamond$), 100 ($\square$) or 200 ($\triangle$) mM. The inset shows the replots of intercepts (a , $\blacklozenge$) and slopes (b , $\diamond$) against the reciprocal of the concentration of D-xylose (50–1000 mM). The values for the slopes were multiplied by a factor of 10. The units of intercept and slope values are $(\mu mol/min \text{ per ml})^{-1}$ and $mM/(\mu mol/min \text{ per ml})$ respectively. The enzyme concentration was 17.4 nM.

Figure 5 Initial-rate analysis of ALR from *C. tenuis* with NAD⁺ as the variable substrate

Double-reciprocal plot from the initial velocity measurements of the oxidation of xylitol by NAD⁺ catalysed by ALR. The xylitol concentration was held constant at 50 ($\blacklozenge$), 100 ($\square$), 200 ($\blacktriangle$), 500 ($\circ$) or 1000 ($\diamond$) mM. The inset shows the replots of intercepts (a , $\blacklozenge$) and slopes (b , $\diamond$) against the reciprocal of the concentration of xylitol (50–1000 mM). The units of intercept and slope values are $(\mu mol/min \text{ per ml})^{-1}$ and $mM/(\mu mol/min \text{ per ml})$ respectively. The enzyme concentration was 580 nM.

of NADH. The secondary plots of the slopes and intercepts against fixed concentrations of NADH were linear. The inhibition patterns and the corresponding inhibition constants are summarized in Table 5.

Kinetic constants

The initial velocity patterns, together with the product inhibition patterns for *C. tenuis* ALR, are consistent with an ordered Bi Bi mechanism in which NADH binds first, followed by the aldehyde substrate, D-xylose. A kinetic scheme for this mechanism is shown in Scheme 1. The data at non-inhibitory levels of D-xylose in D-xylose reduction and those in xylitol oxidation were fitted to eqn. (2). The resulting kinetic constants are summarized in Table 6. The internal consistency of the kinetic parameters in Table 6

Table 5 Product inhibition of *C. tenuis* ALR

The inhibition constants were determined by non-linear regression. See the Experimental section for details. K_{ic} is the competitive inhibitor constant; K_{inc} is the non-competitive inhibitor constant in the mixed-type inhibition pattern. Results are means $\pm$ S.D.

Inhibitor	Variable substrate	Concentration of fixed substrate	Inhibition pattern	Inhibition constants (K_i)
NADP ⁺	NADH	Saturating	Competitive	$1.50 \pm 0.04 \mu\text{M}$
ATP	NADH	Saturating	Competitive	$23.9 \pm 1.2 \mu\text{M}$
NADP ⁺	D-Xylose	Non-saturating	Mixed-type	$K_{ic} 14.3 \pm 1.0 \mu\text{M}$ $K_{inc} 17.1 \pm 0.1 \mu\text{M}$
Xylitol	NADH	Saturating	Uncompetitive	$0.35 \pm 0.04 \text{ M}$
Xylitol	D-Xylose	Non-saturating	Mixed-type	$K_{ic} 1.6 \pm 0.8 \text{ M}$ $K_{inc} 0.30 \pm 0.01 \text{ M}$

Scheme 1 Compulsory-ordered kinetic mechanism of ALR from *C. tenuis*

E is ALR and K_{iA} and K_{iQ} are the dissociation constants of NADH and NAD⁺ respectively.

Table 6 Steady-state kinetic constants for *C. tenuis* ALR

The values were determined by non-linear regression. The letters A, B, P, and Q represent NADH, D-xylose, xylitol and NAD⁺ respectively. K_m is a Michaelis constant, K_i is a dissociation constant, $k_{cat}(f)$ is the catalytic constant in the reaction of NADH and D-xylose, and $k_{cat}(r)$ is the catalytic constant in the reverse reaction. Results are means $\pm$ S.D.

D-Xylose reduction		Xylitol oxidation	
$k_{cat}(f)$ (s ⁻¹)	17.0 ± 0.86	$k_{cat}(r)$ (s ⁻¹)	1.07 ± 0.02
$K_i(A)$ (μM)	16.0 ± 4.0	$K_i(Q)$ (μM)	195 ± 25
$K_m(A)$ (μM)	15.0 ± 1.0	$K_m(Q)$ (μM)	27.0 ± 3.7
$K_m(B)$ (mM)	75.6 ± 8.1	$K_m(P)$ (mM)	208.9 ± 10.2

was checked with the kinetic Haldane relationship for an ordered Bi Bi mechanism:

$$K_{eq}(\text{app}) = k_{cat}(f)K_i(Q)K_m(P)/k_{cat}(r)K_i(A)K_m(B) \quad (4)$$

where $K_{eq}(\text{app})$ is the apparent equilibrium constant, $k_{cat}(f)$ is the catalytic constant in the reaction of NADH and D-xylose, and $k_{cat}(r)$ is the catalytic constant in the reverse reaction. The kinetic constants in Table 6 give a value at pH 7.0 of 535 for this expression, which agrees with the experimentally observed value of 550 at this pH [36]. The thermodynamic K_{eq} is thus estimated to be 0.535×10^{10} l/mol for the expression $[\text{xylitol}][\text{NAD}^+]/[\text{D-xylose}][\text{NADH}][\text{H}^+]$.

The kinetic significance, R , of the central ternary complex was determined as suggested by Segel [31]. An R value of 0.81 was calculated from:

$$R = \{1/k_{cat}(f)[1 - K_m(A)/K_i(A)] + 1/k_{cat}(r)[1 - K_m(Q)/K_i(Q)]\} / [1/k_{cat}(f) + 1/k_{cat}(r)] \quad (5)$$

Because $k_{cat}(f)$ and $k_{cat}(r)$ are unequal, the R value gives the

Table 7 Substrate spectrum of ALR from *C. tenuis*

Reactions in aldehyde reduction were performed with 17.4 nM ALR at 25 °C in 300 mM sodium phosphate buffer, pH 7.0, with saturating (relative activities) and non-saturating substrate concentrations (1 mM; relative catalytic efficiencies). The constant coenzyme concentration was 250 μM NADH. Reactions in alcohol oxidation were performed with 580 nM ALR in 50 mM sodium phosphate buffer, pH 8.9. The constant alcohol concentration was 100 mM; that of the coenzyme was 250 μM NAD⁺. The results are mean values of triplicate experiments. The apparently saturating substrate concentration is given in parentheses in the first column. Abbreviations: —, not determined; n.d., not detectable.

Aldehyde	Relative activity (relative catalytic efficiency)	Alcohol	Relative catalytic efficiency
D,L-Glyceraldehyde (20 mM)	0.9 (37)	Glycerol	< 0.02
D-Erythrose (20 mM)	1.8 (100)	Erythritol	0.2
D-Xylose (300 mM)	1 (1)	Xylitol	1
L-Arabinose (300 mM)	0.7 (2)	L-Arabinol	1.0
D-Ribose (300 mM)	0.3 (0.2)	Ribitol	0.1
D-Glucose (300 mM)	0.2 (< 0.1)	D-Sorbitol	0.6
D-Galactose (300 mM)	0.7 (0.3)	—	—
D-Mannoheptose (240 mM)	< 0.1 (< 0.1)	—	—
D-Xylosone (25 mM)	1.1 (20)	—	—
D-Glucosone (25 mM)	1.1 (22)	D-Fructose	n.d.
Methylglyoxal (50 mM)	1.4 (20)	—	—
Phenylglyoxal (12 mM)	0.6 (17)	—	—
Valeraldehyde (50 mM)	0.8 (13)	—	—
Pyridine-2-aldehyde (10 mM)	1.0 (7)	—	—

fraction of total enzyme present as the central complexes at saturating concentrations of both substrates for the slower direction [31], i.e. in xylitol oxidation. Consequently, the ratio of the central complexes to all other ALR species present, $R/(1 - R)$, is 4.2.

Substrate spectrum

On the basis of the results obtained with the physiological aldehyde D-xylose, various other carbonyl-containing compounds were tested as possible substrates of *C. tenuis* ALR. Each substrate was used in a saturating and a non-saturating concentration range, and the results are given as activities and catalytic efficiencies relative to D-xylose (Table 7). Carbohydrates composed of three to seven carbon atoms, dicarbonyl compounds, aliphatic and aromatic aldehydes are reduced by ALR with significant activity. With the exception of aldohexoses and one aldohexose, the relative activities (measured at apparent substrate saturation) are in a range similar to those for all aldehydes. In contrast, the values representing estimates of the relative catalytic efficiencies (measured at non-saturating substrate concentrations) varied up to 1000-fold. Accordingly, ALR prefers glyceraldehyde, D-erythrose and even some aliphatic and aromatic aldehydes to the pentose sugars D-xylose and L-arabinose. Aldosones such as D-glucosone or D-xylosone are good substrates of ALR (Table 7), whereas the corresponding 2-deoxy-aldose sugars were reduced at hardly detectable rates. D-Glucuronic acid and D-galacturonic acid are typical substrates of aldehyde reductases of the aldo-keto reductase superfamily [1,5]. In concentrations of 300 mM, both compounds were not reduced at appreciable reaction rates as were keto-groups in sugars such as D-fructose. Interestingly, the relative catalytic efficiencies in polyol oxidation show a different picture. At a non-saturating concentration of 100 mM, highest activities were observed with xylitol, whereas those with glycerol or erythritol were low.

Effectors, inhibitors and stability of ALR

The ALR activity increased by 10–15 % in the presence of BSA

or non-ionic detergents such as Tween-20, Tween-80 and Triton X-100 (0.1 %, w/v). In contrast, ionic detergents such as cholic acid or deoxycholic acid in the same concentration caused 30 % inhibition of the enzyme. Approx. 30 % and 40 % inhibition was observed in the presence of EDTA and dithiothreitol respectively (1 mM each). The cations Zn^{2+} and Mn^{2+} are inhibitory and, at a concentration of 25 mM, decreased the original ALR activity by 95 % and 40 % respectively. Other mono- or bivalent cations (Na^+ , K^+ , NH_4^+ , Mg^{2+} , Ca^{2+} , Co^{2+}) in the form of the chloride salt in 50 mM Tris, pH 7.0, as well as several anions (Cl^- , PO_4^{3-} , SO_3^{2-} , NO_3^- , CO_3^{2-} , citrate, tetraborate) in the form of the sodium salt in 50 mM P_i, pH 7.0, did not affect ALR activity to an appreciable extent. The effect of sulphate, known to activate human ALR by more than 2.5-fold at a concentration of 200 mM [37], was moderate, resulting in at most a 10 % activation of the enzyme from *C. tenuis* (50 mM Tris buffer, pH 7.0). A number of metabolic intermediates that are found to be potent inhibitors or effectors of key enzymes in carbohydrate metabolism were investigated with regard to their effect on ALR activity. Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, 6-phosphogluconate, phosphoenolpyruvate, oxaloacetate (5 mM each) had no effect. AMP, ATP and especially NADP⁺ but not NAD⁺ were potent inhibitors of the ALR-mediated D-xylose reduction. NADP⁺ inhibited both the NADH-dependent and the NADPH-dependent ALR activity and, at a concentration of 2 mM, completely abolished D-xylose reduction in the activity assay.

Pure ALR from *C. tenuis* is a stable enzyme at 25 °C in phosphate and Tris buffer of various ionic strengths between pH 6.0 and 7.0. The half-life of the enzyme at this temperature is longer than 2 months. At pH values below 5.0 and above 8.0, however, ALR was inactivated within 3–6 days. Non-ionic detergents and BSA were found to stabilize ALR to a significant extent during long-term incubation at 25, 30 or 38 °C.

Oxidation state of ALR

ALR from mammalian sources was shown to undergo thiol oxidation during storage or purification. The oxidized form of the enzyme exhibited altered kinetic properties, that is a higher k_{cat} and a higher K_m for the aldehyde substrate [38–40]. We determined the kinetic constants of *C. tenuis* ALR as a function of storage time (in the presence of a water/air interface) and found no time-dependent modification of the enzyme's kinetic properties. No free, oxidation-prone thiols were detectable in the native protein from the very beginning after the isolation.

Chemical modification with PLP

PLP has been used for the specific modification of ϵ -amino groups of lysine residues in various NAD(P)⁺-dependent enzymes including aldehyde reductase and ALR from human muscle [37,41]. We found that ALR from *C. tenuis* was gradually inactivated when incubated in the presence of PLP. The rate and the final extent of enzyme inactivation was determined by the concentration of the reagent (Figure 6). NADH and its analogues and the substrate D-xylose were tested for their ability to protect against inactivation by PLP (0.4 mM). Complete protection was achieved after saturation of ALR with the coenzyme NADH (0.1–0.2 mM). ATP and 2'-AMP (each at a concentration of 0.1 mM) were equally effective. In contrast, saturating concentrations of D-xylose (700 mM) did not provide any protection. After removal of excess of PLP by a two-step gel filtration of the PLP-modified enzyme, no original enzyme activity could be recovered. Hence the inactivation of ALR from *C. tenuis* is irreversible. PLP is not an instantaneous inhibitor of the *C. tenuis* ALR as it had

Figure 6 Modification of ALR with PLP

The concentration of ALR was 0.37 μM ; the modification was carried out at 25 °C and pH 7.0. Upper panel: the time course of enzyme inactivation is shown with a 50-fold (■) and 130-fold (□) molar excess of reagent over protein. Protection is shown for NADH (○), ATP (▲) and 2'-AMP (●) in concentrations of 0.1 mM. Lower panel: apparent pseudo-first-order rate constant (■) and final extent (□) of ALR inactivation in dependence on the molar ratio reagent to protein. The rate constants were derived as described in the text.

no effect on the NADH-linked reduction of D-xylose in a steady-state assay.

The kinetics of inactivation is complex and does not follow a pseudo-first-order reaction. When the natural logarithm of the remaining fraction of ALR activity is plotted against incubation time, time courses are obtained that seem at least biphasic (Figure 6, upper panel). We used the fast initial phase of inactivation for the calculation of an apparent pseudo-first-order rate constant. This rate constant is not a linear function of the molar ratio of reagent to protein. Instead, a saturation characteristic is observed yielding a k_{max} of $0.37 \pm 0.02 \text{ min}^{-1}$ and a half-saturation constant, K , of $30.8 \pm 4.1 \mu\text{M}$ PLP or $82.1 \pm 10.9 \mu\text{M}$ PLP/ μM ALR. The minimal mechanism consistent with such a type of kinetic behaviour is described by eqn. (6) and suggests that the reagent forms a reversible complex before inactivation:

where E is the enzyme, E·PLP is a rapidly formed reversible complex and E–PLP is the inactive enzyme with the Schiff base formed between PLP and a lysine residue. From eqn. (1) the constant K is $(k_{-1} + k_2)/k_1$ and k_{max} is k_2 . The second, slow phase of inactivation observed in Figure 6 (upper panel) occurs for

unknown reasons but might involve conformational transitions of the enzyme as well as other secondary reactions [42].

Only after exhaustive incubation in the presence of a 300–500-fold excess of reagent, was complete inactivation of ALR observed. The extent of inactivation obeyed a saturation function in dependence on the ratio of PLP to ALR, and the resulting curve is actually superimposable on that seen for the rate of inactivation (Figure 6, lower panel). The extent of incorporation of reagent and thus the stoichiometry of the modification reaction has not been completely resolved. Ten amino groups were accessible to titration with TNPS in native but NADH-protected ALR as well as in the inactive, PLP-modified enzyme. This result suggests a high selectivity of the reagent for a reactive lysine residue at or close to the coenzyme-binding site. Treatment of the E–PLP complex with NaBH_4 caused the appearance of a new peak in the absorbance spectrum of the protein at 325 nm that corresponds to ϵ -pyridoxyl-lysine [34]. According to this peak, 2.5 mol of PLP per mol of ALR had been incorporated after 60 min of incubation with 0.5 mM PLP.

DISCUSSION

The yeast *C. tenuis* grown on D-xylose as a carbon source contains one ALR that has a dual specificity for the phosphorylated and the non-phosphorylated nicotinamide coenzyme. We have found no evidence of multiple forms of ALR in *C. tenuis* like those found in other yeasts such as *Pachysolen tannophilus* [22] or *C. tropicalis* [43,44]. The enzyme activities catalysing the first two steps in yeast D-xylose conversion, ALR and xylitol dehydrogenase, are 3–10-fold higher than those of the pentose phosphate pathway, transaldolase and transketolase. Hence the flux of carbon metabolite through the pentose phosphate pathway could represent a bottleneck in the metabolism of D-xylose by *C. tenuis*. Studies dealing with the metabolic engineering of baker's yeast have demonstrated that transketolase might indeed be a limiting factor of the growth rate on D-xylose [45]. Other yeasts, such as *P. stipitis* and *C. shehatae*, capable of fermenting D-xylose were shown to grow faster on the pentose sugar than can *C. tenuis* [18].

We have established a simple and efficient three-step purification procedure that allows the isolation of ALR from *C. tenuis* in a homogeneous form on a 50 mg scale in a short time. Our results as well as other examples in the literature show that the combination of dye-ligand pseudo-affinity chromatography and anion-exchange chromatography is a well-suited strategy for purifying ALR from different sources (see, for example, [9,37,46]). Comparative characterization of the enzyme from *C. tenuis* revealed a variety of common aspects but also clear dissimilarities to ALRs from other yeasts and from mammalian sources.

In yeast, one major distinguishing property of different ALRs is the subunit composition of the respective protein. The ALR from *C. tenuis* is a monomer of 43 kDa, whereas those from *P. stipitis* [9], *P. quercuum* [47], *C. tropicalis* [43] or *Cryptococcus lactivorius* [48] are dimers with a considerably smaller subunit of approx. 32 kDa. The *C. tenuis* enzyme seems to be quite similar to the ALRs from *C. shehatae* [19] and *P. tannophilus* [20] as regards its monomeric structure, pI and total amino acid composition. The same holds true when comparison is made with mammalian ALRs, all of which are monomers of 34–41 kDa. However, ALR monomers (except that from *C. shehatae*) have a strong preference for NADPH, and their NADH-linked activities are marginal. A structural basis of enzyme–coenzyme interaction in ALR has become available from the completed crystallographic studies on the ALR–NADPH binary complex [13–15]. NADPH is bound in an extended conformation; an 11-residue loop is responsible for the interactions with the adenosine-

2'-monophosphate moiety of the coenzyme. The loop is thought to determine the preference of mammalian ALR for NADPH over NADH [15]. The coenzyme preference in human ALR is manifested by a more than 100-fold better apparent binding of NADPH than NADH. In contrast, the corresponding catalytic constants are closely comparable [49]. Hence a dual coenzyme specificity in ALR would require improved binding of the non-phosphorylated form of the coenzyme. This is exactly the situation found with the ALRs from *C. tenuis* (this study) and *P. stipitis* [9]. The affinities of the *C. tenuis* enzyme for NADPH and NADP^+ are only 5- and 2-fold higher than for NADH and NAD^+ in the forward and reverse directions of reaction, respectively. It is interesting to note that the catalytic-centre activities seen with yeast ALRs from *C. tenuis* and *P. stipitis* [9] are identical, but are at least 10-fold higher than that of the mammalian enzymes [49,50]. In aldehyde reduction by *C. tenuis* ALR, k_{cat} is 1.2-fold higher with NADPH than NADH, whereas, in xylitol oxidation, the catalytic constants are identical for both forms of the oxidized coenzyme, NADP^+ and NAD^+ . This result indicates that (1) the nature of the coenzyme contributes to rate-limitation in D-xylose reduction, and (2) non-identical rate-limiting factors exist in the forward and reverse directions of reaction.

A number of experiments suggest that the kinetic mechanism of ALR from *C. tenuis* is compulsory-ordered, with coenzyme binding first and leaving last. Whereas initial velocity studies were consistent with at least two sequential kinetic mechanisms other than ordered Bi Bi, the product inhibition pattern ruled out a rapid-equilibrium random Bi Bi or a Theorell–Chance mechanism. Furthermore kinetic analysis showed that the central ternary complex (Scheme 1) is kinetically significant; thus a Theorell–Chance mechanism for ALR is very unlikely. Uncompetitive substrate inhibition by D-xylose indicates the formation of a non-productive D-xylose– NAD^+ –enzyme complex that is only found in ordered, but not random, enzyme systems. Protection of ALR by NADH, but not D-xylose, against inactivation by PLP proved the significance of the binary enzyme–coenzyme complex and reinforced a compulsory-order kinetic mechanism in which the aldehyde will only bind to the binary enzyme–coenzyme complex and not to the free enzyme (Scheme 1). The proposed kinetic mechanism is in agreement with previous kinetic studies performed with other ALRs from a mammalian source [50–52] and the yeast *P. stipitis* [36].

Functionally important lysine residues at the active site of ALR have been identified by chemical modification with PLP, site-directed mutagenesis and crystallographic analysis [15,37,41]. These lysine residues participate in coenzyme binding and catalysis. PLP modification in human ALR was targeted to Lys-262, which belongs to a well-conserved Ile-Pro-Lys-Ser motif involved in the binding of NADP(H) [41]. The proposed role of this lysine residue is to form a hydrogen-bonding/salt-linking interaction with the 2'-phosphate group of the adenosine mononucleotide and the pyrophosphate bridge of NADPH [15]. Strikingly, human ALR was activated more than 4-fold by modification with PLP, and the coenzyme-binding site of this enzyme was shown to accommodate both PLP and an NADH, but not an NADPH, molecule [41]. In complete contrast with the effect of PLP on human ALR, the reagent inactivated the ALR from *C. tenuis*, which clearly reflects differences in the topology at the active sites of these two enzymes. Protection studies have demonstrated that inactivation of the yeast enzyme results from lysine modification in or at least near the adenosine portion of the NAD(P)H -binding site. The enzyme from *C. tenuis*, unlike human ALR, does not allow NADH plus PLP to bind at the coenzyme-binding site. It is noteworthy in this context that the

yeast enzyme has a real dual coenzyme specificity (this study), whereas the human ALR exhibits a nearly exclusive preference for NADPH, judged from its lower affinity (1/100) for NADH [49].

Substrate specificity is an extensively studied topic with each newly isolated member of the ALR group. The ALR from *C. tenuis* is active with three-carbon to seven-carbon aldose sugars (but not di- and oligosaccharides), and aliphatic and aromatic aldehydes. This very broad substrate specificity is a common feature of ALRs from both yeast and mammals. On the basis of a comparison of estimates of the catalytic efficiencies with various aldehydes, the four-carbon sugar D-erythrose seems to be the preferred substrate in aldehyde reduction catalysed by ALR from *C. tenuis*. The C₂ atom in aldoses has an important effect with regard to determining the ALR-sugar interaction. (1) The activities found with dicarbonyl compounds such as D-glucosone, phenylglyoxal or methylglyoxal confirm previous results obtained with human ALR [53,54]. (2) In contrast, 2-deoxy sugars are not reduced at significant rates. ALR is known to contain an extremely hydrophobic substrate-binding pocket [15], which could explain why a non-polar molecule such as valeraldehyde is a good substrate of the *C. tenuis* enzyme. Substrate binding and catalysis are more efficient in the forward direction of reaction, thus reflecting the reductase properties of the *C. tenuis* enzyme. In agreement with the specificity of human ALR [10], the discrimination between different aldehyde substrates by the yeast enzyme seems to be mediated by widely varying apparent binding constants rather than catalytic-centre activities (W. Neuhauser and B. Nidetzky, unpublished work).

Activation by oxidative modification or by interaction with sulphate ions has been considered a typical feature of ALR [38–40]. It has recently been shown that the oxidation of one cysteine residue (Cys-298 in human ALR) has a key role in the conversion from the unactivated to the activated enzyme form. Furthermore this cysteine residue is critical for activation by sulphate [40]. The ALR from *C. tenuis* does not contain oxidation-prone thiols in the native protein and is activated neither by sulphate nor by oxidation, for example during storage. It is noteworthy that the critical cysteine residues (Cys-298 and Cys-303) are well conserved in the C-terminal part of mammalian ALRs [40,55], but are not found in those from yeast [44,46]. Oxidation-mediated activation might thus be a characteristic typical of the ALRs from mammalian sources.

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