Biodegradation (2011) 22:1227–1237
DOI 10.1007/s10532-011-9477-x

ORIGINAL PAPER

Biochemical characterization of ethanol-dependent reduction of furfural by alcohol dehydrogenases

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Received: 8 January 2011 / Accepted: 18 April 2011 / Published online: 28 April 2011

Abstract Lignocellulosic biomass is usually converted to hydrolysates, which consist of sugars and sugar derivatives, such as furfural. Before yeast ferments sugars to ethanol, it reduces toxic furfural to non-inhibitory furfuryl alcohol in a prolonged lag phase. Bioreduction of furfural may shorten the lag phase. *Cupriavidus necator* JMP134 rapidly reduces furfural with a Zn-dependent alcohol dehydrogenase (FurX) at the expense of ethanol (Li et al. 2011). The mechanism of the ethanol-dependent reduction of furfural by FurX and three homologous alcohol dehydrogenases was investigated. The reduction consisted of two individual reactions: ethanol-dependent reduction of NAD^+ to NADH and then NADH-dependent reduction of furfural to furfuryl alcohol. The kinetic parameters of the coupled reaction and the individual reactions were determined for the four enzymes. The data indicated that limited NADH was released in the coupled reaction. The enzymes had high affinities for NADH (e.g., K_d of 0.043 μM for the FurX-NADH complex) and relatively low affinities for NAD^+ (e.g., K_d of 87 μM for FurX-NAD^+). The kinetic data suggest that the four enzymes are efficient "furfural reductases" with either ethanol or NADH as the reducing power. The standard free energy change ($\Delta G^{o\prime}$) for ethanol-dependent reduction of furfural was determined to be -1.1 kJ mol^{-1}. The physiological benefit for ethanol-dependent reduction of furfural is likely to replace toxic and recalcitrant furfural with less toxic and more biodegradable acetaldehyde.

Keywords Zn-dependent alcohol dehydrogenase · Ethanol · Furfural · Furfuryl alcohol · Furfural reductase

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Introduction

Furfural is a natural product, common in certain foods. Most of the information about furfural has recently been reviewed (Hoydonckx et al. 2007). It is also present at high concentrations in the waste of pulp and paper industry. Industrial production of furfural is mainly from agricultural residues through heating in dilute sulfuric acid solutions. Its derivatives have broad usages as braking pad resin, solvents, nematode control agent, rocket fuel, etc. Since furfural is readily biodegraded in activated sludge (Ellis and Eliosov 2004), it is not a major concern of environmental pollution. A resent surge of interest in furfural is because of the inhibitory effect of furfural to yeast in ethanol production from lignocellulosic biomass, including agricultural residues. Furfural and 5-hydroxymethylfurfural are produced from pentose and hexose during the conversion of the plant structural materials

into hydrolysates in dilute acids at high temperatures, a common pretreatment before fermentation (Antal et al. 1991). They inhibit yeast growth. Furfural is more toxic than 5-hydroxymethylfurfural to yeast, and it is a key inhibitor for yeast fermentation in four different lignocellulosic hydrolysates, as it prolongs the lag phase of yeast growth (Heer and Sauer 2008).

Escherichia coli and brewer's yeast (*Saccharomyces cerevisiae*) gradually reduce furfural to less toxic furfuryl alcohol during fermentation (Boopathy et al. 1993; Heer and Sauer 2008). Several enzymes capable of reducing furfural have been identified from *E. coli* and the yeast. Two NADPH-dependent furfural reductases, EcYqhD and EcDkgA, have been identified from *E. coli*. They have high K_m values for furfural at 9 mM (EcYqhD) and >130 mM (EcDkgA). However, EcYqhD and EcDkgA have low K_m values for NADPH at 8 and 23 μM, respectively. Their activities of depleting NADPH have been attributed to the slow growth of *E. coli* at high furfural concentrations (Miller et al. 2009). Sequence analysis places EcYqhD in the "iron-containing alcohol dehydrogenase" family and EcDkgA in the "aldose-ketose reductases" family. Another NADPH-dependent furfural reductase has been purified from *E. coli*, but the corresponding gene is not reported (Gutierrez et al. 2006). Yeast has two cinnamyl alcohol dehydrogenases (YADH6 and YADH7), and both catalyze NADPH-dependent reduction of furfural (Larroy et al. 2002a, b). In addition, a yeast NADPH-dependent aldehyde reductase (YTPA), a member of the "short-chain dehydrogenase/reductase" family, is able to reduce furfural (Liu and Moon 2009). The yeast alcohol dehydrogenase (YADH1), responsible for ethanol fermentation, has been shown to reduce furfural with NADH as the reducing power, and furfural is a competitive inhibitor for acetaldehyde reduction by YADH1 (Modig et al. 2002). A mutant YADH1 is discovered from a furfural resistant yeast strain, and the mutant enzyme is more efficient in reducing furfural (Laadan et al. 2008). YADH1, YADH6 and YADH7 belong to "Zn-dependent alcohol dehydrogenase" superfamily. Further, yeast aldehyde dehydrogenase (YAld4) and aldose reductase (YGRE3) are likely to catalyze NAD(P)H-dependent furfural reduction (Liu et al. 2008). The diversity of "furfural reductases" is reflected in the phylogenetic tree of the reported enzymes (Fig. 1). Thus, various types of reductases can reduce furfural.

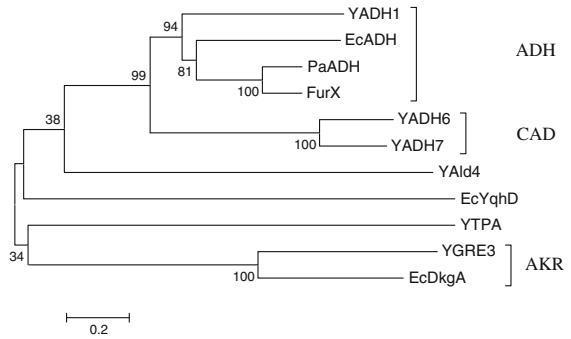

Fig. 1 Dendrogram of inferred phylogenetic relationship among "furfural reductases". The tree was constructed using Neighbor-Joining method. The numbers on the branches are bootstrap values (percentage of 1000 runs), indicating the frequency of grouping in the cluster. The distance correlates to the number of amino acids substituted per site. The "furfural reductases" and corresponding NCBI protein accession numbers are as follows: YADH1, NP_014555; EcADH, NP_415995; PaADH, NP_254114; FurX, YP_297879; YADH6, NP_014051; YADH7, NP_010030; YAld4, NP_015019; EcYqhD, NP_417484; YTPA, DAA07955; YGRE3, NP_011972; EcDkgA, NP_417485. *ADH* alcohol dehydrogenase, *CAD* cinnamyl alcohol dehydrogenase, *AKR* aldose-ketose reductase. Both ADH and CAD belong to Zn-dependent alcohol dehydrogenase superfamily

The contribution of these "furfural reductases" to furfural reduction in *E. coli* or the yeast is likely collective, as mutations of individual genes did not significantly affect furfural reduction by the mutant organisms (Liu et al. 2008). In the companion article (Li et al. 2011), we report a "furfural reductase" (FurX) that rapidly reduces furfural with ethanol as the reducing power in *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134. FurX, YADH1 and two homologous bacterial alcohol dehydrogenases (EcADH of *Escherichia coli* and PaADH of *Pseudomonas aeruginosa*) are grouped together by phylogenetic analysis (Fig. 1).

Here, we report that FurX, EcADH, PaADH and YADH1 all catalyze ethanol-dependent and NADH-dependent reduction of furfural. Biochemical characterization of the four enzymes revealed that they may play significant roles in reducing furfural in bacteria and yeast. The reducing power could be NADH from metabolism of other organic compounds or could be ethanol in a coupled reaction, in which the enzymes first used ethanol to reduce NAD^+ and then they use the produced NADH to reduce furfural to furfuryl alcohol.

Materials and methods

Chemicals and enzymes

All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Co. (St. Louis, MO). Alcohol dehydrogenase (YADH1) of the brewer's yeast was obtained from Sigma Co. (Cat. No. A7011-7.5KU). Restriction enzymes and *Taq* DNA polymerase were obtained from New England Biolabs (Beverly, MA). PCR primers were purchased from Invitrogen (Carlsbad, CA).

Bacterial strains and culture conditions

Escherichia coli strains Nova Blue and BL21(DE3), *C. necator* JMP134 and *Pseudomonas aeruginosa* PAO1 were grown in Luria–Bertani (LB) medium (Sambrook et al. 1989) at 30°C. When needed, kanamycin was added to 30 μg ml^{-1}.

Gene cloning and expression

Genomic DNA of *E. coli* BL21(DE3) and *P. aeruginosa* PAO1 were extracted with a DNA isolation kit (Puregene, Minneapolis, MN). The *adhP* gene coding for PaADH (NP_254114.1) was amplified from *Pseudomonas aeruginosa* PAO1 genome with primers PaADH1F (CATGGTTACATATGACCCTGCCAC AG) and PaADH1R (CCAGAAGCTTCATCTCAAG GACGATG); the *adhP* gene encoding EcADH (NP_415995.4) was amplified from *E. coli* genomic DNA with primers EcADHF (AAGGAGGACATATG AAGGCTGCAG) and EcADHR (GGCAAGCTTGT-GACGGAAATCAATC). The PCR products were digested with NdeI and HindIII and cloned into pET-30 Ek/LIC (Novagen, Madison, WI) previously digested by the same enzymes. The genes coding for PaADH and EcADH were cloned with a C-terminal 6 × His-tag. The ligation products were electroporated into *E. coli* Nova Blue. The correct clones were identified by colony PCR. The plasmids with the correct clones were purified and transformed into *E. coli* BL21(DE3) for recombinant protein production.

Purification of recombinant proteins

E. coli strains carrying the cloned gene on an expression vector was grown in one liter of LB medium at 37°C to turbidity of 0.6 at 600 nm, induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside in the presence of 0.2 mM $ZnSO_4$, and then incubated at room temperature for 5 h. PaADH and EcADH were purified with Ni–NTA agarose resin, according to the manufacture's instructions (Qiagen). The targeted protein was concentrated and the buffer was exchanged to 40 mM potassium phosphate (KPi) buffer (pH 7) containing 2 mM dithiothreitol (DTT) with Centriprep Ultracel YM-10 (Millipore, Bedford, MA). FurX was purified from *C. necator* JMP134 as reported (Li et al. 2011). The purchased YADH1 showed up as a single band about 36 kDa on SDS-PAGE gel and used without further purification. All proteins were stored in 40 mM KPi buffer (pH 7) with 2 mM DTT and 10% glycerol at −80°C. No apparent loss of activity was noticed after 3 months for all four proteins.

Kinetic analysis

The kinetic parameters of ethanol-dependent reduction of furfural and related reactions were determined with FurX, PaADH, EcADH, and YADH1. Most of the assays were done in 40 mM KPi (pH 7.0) with a tested enzyme at 23°C, and reaction rates were measured with a UV/Vis spectrophotometer. For ethanol-dependent reduction of furfural, the assay contained ethanol, NAD^+, furfural and several μg of the tested enzyme. Furfural reduction was directly monitored at 278 nm. Because YADH1 had a high K_m value for furfural, its kinetic parameters for ethanol-dependent reduction of furfural were determined by analyzing the production of furfuryl alcohol after 1 min incubation. The reactions were terminated by adding equal volume of an acetonitrile-acetic acid (9:1) mixture. The sample was centrifuged and the supernatant was analyzed by HPLC as described in the companion article (Li et al. 2011). For NAD^+ reduction with ethanol or furfuryl alcohol, NADH production was directly monitored at 340 nm. For NADH-dependent reduction of furfural or acetaldehyde, NADH consumption was directly monitored at 340 nm. The substrates were tested in the range of less than its K_m value to four times of the K_m value, and the other substrate was fixed at five times of the corresponding K_m value. The initial rate was used to obtain the kinetic parameters by fitting the data into Michaelis–Menten equation, using Grafit 5

(R. J. Leatherbarrow, Erithicus Software Ltd., Staines, England, 2001).

Measurement of the dissociation constant (K_d) values for NAD^+ and NADH

Fluorescence spectroscopy measurements were used to determine the binding of NAD^+ and NADH to FurX, PaADH, EcADH, and YADH1. All measurements were done in a spectrofluorometer (HORIBA Jobin-Yvon, Inc., New Jersey, NJ). Fluorescence emission changes were followed from 300 to 400 nm using an excitation wavelength of 280 nm for protein. Emission and excitation bandwidths were set at 2 nm. For titration experiments, microliter aliquots of the NAD^+ or NADH were added to 2 ml of the protein solution. The protein concentrations were 0.1 μM for NAD^+ measurements and 0.5 μM for NADH measurements. The minor changes in fluorescence due to dilution during titration were corrected in calculations. The fluorescence changes of protein at 340 nm after incremental addition of NAD^+ or NADH were used to determine the dissociation constant. For example, the concentration of FurX-NADH complex was estimated by the following equation:

$$[\text{FurX} - \text{NADH}] = [\text{FurX}] \times \left\{(I_0 - I_c)/\left(I_0 - I_f\right)\right\} \quad (1)$$

In the equation, [FurX] represented the initial concentration of FurX, I_0 was the fluorescence intensity of FurX at the initial titration point, I_c was the fluorescence intensity of FurX at a specific titration point, and I_f was the fluorescence intensity at saturating concentrations of NADH. The K_d was determined from a plot of [Bound NADH] (y-axis) vs. [Total NADH] (x-axis) fitted with Eq. 2, using Grafit 5.0 (Erithacus Software, Horley, UK). *Cap* was the binding capacity of FurX.

$$y = \frac{-(K_d + x + \text{Cap}) + \sqrt{(\text{Cap} + x + K_d)^2 - 4x\text{Cap}}}{2} \quad (2)$$

Determine the standard Gibbs free energy change ($\Delta G^{\circ\prime}$)

Relatively high amounts of FurX (130 μg per ml) and PaADH (162 μg per ml) were used to catalyze the ethanol-dependent reduction of furfural to completion over night (20–24 h) in 1 ml of 50 mM KPi buffer (pH 7) at 25°C with 11.2 mM furfural, 17 mM ethanol, and 10 μM NAD^+. The reaction was performed in sealed tubes. The concentrations of furfural, furfuryl alcohol and acetaldehyde were determined at the end of the experiment using HPLC. Ethanol concentration was calculated by subtracting the amount consumed for furfural reduction. The concentrations were used to calculate the standard Gibbs free energy change ($\Delta G^{\circ\prime}$) according to Eq. 3.

$$\Delta G' = \Delta G^{\circ\prime} + \text{RT}ln([\text{furfuryl alcohol}][\text{acetaldehyde}]/[\text{furfural}][\text{ethanol}]) \quad (3)$$

where R is the gas constant (8.314 J mol^{-1} T^{-1}) and T is the absolute temperature (298 K = 25°C). At equilibrium, the $\Delta G'$ is 0.

Protein purity and concentration analyses

SDS-PAGE was done by the method of Laemmli (1970) to check protein purity. Protein concentrations were determined with a protein dye reagent (Bradford 1976) with bovine serum albumin as the standard.

Protein sequence analysis

Protein sequences were aligned using CLUSTALW (Larkin et al. 2007). The evolutionary distances were computed with pairwise deletion for gaps using Poisson correction and were presented in units of the number of amino acid substitutions per site. Optimal global alignment of two protein sequences was performed with ALIGN program at website of Biology Workbench (http://workbench.sdsc.edu). All the analyses were performed with default parameters.

Results and discussion

BLAST search of FurX homologs

BLASTP searches revealed that FurX (encoded by Reut_B3677) had extensive homologs in the non-redundant protein sequence database at NCBI. The best homolog is from *Cupriavidus metallidurans* CH34 with 88% sequence identity, and the last one on the top 100 list is an alcohol dehydrogenase of *Acetobacter pasteurianus* IFO 3283-01 with 66%

sequence identity. The alcohol dehydrogenase (PaADH)(NP_254114.1) of *Pseudomonas aeruginosa* PAO1 is on the top 100 list with 77% sequence identify and the alcohol dehydrogenase (EcADH) (NP_415995.4) of *E. coli* is not on the list with 45% sequence identify. Yeast alcohol dehydrogenase (YADH1) responsible for ethanol fermentation shared 40% sequence identify with FurX.

Multiple sequence alignment and amino acid residues in substrate binding

FurX, PaADH, EcADH, and YADH1 were aligned by ClustalW. The crystal structures of PaADH and EcADH are known (Karlsson et al., 2003; Levin et al., 2004). Each protein consists of two domains, the catalytic domain at the N-terminus and the NAD^+-binding domain at the C-terminus. The catalytic domain contains a catalytic Zn^{2+} and a structural Zn^{2+}. The secondary structures and amino acid residues of PaADH were presented as reference (Fig. 2). The amino acid residues for binding the catalytic Zn^{2+} (44-Cys, 67-His, 154-Cys, numbers according to PaADH and marked with *circled plus*) and structural Zn^{2+} (98-Cys, 101-Cys, 104-Csy, 112-Cys, marked with *inverted open triangle*) were well conserved. The amino acid residues (45-His, 49-His, 181-Gly, 182-Leu, 201-Asp, 266-Val, 268-Leu, 290-Ser, 292-Val, 337-Arg, marked with *filled diamond*) positioned for the binding of NAD^+ were also conserved. Further, the amino acid residues (46-Thr, 49-His, highlighted with gray) involved in transferring the proton abstracted from the alcohol substrate to the free solvent were conserved. Since the catalytic zinc ion helps coordinate the small alcohol (PaADH structure), it is expected to be involved in furfural-binding.

Ethanol-dependent reduction of furfural and reverse reaction

FurX, PaADH, EcADH and YADH1 all catalyzed ethanol-dependent reduction of furfural with NAD^+ as the cofactor. The apparent kinetic parameters were determined (Table 1). Although the four proteins were homologous with sequence identity higher than 40%, they had different activities for furfural reduction. On the basis of $k_{cat}/K_{m.furfural}$ values, both FurX and PaADH were much more efficient than EcADH in catalyzing ethanol-dependent furfural reduction. YADH1 had the smallest $k_{cat}/K_{m.furfural}$ value. The K_m values for NAD^+ were low, indicating that NAD^+ was required at low concentrations during ethanol-dependent furfural reduction. No apparent accumulation of NADH was observed during ethanol-dependent reduction of furfural.

Since most alcohol dehydrogenases catalyze reversible reactions, we also tested the furfuryl alcohol-dependent reduction of acetaldehyde (Table 1, reverse reaction). Direct comparison of the kinetic parameters suggested that the enzymes catalyzed forward reaction more efficiently than the reverse reaction for each enzyme. According to the $k_{cat(r)}/K_{m.furfuryl\ alcohol}$ values, the enzyme that catalyzed efficient forward reaction was also efficient in the reverse reaction. YADH1 was the least efficient enzyme. No apparent accumulation of NADH was observed during furfuryl alcohol -reduction of acetaldehyde.

Ethanol-dependent reduction of NAD^+ and reverse reaction

The reactions presented in Table 1 could be due to two individual reactions. First, the enzymes catalyze the ethanol-dependent reduction of NAD^+. Then, the enzymes catalyze NADH-dependent reduction of furfural. When tested, the enzymes catalyzed the individual reactions. When NAD^+ was the electron acceptor, the reactions were significantly slower with decreased k_{cat} values for FurX, PaADH, and EcADH (Table 2). While the $K_{m.ethanol}$ values showed minimal changes, the K_{mNAD^+} values increase dramatically from ethanol-dependent reduction of furfural (Table 1) to ethanol-dependent reduction of NAD^+ (Table 2). For FurX, the K_{mNAD^+} value changed from 0.47 μM (Table 1) to 1 mM (Table 2), an increase of 2174 fold. A likely explanation is that FurX does not release the produced NADH during ethanol-dependent reduction of furfural. The kinetic parameters for ethanol-dependent reduction of NAD^+ by YADH1 have been reported (Ganzhorn et al. 1987), and we determined them again under our assay conditions (Table 2). Our k_{cat} value (152 s^{-1}) was done at 23°C, and the reported k_{cat} value (340 s^{-1}) is determined at 30°C. Thus, it is expected that our k_{cat} value is lower than the reported k_{cat} value. The K_{mNAD^+} values are similar, but our $K_{m.ethanol}$ value is about 3-fold higher

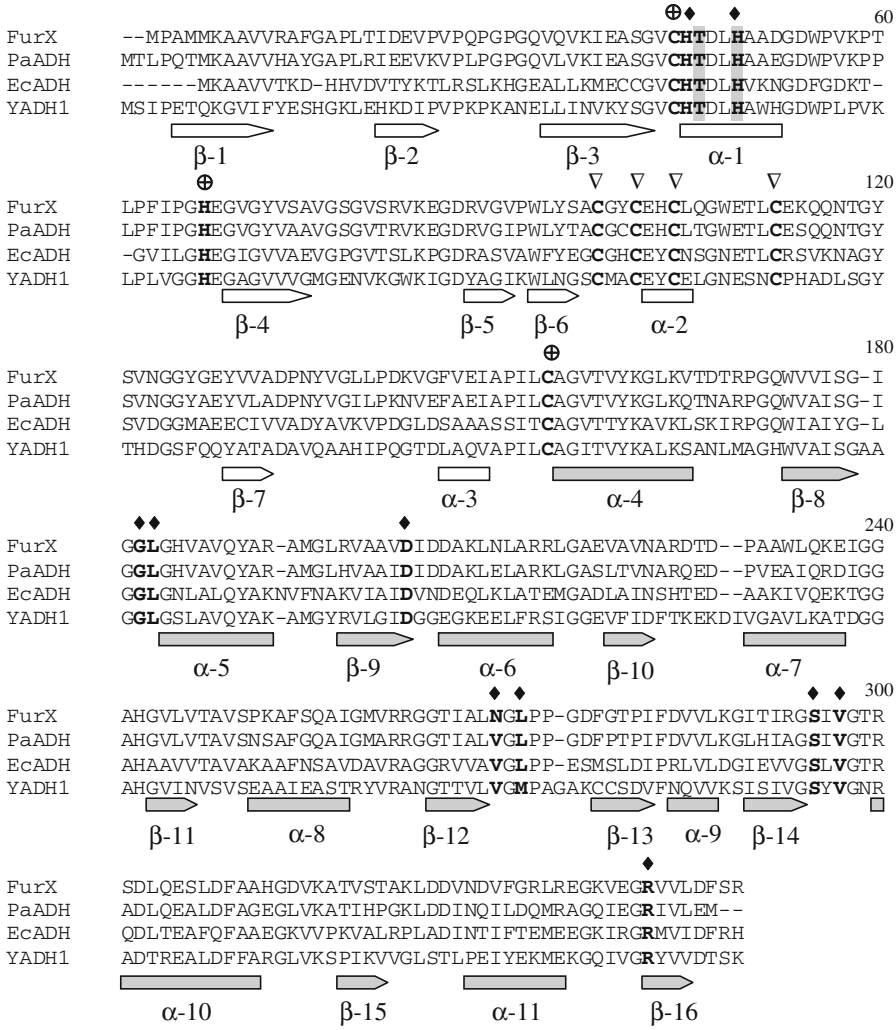

Fig. 2 Alignment of peptide sequences of FurX and related Zn-dependent alcohol dehydrogenases. The proteins and corresponding NCBI protein accession numbers are as follows: FurX, YP_297879; PaADH, NP_254114; EcADH, NP_415995; YADH1, NP_014555. The secondary structures (alpha helixes and beta sheets) of PaADH are shown for reference. The secondary structures of the N-terminal domain and C-terminal domain are shown in *white* and *grey*. The amino acid residues for binding the catalytic Zn^{2+} (marked with *circled plus*), structural Zn^{2+} (marked with *open inverted triangle*), and NAD^{+} (marked with *filled diamond*) are conserved. The amino acid residues (highlighted with *gray*) involved in transferring the proton abstracted from the alcohol substrate to the free solvent are also conserved

than the reported value. The kinetic parameters of EcADH for ethanol oxidation have been reported, but the assay is done at pH 10, which favors ethanol oxidation (Shafqat et al. 1999).

YADH1 had the highest $k_{cat(r)}$ value for NADH-dependent reduction of acetaldehyde, but its high $K_{m.acetaldehyde}$ value decreased its $k_{cat(r)}/K_{m.acetaldehyde}$ value to less than those of the other enzymes (Table 2). The measured kinetic parameters of YADH1 for NADH-dependent reduction of acetaldehyde are in agreement with reported values (Ganzhorn et al. 1987), and our $k_{cat(r)}$ value was lower due to the lower temperature used. EcADH was the most efficient for NADH-dependent reduction of acetaldehyde on the basis of $k_{cat(r)}/K_{m.acetaldehyde}$ values, and our data are similar to the reported kinetic parameters of EcADH for NADH-dependent reduction of acetaldehyde at pH 7.5 (Shafqat et al. 1999).

NADH-dependent reduction of furfural and reverse reaction

The $K_{m.furfural}$ values for NADH-dependent reduction of furfural (Table 3) were similar to those for ethanol-dependent reduction of furfural (Table 1). Although YADH1 had a relatively high k_{cat} value for NADH-dependent reduction of furfural, it had a low $k_{cat}/K_{m.furfural}$ value in comparison to other three enzymes because of its extremely high $K_{m.furfural}$ value (Table 3). The data suggest that YADH1 is not an efficient furfural reductase at low furfural concentrations; however, YADH1 may significantly

Table 1 Ethanol-dependent reduction of furfural and reverse reaction kinetics

Ethanol + Furfural + NAD^+(f) ⇔ Acetaldehyde + Furfuryl alcohol + NAD^+(r)

Parameter	FurX	PaADH	EcADH	YADH1[a]
Forward reaction				
$K_{m.\text{ethanol}}$ (μM)	1600 ± 170	5500 ± 580	2600 ± 490	39000 ± 8400
$K_{m.\text{furfural}}$ (μM)	23 ± 2	20 ± 3.2	78 ± 15	11000 ± 3600
$K_{m.\text{NAD}^+\text{(f)}}$ (μM)	0.46 ± 0.08	4.7 ± 0.6	6.5 ± 2.0	13 ± 2.9
$k_{\text{cat(f)}}$ (s^{-1})	27 ± 1	29 ± 1.0	14 ± 1.3	6.3 ± 0.9
$k_{\text{cat(f)}}/K_{m.\text{furfural}}$ (s^{-1} mM^{-1})	1174	1450	179	0.57
Reverse reaction				
$K_{m.\text{acetaldehyde}}$ (μM)	87 ± 21	39 ± 8.9	22 ± 2.8	22 ± 5.5
$K_{m.\text{fur-alco}}$ (μM)[b]	570 ± 160	370 ± 39	2600 ± 220	14000 ± 2300
$K_{m.\text{NAD}^+\text{(r)}}$ (μM)	3.7 + 1.0	11 + 2.6	36 ± 5.0	150 ± 36
$k_{\text{cat(r)}}$ (s^{-1})	11 ± 0.9	14 ± 0.8	15 ± 0.9	0.15 ± 0.03
$k_{\text{cat(r)}}/K_{m.\text{fur-alco}}$ (s^{-1} mM^{-1})	19	38	5.8	0.011

The assays were done with NAD^+ as the cofactor at 23°C in 50 mM KPi (pH 7). $K_{m.\text{NAD}^+\text{(f)}}$ was for the forward reaction, and $K_{m.\text{NAD}^+\text{(r)}}$ was for the reverse reaction

[a] YADH1 had a high K_m for furfural and furfuryl alcohol. The kinetic parameters were determined with the HPLC method

[b] $K_{m.\text{fur-alco}}$ is short for $K_{m.\text{furfuryl alcohol}}$

contribute to furfural reduction at furfural concentrations higher than its $K_{m.\text{furfural}}$ value (Table 3).

In the reverse reaction, the $K_{m.\text{NAD}^+}$ values for the furfuryl alcohol-dependent reduction of NAD^+ (Table 3) were much higher than those for the furfuryl alcohol-dependent reduction of acetaldehyde (Table 2). Apparently, the enzymes required less NAD^+ in the coupled reactions (Table 1) than alcohol-dependent reduction of NAD^+ (Tables 2 and 3).

The enzymes' affinity for NADH and NAD^+

An enzyme's affinity for a substrate is reversely proportional to the dissociation constant (K_d) for the substrate. The K_d values for NADH were 0.043 ± 0.002, 0.64 ± 0.037, 0.11 ± 0.031, and 0.099 + 0.026 μM for FurX, PaADH, EcADH, and YADH1, respectively. The K_d values for NAD^+ were 87 ± 3.4, 115 ± 3.8, 114 ± 4.2, and 161 ± 6.9 μM for FurX, PaADH, EcADH, YADH1, respectively. The K_d values are in good agreement with the high $K_{m.\text{NAD}^+}$ values during alcohol-dependent reduction of NAD^+ (Tables 2 and 3) and low $K_{m.NADH}$ values for NADH-dependent reduction of aldehydes (Tables 2 and 3). The $K_{m.\text{NAD}^+}$ values became significantly lower for the coupled reactions (Table 1), suggesting NADH was not released.

Thermodynamics of furfural reduction

When enzymes catalyze a reversible reaction, the reaction equilibrium is reached according to the thermodynamics. The equilibrium experiment was done with 17 mM ethanol, 11.2 mM furfural, 10 μM NAD^+ and 0.13 mg of FurX or PaADH per ml of reaction in 50 mM KPi buffer (pH 7). The reaction was incubated at 25°C and monitored every hour, and the reaction was 70–80% completed within 1 h. To reach equilibrium the reaction was incubated for 24 h, and the concentrations of furfural, furfuryl alcohol, and acetaldehyde in the reaction mixture after 24 h were 3.3 ± 0.37, 7.6 ± 0.0.16, and 6.3 ± 0.12 mM, respectively. The combined furfural and furfuryl alcohol concentration at equilibrium was 10.9 mM, which was close to the expected value (the initial furfural concentration). Acetaldehyde concentration was expected to be the same as furfuryl alcohol concentration, but it was only 83% of the expected value. Acetaldehyde has a boiling point of 20.2°C, and some evaporation was likely. Ethanol concentration was estimated by subtracting the produced furfuryl alcohol concentration from the initial ethanol concentration. Using the obtained equilibrium concentrations, the standard Gibbs free energy change ($\Delta G^{\circ\prime}$) was estimated as −1.1 ± 0.29 kJ mol^{-1} (average of

Table 2 Ethanol-dependent reduction of NAD^+ and reverse reaction kinetics

Ethanol + NAD^+ ⇔ Acetaldehyde + NADH + H^+

Parameter	FurX	PaADH	EcADH	YADH1	YADH1[a]
Forward reaction					
$K_{m.\text{ethanol}}$ (μM)	1500 ± 280	3600 ± 890	2700 ± 730	49000 ± 12000	17000
$K_{m.\text{NAD}^+}$ (μM)	1000 ± 190	250 ± 65	2100 ± 730	180 ± 41	170
$k_{\text{cat(f)}}$ (s^{-1})	4.8 ± 0.5	8.0 ± 0.3	1.3 ± 0.2	150 ± 15	340
$k_{\text{cat(f)}}/K_{m.\text{ethanol}}$ (s^{-1} mM^{-1})	3.2	2.2	0.48	3.1	20
Reverse reaction					
$K_{m.\text{acetaldehyde}}$ (μM)	34 ± 5	43 ± 13	35 ± 7	764 ± 97	1100
$K_{m.\text{NADH}}$ (μM)	16 ± 5	7.1 ± 2.8	40 ± 5	71 ± 8.5	110
$k_{\text{cat(r)}}$ (s^{-1})	73 ± 7	77 ± 1	140 ± 5	1200 ± 48	1700
$k_{\text{cat(r)}}/K_{\text{m.acetal}}$ (s^{-1} mM^{-1})	2147	1791	4000	1571	1545

[a] The values are from literature and obtained at 30°C at pH 7.3 (Ganzhorn et al. 1987), and our assay was performed at 23°C at pH 7

Table 3 NADH-dependent reduction of Furfural and reverse reaction kinetics

Furfural + NADH + H^+ ⇔ Furfuryl alcohol + NAD^+

Parameter	FurX	PaADH	EcADH	YADH1
Forward reaction				
$K_{m.\text{furfural}}$ (μM)	20 ± 5.4	13 ± 3.4	54 ± 12	11000 ± 2100
$K_{m.\text{NADH}}$ (μM)	5 ± 2	25 ± 3.6	25 ± 8.1	38 ± 112
$k_{\text{cat(f)}}$ (s^{-1})	13 ± 2	22 ± 0.7	70 ± 7.0	39 ± 3.1
$k_{\text{cat(f)}}/K_{m.\text{furfural}}$ (s^{-1} mM^{-1})	650	1692	1296	3.5
Reverse reaction				
$K_{m.\text{fur-alco}}$ (μM)	150 ± 50	200 ± 80	900 ± 170	14000 ± 2,300
$K_{m.\text{NAD}^+}$ (μM)	370 ± 69	480 ± 140	1200 ± 180	640 ± 89
$k_{\text{cat(r)}}$ (s^{-1})	4.0 ± 0.3	1.3 ± 0.1	3.8 ± 0.2	0.37 ± 0.02
$k_{\text{cat(r)}}/K_{m.\text{fur-alco}}$ (s^{-1} mM^{-1})	26.7	6.5	4.2	0.026

six equilibria catalyzed by FurX or PaADH) for ethanol-dependent reduction of furfural at pH 7 and 25°C (Eq. 4).

The $\Delta G^{\circ\prime}$ was slightly negative, indicating the forward reaction is thermodynamically favorable. On the basis of the experimentally determined $\Delta G^{\circ\prime}$, 170 mM ethanol and 17 mM furfural can be catalyzed by FurX or another alcohol dehydrogenase to an equilibrium of 154 mM ethanol, 1.1 mM furfural, 16 mM furfuryl alcohol and 16 mM acetaldehyde. When we used *C. necator* JMP134 cells containing FurX to catalyze 17 mM Furfural reduction with 170 mM ethanol, 2.7 mM furfural remained after 80 min (Li et al. 2011). The number is close but slightly higher than the theoretical value of 1.1 mM. The $\Delta G^{\circ\prime}$ value for ethanol-dependent reduction of NAD^+ is available from literature (Thauer et al., 1977) (Eq. 5), and it is thermodynamically unfavorable. Eq. 6 is obtained by combining Eqs. 4 and 5. The $\Delta G^{\circ\prime}$ value for NADH-dependent reduction of furfural is thermodynamically favorable. Clearly, the coupled reaction (Eq. 4) is thermodynamically more favorable than either ethanol-dependent (Eq. 5) or furfuryl alcohol-dependent reduction of NAD^+ (Eq. 6 reverse reaction).

$$\text{Ethanol} + \text{Furfural} \leftrightarrow \text{Acetaldehyde} + \text{Furfuryl alcohol} \quad (4)$$
$$\Delta G^{\circ\prime} = -1.1 \pm 0.3 \text{ kJ mol}^{-1}$$

$$\text{Ethanol} + \text{NAD}^+ \leftrightarrow \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \quad (5)$$
$$\Delta G^{\circ\prime} = 23.7 \text{ kJ mol}^{-1}$$

$$\text{Furfural} + \text{NADH} + \text{H}^+ \leftrightarrow \text{Furfuryl alcohol} + \text{NAD}^+$$
$$\Delta G^{\circ\prime} = -24.8\ \text{kJ mol}^{-1} \quad (6)$$

The coupled reaction mechanism

When a coupled reaction consists of two individual reactions, the overall rate is limited by the slow reaction. The k_{cat} values for ethanol-dependent reduction of furfural (Table 1) were much higher than those for ethanol-dependent reduction of NAD^+ for FurX, PaADH and EcADH (Table 2). The data suggest that the coupled reaction is not made of two individual reactions. Further, the low $K_{m.NADH}$ values also suggested that the produced NADH was not released in the coupled reactions. If releasing NADH slows down ethanol-dependent reduction of NAD^+, transferring the reducing power to furfural should stimulate the rate in the coupled reaction. For FurX, it is reasonable to assume that the produced NADH remains bound to the enzyme during the ethanol-dependent reduction of furfural because of the small $K_{m.NAD^+}$ (0.46 μM) in comparison and the high $K_{m.NAD^+}$ (1 mM) for ethanol-dependent reduction of NAD^+. The same argument can also apply to PaADH and EcADH. The structure of PaADH with NAD^+ and ethylene glycol has been determined and the ethanol-dependent reduction follows the established scheme for Zn-dependent alcohol dehydrogenases (Fig. 3, steps 1–5) (Levin et al. 2004). Since NADH is not accumulated during the ethanol-dependent reduction of furfural, the dissociation of E.NADH (Fig. 3, step 5) should be limited if any. Instead, furfural comes in to form E.NADH.furfural (Fig. 3, step 6). Further catalysis leads to furfural reduction (step 7) and furfuryl alcohol release (step 8). Given the enzymes' high affinity (low K_d values) for NADH and relatively low affinity (high K_d values) for NAD^+, more E.NAD^+ dissociation (Fig. 3, step 1 reverse) is expected than E.NADH dissociation (Fig. 3, step 5). Figure 3 shows the reversible nature of the reactions catalyzed by the alcohol dehydrogenases. During furfuryl alcohol-dependent reduction of acetaldehyde, limited dissociation of E.NADH (step 5) is also expected. A clear conclusion for the coupled reactions cannot be drawn for YADH1 using the kinetic data.

Physiological implications

Nicotinoprotein (NADH-containing) alcohol dehydrogenase from *Rhodococcus erythropolis* DSM 1069 has been reported to catalyze the interconversion of alcohols and aldehydes (Schenkels and Duine 2000). The enzyme has a bound NADH and does not require the addition of NAD^+ as cofactor during its catalysis. Further, the enzyme does not catalyze NADH-dependent reduction of acetaldehyde. The sequence of the first 22 amino acid residues at the N-terminus of the enzyme has been reported, and BLASTP search revealed a 100% match with a Zn-dependent alcohol dehydrogenase (Protein ID: ABG94302) in *Rhodococcus jostii* RHA1. ABG94302 and FurX share 31.3% sequence identity. *R. erythropolis* NCIMB 13064 resting cells have been used as the catalyst for the coupled butanol-dependent reduction of hexanal (Marchand et al. 2008). The reaction is believed to be catalyzed by the nicotinoprotein alcohol dehydrogenase.

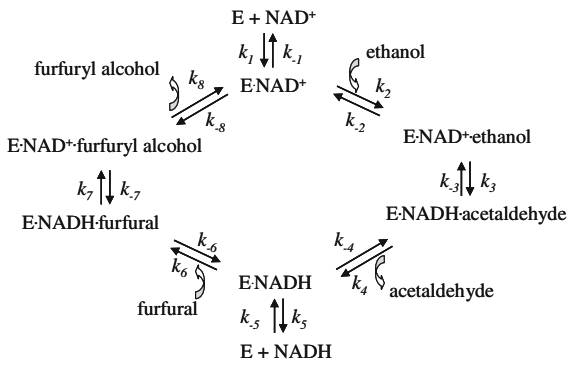

Fig. 3 The proposed scheme of the coupled reactions of ethanol-dependent reduction of furfural and furfuryl alcohol-dependent reduction of acetaldehyde. The rate constants of individual steps are marked. The reversible nature of each reaction is shown with *reversible arrows*

The ethanol-dependent reduction of furfural catalyzed by FurX and *C. necator* JMP134 cells is similar to the coupled reactions of alcohol oxidation and aldehyde reduction by the nicotinoprotein alcohol dehydrogenase and *R. erythropolis* NCIMB cells. Although FurX is not a nicotinoprotein, it has a high affinity (K_d of 0.043 μM) for NADH. Therefore, small amount of NAD^+ is required for FurX during ethanol-dependent reduction of furfural. Since nicotinoprotein alcohol dehydrogenases do not release NAD^+/NADH, they may be more efficient in catalyzing the interconversion of alcohols and aldehydes. It remains to be tested whether the nicotinoprotein alcohol dehydrogenase of *R. erythropolis* can catalyze the ethanol-dependent reduction of furfural and whether it is more efficient than FurX.

The ability of nicotinoprotein alcohol dehydrogenases to interconvert alcohols and aldehydes is believed to be physiologically important when alcohols and aldehydes coexist. For example, the interplay of alcohols and aldehydes is involved in generating H_2O_2 used by peroxidase to breakdown lignin (Schenkels and Duine 2000). Microorganisms containing nicotinoprotein alcohol dehydrogenases or FurX and related alcohol dehydrogenases may use the interconversion to reduce the aldehydes that they cannot oxidize or that they can only slowly oxidize and to convert alcohols to the corresponding aldehydes that can be easily oxidized for energy production. Given the physical association of *furX* (Reut_B3677) with an aldehyde dehydrogenase gene (Reut_B3678) on the chromosome, the two corresponding proteins are likely responsible for the oxidization of alcohols to aldehydes and then to fatty acids that can be used as the carbon and energy source. If the aldehyde dehydrogenase could not oxidize furfural to furoic acid, then it is advantageous for FurX to couple the oxidation of ethanol or other alcohols with furfural reduction to non-inhibitory furfuryl alcohol. The produced aldehydes could be further oxidized by the aldehyde dehydrogenase. Since aldehydes are generally toxic (O'Brien et al. 2005), rapidly minimizing the concentrations of aldehydes offers a detoxification mechanism for microorganisms.

FurX and related enzymes can also use NADH generated from metabolism of other carbon sources, such as sugar, to reduce furfural. YADH1 is constitutively expressed and is the main enzyme responsible for acetaldehyde reduction during ethanol fermentation. Its kinetic parameters for NADH-dependent reduction of furfural suggest that it can effectively catalyze furfural reduction at high furfural concentrations. The k_{cat} value of 39 s^{-1} (Table 3) corresponds to a V_{max} of 63.5 μmol min^{-1} mg^{-1} of protein, which is significantly higher than the recently reported NADPH-dependent aldehyde reductase that reduces furfural with a specific activity of 4.19 μmol min^{-1} mg^{-1} of protein under similar assay conditions (Liu and Moon 2009). Since YADH1 is responsible for ethanol fermentation, it is a major protein in yeast (de Smidt et al. 2008). The NADPH-dependent aldehyde reductase may reduce furfural at low furfural concentrations, the kinetic parameters should be determined to confirm the possibility. The ability of FurX to reduce furfural to furfuryl alcohol in *C. necator* JMP134 has been reported in the companion article (Li et al. 2011). Thus, FurX and related Zn-dependent alcohol dehydrogenases may play significant roles in reducing furfural in bacteria and the yeast.

Acknowledgments Q. Li was partially funded by a scholarship from the Ministry of Education of the People's Republic of China. L. K. M. Lam was partially funded by College of Science, Washington State University.

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