Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*

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A recombinant *Saccharomyces cerevisiae* strain transformed with xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *Pichia stipitis* (*PsXR* and *PsXDH*, respectively) has the ability to convert xylose to ethanol together with the unfavourable excretion of xylitol, which may be due to intercellular redox imbalance caused by the different coenzyme specificity between NADPH-preferring XR and NAD⁺-dependent XDH. In this study, we focused on the effect(s) of mutated NADH-preferring *PsXR* in fermentation. The R276H and K270R/N272D mutants were improved 52- and 146-fold, respectively, in the ratio of NADH/NADPH in catalytic efficiency [$(k_{\text{cat}}/K_m \text{ with NADH})/(k_{\text{cat}}/K_m \text{ with NADPH})$] compared with the wild-type (WT), which was due to decrease of k_{cat} with NADPH in the R276H mutant and increase of K_m with NADPH in the K270R/N272D mutant. Furthermore, R276H mutation led to significant thermostabilization in *PsXR*. The most positive effect on xylose fermentation to ethanol was found by using the Y-R276H strain, expressing *PsXR* R276H mutant and *PsXDH* WT: 20 % increase of ethanol production and 52 % decrease of xylitol excretion, compared with the Y-WT strain expressing *PsXR* WT and *PsXDH* WT. Measurement of intracellular coenzyme concentrations suggested that maintenance of the of NADPH/NADP⁺ and NADH/NAD⁺ ratios is important for efficient ethanol fermentation from xylose by recombinant *S. cerevisiae*.

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INTRODUCTION

Xylose is one of the major fermentable sugars present in lignocellulosic biomass, the second most abundant carbohydrate polymer in nature after glucose. The efficient fermentation of xylose is required to develop economically viable processes for producing biofuels such as ethanol from biomass (Jeffries & Jin, 2004). Although a few xylose-fermenting yeasts are found in nature (Jeffries, 1983; Kurtzman, 1994), *Saccharomyces cerevisiae* is used universally

for industrial ethanol production because of its ability to produce high concentrations of ethanol and high inherent ethanol tolerance; however, native *S. cerevisiae* can not ferment xylose, so engineering *S. cerevisiae* for xylose utilization has focused on adapting the xylose metabolic pathway from the xylose-utilizing yeast *Pichia stipitis*. In this organism, xylose is converted to xylulose by two oxidoreductases: xylose is initially reduced to xylitol by NAD(P)H-linked xylose reductase (XR; EC 1.1.1.21) (Verduyn *et al.*, 1985), and then xylitol is oxidized to xylulose by NAD⁺-linked xylitol dehydrogenase (XDH; EC 1.1.1.9) (Rizzi *et al.*, 1989). Finally, xylulokinase (EC 2.7.1.17) phosphorylates xylulose into xylulose 5-phosphate, which is metabolized further via the pentose-phosphate pathway.

Although *S. cerevisiae* transformed with native *XYL1* and *XYL2* genes encoding XR and XDH from *P. stipitis*

Abbreviations: AKR, aldo-keto reductase; CD, circular dichroism; PGK, phosphoglycerate kinase; XDH, xylitol dehydrogenase; *PsXDH*, XDH from *Pichia stipitis*; XI, xylose isomerase; XR, xylose reductase; CIXR, XR from *Candida tenuis*; *PsXR*, XR from *Pichia stipitis*; T_m , thermal unfolding transition temperature; WT, wild-type.

Two supplementary tables are available with the online version of this paper.

(referred to as PsXR and PsXDH, respectively) and the endogenous *XYL3* gene encoding xylulokinase was the most potent recombinant strain, its ethanol production was not sufficient for application in the industrial bio-process. One of the main reasons is the unfavourable excretion of xylitol, which may be due to intracellular redox imbalance caused by the different coenzyme specificity between XR and XDH (Jeffries & Jin, 2004). On the other hand, in bacteria, xylose is directly converted with no coenzyme by xylose isomerase (XI, EC 5.3.1.5): this is one solution to the redox balance issue. However, no significant XI activity had been attained in transformed *S. cerevisiae* cells, except a thermophilic enzyme. Furthermore, the energetics of isomerization between xylose and xylulose favours xylose formation (Jeffries, 1985). Therefore, modifying the coenzyme specificity of XR and/or XDH by protein engineering is one of the attractive challenges for achieving efficient ethanol fermentation from xylose using *S. cerevisiae*. In the case of XDH, Metzger & Hollenberg (1995) first attempted to identify a set of amino acid residues in PsXDH responsible for specificity to NAD⁺. They introduced the potential NADP⁺(H)-recognition sequence of *Escherichia coli* glutathione reductase (no homology with PsXDH) and thermophilic alcohol dehydrogenase (~30 % homology) into the homologous sequence in PsXDH. However, the mutant enzyme(s) had decreased XDH activity (~40 %) and still preferred NAD⁺ to NADP⁺, although an approximately 10-fold increase in K_m with NAD⁺ was found. In an alternative approach, we used the unique NADP⁺(H)-dependent sorbitol dehydrogenase as a reference enzyme and achieved complete reversal of coenzyme specificity toward NADP⁺ (Watanabe *et al.*, 2005). Furthermore, when the novel NADP⁺-dependent XDH mutant was co-expressed with PsXR in *S. cerevisiae* cells, effective ethanol fermentation and a reduction in xylitol excretion were found, probably due to maintenance of the intracellular redox balance (Watanabe *et al.*, 2007). To our knowledge, this is the first report of improving ethanol fermentation from xylose by the protein engineering of XDH.

Jeppsson *et al.* (2006) reported that enhanced ethanol yield accompanied by decreased xylitol excretion was found in recombinant *S. cerevisiae* carrying the K270M PsXR mutant with increased K_m for NADPH (Kostrzynska *et al.*, 1998) together with PsXDH WT. This is the first improvement of ethanol fermentation from xylose by the protein engineering of XR. In the protein engineering of XR, the enzyme from *Candida tenuis* (CtXR) has recently been studied extensively (Kavanagh *et al.*, 2002, 2003; Leitgeb *et al.*, 2005; Petschacher & Nidetzky, 2005; Petschacher *et al.*, 2005), although the effect of expression of the mutant(s) generated has not been reported. XR belongs to the aldoketo reductase (AKR) superfamily (Ellis, 2002). Although most members of this superfamily show strong dependence on NADPH, a few members including XR (AKR2B5), 3 α -hydroxysteroid dehydrogenase (AKR1C9) and 3-dehydroecdysone 3 β -reductase (AKR2E1) can utilize both

NADH and NADPH (Ellis, 2002). Furthermore, the crystal structures of CtXR bound to NAD⁺ or NADP⁺ were the first to be resolved among AKR superfamily enzymes (Kavanagh *et al.*, 2002, 2003). Based on this useful information, we generated several NADH-preferring PsXR mutants by site-directed mutagenesis and then constructed recombinant yeasts co-expressing *XYL1* genes encoding mutated PsXR and *XYL2* genes encoding WT PsXDH. The resultant recombinant yeasts were characterized for their enzyme activity and the ability to ferment xylose to ethanol.

METHODS

Materials and media. *S. cerevisiae* D452-2 (*MATa leu2 his3 ura3 can1*) (Kurtzman, 1994) was used as the recipient yeast strain for the expression of PsXDH and PsXR. *S. cerevisiae* was grown in yeast-peptone-glucose [dextrose] (YPD) medium (10 g yeast extract, 20 g peptone and 20 g glucose per litre) and in a defined minimal medium (6.7 g l⁻¹ yeast nitrogen base without amino acids). Glucose (5 g l⁻¹) and/or xylose (15 g l⁻¹) was added to the minimal medium in fermentation experiments. Leucine, histidine and uracil were added at 20, 20 and 5 mg l⁻¹, respectively, when necessary.

Enzyme assays. Activities of XR and XDH were assayed by the method described previously (Watanabe *et al.*, 2007, 2005). The kinetic parameters K_m and k_{cat} were calculated by Lineweaver–Burk plots. Protein concentrations were determined by the Lowry method with BSA as the standard.

SDS-PAGE and Western blot analysis. SDS-PAGE was carried out by the method of Laemmli (1970) with 12 % acrylamide gels at 25 mA. For Western blot analysis, cell-free extracts of *E. coli* and/or purified PsXRs were separated by SDS-PAGE, and the proteins on the gels were transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences). Western blot analysis was carried out with the ECL-Western blotting detection system (Amersham Biosciences) and RGS-His HRP antibody, a horseradish-peroxidase-fused mouse monoclonal antibody against Arg-Gly-Ser-(His)₆ in the N-terminal additive peptide of expressed recombinant proteins (Qiagen).

Cloning of the *P. stipitis* *XYL1* gene and construction of recombinant plasmids. *Pichia stipitis* (*Yamadazyma stipitis* NBRC 1687) was purchased from the National Institute of Technology and Evaluation (Chiba, Japan). *P. stipitis* genomic DNA was isolated according to the method described previously (Watanabe *et al.*, 2005). Based on the published sequence of the *P. stipitis* *XYL1* gene (GenBank accession no. X59465), two primers were designed as follows: XR-UP1 (5'-ATGCCTTCTATTAAGTTGAAGTCTGG-3') and XR-DOWN1 (5'-TTAGACGAAGATAGGAATCTTGTC-3'). PCR was carried out using a PCR Thermal Cycler PERSONAL (TaKaRa) in a 50 μ l reaction mixture containing 10 pmol primers, 1 U KOD-plus DNA polymerase (Toyobo) and 100 ng *P. stipitis* genomic DNA under the following conditions: denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and extension at 68 °C for 1.5 min. The amplified DNA fragment was introduced into the *Sma*I site in the plasmid pBluescript SK(-) (Stratagene), to yield pBS-XYL1. To introduce the restriction sites for *Bam*HI and *Pst*II at the 5'- and 3'-termini, respectively, of the *XYL1* gene, PCR was carried out using pBS-XYL1 as template DNA and the following two primers: XR-UP2 (5'-catagcgatccTTCTATTAAGTTGAAC-3') and XR-DOWN2 (5'-cttgctgcagTTAGACGAAGATAGG-3') [small letters indicate additional bases for introducing *Bam*HI and *Pst*II digestion sites (underlined)]. The amplified DNA fragment was introduced into the

*Bam*HI/*Pst*I sites in pQE-81L (Qiagen), a plasmid vector for adding an N-terminal (His)₆-tag on expressed proteins, to obtain pHis(WT).

Site-directed mutagenesis of *PsXR*. The mutations were introduced by single-round PCR with a small modification. The synthetic oligonucleotide primer sequences used for the *PsXR* mutations are shown in Supplementary Table S1, available with the online version of this paper. Briefly, the codons used for single mutants are as follows: K270R (AAG→AGA), K270G (AAG→GGT), N272D (AAC→GAC) and R276H (AGA→CAC). Every double and triple mutant was made using these single mutations. The nicked circular strands with mutation in the *XYL1* gene, which is contained in a plasmid template, were amplified using 22–25 bp sense and antisense primers containing the mutation (described above) with *LA Taq* DNA polymerase (TaKaRa) under the following conditions: denaturation at 94 °C for 10 s, annealing and extension at 68 °C for 6 min (30 cycles). PCR products were incubated with *Dpn*I at 37 °C for 1 h. This treatment ensured the digestion of the Dam-methylated parental strand, which was used as the PCR template. The remaining nicked circular mutagenic strands were transformed into *E. coli* DH5 α , where bacterial DNA ligase repaired the nick and allowed normal replication to occur. The whole coding region of all mutated genes was sequenced in both directions using a Dual CyDye Terminator Sequencing kit (Veritas) and appropriate primers with a Long-Read Tower, UBC DNA sequencer (Amersham Biosciences); only appropriate mutation(s) were found.

Overexpression and purification of (His)₆-tagged enzymes. *E. coli* DH5 α harbouring the expression plasmid for the (His)₆-tagged WT and mutated *PsXRs* were grown at 37 °C to OD₆₀₀ 0.6 in Super broth medium (per litre: 12 g tryptone, 24 g yeast extract, 5 ml glycerol, 3.81 g KH₂PO₄ and 12.5 g K₂HPO₄; pH 7.0) containing 50 mg ampicillin l⁻¹. The cultures were then rapidly cooled on ice, added to 1 mM IPTG and further incubated for 24 h at 18 °C to induce the expression of *PsXR* protein. Cells were harvested and resuspended in 20 ml buffer A (50 mM sodium phosphate, pH 6.0, containing 0.3 M NaCl, 10 mM xylose, 5 mM 2-mercaptoethanol and 10 mM imidazole) per litre of culture. The cells were then disrupted by sonication for a total of 4–5 min, with cooling intervals on ice, using an ASTRASON Ultrasonic Liquid Processor XL2020 (Misonix), and the cell lysate was centrifuged to remove cell debris. All chromatography was carried out using the ÄKTA purifier system (Amersham Biosciences) at 4 °C. The resultant supernatant was loaded onto a Ni-NTA Superflow column (Qiagen) equilibrated with buffer A. After washing with the same buffer, the column was further washed with buffer B (buffer A containing 10%, v/v, glycerol and 50 mM instead of 10 mM imidazole). The enzymes were then eluted with buffer C (buffer B containing 250 mM instead of 50 mM imidazole). The eluate was treated by ultrafiltration with Centriplus YM-30 (Millipore) and loaded onto a HiLoad 16/60 Superdex 200 pg column (Amersham Biosciences) equilibrated with buffer D (50 mM sodium phosphate, pH 6.0, containing 10 mM xylose and 5 mM 2-mercaptoethanol). The peak fraction corresponding to *PsXR* was pooled, treated by ultrafiltration and dialysed against buffer E (50 mM sodium phosphate, pH 6.0, containing 10 mM xylose, 5 mM 2-mercaptoethanol and 50%, w/v, glycerol). All (His)₆-tagged recombinant *PsXRs* were stored at –35 °C until use.

Enzyme stability. To estimate the effect of site-directed mutagenesis in thermostability, circular dichroism (CD) and heat-inactivation analysis were carried out. Enzyme samples dialysed overnight against buffer F (50 mM sodium phosphate, pH 6.0, containing 5 mM 2-mercaptoethanol) were diluted to concentrations of 2 mg ml⁻¹ with the same buffer. CD at 220 nm was measured between 10 °C and 50 °C with a Jasco spectropolarimeter model J-720 (Japan Spectroscopic Co.), equipped with temperature-controlled cell holders, using a quartz cuvette with a path length of 2 mm under

constant N₂ flush. Temperature was increased at a rate of 1 °C min⁻¹. The thermal transition temperature (*T*_m) was determined using Jasco software. For heat-inactivation analysis, the enzyme sample was incubated at 30 °C for 10 min and the remaining activity was measured under standard assay conditions.

Construction of vectors overexpressing *XYL1* and *XYL2* genes in *S. cerevisiae*. The *XYL1* and *XYL2* genes were expressed constitutively under the control of the phosphoglycerate kinase (PGK) promoter and terminator. To introduce a restriction site for *Hind*III and *Bam*HI at the 5'- and 3'-termini, respectively, of the *XYL1* gene, PCR was carried out using pBS-*XYL1* as template DNA and the following two primers: XR-UP3 (5'-catcgacaagcttATG-CCTTCTATTAAGTTGAACCTCTGG-3') and XR-DOWN3 (5'-gtcgatgggatccTTAGACGAAGATAGGAATCTTGTC-3') [small letters indicate additional bases for introducing *Hind*III and *Bam*HI digestion sites (underlined)]. The amplified DNA fragment was introduced into the *Hind*III/*Bam*HI sites between the PGK expression cassettes in plasmid pPGK (Kang *et al.*, 1990) to obtain pPGK-XR(pre-WT). To eliminate two restriction sites for *Sal*I in the *XYL1* gene, single-round PCR was performed by *LA Taq* DNA polymerase using pPGK-XR(pre-WT) as template DNA and the following two primers: Δ *Sal*I-S (5'-GTTGGAAAGTTGACGTTGACACCTGTTC-3') and Δ *Sal*I-AS (5'-GAACAGGTGTCACGTCACCTTTCCAAC-3') (underlining indicates mutated regions). The substitution of *Sal*I sites in the *XYL1* gene resulted in no change of amino acid residues. After treatment with *Dpn*I, PCR products were transformed into *E. coli* DH5 α to obtain plasmid pPGK-XR(WT). The ~2.0 kbp DNA fragment comprising the *XYL1* gene between the PGK expression cassettes was excised from pPGK-XR(WT) by *Xho*I and *Sal*I, and introduced into the *Sal*I site of plasmid YEpm4 (Nikawa *et al.*, 1987), resulting in YEpm4-XR(WT). Site-directed mutagenesis of the *XYL1* gene in plasmid YEpm4-XR(WT) (K270R, R276H and K270R/N272D) was performed by single-round PCR with the same procedure as for the pQE-81L series.

To introduce a restriction site for *Eco*RI and *Bam*HI at the 5'- and 3'-termini, respectively, of the *XYL2* gene, PCR was carried out using pBS-*XYL2* [pBluescript SK(-) containing the *XYL2* gene] (Watanabe *et al.*, 2005) as a template DNA and the following two primers: XDH-UP (5'-catcgacaagcttATGACTGCTAACCCCTTCCTTGGTGTG-3') and XDH-DOWN (5'-gtcgatgggatccTTACTCAGGGCCGTCATG-AGACACTTG-3') (small letters indicate additional bases for introducing *Eco*RI and *Bam*HI digestion sites (underlined)]. The amplified DNA fragment was introduced into the *Eco*RI/*Bam*HI sites between the PGK expression cassettes in plasmid pPGK to obtain pPGK-XDH.

Yeast transformation. This was performed by the lithium acetate method (Gietz *et al.*, 1992). *S. cerevisiae* D452-2 was transformed with pPGK-XDH and then further transformed with YEpm4-XR(WT), YEpm4-XR(R276H) and YEpm4-XR(K270R/N272D) to construct recombinant yeast strains Y-WT, Y-R276H and Y-K270R/N272D, respectively. Both empty vectors, pPGK and YEpm4, were transformed into *S. cerevisiae* D452-2 to construct a control strain (Y-Vector).

Preparation of cell-free extract. The recombinant yeast strains were grown in minimal medium supplemented with glucose as sole carbon source at 30 °C. The cells were harvested, resuspended in 100 mM sodium phosphate, pH 7.0, containing 1 mM MgCl₂, 0.5 mM EDTA and 0.5 mM dithiothreitol, and vortexed together with an equal volume of glass beads (0.5 mm diameter). Cell debris and glass beads from the cell extract were separated by centrifugation and the remaining supernatant was used for enzyme determinations.

Preliminary batch fermentation in shake flasks. After precultivation of recombinant yeast strains in 3 ml minimal medium for 3 days,

yeast cells were aerobically cultivated for 3 days at 30 °C in 100 ml minimal medium supplemented with 5 g glucose l⁻¹ and 15 g xylose l⁻¹ with shaking at 200 r.p.m. Cell pellets were collected by centrifugation, washed with NaCl solution (9 g l⁻¹) and inoculated into 200 ml fermentation medium. Ethanol fermentation was carried out as batch cultures in a 200 ml flask sealed with two layers of Saran wrap (under oxygen-limiting conditions) in an incubator at 150 r.p.m. Samples (1 ml) of the fermentation broth were removed at intervals and stored at -35 °C to analyse substrates and fermentation products.

Batch fermentation in a bioreactor. A high-performance bioreactor (BioFlo 110, New Brunswick Scientific Co.) was used for oxygen-limiting batch fermentation. *S. cerevisiae* cells were prepared by similar procedures to those for fermentation in shake flasks except that the culture volume was 300 ml. Cell pellets obtained by centrifugation were inoculated into 1 litre minimal medium supplemented with 5 g glucose l⁻¹ and 15 g xylose l⁻¹ with shaking at 200 r.p.m. and transferred into a 1.3 litre bioreactor. Temperature was maintained at 30 °C and pH was controlled at 5.5 by the addition of 2 M NaOH and 1 M H₂SO₄. The agitation speed was constant at 500 r.p.m.

Measurement of intracellular coenzyme contents. Intracellular coenzymes were extracted from cells growing under oxygen-limiting conditions in minimal medium with 10 g xylose l⁻¹ plus 10 g glucose l⁻¹. A 20 ml sample of yeast culture was withdrawn and sprayed into 80 ml 60 % (v/v) methanol at -40 °C. After this quenching step, coenzymes were extracted in 50 mM potassium phosphate (pH 5.0) (for NAD⁺ and NADP⁺) or 50 mM Tris/HCl (pH 9.0) (for NADH and NADPH). The contents of NAD⁺, NADH, NADP⁺ and NADPH in the samples were determined by monitoring the absorbance difference at 339 nm using the followed coupling enzymes at room temperature (Bergmeyer, 1985): for NAD⁺, alcohol dehydrogenase (from baker's yeast, Sigma); for NADP⁺, glucose-6-phosphate dehydrogenase (type XV from baker's yeast, Sigma); for NADH, glycerol-3-phosphate dehydrogenase (from rabbit muscle, Wako); for NADPH, glutamate dehydrogenase (from beef liver, Oriental Yeast).

Analysis of fermentation products. Ethanol concentration was measured by gas chromatography (model GC-14B, Shimadzu; fitted with a flame-ionization detector) under the following conditions: glass column packed with Thermon-3000 (2.0 m × 3.2 mm, Shimadzu); temperature of column, 70 °C; injector, 200 °C; detector, 250 °C; and nitrogen carrier gas flow rate, 25 ml min⁻¹. The concentrations of glucose, xylose, xylitol, glycerol and acetic acid were estimated by HPLC with a Multi-Station LC-8020 model II system (TOSOH). Samples (100 µl) were applied at 30 °C to an Aminex HPX-87H Organic Analysis column (300 × 7.8 mm,

Bio-Rad) linked to an RID-8020 refractive index detector (TOSOH) and eluted with 5 mM H₂SO₄ at a flow rate of 0.4 ml min⁻¹. Cell growth was monitored by measuring OD₆₀₀ with a Hitachi model U-2001 spectrophotometer.

RESULTS

Modification of coenzyme specificity of PsXR

XR (AKR2B5) is a unique member, with dual coenzyme specificity, of the aldo-keto reductase (AKR) superfamily (Ellis, 2002), although significant NADPH preference is found in most XRs, similar to most AKR superfamily enzymes. In this study, we designed NADH-preferring PsXR mutants by three different strategies based on the following observations.

(1) XR from *Candida parapsilosis* shows 100-fold higher utilization of NADH than NADPH in terms of catalytic efficiency; it possesses a unique arginine residue in the coenzyme-binding pocket instead of Lys²⁷⁰ in PsXR (Table 1) (Lee *et al.*, 2003).

(2) Several site-directed mutagenetic and crystallographic studies for modifying coenzyme specificity toward NADH, using NADPH-preferring XR from *C. tenuis* (CtXR), were recently reported by B. Nidetzky and coworkers (Kavanagh *et al.*, 2002, 2003; Leitgeb *et al.*, 2005; Petschacher & Nidetzky, 2005; Petschacher *et al.*, 2005). This fungal XR shows ~77 % sequential similarity to PsXR. The K274R/N276D mutations lead to the highest ratio of NADH/NADPH preference in terms of k_{cat}/K_m among the series of CtXR mutants investigated, and correspond to the K270R/R272D mutations in PsXR (Table 1).

(3) 2,5-Diketo-D-gluconic acid reductase, a member of the AKR superfamily (AKR5C1), also shows inherent NADPH specificity. However, mutations equivalent to K270G and R276H in PsXR lead to efficient NADH preference (Banta *et al.*, 2002a, b).

On the basis of the above, we generated three single mutants (K270R, K270G and R276H), three double mutants (K270R/N272D, K270R/R276H and N272D/

Table 1. Partial amino acid sequence alignment of AKRs

Enzyme	Accession no.	Organism	Coenzyme	Sequence§
XR	CAA42072	<i>P. stipitis</i>	NADPH	268 IPK S NTVP R LL
XR	AAC25601	<i>C. tenuis</i>	NADPH	272 IPK S NLP R LV
			NADH†	174 IPR S DL P ERLV
XR	AY193716	<i>C. parapsilosis</i>	NADH	274 IPR S NS P DRMA
2,5-DKGRA*	AAA83534	<i>Corynebacterium</i> sp.	NADPH	230 FPK S VRRERLE
			NADH‡	230 FPG S VRR H LE

*2,5-Diketo-D-gluconic acid reductase A.

†K274R/N276D mutant.

‡K232G/R238H mutant.

§Bold letters correspond to mutation sites in this study.

R276H) and one triple mutant (K270R/N272D/R276H) to modify the coenzyme specificity of PsXR.

Overexpression and purification of recombinant PsXR

We initially attempted to express PsXR in *E. coli* cells at 37 °C because PsXR is a typical mesophilic enzyme, but after induction by IPTG, no expression was detectable by enzyme activity assay or Western blotting using an antibody against the (His)₆-tag in cell-free extracts of *E. coli* (data not shown). However, the *XYL1* gene was significantly induced at 18 °C by IPTG. (His)₆-tagged PsXR (WT) may be relatively thermolabile, at least *in vitro* (see below); therefore, all recombinant PsXRs were over-expressed under this condition and purified with a Ni²⁺-chelating affinity column and subsequent gel filtration. SDS-PAGE analysis revealed that the purified recombinant enzymes were almost homogeneous and the apparent molecular mass of the PsXRs was ~38,000 Da, in good agreement with the calculated molecular mass of the enzyme with a (His)₆-tag (37,261.77 Da) (data not shown). Gel filtration revealed the homodimeric form of the purified recombinant enzyme as well as the native form (data not shown) (Verduyn *et al.*, 1985). Kostrzynska *et al.* (1998) successfully expressed PsXR in *E. coli* cells even at 37 °C, although the recombinant enzyme was a non-tagged form. Recently, we also constructed an alternative expression

system for (His)₆-tagged PsXR at 37 °C, in which a plasmid vector based on the T7 promoter (the T5 promoter was used in the present study) and *E. coli* BL21(DE3) as a host cell (DH5 α was used in the present study) were used. Therefore, the expression of PsXR found only at low temperature in the present study may be due to the expression system rather than the inherent thermolability of the protein.

Characterization of PsXR mutants *in vitro*

Specific activities of the recombinant PsXR WT enzyme in the presence of NADH and NADPH (Table 2) were 7.2 and 15.7 U mg⁻¹, respectively, comparable to those of the native enzyme (16.7 and 23.2 U mg⁻¹, respectively) (Verduyn *et al.*, 1985). Among the five single mutants, R276H substitution was the best for acquired NADH preference: specific activity in the presence of NADPH decreased 39-fold compared to WT, while NADH-dependent activity was similar to NADH-dependent activity in WT. The K270R mutant showed higher specific activity with NADPH, and the K270G mutant had dramatically decreased activity, although some reversal of coenzyme specificity was found. The positive effect of the R276H mutation was also observed when introduced into other single mutants: K270R/R276H and N272D/R276H. Since the K270R/N272D mutant corresponds to the best NADH-preferring CrXR mutant, K274R/N276D (Petschacher *et al.*,

Table 2. Specific activity and thermostability of WT and mutant PsXR

Enzyme	Coenzyme	Specific activity* (U mg ⁻¹)	NADH/ NADPH ratio†	Thermostability	
				<i>T</i> _m (°C)‡	Activity (%)§
WT	NADH	7.2 ± 0.4	0.5	27.67 ± 0.04	4.1 ± 0.3
	NADPH	15.7 ± 0.3			
K270R	NADH	6.7 ± 0.0	0.9	29.86 ± 0.05 (+ 2.19)	2.4 ± 0.1 (-1.7)
	NADPH	7.7 ± 0.1			
K270G	NADH	1.0 ± 0.0	2.0	32.40 ± 0.08 (+ 4.73)	35.5 ± 0.4 (+ 31.4)
	NADPH	0.5 ± 0.0			
R276H	NADH	9.3 ± 0.1	23.1	36.43 ± 0.06 (+ 8.76)	92.0 ± 3.0 (+ 87.9)
	NADPH	0.4 ± 0.0			
K270R/R276H	NADH	3.5 ± 0.0	12.3	37.68 ± 0.05 (+ 10.01)	79.9 ± 1.3 (+ 75.8)
	NADPH	0.3 ± 0.0			
N272D/R276H	NADH	9.3 ± 0.5	3.9	34.00 ± 0.06 (+ 6.33)	52.8 ± 1.3 (+ 48.7)
	NADPH	2.4 ± 0.0			
K270R/N272D	NADH	8.6 ± 0.2	2.9	32.75 ± 0.05 (+ 5.08)	34.9 ± 1.9 (+ 30.8)
	NADPH	3.0 ± 0.0			
K270R/N272D/R276H	NADH	7.4 ± 0.0	3.4	34.86 ± 0.05 (+ 7.19)	53.6 ± 4.6 (+ 49.5)
	NADPH	2.2 ± 0.0			

*Under standard assay conditions (see Methods).

†Specific activity with NADH/specific activity with NADPH.

‡CD measurement. The difference from WT in *T*_m is shown in parentheses.

§Remaining activity after incubation at 30 °C for 10 min. Relative value expressed as a percentage of the controls without heat treatment (100%). For WT and K270R, NADPH was used as a coenzyme, while NADH was used for other enzymes. The difference from WT in remaining activity is shown in parentheses.

2005), the significant acquisition of NADH preference is not unexpected.

Kinetic constants determined for coenzymes of WT and mutant PsXRs (R276H, K270R/N272D, K270R/R276H and K270R/N272D/R276H) are shown in Table 3. NADPH preference in the recombinant WT enzyme was mainly due to much higher affinity (lower K_m) for NADPH than NADH. A high ratio of NADH/NADPH in k_{cat}/K_m was found for the R276H and K270R/N272D mutants: 2.62 and 7.29, respectively. Their kinetic constants for NADH were almost same as those of WT, although the K270R/N272D mutant showed an approximately 5-fold higher K_m value with NADH. On the other hand, the K_{cat}/K_m values of the R276H and K270R/N272D mutants with NADPH decreased 28- and 370-fold compared with WT; this was mainly due to a 40-fold decrease in K_{cat} and 1120-fold increase in K_m , respectively. When the R276H mutation was introduced into the K270R/N272D mutant, no further improvement of NADH preference was seen. This may be due to an antagonistic effect by the introduction of two arginine residues, which may also account for a similar result found in the corresponding triple mutant of CtxR, K274R/N276D/R280H (Petschacher & Nidetzky, 2005; Petschacher *et al.*, 2005): the effect of steric hindrance on the 2'-phosphate group of NADP⁺, generated by the introduction of the side chain of Arg²⁷⁰, could be strongly reduced when the side chain of Arg²⁷⁶ is substituted by the smaller side chain of a histidine residue. By contrast, in our previous study using site-directed mutagenesis of NAD⁺-dependent PsXDH for reversal of coenzyme specificity, novel NADP⁺-dependent activity in the best mutant, D207A/I208R/F209S/N211R, increased additionally via single, double and triple mutations (Watanabe *et al.*, 2005). When compared with the WT, the R276H mutant showed similar K_m values with xylose in the presence of NADH or NADPH (Table 3). On the other hand, the

K270R/N272D mutant showed a 7.4-fold higher K_m value for xylose in the presence of NADPH.

Effect of the modification of coenzyme specificity on thermostability

The thermostability of WT and mutant PsXRs was estimated by monitoring the change in the CD signal at 220 nm at the thermal unfolding transition temperature (T_m). Overall, modification of coenzyme specificity towards NADH led to stabilization of PsXR, and the greatest thermostabilization was found in the R276H single mutant (increase of 8.8 °C in T_m) (Table 2). Furthermore, this mutation is also likely to stabilize when present with other single and double mutations. Similar results were obtained by heat treatment of the enzyme: the activity of the WT enzyme decreased to only 4.1 % after incubation for 10 min at 30 °C, whereas mutants containing the R276H mutation maintained 50–90 % of activity after the same treatment (Table 2). These results were unexpected because, as observed in several other dehydrogenases/reductases (Chen *et al.*, 1991; Grimshaw *et al.*, 1992; Steen *et al.*, 2002; Serov *et al.*, 2002), the modification(s) of coenzyme specificity leads to a significant loss of thermostability. In fact, most NADP⁺-dependent mutants of PsXDH are more thermolabile than WT (Watanabe *et al.*, 2005).

Expression of PsXR mutants in *S. cerevisiae*

To estimate the effect of modifying coenzyme specificity in PsXR on ethanol fermentation from xylose, the respective *XYL1* genes encoding the WT, R276H and K270R/N272D enzymes were each expressed in *S. cerevisiae* together with the *XYL2* gene encoding WT PsXDH. Initially, both *XYL1* and *XYL2* genes were ligated to a yeast expression vector

Table 3. Kinetic parameters of WT and mutant PsXR

Enzyme	Coenzyme	K_m^* (μM)	k_{cat}^* (min ⁻¹)	k_{cat}/K_m (μM ⁻¹ min ⁻¹)	NADH/ NADPH ratio in $k_{cat}/K_m^\dagger$	K_m (xylose) (mM)
WT	NADH	30.5 ± 0.7	415 ± 8	13.6 ± 0.1	0.05	31.5 ± 0.1
	NADPH	2.5 ± 0.1	630 ± 1	260 ± 11		47.0 ± 0.1
R276H	NADH	17.0 ± 2.1	408 ± 15	24.4 ± 3.9	2.62	45.5 ± 0.2
	NADPH	1.7 ± 0.1	16.0 ± 0.2	9.3 ± 0.4		53.2 ± 0.5
K270R/N272D	NADH	138 ± 17	706 ± 75	5.1 ± 0.1	7.29	67.5 ± 0.4
	NADPH	2810 ± 480	1850 ± 310	0.7 ± 0.0		350 ± 20
K270R/R276H	NADH	66 ± 5	58 ± 4	0.9 ± 0.1	0.30	ND
	NADPH	2.7 ± 0.2	8.4 ± 0.2	3.1 ± 0.3		ND
K270R/N272D/R276H	NADH	41.8 ± 9.1	303 ± 46	7.4 ± 0.5	1.01	ND
	NADPH	10.2 ± 1.2	74 ± 3.9	7.3 ± 0.5		ND

ND, Not determined.

*Six different concentrations of NAD(P)H between 50 and 300 μM were used. The xylose concentration was 133 mM.

†(k_{cat}/K_m with NADH)/(k_{cat}/K_m with NADPH).

(pPGK) with the constitutive *PGK* promoter (Kang *et al.*, 1990). In the case of the *XYL1* gene, the *PsXR* coding region with *PGK* promoter and terminator was excised from the plasmid and reinserted into the YEpm4 vector (Nikawa *et al.*, 1987). The resultant plasmids, pPGK containing the *XYL2* gene and YEpm4 containing the *XYL1* gene, were transformed into *S. cerevisiae*. XR and XDH activities in cell-free extracts prepared from yeast cells grown in minimal medium were measured spectrophotometrically (Fig. 1). Overall, significant NADH preference in reductase activity for xylose was found in cell-free extract prepared from recombinant *S. cerevisiae* cells expressing the NADH-preferring XR mutants, R276H or K270R/N272D. In contrast with the comparison using purified enzyme, the novel NADH-dependent activity in these mutants was higher than the NADPH-dependent activity in WT; this may be due to their thermostabilization (Table 2). XDH activities in these recombinant yeasts were observed at almost the same levels (Fig. 1). Little NADPH-preferring reduction of xylose and NAD⁺-dependent oxidization of xylitol were observed in the Y-Vector control, probably due to endogenous XR (YHR104W) (Träff *et al.*, 2002) and XDH (YLR070C) (Richard *et al.*, 1999).

Batch fermentation

Ethanol production and xylitol excretion of recombinant yeasts co-expressing NADH-preferring XR mutant (R276H or K270R/N272D) and XDH (WT) were preliminarily estimated by fermentation using shake-flask culture. One fermentation set (100 ml) was started with 5 g glucose l⁻¹

Fig. 1. Activities of XR (top) and XDH (bottom) in recombinant yeast strains. Dark-grey and light-grey bars indicate NADH- and NADPH-dependent XR activities, respectively. NAD⁺ was used as a coenzyme for XDH activity. Values are means $\pm$ SD, $n=3$.

plus 15 g xylose l⁻¹ (Fig. 2a). There was no significant difference between these recombinant yeast strains in the rate of cell growth (data not shown). In all these recombinant yeast strains, the glucose was initially consumed within 35 h, and 37–46% of the xylose was fermented in 72 h. Y-WT produced ethanol at 2.15 g l⁻¹ and excreted a relatively higher amount of xylitol (1.45 g l⁻¹). Y-R276H produced the highest amount of ethanol at 2.30 g l⁻¹ and excreted the lowest amount of xylitol at 0.97 g l⁻¹. It is noteworthy that a more positive effect, particularly on xylitol excretion, was observed in Y-R276H rather than Y-K270R/N272D; this may be due to different adaptation for NADH of these enzymes *in vitro* as described above.

Fig. 2. Ethanol fermentation by recombinant *S. cerevisiae* in minimal medium supplemented with glucose (5 g l⁻¹) and xylose (15 g l⁻¹). Values are the means of the results of duplicate experiments which differed by less than 10%. (a) Ethanol (top) and xylitol (bottom) production profiles during batch fermentation in a shake flask by Y-WT (white), R276H (black) and K270R/N272D (grey). (b) Batch fermentation in a bioreactor under the same conditions as in (a) by Y-WT (white symbols) and Y-R276H (black symbols). Concentrations of glucose (squares), xylose (circles), xylitol (triangles) and ethanol (diamonds) are shown.

The fermentation by Y-R276H was further investigated using a bioreactor under the same conditions as for the shake-flask culture and compared with that by Y-WT (Fig. 2b). Y-R276H produced ethanol at 5.94 g l⁻¹ with a yield of 0.43 g ethanol per g total consumed sugars, which reached 84 % of the theoretical yield. Of the xylose, 59 % was consumed in 72 h and xylitol excretion was 1.29 g l⁻¹. Compared with Y-WT, a 20 % increase in ethanol production and a 52 % decrease in xylitol excretion were observed in fermentation by Y-R276H. These results indicated that this protein-engineering approach is useful for the fermentation of xylose to ethanol using *S. cerevisiae*.

Intracellular coenzyme levels

We measured the intracellular concentrations of NADH, NAD⁺, NADPH, and NADP⁺ in cells of recombinant strains in the late exponential growth phase (Table 4). No significant differences in the concentrations of the four coenzymes were observed between samples in early, middle and late exponential growth phases (data not shown). Intracellular redox states were estimated by the ratios of NADH/NAD⁺ and NADPH/NADP⁺ (referred to as $R_{\text{NAD(H)}}$ and $R_{\text{NADP(H)}}$, respectively). When compared with Y-Vector, the $R_{\text{NADP(H)}}$ of Y-WT was threefold higher, while $R_{\text{NAD(H)}}$ was twofold less. It is noteworthy that Y-R276H has $R_{\text{NAD(H)}}$ and $R_{\text{NADP(H)}}$ values more similar to Y-Vector than does Y-WT, indicating that the NADH-preferring mutation in *PsXR* led to maintenance of redox balance in yeast cells.

DISCUSSION

Reversal of coenzyme specificity of *PsXR*

Since *PsXR* shows high sequence similarity to *CtXR* (~77 %), the kinetic and crystallographic characterization of NADH-preferring *CtXR* is useful for estimating the effects of K270R/N272D and R276H mutations, which gave the most significant reversal of coenzyme specificity in this study. The K274R/N276D *CtXR* mutant corresponds to the K270R/N272D *PsXR* mutant and shows the best ratio of NADPH/NADH in $k_{\text{cat}}/K_{\text{m}}$ (0.2), compared with that of WT (33) (Petschacher *et al.*, 2005). This improvement is

mainly due to a dramatic decrease of binding affinity to NADPH, compared with that in WT. A similar tendency is also found in the K270R/N272D mutant *PsXR* (Table 3). Crystallographic studies revealed that both the native enzyme and K274R/N276D *CtXR* mutant interact with NAD⁺ (probably also with NADH) in a very similar manner (Kavanagh *et al.*, 2003; Petschacher *et al.*, 2005): the responsibility for the NADH preference in these mutations of *CtXR* and *PsXR* remains unclear. However, XR from *C. parapsilosis* (Lee *et al.*, 2003) natively possesses arginine at a position equivalent to Lys²⁷⁴ and Lys²⁷⁰ in *C. tenuis* and *P. stipitis* XRs, respectively, indicating that this Lys→Arg substitution may at least be physiological (Table 1). The R280H mutation in *CtXR*, corresponding to the R276H mutation in *PsXR*, leads to an eightfold decrease in the ratio of NADPH/NADH in $k_{\text{cat}}/K_{\text{m}}$, although the enzyme still shows a fourfold NADPH preference (Petschacher & Nidetzky, 2005). Since the side-chain of Arg²⁸⁰ interacts with the 2'-position of adenosine ribose more strongly in the *CtXR*-NADP⁺ complex than in the *CtXR*-NAD⁺ complex (Kavanagh *et al.*, 2003, 2002), the R280H mutation, which removes the contact mediated by the guanidinium group of arginine, may effect an NADPH-linked rather than NADH-linked enzyme reaction. In fact, when compared with WT, the kinetic parameters with NADH of the R276H mutant of *PsXR* are similar, while the k_{cat} value for NADPH decreases dramatically, by 40-fold (Table 3). Overall, it is likely that the K270R/N272D and R276H mutants of *PsXR* are responsible for NADPH preference in a similar manner to the K274R/N276D and R280H mutants of *CtXR*.

Thermostabilization of *PsXR*

Most of the modifications of coenzyme specificity of *PsXR* led to simultaneous stabilization of the mutant enzymes (Table 2). In particular, the R276H single mutant maintains almost full activity after heat treatment at 30 °C for 10 min, while the WT enzyme is inactivated completely under the same conditions. This result was unexpected because most mutations tend to affect the stability of the protein neutrally or negatively. Since XR is composed of two identical subunits, thermostabilization may potentially be achieved by strengthening intermolecular and/or intersubunit interaction(s). In the crystal

Table 4. Intracellular concentration of coenzymes in recombinant yeast cells

Strain	Intracellular concn [μmol (g dry wt of biomass) ⁻¹]				$R_{\text{NAD(H)}}^*$	$R_{\text{NADP(H)}}^\dagger$
	NAD ⁺	NADH	NADP ⁺	NADPH		
Y-Vector	6.58 ± 0.11	3.19 ± 0.29	3.41 ± 0.18	5.24 ± 0.01	0.49	1.54
Y-WT	8.26 ± 0.16	2.69 ± 0.08	1.68 ± 0.04	8.24 ± 0.14	0.33	4.92
Y-R276H	7.39 ± 0.28	4.06 ± 0.04	2.02 ± 0.01	7.15 ± 0.09	0.55	3.54

*Ratio of NADH/NAD⁺.

†Ratio of NADPH/NADP⁺.

structure of the CtxR apo-form, a side-chain of the equivalent arginine to Arg²⁷⁶ in PsXR forms no interaction with another amino acid residue, although there is a hydrogen bond with –NH of Arg²⁷⁶ C α and –CO of Leu²⁷⁷ C α (Kavanagh *et al.*, 2002). This arginine is contained within a very flexible coenzyme-binding loop, which is located separately from the subunit–subunit interface in dimeric CtxR. On the other hand, Klimacek *et al.* (2003) reported that the R180A mutation of CtxR, which is located within the subunit–subunit interface, leads to destabilization of the protein without changing the dimeric form. Interestingly, the NADH-dependent activity of this mutant is one-third of the WT level, mainly due to a 2.5-fold decrease of k_{cat} for NADH, although significant NADPH preference was still maintained as well as WT. These results suggest that modifications at the subunit–subunit interface have long-range effects on enzyme catalytic function(s) and that the R276H mutant of PsXR in this study is stabilized by such a mechanism(s) because this mutation leads to the modification of coenzyme specificity toward NADH (but not NADPH) (Tables 2 and 3).

Effect of NADH-preferring XR mutant on ethanol fermentation

The typical unfavourable excretion of xylitol in ethanol fermentation from xylose using recombinant *S. cerevisiae* has been ascribed to the difference in coenzyme utilization in NADPH-preferring XR and strict NAD⁺-dependent XDH reactions (Jeffries & Jin, 2004). Therefore, many researchers have targeted the production and consumption of NADPH/NADP⁺ and NADH/NAD⁺ using recombinant yeast co-expressing WT enzymes, PsXR and PsXDH: disruption of the *ZWF1* gene encoding glucose-6-phosphate dehydrogenase (Jeppsson *et al.*, 2002); overexpression of a cytoplasmic transhydrogenase, catalysing the conversion of NAD⁺ and NADPH into NADH and NADP⁺, respectively (Nissen *et al.*, 2001); overproduction of NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (Verho *et al.*, 2003); and disruption of the *GDH1* gene encoding NADPH-dependent glutamate dehydrogenase in ammonium assimilation (Roca *et al.*, 2003). These metabolic modifications generally lead to reduction of the intracellular NADPH pool to improve ethanol production and xylitol excretion.

Alternatively, for the same purpose, there are few reports on the protein-engineering approach to XR and XDH, as described in the Introduction; therefore, one of the most significant insights in this study is the relationship between the modification of coenzyme specificity of xylose-metabolizing enzyme (*in vitro*) and ethanol fermentation from xylose using the mutated enzyme(s) (*in vivo*). Jeppsson *et al.* (2006) estimated the effect on xylose fermentation of a PsXR K270M mutant with a higher K_m for NADH. In the recombinant *S. cerevisiae* carrying the mutated PsXR (referred to as Y-K270M), xylitol and glycerol yields decreased 24 % and 50 %, respectively, while

only 11 % increase in ethanol yield was observed. On the other hand, in *S. cerevisiae* Y-R276H, improvement of xylitol and ethanol yields was much higher than that in Y-K270M (32 % and 58 %, respectively), while no significant change was found in glycerol yield (Supplementary Table S2). The main reason for this difference must be different adaptation for NADPH between the R276H and K270M PsXR mutants: the former shows 26-fold lower k_{cat} for NADPH than NADH in the R276H mutant (Table 3), while the latter shows 17-fold higher K_m for NADH and unchanged K_m for NADPH (Kostrzynska *et al.*, 1998), which is analogous to the K270R/N272D mutant. In fact, Y-K270R/N272D has no advantage over Y-R276H in xylose fermentation (Fig. 2a). The intracellular concentration of NADPH decreases by 13 % in Y-R276H, while that of NADH increases by 51 %, compared with that of Y-WT (Table 4). Similar tendencies are observed in Y-K270M (Jeppsson *et al.*, 2006). These results indicate that the two strains harbouring the NADH-preferring XR mutant utilize a larger fraction of NADH for the reduction of xylose.

As described in the Introduction, an alternative strategy for maintaining intracellular redox balance is to utilize the xylose-fermenting pathway via XI. Recently, it was first reported that XI from the fungus *Piromyces* sp. strain E3 resulted in significant activity in *S. cerevisiae* cells (Kuyper *et al.*, 2003). However, comparison of xylose-fermenting ability by XR-XDH- and XI-carrying recombinant *S. cerevisiae* strains revealed that the XR-XDH xylose utilization pathway is much better than the XI pathway (Karhumaa *et al.*, 2007). In this study, we introduced only exotic *XYL1* and *XYL2* genes into *S. cerevisiae*. On the other hand, in addition to the metabolic engineering described above, the introduction and overexpression of other endogenous genes, including xylulokinase (*XK*), transketolase (*TKL1*), transaldolase (*TAL1*) and several hexose-transporter (*HXT1*–*7*) genes, have been attempted to enhance the pentose-phosphate pathway and/or xylose uptake (Jeffries & Jin, 2004). Combined approaches of these strategies and our strategy using the protein-engineered enzyme should be considered to achieve more effective ethanol production from xylose by recombinant *S. cerevisiae*.

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