Biochemical and Biophysical Research Communications 404 (2011) 634–637

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel strictly NADPH-dependent *Pichia stipitis* xylose reductase constructed by site-directed mutagenesis

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ARTICLE INFO

Article history:
Received 26 November 2010
Available online 10 December 2010

Keywords:
Coenzyme specificity
Xylose reductase
Site-directed mutagenesis

ABSTRACT

Xylose reductase (XR) and xylitol dehydrogenase (XDH) are the key enzymes for xylose fermentation and have been widely used for construction of a recombinant xylose fermenting yeast. The effective recycling of cofactors between XR and XDH has been thought to be important to achieve effective xylose fermentation. Efforts to alter the coenzyme specificity of XR and HDX by site-directed mutagenesis have been widely made for improvement of efficiency of xylose fermentation. We previously succeeded by protein engineering to improve ethanol production by reversing XDH dependency from NAD^+ to $NADP^+$. In this study, we applied protein engineering to construct a novel strictly NADPH-dependent XR from *Pichia stipitis* by site-directed mutagenesis, in order to recycle NADPH between XR and XDH effectively. One double mutant, E223A/S271A showing strict NADPH dependency with 106% activity of wild-type was generated. A second double mutant, E223D/S271A, showed a 1.27-fold increased activity compared to the wild-type XR with NADPH and almost negligible activity with NADH.

1. Introduction

Xylose is the second most abundant pentose sugar constituting the lignocellulosic renewable biomass after glucose, and its complete fermentation is economically valuable for producing biofuel from lignocellulosic biomass [6]. Recombinant *Saccharomyces cerevisiae* can ferment xylose through a fungal pathway involving two heterologous oxidoreductase genes. In this pathway, *Pichia stipitis* xylose reductase (PsXR) (XR; EC 1.1.1.21) [18], which prefers NADPH, reduces xylose to xylitol followed by *P. stipitis* xylitol dehydrogenase (PsXDH), which exclusively requires NAD^+ (XDH; EC 1.1.1.9) [17], oxidizes xylitol into xylulose. *S. cerevisiae* xylulokinase (XK) (EC 2.7.1.17) naturally phosphorylates xylulose to xylulose-5-phosphate, which is then metabolized by the glycolytic pathway via the pentose phosphate pathway [7]. XK overexpression improves the efficiency of xylose fermentation [4,5,15]. Although this fungal pathway is highly expressed in *S. cerevisiae*, the efficiency of ethanol production is somewhat obstructed by the unfavorable accumulation of xylitol due to the imbalance of coenzyme specificities between XR and XDH [6].

Xylose reductase is a member of the aldo–keto reductase (AKR) superfamily which is made up of 14 different families and approximately 120 members with a majority of dual cofactor type enzymes [8]. *Candida tenuis* XR (CtXR) is one of these enzymes. Its crystal structure has been determined at different levels of resolution and its binding sites with NAD(P)H were also determined [10,12,25]. Although only little structural information of PsXR is available, it has about 76% homology with the CtXR. This high percentage of similarity should provide some clues for manipulation of PsXR [13].

Protein engineering has been widely used to alter the coenzyme specificity of XR and XDH. Since PsXDH accepts only NAD^+, many researchers reversed the preference of XR to NADH in order to achieve NAD^+/NADH cofactor recycling [1,13,16,20]. On the other hand, we have been working on converting cofactor usage of XDH to $NADP^+$ from NAD^+ [21]. We previously succeeded to improve the fermentation process and ethanol production by using these XDH mutants [23]. In this study, site-directed mutagenesis of PsXR was performed to construct a strictly NADPH-dependent XR, expecting decreasing or preventing xylitol accumulation and subsequently improving ethanol production.

2. Materials and methods

2.1. Cloning of the P. stipitis xylose reductase gene and site-directed mutagenesis

A plasmid, named pHis (WT) harboring the His-tagged wild-type (WT) PsXR gene was constructed as described previously [20]. All XR mutations were introduced by site-directed mutagenesis, using the single round PCR method with *PfuTurbo* DNA

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doi:10.1016/j.bbrc.2010.12.028

polymerase (Stratagene) and the PCR Thermal Cycler PERSONAL (TaKaRa, Otsu, Japan). The codons used for mutations introduced in this study were as follows: E223A (GAA → GCA), E223D (GAA → GAC), and S271A (TCC → GCC). The PCR products were subjected to DpnI restriction enzyme treatment in order to digest the parent DNA strands to prevent transformation of the template plasmid. Only nicked circular mutagenic strands were transformed into *Escherichia coli* DH5α. Electroporation method was used to transform plasmids and the mutations were confirmed by DNA sequencing using Applied Biosystems 3031 genetic analyzer and ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing kit.

2.2. Overexpression and purification of $(His)_6$-tagged enzymes

P. stipitis xylose reductase wild-type and mutated genes were expressed in *E. coli* DH5α and purified as described previously [20]. Purified enzymes were confirmed on 10% acrylamide SDS–PAGE. Protein concentrations were determined using the Bio-RAD Quick Start Bradford 1× dye reagent (Bio-Rad Laboratories, CA, USA) by measuring the absorbance at 595 nm with γ-globin as a standard.

2.3. Enzyme assays and kinetic parameters

Enzyme activities were measured spectrophotometrically as described previously [20] with modification in xylose concentration to 400 mM. The kinetic parameters were calculated by Line Weaver–Burk plots.

3. Result and discussion

3.1. Speculation and prediction of NAD(P)H binding sites

Crystallographic analyzes of many AKRs have revealed that they share a common $(\alpha/\beta)_8$ barrel fold, with a highly conserved coenzyme binding pocket at the C-terminus. 90.9% of the residues are located in the core area and 9.1% are in the allowed regions [11]. The nicotinamide ring of NAD(P)H is resides in the core of the barrel. Residues Glu^{227} and Asn^{276} in CtXR, which equal to Glu^{223} and Asn^{272} in PsXR, primarily mediate the interactions with the adenosine ribose 2′- and 3′-hydroxy groups. As shown in Fig. 1, Glu^{223} represents the essential part NADH binding where contacts by bidentate hydrogen bond with both of the hydroxy groups. Similar interactions have been seen in many other NADH-binding protein structures [2,3]. However, The structurally equivalent residues Asp^{216} and Val^{264} in aldose reductase in human (AR) are unable to fulfill the equivalent roles, Asp^{216} is required for high affinity binding of NADPH by forming two salt linkages with Lys^{21} and Lys^{262} and fastening the loop over the co-substrate [24].

Glu^{227} and Lys^{274} in CtXR make water-mediated interactions each other and with the 3′-hydroxy group in the case of $NADP^+$-bound structures. In the absence of a negatively charged phosphate, Glu^{227} side chain is able to rotate into a favourable conformation to accept a 2.64 Å hydrogen bond contact with the 2′-hydroxy group and a 2.65 Å hydrogen bond with the 3′-hydroxy group when NAD^+ is bound. The root mean square deviations of the Cα values between NAD^+- and $NADP^+$-bound models was calculated in CtXR. The largest conformational change is seen in residues 274–280, which corresponding to 270–276 in PsXR, and then residues 225–229, which corresponding to 221–225 in PsXR, a short helical region that appears at the end of β7. The largest main-chain shift is seen in Ser^{275}, which corresponding to Ser^{271} in PsXR, moves 2.0 Å in response to the miss contact of the phosphate group of NADPH [10]. Furthermore, Glu^{223} of PsXR was subjected to a mutation trial and the result revealed that alteration of this site might further inhibit NADH binding [13]. In addition, from the 3D structure model of PsXR, it was reported that Glu^{223} and Phe^{236} can form 3 and 2 hydrogen bonds with NAD^+, respectively [19].

Considering the property as described above, the mutations were designed based on sequence alignment of some strictly NADPH dependent analogous enzymes in the AKR family, such as AR, as shown in Table 1, where glutamic acid 223 was substituted by aspartic acid. Both glutamic and aspartic acid are acidic side chain and fully ionized at neutral pH and able to engage in hydrogen bonds, which is a necessary component for a high affinity xylose binding site [9]. Alanine is a nonpolar side chain that does not bind or give off protons, or participate in hydrogen or ionic bonds. Alanine can be worked as oily or lipid-like that promotes hydrophobic interactions. Accordingly, we apply aspartic acid and alanine to mutation trials instead of PsXR glutamic acid 223.

3.2. Strictly NADPH dependency on Glu^{223} mutants

We applied Glu^{223} residue for mutation trails in order to delete NADH dependency. Although this residue is also shared in NADPH binding, some reports reveal that it contributes more to the affinity of NADH, where it plays a role in the binding site by binding two hydrogen bonds with 2′ and 3′ hydroxy groups of the adenosine ribose. In addition to changes in hydrogen-bonding of the adenosine, the ribose unmistakably adopts the 3′-*endo* conformation rather than the 2′-*endo* conformation seen in the $NADP^+$-bound form

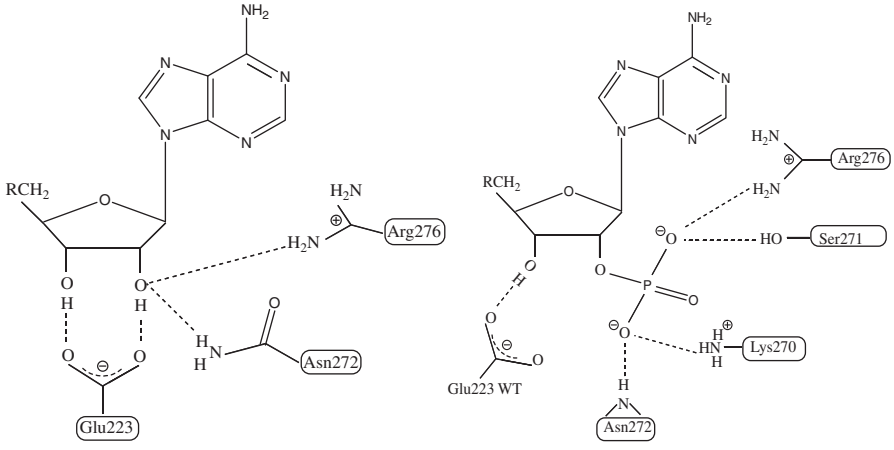

Fig. 1. Schematic diagrams showing the predicted interactions of wild-type PsXR; Left-hand panel: adenosine 2′- and 3′-hydroxy groups in the complex with NAD^+ and Right-hand panel: adenosine 2′- and 3′-hydroxy groups in the complex with $NADP^+$ based on the coenzyme binding sites in CtXR [10].