Complete Reversal of Coenzyme Specificity of Xylitol Dehydrogenase and Increase of Thermostability by the Introduction of Structural Zinc*

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Pichia stipitis NAD⁺-dependent xylitol dehydrogenase (XDH), a medium-chain dehydrogenase/reductase, is one of the key enzymes in ethanol fermentation from xylose. For the construction of an efficient biomass-ethanol conversion system, we focused on the two areas of XDH, 1) change of coenzyme specificity from NAD⁺ to NADP⁺ and 2) thermostabilization by introducing an additional zinc site. Site-directed mutagenesis was used to examine the roles of Asn²⁰⁹, Ile²⁰⁹, Phe²⁰⁹, and Asn²¹¹ in the discrimination between NAD⁺ and NADP⁺. Single mutants (D207A, I208R, F209S, and N211R) showed more than 4500-fold higher values in k_{cat}/K_m with NADP⁺ compared with the wild type but retained substantial activity with NAD⁺. The double mutants (D207A/I208R and D207A/F209S) improved by 3 orders of magnitude in k_{cat}/K_m with NADP⁺, but they still preferred NAD⁺ to NADP⁺. The triple mutant (D207A/I208R/F209S) and quadruple mutant (D207A/I208R/F209S/N211R) showed more than 4500-fold higher values in k_{cat}/K_m with NADP⁺ than the wild-type enzyme, reaching values comparable with k_{cat}/K_m with NAD⁺ of the wild-type enzyme. Because most NAD⁺-dependent XDH mutants constructed in this study decreased the thermostability compared with the wild-type enzyme, we attempted to improve the thermostability of XDH mutants by the introduction of an additional zinc atom. The introduction of three cysteine residues in wild-type XDH gave an additional zinc-binding site and improved the thermostability. The introduction of this mutation in D207A/I208R/F209S and D207A/I208R/F209S/N211R mutants increased the thermostability and further increased the catalytic activity with NADP⁺.

Xylose is one of the major components of hemicellulose, the second most abundant carbohydrate polymer in nature. Efficient utilization of xylose is required to develop economically viable processes for producing biofuels such as ethanol from biomass (for recent reviews, see Refs. 1–4). Yeasts have long been used for the production of alcoholic beverages such as wine, beer, and Japanese sake. In particular, Saccharomyces cerevisiae has been used widely because of the ability to produce high concentrations of ethanol and high inherent ethanol tolerance. The native strains, however, cannot ferment xylose as a carbon source. The major strategy for the generation of xylose-fermenting S. cerevisiae is to introduce genes involved in xylose metabolism from other organisms (Scheme 1). In xylose-fermenting fungi such as Pichia stipitis, xylose is converted into xylulose by the sequential action of two oxidoreductases. First, xylose reductase (alditol:NAD⁺ 1-oxidoreductase, EC 1.1.1.21) catalyzes reduction of the C1 carbonyl group of xylose, yielding xylitol as the product. Xylitol is then oxidized by xylitol dehydrogenase (XDH; EC 1.1.1.9) to give xylulose. S. cerevisiae transformed with these two genes from P. stipitis could ferment xylose to ethanol. There is another problem; the excretion of xylitol occurs unless a co-metabolizable carbon source such as glucose is added. This is probably caused by several combined factors. In particular, intercellular redox imbalance due to a different coenzyme specificity of xylose reductase (with NADPH) and XDH (with NAD⁺) has been thought to be one of the main factors (2). The generation of an NAD⁺-dependent XDH by protein engineering would avoid this problem.

XDH is a medium-chain dehydrogenase/reductase (MDR), which constitutes a large enzyme superfamily consisting of about 1000 members (for reviews, see Refs. 5 and 6). The superfamily is classified into eight subfamilies based on amino acid sequence alignment and the structural similarity of substrates. XDH belongs to the polyol dehydrogenase (PDH) subfamily that contains sorbitol dehydrogenase (SDH; EC 1.1.1.14) and L-arabinitol 4-dehydrogenase from several organisms. Most PDHs catalyze strict NAD⁺(H)-dependent interconversion between alcohols and their corresponding ketones or aldehydes. The alcohol dehydrogenase (ADH; EC 1.1.1.1) subfamily, whose enzymes have been studied most extensively, are either NAD⁺(H)- or NADP⁺(H)-dependent enzymes. A number of crystallographic analyses of MRs, such as ADHs (7–13) and SDHs (14, 15), have revealed that their protein folds are very similar to that of XDH from P. stipitis; MDR, medium-chain dehydrogenase/reductase; PDH, polyol dehydrogenase; ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; WT, wild-type enzyme; AR, D207A/I208R mutant; ARSdR, D207A/I208R/F209S/N211R mutant; C4, S96C/S99C/Y102C mutant; C4/ARS, mutant containing C4 and ARSdR mutations; C4/ARSdR, mutant containing C4 and ARSdR mutations; CD, circular dichroism; T_m, thermal unfolding transition temperature; M. morganii, Morganella morganii; S. pombe, Schizosaccharomyces pombe; E. japonica, Eriobotrya japonica; H. jecorina, Hypocrea jecorina.

The abbreviations used are: XDH, xylitol dehydrogenase; PoXDH, XDH from P. stipitis; MDR, medium-chain dehydrogenase/reductase; PDH, polyol dehydrogenase; ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; WT, wild-type enzyme; AR, D207A/I208R mutant; ARSdR, D207A/I208R/F209S/N211R mutant; C4, S96C/S99C/Y102C mutant; C4/ARS, mutant containing C4 and ARSdR mutations; C4/ARSdR, mutant containing C4 and ARSdR mutations; CD, circular dichroism; T_m, thermal unfolding transition temperature; M. morganii, Morganella morganii; S. pombe, Schizosaccharomyces pombe; E. japonica, Eriobotrya japonica; H. jecorina, Hypocrea jecorina.

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1 The abbreviations used are: XDH, xylitol dehydrogenase; PoXDH, XDH from P. stipitis; MDR, medium-chain dehydrogenase/reductase; PDH, polyol dehydrogenase; ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; WT, wild-type enzyme; AR, D207A/I208R mutant; ARSdR, D207A/I208R/F209S/N211R mutant; C4, S96C/S99C/Y102C mutant; C4/ARS, mutant containing C4 and ARSdR mutations; C4/ARSdR, mutant containing C4 and ARSdR mutations; CD, circular dichroism; T_m, thermal unfolding transition temperature; M. morganii, Morganella morganii; S. pombe, Schizosaccharomyces pombe; E. japonica, Eriobotrya japonica; H. jecorina, Hypocrea jecorina.
similar, and their coenzyme binding mode is followed a classical "Rossmann fold." Many MDRs have one zinc atom at the catalytic site, which is necessary for enzyme activity. Some possess an additional second zinc atom, which is known as a structural zinc atom (see Fig. 1). Although the role of the structural zinc atom has been proposed to maintain the quaternary structure of the protein, its role has not yet been clarified (16).

A large body of literature has described the alteration of nicotinamide coenzyme specificity (17–38). Because NAD$^+$ (H) only differs from NADP$^+$ (H) in the phosphate group esterified at 2'-position of adenosine ribose, a limited number of amino acid residues interacting with these portions are the first candidates for protein engineering using site-directed mutagenesis. Most of these studies are Rossmann-fold type oxidoreductases (17–23, 25, 28, 30–32, 34, 36–38), and "landmark" amino acid residues have already been proposed for the discrimination between NAD$^+$ (H) and NADP$^+$ (H) (39). Generally, subunit structures in the MDR superfamily are composed of two domains, namely a coenzyme binding domain within the intermediate segment and a catalytic domain consisting of N- and C-terminal segments (Fig. 1). The coenzyme binding domains possess a similar β-α-β motif centered around a highly conserved Gly-X-Gly-X-Gly sequence (where X is any amino acid) that constitutes a tight turn at the end of the first β-strand and marks the beginning of the succeeding α-helix (Table I). The ability of dehydrogenases to discriminate between NAD$^+$ and NADP$^+$ lies in the amino acid sequence of this β-α-β motif. The primary determinant of NAD$^+$ specificity is the presence of an aspartate residue, which forms double-hydrogen bonds to both the 2'- and 3'-hydroxyl groups in the ribosyl moiety of NAD$^+$ and induces negative electrostatic potential to the binding site. Commonly, this residue in NADP$^+$-dependent dehydrogenases is replaced by a smaller and uncharged residue such as Gly, Ala, and Ser, accompanied by the concurrent presence of an arginine residue that forms a positive binding pocket for the 2'-phosphate group of NADP$^+$. Despite the prevalence of this information, the full reversal of coenzyme specificity, in terms of having a mutant enzyme catalytically efficient as the wild type, has rarely been achieved. Bemisia argentifolii (whitefly) SDH is the only enzyme showing a strict preference to NADP$^+$ (H) among all characterized PDHs (14, 40). Structural information regarding coenzyme recognition may be very useful to generate NADP$^+$-dependent XDH.

In this study we used NAD$^+$-dependent XDH from P. stipitis (PsXDH) as a target protein (41, 42) and completely reversed the coenzyme specificity to generate a novel NADP$^+$-dependent XDH mutant by multiple site-directed mutagenesis. Because the mutations produced decreased thermostability compared with the wild-type enzyme, we focused on a structural zinc atom that is not contained in native PsXDH (see Fig. 1). Introduction of specific cysteine ligands into the enzyme gave an additional zinc-binding site and improved thermostability. Furthermore, the catalytic efficiency of NADP$^+$-dependent PsXDH was also improved by the introduction of an additional zinc atom.

**EXPERIMENTAL PROCEDURES**

Cloning of the PsXDH Gene—P. stipitis (Yamazakzyma stipitis NBRC 1687) was purchased from the National Institute of Technology and Evaluation (Chiba, Japan) and cultured at 25 °C in YM medium (10 g of glucose, 5 g of peptone, 3 g of yeast extract, and 3 g of malt extract per liter (pH 5.6)). The isolation of P. stipitis genomic DNA was carried out according to the methods of Kotter et al. (42). Based on the published sequence of the P. stipitis Xyl2 gene (GenBankTM accession number X55392), the following two primers were designed: Xyl2UP, 5'-ATGACT-GCTAAACCTCTCCTGGTGTTG-3' (27-mer) and Xyl2DOWN, 5'-TTACT-CAGGGCCGTCATTAGACACTTG-3' (28-mer). PCR was carried out using PCR Thermal Cycler PERSONAL (TaKaRa) in a 50-μl reaction mixture containing 10 pmol of primers, 1 unit of KOD-plus DNA polymerase (Toyobo), and 100 ng of P. stipitis genomic DNA under conditions of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, and extension at 68 °C for 1.5 min. A single PCR product was introduced.
into the SmaI site in the plasmid pBluescript SK(−) (Stratagene) to yield pBS[H11001].

To introduce the restriction site for BamHI and PstI at 5′ and 3′ termini of the DNA fragments respectively, PCR was carried out using pHisWT as a template DNA and the following two primers: XDH[BamHI], 5′-cataggtcAGCTGTAACCTTCTGG-3′ (32-mer), and XDH[BstEII], 5′-ctgcatcaTTACTAAGGCGCTGTAAGGAC-3′ (36-mer) (lowercase letters indicate additional bases for introducing digestion sites of BamHI and PstI (underlined letters)). The amplified DNA fragment was introduced into BamHI-PstI sites in pQE-81L (Qiagen), a plasmid vector for conferring N-terminal His6 tag on the expressed proteins, to obtain pHs[W11001].

Amino Acid Sequence Alignment of PsXDH—The protein sequence of PsXDH was analyzed using the Protein BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and only proteins that possessed highly significant similarity and had already been characterized were used for multiple-sequence alignment using the ClustalW program distributed by the Bioinformatics Center of Kyoto University (www.genome.jp).

Site-directed Mutagenesis—The mutations were introduced by sequential steps of PCR (43) with a small modification. The synthetic oligonucleotide primer sequences involving the mutations are shown in Table II. In the first round, two reactions, I and II, were performed with the following primers, XDH[BamHI] and one of the antisense primers containing the mutations (reaction I) and one of the sense primers and XDH[BstEII] (reaction II). In the first amplification step purified overlapping PCR products were used as templates, and XDH[BamHI] and XDH[BstEII] were used as primers. D207A, I208R, F209S, N211R, and C (S96C/S99C/Y102C) mutants were obtained using pHs[W11001] as a template. All the final PCR products were cloned into pQE-81L to obtain plasmids pHs[WT], pHs[D207A], pHs[I208R], pHs[F209S], pHs[N211R], and pHs[C], respectively.

Furthermore, ARS mutant (D207A/I208R/F209S) was obtained using pHisWT as a template DNA and the following two primers: XDHBamHI, 5′-cataggtcAGCTGTAACCTTCTGG-3′ (32-mer), and XDHPstI, 5′-ctgcatcaTTACTAAGGCGCTGTAAGGAC-3′ (36-mer) (lowercase letters indicate additional bases for introducing digestion sites of BamHI and PstI (underlined letters)). The amplified DNA fragment was introduced into BamHI-PstI sites in pQE-81L (Qiagen), a plasmid vector for conferring N-terminal His6 tag on the expressed proteins, to obtain pHisWT.

The native molecular mass of PsXDH was estimated by gel filtration, which was carried out using an AKTA purifier system at a flow rate of 1 ml/min. The purified enzyme was loaded onto a HiLoad 16/60 Superdex 200 pg column (Amersham Biosciences) equilibrated with Buffer A containing 2 mM MgCl2, 0.3M NaCl, 10 mM 2-mercaptoethanol, and 50% (w/v) glycerol. All His-tagged recombinant PsXDHs were stored at −35 °C until use.

The zinc content of PsXDH was determined using an inductively coupled argon plasma emission spectrophotometer (ICP-500, Nippon Jarrell Ash Co., LTD, Kyoto, Japan). The protein sample was gel-filtered on a HiLoad 16/60 Superdex 200 pg column with 50 mM sodium phosphate (pH 8.0) and adjusted at concentrations of 0.5 mg/ml with the same buffer. A standard solution of Zn(NO3)2 in 0.1 x HNO3 was used for the calibration curve.

Circular Dichroism (CD) Measurements—For comparisons with the thermostability of the PsXDH, CD measurements at 220 nm were carried out between 10 and 70 °C with a Jasco J-720 (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a quartz cuvette with a path length of 2 mm under constant N2 flush. The temperature was increased at a rate of 1 °C/min. Enzyme samples dialyzed overnight against Buffer E were diluted at concentrations of 1 mg/ml with the same buffer.

RESULTS

Mutations for Reversal of Coenzyme Specificity—A search of protein databases revealed that PsXDH showed higher homology with XDHs and/or SDHs from several organisms than other MDRs and that most of these proteins have been reported as strict NAD(+) dependent enzymes (Table I). B. argentifolii SDH is the only NADP(+) dependent enzyme among the characterized PDHs (40). Although the crystal structure of B. argentifolii SDH resolved recently is an apo form (14), a phosphate ion from the crystallization buffer is found adjacent to Ala199, Arg202, and Arg201 (Fig. 1). There is a sequence gap around the putative coenzyme binding region (Table I), but Ala199-Arg202 is obviously homologous to Asp207-Ile208 in PDHs (position 11). On the other hand, the adjacent basic residues (Lys211 in PsXDH) is highly conserved regardless of the coenzyme specificity of PDHs (position 12). It is difficult to judge whether the position of 209 should be involved in the mutation sites because there is a homologous Ser residue regardless of NAD(+) and NADP(+) dependent enzyme(s) (position 9). We decided to choose this position as an additional mutation site, and Asp207, Ile208, His209 and Asn211 were chosen as amino acid residues to attempt the switch of coenzyme specificity of PsXDH. Four single mutants, D207A, I208R, F209S, and N211R, three double mutants, AR (D207A/I208R), RS (D207A/F209S), and ARS (D207A/I208R/F209S), and all three mutants with the reverse substitutions (D207A/I208R/F209S) were made (Fig. 1).
and AiS (D207A/F209S), a triple mutant, ARS (D207A/I208R/F209S), and a quadruple mutant, ARSdR (D207A/I208R/F209S/N211R), were generated by site-directed mutagenesis (Table II).

Purification of Recombinant PsXDHs—All recombinant PsXDHs-attached His6 tags at their N termini were expressed in E. coli (Fig. 2A) and purified with a Ni2+-chelating affinity column. SDS-PAGE analysis (Fig. 2B) revealed that the puri-
ties of recombinant enzymes were at least 90–95%, and each apparent molecular mass of PsXDHs was ~39,000 Da, in good agreement with the calculated molecular mass of the enzyme with His6 tag (39,956.79 Da for the WT). To estimate the native molecular mass, the purified WT enzyme was loaded on HiLoad 16/60 Superdex 200 pg column (Fig. 2C). When sodium phosphate was used as a buffer, the estimated molecular mass was 82,000 ± 1,000 Da, indicating that the enzyme existed as a dimer of the native enzyme (41). On the other hand, the value was 188,900 ± 10,000 Da in deionized water, indicating that the enzyme existed as a tetrameric form. Other recombinant PsXDH mutants also showed a similar tendency (data not shown). Furthermore, the WT in water maintained similar specific activity compared with that in sodium phosphate buffer (see Fig. 5B). This analysis indicated that recombinant PsXDH can form either a dimer or tetramer without loss of activity, in contrast with Galactocandida mastotermitis XDH; this enzyme is natively a tetramer and lost half its activity under deionized conditions caused by loss of the catalytic zinc atom (46). Generally, all MDRs are a dimer or tetramer consisting of identical subunits (5, 6).

Kinetics Constants for NAD⁺, NADP⁺, and Xylitol—Kinetic constants determined for coenzymes of WT and mutant PsXDHs are shown in Table III and Fig. 3. The kinetic constant for NAD⁺ of the recombinant PsXDH WT were similar to those of the native enzyme purified from P. stipitis (41). Although kinetic constants of the native enzyme for NADP⁺ have not been reported probably due to low activity, we determined the kinetic constant for NADP⁺ by using large amounts of the enzyme. Comparison with NAD⁺ in catalytic efficiency indicated that the enzyme possesses strict dependence for NAD⁺. The $K_{m}^{\text{xylo}}$ value of the WT in the presence of NAD⁺ was 21.7 mM, comparable with that of the native enzyme (26 mM) (41).

Single substitutions produced a more positive effect on NADP⁺ kinetics than those of NAD⁺; their $k_{cat}/K_{m}^{\text{NADP⁺}}$ values showed an ~5–45-fold increase. However, the mutant enzymes still preferred NAD⁺ to NADP⁺. These results were not unexpected considering that the only example of a single amino acid replacement resulting in a pronounced change in coenzyme specificity, i.e., NAD⁺ to NADP⁺, was the case of ADH from Drosophila (18). Double mutations, AR and AiS, led to a moderate increase of $k_{cat}/K_{m}^{\text{NADP⁺}}$ compared with single mutations. In particular, there was a complete synergistic effect in the AR mutant; the increase in $k_{cat}/K_{m}^{\text{NADP⁺}}$ for AR was 250-fold, the same as the product of the ratios of the increase in $k_{cat}/K_{m}^{\text{NADP⁺}}$ for D207A and for I208R. A similar synergistic effect was also observed for phosphite dehydrogenase (38). On the other hand, $k_{cat}/K_{m}^{\text{NADP⁺}}$ values did not significantly change in the mutants. The RS double mutant displayed low $k_{cat}$ values with either NAD⁺ or NADP⁺. Overall, the double mutants improved the catalysis for NADP⁺, although $k_{cat}/K_{m}^{\text{NADP⁺}}$ did not reach the $k_{cat}/K_{m}^{\text{NADP⁺}}$ of WT.

More striking effects of the triple mutant ARS were observed in kinetic constants for both NAD⁺ and NADP⁺. The $k_{cat}/K_{m}^{\text{NAD⁺}}$ dropped 15-fold compared with WT by an increase of $K_{m}^{\text{NAD⁺}}$ and a decrease of $k_{cat}$. On the other hand, $k_{cat}/K_{m}^{\text{NADP⁺}}$ increased dramatically (up to 4100-fold), caused by a large decrease of $K_{m}^{\text{NADP⁺}}$ value and increase of $k_{cat}$ value. It is noteworthy that $k_{cat}/K_{m}^{\text{NADP⁺}}$ was almost identical to that of WT for NAD⁺. There was no difference in the $K_{m}^{\text{xylo}}$ values in the presence of NAD⁺ or NADP⁺, although the $K_{m}^{\text{xylo}}$ values were slightly higher than that of WT in the presence of NAD⁺. Further introduction of N211R mutation into AR (ARSdR mutant) produced about a 2-fold decrease in the $k_{cat}/K_{m}^{\text{NAD⁺}}$ value mostly because of a dramatic decrease of $K_{m}^{\text{NAD⁺}}$ value compared with WT, whereas no additional change in the kinetics constant for NADP⁺ was observed. Overall, the N211R mutation was not necessary to switch the coenzyme specificity, whereas a more important effect of this mutation was the stabilization of the ARS mutant (see next section).

Effect of the Modification of Coenzyme Specificity on the Thermostability of the Mutant—The thermostability of WT and the 11 mutants was estimated by monitoring the change in the CD signal at 220 nm as the thermal unfolding transition temperature ($T_{m}$) (Fig. 4A). Four single mutations produced little effect on thermal denaturation. AR, RS, AiS, and ARS mutants displayed lower $T_{m}$ values, ranging between 4.1 and 6.3 °C compared with WT. The ARSdR mutant showed almost the same thermostability as WT despite similar kinetic constants to the ARS mutant, suggesting that the addition of the N211R mutation to the ARS mutant would contribute to maintaining thermostability rather than the catalytic property of this enzyme.
Introduction of Additional Structural Zinc Atom—Because there have been no reports about the XDH of thermophilic microorganisms, we cannot undertake site-directed mutagenesis based on the structural comparison of thermophilic XDH and mesophilic PsXDH and/or the random mutagenesis method by using a Xyl2− thermophilic strain (43). Alternatively, we attempted to increase the thermostability of PsXDH by introducing a metal ion.

All enzymes of the ADH and PDH subfamily contain one zinc atom involved in catalytic function, whose mechanistic role has been well known (5, 6, 16). There are several enzymes with a second zinc atom (structural zinc atom), although the exact function of the structural zinc atom is less clear. The structural zinc atom is coordinated with four cysteine residues (Cys96, Cys99, Cys102, and Cys110) in the Thermus aquaticus XDH as well as many other structural zinc-containing ADHs (a pattern of CCCC) (Fig. 1 and Table I) (14). There are some substitution patterns of these four cysteines in the PDHs (Table I). Among them, enzymes with the pattern of DSMD, RDCT, RDCT have been shown to have no structural zinc by enzymatic or crystallographic analyses (15, 46, 47). These analyses suggested that four fully conserved cysteine residues are necessary for binding to the structural zinc atom and that PsXDH having a pattern of SSSC has no structural zinc atom. We attempted to generate a C4 mutant in which the zinc atom was introduced by substituting Ser96, Ser99, and Tyr102 by cysteine residues, expecting that introduction of the structural zinc atom may increase the thermostability of PsXDH.

Characterization of C4 Mutant—By using a set of two synthetic primers involving the mutations, cysteine ligands for structural zinc were introduced into PsXDH as described under “Experimental Procedures.” We determined the binding stoichiometry of zinc for the WT and C4 mutant by atomic absorption spectroscopy. The zinc content value of WT was very close to 1.0 mol of zinc/mol of subunit (Fig. 5A), suggesting that wild-type XDH contains only a catalytic zinc atom. This result was also supported by the observation that the putative binding site for the catalytic zinc atom is conserved completely in PsXDHs (Cys41, His66, and Glu67) compared with other MDRs. The zinc content value of the C4 mutant was 1.9 ± 0.1 mol of zinc/mol of subunit, close to 2.0, indicating the acquirement of an additional zinc atom (Fig. 5A). The C4 mutant displayed almost the same kinetic constants for NAD+ and substrate as WT. Although the C4 mutant had a 20-fold decrease in the Km value for NADP+ compared with that for WT, the enzyme significantly preferred NAD+ to NADP+, similar to WT (Table III and Fig. 3). The thermostability of the C4 mutant was estimated from the Tm value by CD measurements and compared with that of WT. The introduction of the structural zinc atom increased the thermostability of the C4 mutant (4.5 °C increase of the Tm value compared with WT) (Fig. 4A). Similar results were obtained by heat treatment of the enzyme; the enzyme activity of WT decreased to 15% after incubation for 10 min at 40 °C, whereas no inactivation of the C4 mutant was detected by the same treatment (Fig. 4B). The level of translational expression of the C4 mutant was ~3-fold higher than that of WT (Fig. 2A), suggesting that the stability of the C4 mutant was also increased in vivo. The removal of Zn2+ and concomitant enzyme inactivation has been observed in several zinc-containing ADHs and SDHs (47–49). The enzyme activity of WT and the C4 mutant decreased to 50 and 90%, respectively, by treatment with 1 mM EDTA (pH 7.5) (Fig. 5B), suggesting that introduction of the structural zinc atom also provided resistance against the chelating agent for the catalytic zinc atom. The subunit of the G. mossustitermis XDH was found to contain 6 Mg2+ ions, although the role is unclear (46). Thus, we also measured the content of magnesium in WT and the C4 mutant of PsXDH, and obtained insignificant values; 0.26 and 0.2 mol of magnesium/mol of subunit (data not shown).

Characterization of C4/ARS and C4/ARSdR Mutants—We attempted to introduce structural zinc into ARS and ARSdR mutants to improve the thermostability of these mutants. By using one KpnI digestion site in the Xyl2 gene, as described under “Experimental Procedures,” the C4 mutation was combined with each ARS and ARSdR mutation to construct the C4/ARS (S96C/S99C/Y102C/D207A/I208R/F209S) and C4/ARSdR (S96C/S99C/Y102C/D207A/I208R/F209S/N211R) mutants, respectively (Table II). Their translational expression levels in E. coli were almost the same as the C4 mutant (Fig. 2A), estimating that the stability in vivo might increase compared with their parent enzymes, ARS and ARSdR. In fact, their Tm values were almost the same as that of the C4 mutant, and in particular, C4/ARS was dramatically stabilized by im-
specificity was achieved by multiple site-directed mutagenesis of mutant(s). Complete reversal of the coenzyme specificity in the literature (17–23, 25, 26) is homologous Ser residue regardless of NAD$^+$ and NADP$^+$ dependence in other NAD(P)H-dependent wild-type XDH by using NADP$^+$-specific SDH as a reference. Furthermore, the introduction of a structural zinc atom to a NADP$^+$-dependent mutant(s) produced significant thermostabilization and enhancement of the catalytic activity with NADP$^+$.

**Complete Reversal of Coenzyme Specificity of PsXDH**—The homologous positions in dehydrogenases to Asp$^{207}$-Ile$^{208}$ in PsXDH have often been used as candidates for the modification of nicotinamide cofactor specificity in the literature (17–23, 25, 28, 30–32, 34, 36–38). We also observed some significant effects in the AR (D207A/I208R) mutant, but the acquired NADP$^+$-dependent activity could not be satisfied compared with the NAD$^+$-dependent activity of WT (Table III and Fig. 3).

**Dramatic change of the kinetics constants for NADP$^+$ between AR and ARS is very interesting (Table III and Fig. 3).** The homologous position of Ser$^{209}$ has not been targeted by site-directed mutagenesis for the reversal of coenzyme specificity in other NAD(P)H-dependent dehydrogenases. In fact, there is homologous Ser residue regardless of NAD$^+$ and NADP$^+$-dependent PDHs (Table I). What is an important factor(s) for the change of the kinetics constants for NADP$^+$ between AR and ARS? Because both phenylalanine and tyrosine possess phenyl rings, construction of AR (D207A/I208R/F209Y) mutant makes it possible to clarify the effect of the introduction of hydroxyl group at position 209 (Table II). Some positive effects were observed in the kinetics constants for NADP$^+$ in the ARY but not reached the same level as those of ARS (Table III and

| Enzyme   | Specific activity | Kinetic parameters |
|----------|------------------|--------------------|
|          | NAD$^+$ | NADP$^+$ | Km | kcat | kcat/Km | Kcat | kcat | kcat/Km | Kcat |
|          |  | min$^{-1}$ | min$^{-1}$ | min$^{-1}$/max$^{-1}$ | min$^{-1}$ | min$^{-1}$ | min$^{-1}$/max$^{-1}$ | min$^{-1}$ | min$^{-1}$ |
| WT       | 1110 ± 90 | (1.20 ± 0.10)$^b$ | 0.381 ± 0.030 | 1050 ± 30 | 2760 ± 130 | 21.7 ± 0.1 | 170 ± 16 | 110 ± 10 | 0.650 ± 0.002 |
| D207A    | 430 ± 7  | (2.19 ± 0.04) | 0.403 ± 0.003 | 310 ± 5  | 765 ± 17  | 22.2 ± 0.1 | 120 ± 5  | 340 ± 25 | 2.80 ± 0.10 |
| I208R    | 1630 ± 20 | (32.2 ± 1.5) | 0.498 ± 0.008 | 1430 ± 10 | 2850 ± 20 | 29.5 ± 0.1 | 21.1 ± 2.3 | 600 ± 51 | 28.6 ± 0.7 |
| F209S    | 1330 ± 50 | (25.3 ± 3.5) | 0.848 ± 0.076 | 1220 ± 90 | 1440 ± 20 | 34.1 ± 1.6 | 28.9 ± 2.9 | 590 ± 41 | 20.4 ± 0.6 |
| N211R    | 1650 ± 20 | (9.03 ± 0.01) | 0.538 ± 0.004 | 1370 ± 10 | 2550 ± 50 | 27.4 ± 1.0 | 56.5 ± 6.1 | 280 ± 29 | 4.92 ± 0.02 |
| AR       | 640 ± 27 | (223 ± 1) | 0.568 ± 0.042 | 620 ± 44 | 1100 ± 10 | 24.2 ± 0.1 | 11.3 ± 1.2 | 1680 ± 130 | 149 ± 4 |
| RS       | 52.0 ± 2.4 | (2.20 ± 0.01) | 0.665 ± 0.022 | 60.3 ± 0.1 | 90.0 ± 3.0 | 45.4 ± 0.6 | 9.19 ± 0.42 | 8.70 ± 0.30 | 0.95 ± 0.01 |
| ARS      | 210 ± 8  | 491 ± 6 | 0.162 ± 0.022 | 200 ± 14 | 1250 ± 90 | 45.2 ± 4.4 | 9.96 ± 0.11 | 480 ± 34 | 484 ± 21 | 59.1 ± 0.1 |
| ARS+     | 320 ± 6  | 2350 ± 110 | 1.30 ± 0.13 | 240 ± 17 | 181 ± 5 | 55.7 ± 0.6 | 0.897 ± 0.036 | 2500 ± 50 | 2790 ± 50 | 31.1 ± 4.1 |
| C4/ARS   | 280 ± 9  | 1900 ± 40 | 0.285 ± 0.016 | 310 ± 13 | 1170 ± 20 | 97.8 ± 6.2 | 0.638 ± 0.031 | 1970 ± 50 | 3090 ± 70 | 46.3 ± 2.0 |
| ARY      | 130 ± 6  | 370 ± 4 | 0.152 ± 0.002 | 61.0 ± 4.0 | 402 ± 28 | 50.1 ± 3.4 | 0.731 ± 0.010 | 370 ± 35 | 512 ± 20 | 58.6 ± 4.4 |
| ARS/DR   | (259 ± 1) | 2570 ± 160 | 17.3 ± 2.1 | 1430 ± 160 | 840 ± 1.0 | — | 1.38 ± 0.11 | 3840 ± 270 | 2790 ± 30 | 72.6 ± 1.7 |
| C4/ARS+  | 1440 ± 30 | (6.85 ± 0.19) | 0.739 ± 0.031 | 1820 ± 80 | 2460 ± 10 | 30.3 ± 1.4 | 9.56 ± 0.01 | 54.6 ± 0.3 | 5.70 ± 0.02 |
| C4/ARSdR | (160 ± 2) | 8010 ± 70 | 23.5 ± 1.4 | 1770 ± 100 | 75.3 ± 0.1 | — | 1.18 ± 0.06 | 12600 ± 400 | 10700 ± 200 | 111 ± 5 |
| C4/ARSdR | (200 ± 3) | 7210 ± 200 | 7.60 ± 0.50 | 790 ± 40 | 104 ± 2 | — | 1.04 ± 0.04 | 11000 ± 400 | 10500 ± 100 | 119 ± 6 |

$^a$ Specific activity was measured under standard assay conditions described under “Experimental Procedures.”
$^b$ Values reported in parentheses are apparent values since saturation of the cofactor was not reached.
$^c$ Dash indicates not determined due to remarked high Km with coenzyme.

**FIG. 3.** Catalytic efficiency ($k_{cat}$/Km) with NAD(P)$^+$ for WT and mutant PsXDH. Enzyme activities were assayed in the direction of polyol oxidation by measuring the reduction of NAD$^+$ (blue bar) or NADP$^+$ (red bar).
Fig. 3). ART (D207A/I208R/F209T) mutant was used for analysis of the effect of side chain volume because the side chain volume of threonine is intermediate between serine and tyrosine (Table II). The $k_{cat}/K_m$ value was almost the same as that of ARS. The $K_m$ for NADP$^+$ was reduced by an order of magnitude from ARS to ART in agreement with the order of the side-chain volume at position 209 (Table III). These results indicated that not only the hydroxyl group but also the relatively small volume group was required for the side chain of the residue at this position to make hydrogen bonds with amino moiety(s) of the side chain of neighbor amino acid residues (probably Arg208 and Lys212 in the ARS mutant) similar to B. argentifolii SDH. Recently, Rosell et al. (37) reported a study about several NAD$^+$-dependent mutants of a vertebrate NADP$^+$-dependent ADH isozyme 8. For complete reversal of the coenzyme specificity toward NAD$^+$, substitution of three amino acid residues at homologous positions of Asp207-Ile208-Phe209 in PsXDH was necessary. These results indicated that, in some cases of dehydrogenase, an amino acid residue close to the landmark amino acid residues would also be effective for coenzyme recognition.

Metzger and Hollenberg (50) also attempted to identify a set of amino acid residues in PsXDH for specificity toward NAD$^+$. They introduced the potential NADP$^+$ recognition sequence of E. coli glutathione reductase (no homology with PsXDH) and thermophilic ADH (30% homology) into the homologous sequence in PsXDH, Asp207-Ile208-Phe209. Although these recombinant enzymes were not purified, the D207G mutation produced a 10-fold increase in apparent $K_m$ for NAD$. Because alanine substitution at position 207, either single or multiple, provided only a small change in $K_m$ for
Physiological Insight of Structural Zinc in MDR Superfamily—The zinc atom in medium-chain ADHs and SDHs is bound within a protruding loop from the catalytic domain (8, 9, 11, 12) whose corresponding amino acid sequence among all PDHs is conserved regardless of the existence of the zinc atom (Table I). Little insight into the role of the structural zinc atom is known, although the importance for maintaining the quaternary structure of the protein has been proposed (16). For example, human \( \beta \beta \) and \( \chi \chi \) ADHs as well as \( B. \) argentifolii SDH contain structural zinc atoms that are coordinated in four cysteine residues. Jeloková et al. (51) reported no expression in \( E. \) coli cells or measurable activity by the mutation of any of the four structural zinc ligands. Furthermore, the same mutations in bacterial phenylacetalddehyde reductase produced a dramatic decrease of activity, less than 4% activity compared with the wild-type enzyme (52). These results indicate that it is impossible to remove the zinc atom without loss of stable folding or enzyme activity. On the other hand, when yeast ADH and thermophilic archaeal Sulfolobus solfataricus ADH are incubated with a chelating reagent, the structural zinc atom is selectively removed without effect on the catalytic zinc atom or loss of enzyme activity, whereas the enzyme becomes very sensitive to treatment with heat and/or denaturant (48, 53). These different results may reflect the role change of the structural zinc atom in the enzyme during evolution. Human ADHs and bacterial phenylacetalddehyde reductase still depend on the second zinc atom for both stability and enzyme activity. On the other hand, the role is limited for maintaining the stability in yeast and \( S. \) solfataricus ADHs. If there had been no significant contribution to enzyme stability and activity, the structural zinc atom would have been lost. In fact, Bacillus subtilis SDH possesses complete conserved amino acid residues for the structural zinc atom but does not have a zinc atom (Table I) (54). The loss of enzymatic pressure would allow the four cysteine residues to be substituted randomly with other amino acid residues (Table I). An additional structural zinc atom could easily be introduced in such enzymes by substituting the cysteine residues for corresponding amino acid residues. The resultant stability improvement may originate from the inherent role of structural zinc in ancestral MDR involving PDHs. This study is the first report that the increase of enzyme thermostability was accomplished by introducing an additional zinc ion using site-directed mutagenesis. Because we can identify whether a MDR enzyme contains a structural zinc atom by simple analysis of the amino acid sequence alignment (Table I), this novel strategy for protein stabilization would be applicable for other MDRs that contain no structural zinc atom natively.

Production of Further Improved NADP\(^+\)-Dependent XDH Mutants—As observed in other several dehydrogenases/reductases (18, 19, 34, 35), the modification of enzyme specificity toward NADP\(^+\) in PsXDH resulted in a significant loss of thermostability (Fig. 4A) associated with the observation that most mutations tend to affect the stability of protein neutrally and/or negatively. Two novel NADP\(^+\)-dependent PsXDH mutants, ARS and ARS\(_{\text{SI}}\), were stabilized by introducing a second structural zinc atom at the potential binding site (Fig. 4). Surprisingly, this stabilization produced further improvement of NADP\(^+\)-dependent activity. Although protein stabilization generally confers rigidity on the protein and subsequently produces increased ligand affinity (55), there is no such change in the kinetic constants of C4/ARS and C4/ARS\(_{\text{SI}}\) mutants; enhancement of their \( k_{\text{cat}}/K_m \) is mainly due to increased \( k_{\text{cat}} \) (Table III). It is noteworthy that the C4 mutation site is separate from ARS/ARS\(_{\text{SI}}\) sequentially and structurally (Fig. 1 and Table I). Our results suggest the possibility that thermostabilization can enhance the catalytic activity of enzymes, which modified the coenzyme specificity in other dehydrogenases/reductases.

Application of Xylose Fermentation by \( S. \) cerevisiae—It is known that in bacteria xylose is metabolized by xylose isomerase (EC 5.3.1.5), which directly converts xylose into xylulose with no coenzyme (Scheme 1). However, because there has been no report of the significant expression of any bacterial xylose isomerase as an active form in \( S. \) cerevisiae except a thermophilic enzyme (56), we focused on the fungal xylose metabolic pathway in this study. XDH and xylose reductase in this fungal pathway are necessary for \( S. \) cerevisiae to ferment xylose to ethanol because of a lack of genes encoding these enzymes in \( S. \) cerevisiae (Scheme 1). Several studies also reported that the introduction of genes encoding related enzymes such as xylulokinase (XKS1), transketolase (TKL1), transaldolase (TAL1), and several hexasose transporters (HXT1–7) improved the efficiency of ethanol fermentation (2, 4). However, \( S. \) cerevisiae transforming the genes encoding xylose reductase, XDH and xylulokinase, a most potent recombinant strain, has not yet been applied to the industrial bio-process due to the unfavorable excretion of xylitol. Because intercellular redox imbalance caused by the different coenzyme specificity of xylose reductase and XDH has been thought to be one of the main factors of xylitol excretion (2), the introduction of NADP\(^+\)-dependent XDH generated in this study is expected to prevent this excretion by maintaining the intercellular redox balance. Wallrissdon et al. (57) reported that the overexpression of XDH in \( S. \) cerevisiae could prevent xylitol formation, although only a slight enhancement of ethanol yield was achieved. It is well known that there is a relationship between the intercellular expression level and thermostability in vitro mainly due to the resistance against proteolysis. Three thermostable mutants containing C4 mutation (C4, C4/ARS, and C4/ARS\(_{\text{SI}}\)) were expressed much more highly than each parent enzyme in \( E. \) coli cells (Fig. 2A), suggesting the possibility that the introduction of such thermostable enzymes can contribute to increase the expression of XDH in \( S. \) cerevisiae and improve the efficiency of ethanol fermentation.

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