

# Molecular Determinants of the Cofactor Specificity of Ribitol Dehydrogenase, a Short-Chain Dehydrogenase/Reductase

Hee-Jung Moon,<sup>a</sup> Manish Kumar Tiwari,<sup>b</sup> Ranjitha Singh,<sup>b,c</sup> Yun Chan Kang,<sup>b</sup> and Jung-Kul Lee<sup>b,c</sup>

Department of Bioscience and Biotechnology,<sup>a</sup> Department of Chemical Engineering,<sup>b</sup> and Institute of SK-KU Biomaterials,<sup>c</sup> Konkuk University, Gwangjin-Gu, Seoul, Republic of Korea

Ribitol dehydrogenase from *Zymomonas mobilis* (ZmRDH) catalyzes the conversion of ribitol to D-ribulose and concomitantly reduces NAD(P)<sup>+</sup> to NAD(P)H. A systematic approach involving an initial sequence alignment-based residue screening, followed by a homology model-based screening and site-directed mutagenesis of the screened residues, was used to study the molecular determinants of the cofactor specificity of ZmRDH. A homologous conserved amino acid, Ser156, in the substrate-binding pocket of the wild-type ZmRDH was identified as an important residue affecting the cofactor specificity of ZmRDH. Further insights into the function of the Ser156 residue were obtained by substituting it with other hydrophobic nonpolar or polar amino acids. Substituting Ser156 with the negatively charged amino acids (Asp and Glu) altered the cofactor specificity of ZmRDH toward NAD<sup>+</sup> (S156D,  $[k_{\text{cat}}/K_{m,\text{NAD}}]/[k_{\text{cat}}/K_{m,\text{NADP}}] = 10.9$ , where  $K_{m,\text{NAD}}$  is the  $K_m$  for NAD<sup>+</sup> and  $K_{m,\text{NADP}}$  is the  $K_m$  for NADP<sup>+</sup>). In contrast, the mutants containing positively charged amino acids (His, Lys, or Arg) at position 156 showed a higher efficiency with NADP<sup>+</sup> as the cofactor (S156H,  $[k_{\text{cat}}/K_{m,\text{NAD}}]/[k_{\text{cat}}/K_{m,\text{NADP}}] = 0.11$ ). These data, in addition to those of molecular dynamics and isothermal titration calorimetry studies, suggest that the cofactor specificity of ZmRDH can be modulated by manipulating the amino acid residue at position 156.

Short-chain dehydrogenases/reductases (SDRs) constitute a large protein family that catalyzes NAD(P)(H)-dependent oxidation/reduction reactions. At present, nearly 3,000 members of the SDR family are known, and they exhibit a wide substrate spectrum, including alcohols, sugars, steroids, aromatic compounds, and xenobiotics (18). Enzymes belonging to this family contain two important typical structural motifs. The first conserved domain of SDRs is present on the interior surface of an  $\alpha$ -helical structure and contains the universally conserved sequence Ser-Tyr-Lys, which is the confirmed active site of the enzyme. The second domain is located in the vicinity of the N terminus with a Gly-X<sub>3</sub>-Gly-X-Gly sequence (a glycine-rich domain), which is generally found in all dehydrogenases (5). Site-directed mutagenesis and X-ray crystallography studies have shown that the region around the glycine-rich domain forms a  $\beta\alpha\beta$ -fold that is characteristic of the cofactor-binding fold in the SDR family (35).

NAD (NAD<sup>+</sup>) and NADP (NADP<sup>+</sup>), two similar redox cofactors, are found in biological systems. NADP<sup>+</sup> structurally differs from NAD<sup>+</sup> only in terms of the presence of an additional phosphate group in its AMP moiety. The secondary structures of the cofactor-binding domains of both NAD<sup>+</sup> and NADP<sup>+</sup> are very similar (27). SDRs use NAD<sup>+</sup> or NADP<sup>+</sup> as cofactors; all of these contain a single Rossmann fold domain. Mostly, SDRs have dimeric or tetrameric structures, although monomers also exist (15, 25). Understanding the determinants of cofactor specificity for dehydrogenases is important from an enzyme-engineering perspective, since the native cofactor specificity of dehydrogenases is often not ideal for use in synthetic metabolic pathways and other industrial applications. Altering the cofactor specificity of an enzyme in an artificial metabolic pathway can potentially correct the redox imbalance in the process and improve the overall product yield; therefore, cofactor engineering is important in applications ranging from cofactor regeneration to bioelectrocatalysis (3, 4, 12, 17, 19, 21). A large body of literature describing the alteration of nicotinamide cofactor specificity is available (2, 9, 10, 24, 26, 28,

29, 33, 34), including the typical determinants and evolution of nicotinamide binding sites (7, 8). Despite these attempts, alteration of cofactor specificity remains a challenge, since there are very few instances in which the catalytic efficiency of an initially disfavored cofactor has been suitably improved to match substrate specificity (5, 13, 29, 34).

The findings of X-ray crystallography studies of NADH-dependent SDRs have suggested that, in case of the NAD(H)-dependent enzyme, the Asp residue forms a bifurcated hydrogen bond with the adenine ribose (5, 32). In case of the NADP(H)-dependent enzymes in other families of dehydrogenases, the third Gly residue of the NAD(H)-binding fingerprint enzyme is replaced by Ala and a positively charged residue is usually included in the vicinity of the C terminus of the  $\beta\alpha\beta$ -fold (5, 22, 35). However, the residue(s) responsible for NADPH specificity remains unknown. The Asp38 residue is present in the NAD<sup>+</sup>-binding domain, and its substituent, Asn38, allows the *Drosophila* alcohol dehydrogenase to use both NAD<sup>+</sup> and NADP<sup>+</sup> as its cofactors (11).

We have reported the cloning and characterization of a novel enzyme ribitol dehydrogenase from *Zymomonas mobilis* (ZmRDH) (23) and provided experimental evidence for the use of both NAD<sup>+</sup> and NADP<sup>+</sup> as cofactors by ZmRDH. In the present study, we identified an important amino acid residue that is responsible for the cofactor specificity of ZmRDH by a systematic screening process involving sequence alignment and molecular dynamics

Received 2 December 2011 Accepted 7 February 2012

Published ahead of print 17 February 2012

Address correspondence to Jung-Kul Lee, jkrhee@konkuk.ac.kr, or Yun Chan Kang, yckang@konkuk.ac.kr.

H.-J.M. and M.K.T. contributed equally to this article.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.07751-11

(MD) simulation, followed by site-directed mutagenesis. Using the crystal structure of *Bacillus megaterium* glucose-1-dehydrogenase (BmGDH) (1GEE) as a template, we built a three-dimensional (3D) model of ZmRDH. Analysis of this model docked with different cofactor molecules into the active site, followed by a systematic screening process, showed that the S156 residue interacted with the cofactor. We further investigated the role of S156 in cofactor binding and catalytic efficiency of ZmRDH.

## MATERIALS AND METHODS

**Materials, bacterial strains, and culture conditions.** Reagents for PCR, ExTaq DNA polymerase, and T4 DNA ligase were purchased from Takara (Takara Corp., Japan); pGEM-T Easy and pGEX-KG vectors were purchased from Promega (Madison, WI) and the American Type Culture Collection (Manassas, VA), respectively. Restriction enzymes and the thrombin cleavage kit were obtained from New England Biolabs (Beverly, MA), and glutathione-Sepharose 4B was purchased from GE Healthcare (Little Chalfont, United Kingdom). A plasmid isolation kit and oligonucleotide primers were obtained from Bioneer (Daejeon, South Korea), and electrophoresis reagents were obtained from Bio-Rad. All of the chemicals for the assay were obtained from Sigma-Aldrich (St. Louis, MO). The plasmid containing the wild-type *ZmRDH* gene (23) was used for the production of wild-type ZmRDH protein. The *rdh* gene from *Z. mobilis* ZM4 was amplified by PCR by using 2 oligonucleotide primers, 5'-ATTAGGATCCATGATACCGCGCCCGATCA-3' (the BamHI restriction site is underlined) and 5'-TATACTCGAGAAAATCTGGGC GCATCCGGT-3' (the XhoI restriction site is underlined). The *rdh* gene released from the pGEM-T Easy vector was ligated with the pGEX-KG vector to form pGEXKG-*Zmrdh*, which is controlled by the *tac* promoter and expresses RDH as a fusion protein at the N terminus of the glutathione S-transferase (GST) tag. The cloned gene was confirmed to be free of point mutations by DNA sequencing. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) for expression. *E. coli* strains harboring wild-type and mutant *ZmRDH* genes for protein expression were cultured at 37°C in Luria-Bertani medium supplemented with ampicillin (100 µg ml<sup>-1</sup>). Finally, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture medium (final concentration, 0.5 mM), and this culture was incubated in a shaker incubator at 37°C.

**Site-directed mutagenesis of ZmRDH.** Site-directed mutagenesis was carried out using a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The recombinant plasmid, pGEXKG-*Zmrdh* (23), containing the wild-type *rdh* gene, was used as the DNA template. The plasmids containing the correct mutant genes were then used to transform *E. coli* BL21 (DE3), and ampicillin-resistant colonies were used for protein expression.

**Purification and protein quantification.** Purification of the wild-type and mutant enzyme was carried out as follows. Cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.5) supplemented with 25 µg of DNase I ml<sup>-1</sup>. The cell suspension was incubated on ice for 30 min in the presence of 1 mg of lysozyme ml<sup>-1</sup>. Cell disruption was carried out by sonication at 4°C for 5 min, and the lysate was centrifuged at 14,000 × g for 20 min at 4°C to remove cell debris. The resulting crude extract was retained for purification. Purification using glutathione-Sepharose 4B was performed according to the manufacturer's protocol (GE Healthcare). Protein concentrations were determined by the Bradford method by using bovine serum albumin as the standard protein (6).

**Enzyme assay and determination of kinetic parameters.** The activity of RDH was determined spectrophotometrically by monitoring the change in absorbance at 340 nm ( $A_{340}$ ) upon oxidation or reduction of NAD(P)H at 25°C. The RDH assay mixture for oxidation consisted of 2 mM NAD(P)<sup>+</sup>, 50 mM ribitol, 5 mM MnCl<sub>2</sub>, and the enzyme solution in 20 mM Tris buffer (pH 9.5). The reaction was started by adding the substrate. The kinetic parameters of ZmRDH were determined in 20 mM Tris buffer (pH 9.5) containing 5 mM Mn<sup>2+</sup>, 5 to 150 mM substrate solution, and 0.125 to 2.5 mM cofactor (23). One unit of enzyme activity was de-

fined as the amount of enzyme required to produce 1 µmol of NAD(P)H min<sup>-1</sup> under the given assay conditions. The kinetic parameters were obtained from multiple measurement by using the Michaelis-Menten equation:  $v = V_{\max}[S]/([S] + K_m)$ , where  $v$  is the observed initial velocity,  $V_{\max}$  is the maximum rate of the reaction, and  $K_m$  is the Michaelis constant for the substrate. The nonlinear curve directly fitted to the Michaelis-Menten equation using Prism version 5.0 (GraphPad Software, San Diego, CA).

**Protein database search.** The amino acid sequences deduced from the *ZmRDH* gene sequence were compared to those of related enzymes from other sources by using the Basic Local Alignment Search Tool (BLAST) network at the National Center for Biotechnology Information. Multiple sequence alignment was performed using the CLUSTAL W program.

**Homology modeling.** The 3D homology models of the wild-type protein and all forms of the mutant protein were developed using the Build Homology Models module in the MODELER application of Discovery Studio 3.0 (DS 3.0; Accelrys Software, Inc., San Diego, CA). Crystal structure of *B. megaterium* glucose-1-dehydrogenase (PDB accession code, 1GEE) was used as a template. Comparative modeling was used to generate the most probable structure of the query protein by aligning it with the template sequences, simultaneously considering the spatial restraints and local molecular geometry. Sequence identity between *ZmRDH* and the template was found to be 33% according to the BLAST parameters. Fitness of the model's sequences in their existing 3D environment was evaluated using Profiles-3D Score/Verify Protein (MODELER) as implemented in DS 3.0. A discrete optimized protein energy (DOPE) score was also calculated to determine the quality of the protein structures (MODELER). To assess the reliability of the model, the root mean square deviation (RMSD) between the model and the template was calculated by superimposing the model onto the template crystal structure, and the RMSD between the C-alpha atoms of the template and the model was 0.76 Å. The generated structure was improved by subsequent refinement of the loop conformations by assessing the compatibility of an amino acid sequence with the known Protein Data Bank (PDB) structures (Protein Health module, DS 3.0). The geometry of loop region was corrected using the Refine Loop/MODELER. Finally, the best model was chosen for further calculations, molecular modeling, and docking studies. Hydrogen atoms were added to the protein model; these atoms were minimized to have stable energy conformation and to also relax the conformation from close contacts. A sphere with a radius of 4.5 Å around the ligand-binding pocket of the ZmRDH defined its active site (S157, Y170, and K174). NAD<sup>+</sup> and NADP<sup>+</sup> were docked into the active-site pocket of the ZmRDH model by using C-DOCKER, an MD simulated annealing-based algorithm module from DS 3.0. Random substrate conformations were generated using high-temperature MD. Candidate poses were created using random rigid-body rotations, followed by simulated annealing. The structures of the protein, cofactor, and their complexes were subjected to energy minimization using the CHARMM force field in DS 3.0. A full-potential final minimization was used to refine the substrate poses. Using C-DOCKER, the energy-docked conformation of the substrate was retrieved for postdocking analysis. The substrate orientation that showed the lowest interaction energy was chosen for subsequent rounds of docking (31).

**ITC.** Isothermal titration calorimetry (ITC) was performed using a Nano ITC low-volume calorimeter (TA Instruments, USA). Wild-type and mutant ZmRDH subunits were purified and subsequently concentrated to 0.1 mM by using a VivaSpin 20 ultrafiltration spin column (Sartorius Stedim Biotech, Germany) at 3,000 × g at 4°C. The titrations were performed at 40°C and consisted of 20 2.5-µl injections of oat spelt xylan into the wild-type ZmRDH or mutant solutions at 120-s intervals. The instrument was equilibrated at 40°C until the baseline was flat and stable. The titration data were analyzed using the NanoAnalyze software (TA Instruments, USA), which makes use of an independent model to obtain the fit graph and thermodynamic binding data. Enthalpy of binding was determined for three titrations each for the wild type and the mutants, and

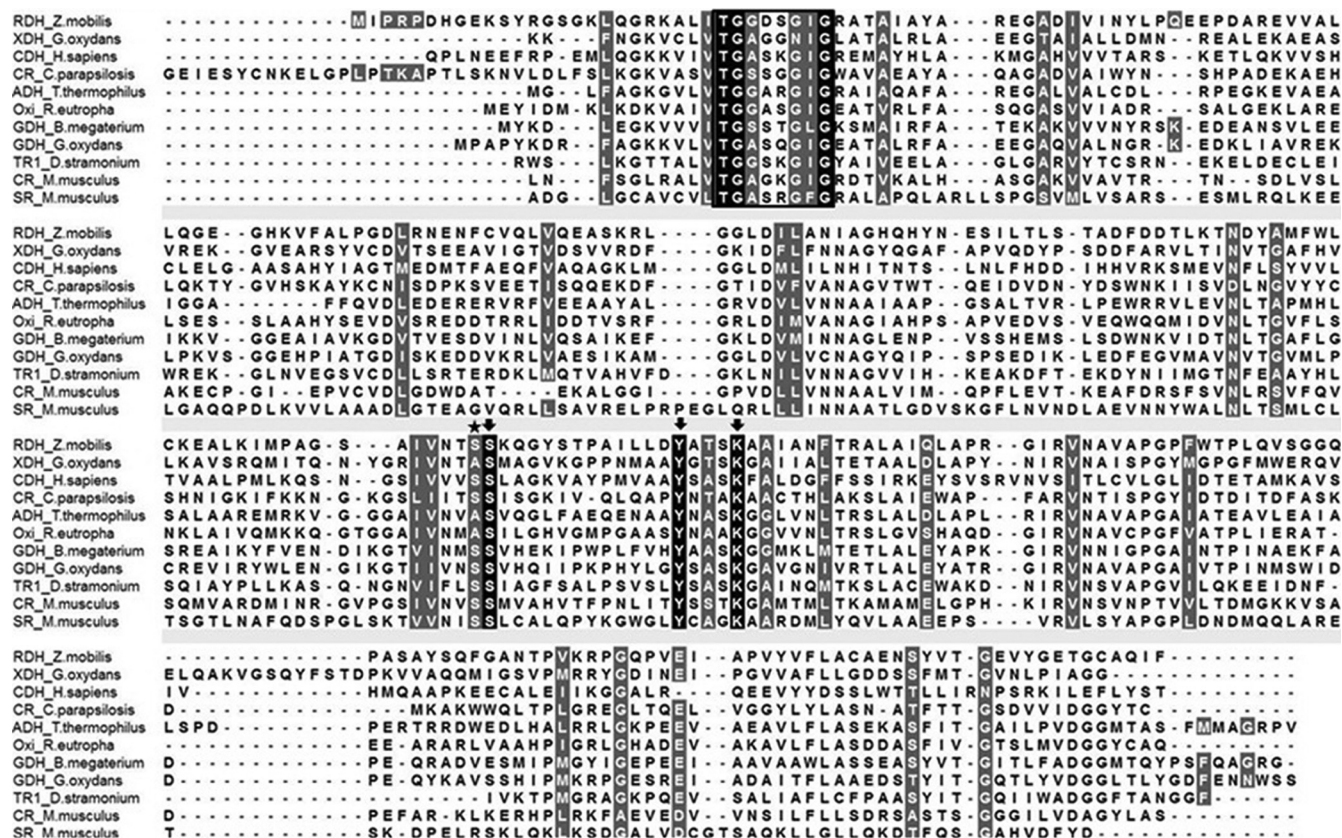


FIG 1 Multiple sequence alignment of *Zymomonas mobilis* subsp. *mobilis* ZM4 RDH (ZmRDH) with selected NAD<sup>+</sup>/NADP<sup>+</sup>-dependent short-chain reductase/dehydrogenases (SDRs). *Z. mobilis* RDH, *Zymomonas mobilis* subsp. *mobilis* ZM4 ribitol dehydrogenase; *G. oxydans* XDH, *Gluconobacter oxydans* xylitol dehydrogenase; *H. sapiens* CDH, *Homo sapiens* corticosteroid 11- $\beta$ -dehydrogenase; *C. parapsilosis* CR, *Candida parapsilosis* carbonyl reductase; *T. thermophilus* ADH, *Thermus thermophilus* alcohol dehydrogenase; *R. eutropha* OXI, *Ralstonia eutropha* oxidoreductase; *B. megaterium* GDH, *Bacillus megaterium* glucose-1-dehydrogenase; *G. oxydans* GDH, *Gluconobacter oxydans* glucose 1-dehydrogenase; *D. stramonium* TR1, *Datura stramonium* tropinone reductase-1; *M. musculus* CR, *Mus musculus* carbonyl reductase; *M. musculus* SR, *Mus musculus* sepiapterin reductase. Residues shaded black are highly conserved, residues shaded gray are homologous in nature, and nonhomologous sequences have a white background. Catalytic active-site residues are indicated by downward-pointing arrows, and coenzyme-binding domain residues are represented in a black box. Target residue (S156) for mutagenesis is indicated by black stars.

the average values were compared using a Student *t* test (Prism v5.0 software). The intrinsic molar enthalpy change ( $\Delta H$ ), the binding stoichiometry (*n*), and the binding constant (*K*) for the binding process were obtained from the best fit of the calorimetric data. The Gibbs free energy of binding ( $\Delta G^b$ ) and the dissociation constant ( $K_d$ ) were calculated from binding affinity measurements by using the formula  $\Delta G^b = -RT \ln(1/K_d)$ , where *R* is the universal gas constant and *T* is the absolute temperature in kelvins. The entropy of the binding was then estimated using the formula  $\Delta S = (\Delta H - \Delta G^b)/T$ , where  $\Delta H$  was the average enthalpy of binding derived from isothermal titrations.

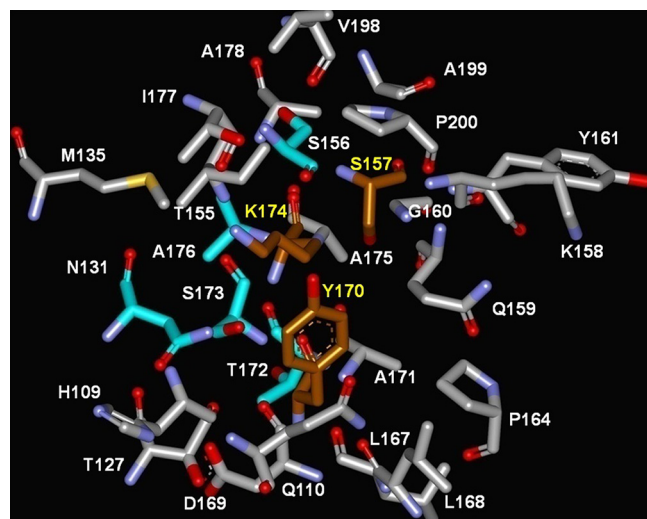
## RESULTS

**Sequence alignment.** To locate the conserved residues in ZmRDH, the amino acid sequence of ZmRDH was aligned with other SDR sequences, such as *Gluconobacter oxydans* xylitol dehydrogenase, *Homo sapiens* corticosteroid 11- $\beta$ -dehydrogenase, *Candida parapsilosis* carbonyl reductase, *Thermus thermophilus* alcohol dehydrogenase, *Mus musculus* carbonyl reductase, *G. oxydans* glucose-1-dehydrogenase, and *Ralstonia eutropha* oxidoreductase. Multiple sequence alignment revealed 42 totally conserved (100% identical) amino acids throughout the sequence (Fig. 1), including the glycine-rich consensus sequences (G27, G31, and G33 in ZmRDH) that have a structural role in cofactor

binding in all of the SDRs and the three active-site residues (S157, Y170, and K174 in ZmRDH).

**Homology modeling.** ZmRDH had a 33% sequence identity with the BmGDH. Using the X-ray crystal structure of BmGDH (PDB entry, 1GEE), we constructed a homology model of ZmRDH. The developed model was then validated by Ramachandran plots (20). In the ZmRDH model, the calculated Ramachandran plot suggested that 91.9, 7.0, and 0.9% of the residues in the derived model lay in the most favored, additional allowed, and generously allowed regions, respectively. In total, 99.8% of the residues were in the favored and allowed regions. A model structure with a high percentage of residues in the favored and allowed regions usually represents a good protein fold. PROCHECK and RAMPAGE were used to validate the folding integrity of our model; the findings indicated that the model structure derived from the 1GEE template was of a better quality in terms of protein fold. The Profiles-3D score of the model was 93 against a maximum expected score of 115, which correlated well with the score of 112 for the 1GEE coordinates.

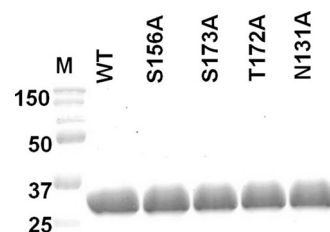
The substrate ribitol and cofactor were docked into the homology model using DS 3.0. In total, 28 amino acid residues were found within a 4.5-Å radius of the substrate-binding pocket (SBP)



**FIG 2** Substrate-binding pocket (SBP) of ZmRDH. A total of 28 amino acid residues, including the three active-site residues (S157, Y170, and K174) were located within the 4.5-Å radius of the SBP. A total of eight conserved residues around the catalytic domain, including active-site residues, were selected for site-directed mutagenesis analysis. The residues located in the SBP are shown in a stick model. Active-site residues are colored with orange carbons, target residues for mutagenesis are colored with cyan carbons, and other residues near the active site are colored with white carbon.

and 8 residues, including the three active-site residues, were partially conserved (Fig. 2). The putative BmGDH active-site residues were proposed to be S145, Y158, and K162 on the basis of the crystal structure of BmGDH (37). On superimposition, residues S157, Y170, and K174 of ZmRDH corresponded with the proposed catalytic residues (S145, Y158, and K162) of BmGDH. Site-directed mutagenesis for each of these three residues was performed by mutating them to Ala. The mutant proteins—S157A, Y170A, and K174A—had no measurable dehydrogenase activity, thereby validating that the corresponding mutated residues are functional active-site residues required for dehydrogenation. Among the other five conserved residues, the roles of four residues—N131, S156, T172, and S173 (but not the neutral A176)—were further investigated by site-directed mutagenesis (Fig. 2).

**Alanine substitution of selected residues.** To probe the functional role of the selected conserved residues, all four selected residues were individually mutated to Ala. The recombinant enzymes carrying N131A, S156A, T172A, and S173A mutations were expressed and purified. After the glutathione *S*-transferase (GST) tag was removed, five amino acid residues (Gly-Ser-Pro-Asn-Ser) were found attached to the N terminus of the ZmRDH sequence. Complete cleavage of the 25.7-kDa GST tag was verified by SDS-PAGE and visualized by staining with Coomassie brilliant blue R-250 (Fig. 3). The wild-type ZmRDH and the N131A, S156A, T172A, and S173A mutant proteins exhibited similar circular dichroism spectra, with ellipticity minima of comparable amplitudes in the 220- to 230-nm range (data not shown). This indicated that all tested wild-type and mutant enzymes had a similar secondary structure, and no major misfolding due to the introduced mutations was observed. When their activities with the cofactor were analyzed and compared to that of the wild-type ZmRDH, except for the substitution at S156, none of these substitutions were found to cause significant change in the ZmRDH



**FIG 3** SDS-PAGE analysis of wild-type ZmRDH and mutants selected for alanine scanning, with a molecular mass indicated in kilodaltons. Lane M contains the protein markers, and lanes N131A, S156A, T172A, and S173A correspond to the purified ZmRDH mutant enzymes (molecular mass, ~28 kDa).

activity (data not shown). The specific activity of mutant S156A for ribitol was 2.58 U mg of protein<sup>-1</sup> with NAD<sup>+</sup> and 0.37 U mg of protein<sup>-1</sup> with NADP<sup>+</sup>. These values corresponded to 45.7 and 15.4% of those obtained for the wild-type enzyme (5.65 U mg of protein<sup>-1</sup> with NAD<sup>+</sup> and 2.42 U mg of protein<sup>-1</sup> with NADP<sup>+</sup>). Therefore, the role of position 156 was further investigated by site-directed mutagenesis (Table 1).

**Site-directed mutagenesis of position 156 and kinetic analysis of ZmRDH variants.** The Ser at position 156 was mutated to nonpolar aromatic and polar/charged residues by site-directed mutagenesis. All mutants were expressed at a level similar to that of the wild type. The activities of the enzymes were determined as a function of the cofactor concentration at saturated ribitol concentrations. This was followed by determination of activities as a function of ribitol concentration in the presence of NAD(P)<sup>+</sup> at saturating concentrations. The kinetic parameters determined for purified wild-type and mutant ZmRDH enzymes are shown in Table 2. The wild-type enzyme has a clear preference for NAD<sup>+</sup> over NADP<sup>+</sup> (by 2.36-fold), on the basis of the ratio of catalytic efficiency ( $[k_{\text{cat}}/K_{m,\text{NAD}}]/[k_{\text{cat}}/K_{m,\text{NADP}}] = 2.36$ , where  $K_{m,\text{NAD}}$  is the  $K_m$  for NAD<sup>+</sup> and  $K_{m,\text{NADP}}$  is the  $K_m$  for NADP<sup>+</sup>). When S156 was replaced with a nonpolar amino acid (S156A or S156G), the mutant enzymes showed a slightly greater preference for NAD<sup>+</sup>. When S156 was replaced with a nucleophilic, uncharged amino acid (S156T or S156C) or an aromatic amino acid (S156Y or S156W), the mutant enzymes showed a slightly greater preference for NADP<sup>+</sup> with the  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value ranging from 1.66 to 1.83 (Table 2).

To further investigate the role of the Ser residue at position 156, it was replaced with Asp, Glu, Arg, His, or Lys. All of these

**TABLE 1** Specific activities for purified ZmRDH wild-type and S156A mutant strains with NAD<sup>+</sup> or NADP<sup>+</sup> as cofactors

| Cofactor and strain     | ZmRDH <sup>a</sup>                      |                       |
|-------------------------|-----------------------------------------|-----------------------|
|                         | Sp act (U mg of protein <sup>-1</sup> ) | Relative activity (%) |
| <b>NAD<sup>+</sup></b>  |                                         |                       |
| Wild type               | 5.65 ± 0.82                             | 100                   |
| S156A                   | 2.58 ± 0.19                             | 45.6                  |
| <b>NADP<sup>+</sup></b> |                                         |                       |
| Wild type               | 2.42 ± 0.05                             | 42.9                  |
| S156A                   | 0.37 ± 0.13                             | 15.4                  |

<sup>a</sup> All assays were performed at 25°C and pH 9.5 in 20 mM Tris-glycine-NaOH. The specific activities are means ± the standard deviations for three experiments.

TABLE 2 Kinetic parameters of purified ZmRDH wild-type and S156 mutant strains<sup>a</sup>

| Strain    | NAD <sup>+</sup>               |                            |                                     |                                                                          | NADP <sup>+</sup>              |                             |                                     |                                                                           |                                                                        |
|-----------|--------------------------------|----------------------------|-------------------------------------|--------------------------------------------------------------------------|--------------------------------|-----------------------------|-------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------|
|           | $K_{m,\text{ribitol}}$<br>(mM) | $K_{m,\text{NAD}}$<br>(mM) | $k_{\text{cat}}$ (s <sup>-1</sup> ) | $k_{\text{cat}}/K_{m,\text{NAD}}$<br>(s <sup>-1</sup> mM <sup>-1</sup> ) | $K_{m,\text{ribitol}}$<br>(mM) | $K_{m,\text{NADP}}$<br>(mM) | $k_{\text{cat}}$ (s <sup>-1</sup> ) | $k_{\text{cat}}/K_{m,\text{NADP}}$<br>(s <sup>-1</sup> mM <sup>-1</sup> ) | $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$ |
| Wild type | 11.8 ± 1.1                     | 0.18 ± 0.02                | 4.83 ± 0.51                         | 27.3                                                                     | 16.4 ± 0.9                     | 0.26 ± 0.02                 | 2.79 ± 0.15                         | 10.8                                                                      | 2.36                                                                   |
| S156A     | 13.7 ± 1.0                     | 0.19 ± 0.02                | 1.93 ± 0.16                         | 10.2                                                                     | 22.6 ± 1.0                     | 0.39 ± 0.13                 | 1.19 ± 0.07                         | 3.05                                                                      | 3.34                                                                   |
| S156G     | 11.1 ± 1.0                     | 0.14 ± 0.01                | 1.04 ± 0.02                         | 7.43                                                                     | 25.4 ± 0.8                     | 0.34 ± 0.07                 | 0.72 ± 0.08                         | 2.12                                                                      | 3.51                                                                   |
| S156T     | 15.9 ± 1.0                     | 0.21 ± 0.06                | 2.85 ± 0.10                         | 13.6                                                                     | 22.2 ± 0.7                     | 0.22 ± 0.03                 | 1.64 ± 0.22                         | 7.45                                                                      | 1.83                                                                   |
| S156C     | 18.3 ± 1.7                     | 0.10 ± 0.02                | 2.00 ± 0.09                         | 20.0                                                                     | 16.3 ± 1.1                     | 0.17 ± 0.02                 | 2.05 ± 0.10                         | 12.1                                                                      | 1.66                                                                   |
| S156Y     | 9.79 ± 0.8                     | 0.15 ± 0.01                | 1.60 ± 0.13                         | 10.7                                                                     | 12.4 ± 1.1                     | 0.23 ± 0.13                 | 1.42 ± 0.16                         | 6.17                                                                      | 1.73                                                                   |
| S156W     | 12.7 ± 1.1                     | 0.16 ± 0.02                | 2.62 ± 0.11                         | 16.4                                                                     | 18.7 ± 0.9                     | 0.21 ± 0.03                 | 2.05 ± 0.11                         | 9.76                                                                      | 1.68                                                                   |
| S156D     | 16.1 ± 0.9                     | 0.02 ± 0.002               | 1.79 ± 0.01                         | 89.5                                                                     | 20.9 ± 1.0                     | 0.30 ± 0.03                 | 2.46 ± 0.21                         | 8.20                                                                      | 10.9                                                                   |
| S156E     | 11.7 ± 0.6                     | 0.05 ± 0.01                | 2.06 ± 0.07                         | 41.2                                                                     | 15.6 ± 0.6                     | 0.35 ± 0.04                 | 2.39 ± 0.15                         | 6.83                                                                      | 6.03                                                                   |
| S156R     | 13.3 ± 0.7                     | 0.27 ± 0.02                | 2.93 ± 0.22                         | 10.9                                                                     | 18.3 ± 0.9                     | 0.05 ± 0.01                 | 2.75 ± 0.11                         | 55.0                                                                      | 0.20                                                                   |
| S156H     | 12.0 ± 1.0                     | 0.40 ± 0.05                | 3.22 ± 0.14                         | 8.05                                                                     | 15.1 ± 1.0                     | 0.03 ± 0.002                | 2.29 ± 0.01                         | 76.3                                                                      | 0.11                                                                   |
| S156H     | 11.8 ± 1.4                     | 0.24 ± 0.03                | 3.10 ± 0.15                         | 12.9                                                                     | 21.7 ± 0.7                     | 0.09 ± 0.01                 | 3.10 ± 0.23                         | 34.4                                                                      | 0.37                                                                   |

<sup>a</sup> All assays were performed at 25°C and pH 9.5 in 20 mM Tris-glycine-NaOH. The  $k_{\text{cat}}$  values were calculated by considering the enzyme as a monomeric form. Results are presented as means ± the standard deviations for three experiments where applicable.

replacements altered the cofactor specificity significantly. When S156 was replaced with a polar, negatively charged amino acid (S156D or S156E), the mutant enzymes showed up to a 3-fold increase in the  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value. The  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value changed from 2.36 for the wild type to 10.9 for the S156D mutant. However, when S156 was replaced with a positively charged polar amino acid (S156R, S156H, or S156K), compared to the wild-type ZmRDH, the S156H mutant showed a significant shift in its cofactor specificity toward NADP<sup>+</sup>:  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}}) = 2.36$  versus  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}}) = 0.11$ . The change in its cofactor specificity occurred due to the drastic decrease of the  $K_{m,\text{NADP}}$  value for the mutant; no significant change was observed in the  $k_{\text{cat}}$  value with NADP<sup>+</sup>.

#### Thermodynamics of cofactor binding by ZmRDH variants.

Thermodynamic parameters for the binding of the cofactor to ZmRDH variants were investigated using a Nano ITC low-volume calorimeter (TA Instruments). The heat output of the enzyme-substrate interaction was recorded, and the data were fitted using the NanoAnalyze software to obtain the values of  $K_d$ ,  $\Delta H$ , and  $\Delta G^b$ . The thermodynamic parameters associated with the interactions of wild-type ZmRDH and the S156 mutants with NAD<sup>+</sup> and NADP<sup>+</sup> are compiled in Table 3. Compared to the wild type, the S156D binding to NAD<sup>+</sup> ( $K_{d,\text{NAD}} = 0.19 \mu\text{M}$ ) showed a decrease of 5.9 kJ mol<sup>-1</sup> in the  $\Delta G^b$ , while binding to NADP<sup>+</sup> ( $K_{d,\text{NADP}} = 30.4 \mu\text{M}$ ) showed an increase of 4.7 kJ mol<sup>-1</sup> in the  $\Delta G^b$ . However, the S156H binding to NAD<sup>+</sup> ( $K_{d,\text{NAD}} = 95.4 \mu\text{M}$ ) showed an increase of 9.2 kJ mol<sup>-1</sup> in the

$\Delta G^b$ , while binding to NADP<sup>+</sup> ( $K_{d,\text{NADP}} = 0.21 \mu\text{M}$ ) showed a decrease of 7.3 kJ mol<sup>-1</sup> in the  $\Delta G^b$ .

#### DISCUSSION

The molecular basis of cofactor specificity is an issue of fundamental interest in the enzymology of dehydrogenases. There is no universal approach for modulating cofactor specificity in dehydrogenases, and the experimental strategy strongly depends on the specific features of the active sites of individual enzymes. In the present study, we used a systematic strategy to identify the molecular determinants of cofactor specificity: we screened for conserved residues by performing multiple sequence alignments, used MD simulation to identify conserved residues binding to the substrate, and individually carried out site-directed mutagenesis for those particular residues.

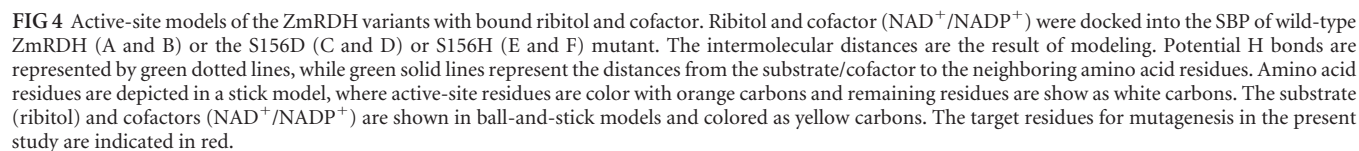
ZmRDH has been reported to be a novel RDH showing dual cofactor specificity. We showed that ZmRDH uses both NAD<sup>+</sup> and NADP<sup>+</sup> as cofactors (23). The molecular docking study and mutational analyses of residues in the SBP of the wild-type ZmRDH showed a significant interaction between the cofactor (NAD<sup>+</sup> or NADP<sup>+</sup>) and S156. A protein sequence BLAST was performed against the PDB, and five SDR sequences showing NAD<sup>+</sup>/NADP<sup>+</sup> dual cofactor specificity were chosen. The chosen SDRs were BmGDH, *G. oxydans* glucose-1-dehydrogenase, *Datura stramonium* tropinone reductase-1, and *M. musculus* carbonyl reductase and sepiapterin reductase. S156 in ZmRDH was completely conserved in the SDRs with dual cofactor specificity (Fig. 1). Replacement of the S156 with a polar negatively charged residue (S156D or S156E) resulted in a significant decrease in  $K_{m,\text{NADP}}$ , whereas that with a polar positively charged residue (S156R, S156H, or S156K) resulted in a decrease in the  $K_{m,\text{NADP}}$ . The  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value changed from 0.11 for the S156H mutant to 10.9 for the S156D mutant. These results suggest that S156 or the position 156 is a crucial determinant of the cofactor specificity of SDRs, especially ZmRDH. The  $K_d$  values of the ZmRDH variants (S156H) indicate the role of the position 156 in the cofactor binding of ZmRDH (Table 3).

The catalytic mechanism of SDRs has been reported in *E. coli* 7 $\alpha$ -hydroxysteroid dehydrogenase on the basis of its crystal structure (30). Based on this, catalytic mechanism of ZmRDH can be

TABLE 3 Thermodynamic parameters of NAD(P)<sup>+</sup> binding determined by ITC for RDH variants<sup>a</sup>

| Strain    | NAD <sup>+</sup>                     |              |                      | NADP <sup>+</sup>                     |              |                      |
|-----------|--------------------------------------|--------------|----------------------|---------------------------------------|--------------|----------------------|
|           | $K_{d,\text{NAD}}$ ( $\mu\text{M}$ ) | $\Delta G^b$ | $\Delta(\Delta G^b)$ | $K_{d,\text{NADP}}$ ( $\mu\text{M}$ ) | $\Delta G^b$ | $\Delta(\Delta G^b)$ |
| Wild type | 2.72                                 | -31.7        | 0                    | 4.26                                  | -30.0        | 0                    |
| S156D     | 0.19                                 | -37.6        | -5.9                 | 30.4                                  | -25.3        | 4.7                  |
| S156H     | 95.4                                 | -22.5        | 9.2                  | 0.21                                  | -37.3        | -7.3                 |

<sup>a</sup> All energy parameters are represented in kJ mol<sup>-1</sup>.  $\Delta G^b$  is the free binding energy. Log  $K$ ,  $K_d$ , and  $\Delta G^b$  values were calculated using the following mathematical relation:  $\Delta G^b = -RT \ln(1/K_d)$ , where  $K_d$  is the dissociation constant,  $R$  is the ideal gas constant, and  $T$  is the absolute temperature in kelvins.



proposed. The S157 residue interacts with the C2 –OH group of the substrate via hydrogen bonding and plays an important role in substrate recognition. Subsequently, the K174 residue lowers the  $pK_a$  of the phenolic –OH group of Y170 via electrostatic interaction, and the resultant deprotonated Y170 residue forms a hydrogen bond with the substrate. The deprotonated Y170 functions as a catalytic base to extract a proton from the substrate. Simultaneously, the cofactor  $\text{NAD(P)}^+$  accepts a hydride ion transferred from the substrate to the C4 of the nicotinamide ring. The interaction of S156 residue with  $\text{NAD}^+$  or  $\text{NADP}^+$  (Fig. 4) probably results in the formation of a stable enzyme-cofactor complex and suitable orientation for the occurrence of a dehydrogenation reaction. Likewise, when  $\text{NAD}^+/\text{NADP}^+$  was docked into the active-site pocket of the wild-type ZmRDH, hydrogen bonds with bond lengths of 2.7/2.9 Å and 3.1/2.7 Å, respectively, were observed between the ribose ring of the  $\text{NAD}^+/\text{NADP}^+$  and the S156 residue (Fig. 4A and B). The mutant S156A showed similar interactions with the wild-type ZmRDH. However, no hydrogen bonding was observed between the residue D156 in the mutant S156D and  $\text{NADP}^+$ , resulting in a longer bond length between the ribose ring of  $\text{NADP}^+$  and the active-site residue Y170 (Fig. 4C and D). This increased bond length in case of the S156D mutant adequately explained the higher  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value (i.e., 10.9) for this mutant. On the other hand, when Ser was replaced with His, the  $(k_{\text{cat}}/K_{m,\text{NAD}})/(k_{\text{cat}}/K_{m,\text{NADP}})$  value (i.e., 0.11) was retained at its minimum, since the length of the hydrogen bond between  $\text{NADP}^+$  and the active-site Y170 residue was less than 2.8 Å (Fig. 4E and F). The role of the Ser residue at position 156 in  $\text{NAD}^+/\text{NADP}^+$  binding was confirmed on the basis of the  $\Delta G^b$  and  $K_d$  values obtained for  $\text{NAD}^+/\text{NADP}^+$  binding of the ZmRDH variants in ITC studies. These results highlight the importance of position 156 in modulating the cofactor specificity of SDRs.

Q362K single mutation of mitochondrial  $\text{NAD(P)}^+$ -dependent malic enzyme shifted its cofactor preference from  $\text{NAD}^+$  to  $\text{NADP}^+$ . The  $(k_{\text{cat}}/K_{m,\text{NADP}})$  value for the Q362K mutant increased by 27-fold compared to the wild-type enzyme (16). The D211S/I212R mutant of *Neurospora crassa* l-arabinitol 4-dehydrogenase displayed a 34-fold increase in  $k_{\text{cat}}/K_{m,\text{NADP}}$  compared to that of the wild type (1). The D39N mutation of *Drosophila* alcohol dehydrogenase showed a 20-fold increase in  $k_{\text{cat}}/K_{m,\text{NADP}}$  compared to that of the wild type (11), and the D53S mutation of NADH-linked lactate dehydrogenase shifted the cofactor specificity by 20-fold toward NADPH (14). Furthermore, a double mutant (E175A A176R) of *Pseudomonas stutzeri* phosphite dehydrogenase uses  $\text{NADP}^+$  with 1,000-fold greater efficiency ( $k_{\text{cat}}/K_{m,\text{NADP}}$ ) and  $\text{NAD}^+$  with 3.6-fold greater efficiency ( $k_{\text{cat}}/K_{m,\text{NAD}}$ ) than the wild type (36). Although many studies on the determinants of cofactor specificity have been reported, the role of S156 in polyol dehydrogenases has never been reported. In the present study, ZmRDH demonstrated significant changes in cofactor specificity upon single mutagenesis at S156 position.

The modulation of cofactor specificity described here was achieved by structural modeling of the SBP with cofactors ( $\text{NAD}^+$  and  $\text{NADP}^+$ ) and subsequent site-directed mutagenesis for the residues bound to the cofactor. On the basis of MD studies, we predicted that the S156 residue binds to the cofactor ( $\text{NAD}^+$  or  $\text{NADP}^+$ ) by hydrogen bond interactions (Fig. 4). The S156 residue was completely conserved in SDRs using both  $\text{NAD}^+$  and  $\text{NADP}^+$ . The enzyme kinetics data in combination with the data

from MD and ITC studies suggest that the cofactor specificity of ZmRDH can be modulated by manipulating the amino acid residue at position 156. These results should help to elucidate SDR enzyme catalysis and ultimately enable the engineering of enzymes with tailor-made cofactor specificity.

## ACKNOWLEDGMENTS

This research was supported by the Converging Research Center Program through the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology (grant 2011-50210). This study also was supported by the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Education, Science, and Technology, Republic of Korea.

## REFERENCES

- Bae B, Sullivan RP, Zhao H, Nair SK. 2010. Structure and engineering of l-arabinitol 4-dehydrogenase from *Neurospora crassa*. *J. Mol. Biol.* 402: 230–240.
- Banta S, Anderson S. 2002. Verification of a novel NADH-binding motif: combinatorial mutagenesis of three amino acids in the cofactor-binding pocket of *Corynebacterium* 2,5-diketo-D-gluconic acid reductase. *J. Mol. Evol.* 55:623–631.
- Banta S, Boston M, Jarnagin A, Anderson S. 2002. Mathematical modeling of in vitro enzymatic production of 2-keto-L-gulonic acid using NAD(H) or NADP(H) as cofactors. *Metab. Eng.* 4:273–284.
- Banta S, Swanson BA, Wu S, Jarnagin A, Anderson S. 2002. Optimizing an artificial metabolic pathway: engineering the cofactor specificity of *Corynebacterium* 2,5-diketo-D-gluconic acid reductase for use in vitamin C biosynthesis. *Biochemistry* 41:6226–6236.
- Bocanegra JA, Scrutton NS, Perham RN. 1993. Creation of an NADP-dependent pyruvate dehydrogenase multienzyme complex by protein engineering. *Biochemistry* 32:2737–2740.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Carugo O, Argos P. 1997. NADP-dependent enzymes. I. Conserved stereochemistry of cofactor binding. *Proteins* 28:10–28.
- Carugo O, Argos P. 1997. NADP-dependent enzymes. II. Evolution of the mono- and dinucleotide binding domains. *Proteins* 28:29–40.
- Chen R, Greer A, Dean AM. 1995. A highly active decarboxylating dehydrogenase with rationally inverted coenzyme specificity. *Proc. Natl. Acad. Sci. U. S. A.* 92:11666–11670.
- Chen R, Greer A, Dean AM. 1996. Redesigning secondary structure to invert coenzyme specificity in isopropylmalate dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.* 93:12171–12176.
- Chen Z, Lee WR, Chang SH. 1991. Role of aspartic acid 38 in the cofactor specificity of *Drosophila* alcohol dehydrogenase. *Eur. J. Biochem.* 202: 263–267.
- Chin JW, Khankal R, Monroe CA, Maranas CD, Cirino PC. 2009. Analysis of NADPH supply during xylitol production by engineered *Escherichia coli*. *Biotechnol. Bioeng.* 102:209–220.
- Danielson UH, Jiang F, Hansson LO, Mannervik B. 1999. Probing the kinetic mechanism and coenzyme specificity of glutathione reductase from the *Cyanobacterium anabaena* PCC 7120 by redesign of the pyridine-nucleotide-binding site. *Biochemistry* 38:9254–9263.
- Feeney R, Clarke AR, Holbrook JJ. 1990. A single amino acid substitution in lactate dehydrogenase improves the catalytic efficiency with an alternative coenzyme. *Biochem. Biophys. Res. Commun.* 166:667–672.
- Ghosh D, et al. 2001. Porcine carbonyl reductase. structural basis for a functional monomer in short chain dehydrogenases/reductases. *J. Biol. Chem.* 276:18457–18463.
- Hsieh JY, Liu GY, Chang GG, Hung HC. 2006. Determinants of the dual cofactor specificity and substrate cooperativity of the human mitochondrial  $\text{NAD(P)}^+$ -dependent malic enzyme: functional roles of glutamine 362. *J. Biol. Chem.* 281:23237–23245.
- Johannes TW, Woodyer RD, Zhao H. 2005. Directed evolution of a thermostable phosphite dehydrogenase for  $\text{NAD(P)H}$  regeneration. *Appl. Environ. Microbiol.* 71:5728–5734.
- Kallberg Y, Oppermann U, Jornvall H, Persson B. 2002. Short-chain dehydrogenases/reductases (SDRs). *Eur. J. Biochem.* 269:4409–4417.

19. Khoury GA, et al. 2009. Computational design of *Candida boidinii* xylose reductase for altered cofactor specificity. *Protein Sci.* 18:2125–2138.
20. Lovell SC, et al. 2003. Structure validation by  $\text{C}\alpha$  geometry:  $\phi$ ,  $\psi$ , and  $\text{C}\beta$  deviation. *Proteins* 50:437–450.
21. Minteer SD, Liaw BY, Cooney MJ. 2007. Enzyme-based biofuel cells. *Curr. Opin. Biotechnol.* 18:228–234.
22. Mittl PR, Berry A, Scrutton NS, Perham RN, Schulz GE. 1994. Anatomy of an engineered NAD-binding site. *Protein Sci.* 3:1504–1514.
23. Moon HJ, Tiwari M, Jeya M, Lee JK. 2010. Cloning and characterization of a ribitol dehydrogenase from *Zymomonas mobilis*. *Appl. Microbiol. Biotechnol.* 87:205–214.
24. Nakanishi M, et al. 1997. Switch of coenzyme specificity of mouse lung carbonyl reductase by substitution of threonine 38 with aspartic acid. *J. Biol. Chem.* 272:2218–2222.
25. Oppermann U, et al. 2003. Short-chain dehydrogenases/reductases (SDR): the 2002 update. *Chem. Biol. Interact.* 143–144:247–253.
26. Schepens I, et al. 2000. Inhibition of the thioredoxin-dependent activation of the NADP-malate dehydrogenase and cofactor specificity. *J. Biol. Chem.* 275:20996–21001.
27. Scrutton NS, Berry A, Perham RN. 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* 343:38–43.
28. Serov AE, Popova AS, Fedorchuk VV, Tishkov VI. 2002. Engineering of coenzyme specificity of formate dehydrogenase from *Saccharomyces cerevisiae*. *Biochem. J.* 367:841–847.
29. Steen IH, Lien T, Madsen MS, Birkeland NK. 2002. Identification of cofactor discrimination sites in NAD-isocitrate dehydrogenase from *Pyrococcus furiosus*. *Arch. Microbiol.* 178:297–300.
30. Tanaka N, et al. 1996. Crystal structures of the binary and ternary complexes of 7  $\alpha$ -hydroxysteroid dehydrogenase from *Escherichia coli*. *Biochemistry* 35:7715–7730.
31. Tiwari M, Lee JK. 2010. Molecular modeling studies of L-arabinitol 4-dehydrogenase of *Hypocrea jecorina*: its binding interactions with substrate and cofactor. *J. Mol. Graph Model.* 28:707–713.
32. Varughese KI, Xuong NH, Kiefer PM, Matthews DA, Whiteley JM. 1994. Structural and mechanistic characteristics of dihydropteridine reductase: a member of the Tyr-(Xaa)<sup>3</sup>-Lys-containing family of reductases and dehydrogenases. *Proc. Natl. Acad. Sci. U. S. A.* 91:5582–5586.
33. Wang H, Lei B, Tu SC. 2000. *Vibrio harveyi* NADPH-FMN oxidoreductase arg203 as a critical residue for NADPH recognition and binding. *Biochemistry* 39:7813–7819.
34. Wiegert T, Sahm H, Sprenger GA. 1997. The substitution of a single amino acid residue (Ser-116→Asp) alters NADP-containing glucose-fructose oxidoreductase of *Zymomonas mobilis* into a glucose dehydrogenase with dual coenzyme specificity. *J. Biol. Chem.* 272:13126–13133.
35. Wierenga RK, Terpstra P, Hol WG. 1986. Prediction of the occurrence of the ADP-binding  $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* 187:101–107.
36. Woodyer R, van der Donk WA, Zhao H. 2003. Relaxing the nicotinamide cofactor specificity of phosphite dehydrogenase by rational design. *Biochemistry* 42:11604–11614.
37. Yamamoto K, et al. 2001. Crystal structure of glucose dehydrogenase from *Bacillus megaterium* IWG3 at 1.7 Å resolution. *J. Biochem.* 129:303–312.