



Alteration of coenzyme specificity of malate dehydrogenase from *Streptomyces coelicolor* A3(2) by site-directed mutagenesis

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ABSTRACT. We describe here for the first time the alteration of coenzyme specificity of malate dehydrogenase (MDH) from *Streptomyces coelicolor* A3(2) (*ScMDH*). In the present study, we replaced four amino acid residues in the Rossmann fold ($\beta\text{B}-\alpha\text{C}$) region of NADH-dependent *ScMDH* by site-directed mutagenesis with those of NADPH-dependent MDH (Glu42Gly, Ile43Ser, Pro45Arg, and Ala46Ser). The coenzyme specificity of the mutant enzyme (*ScMDH*-T4) was examined. Coenzyme specificity of *ScMDH*-T4 was shifted 2231.3-fold toward NADPH using $k_{\text{cat}}/K_{\text{m}}^{\text{coenzyme}}$ as the measurement of coenzyme specificity. Accordingly, the effect of the replacements on coenzyme specificity is discussed. Our work provides further insight into the coenzyme specificity of *ScMDH*.

Key words: Malate dehydrogenase; Coenzyme specificity; *Streptomyces coelicolor*; Site-directed mutagenesis

INTRODUCTION

Malate dehydrogenase (MDH) catalyzes the reversible conversion of malate to oxalacetate using NAD^+ or NADP^+ as the coenzyme (Madern et al., 2004; Hara et al., 2006). It plays crucial roles in many metabolic pathways, including the tricarboxylic acid cycle, amino acid synthesis, gluconeogenesis, maintenance of the oxidation/reduction balance and metabolic stress (Goward and Nicholls, 1994; Lee et al., 2009; Liu et al., 2013; Menckhoff et al., 2013).

MDH can be divided into NAD^+ -dependent MDH (NAD-MDH) (EC 1.1.1.37) and NADP^+ -dependent MDH (NADP-MDH) (EC 1.1.1.82) (Lee et al., 2009), according to preference for cofactors. Most bacterial and archaeal MDHs are NAD-MDH. Eukaryotic MDH isoforms are all NAD-MDH, including mitochondrial MDH (mMDH), cytosolic MDH (cMDH), glyoxysomal MDH (gMDH), and chloroplastic NAD-MDH (cnMDH), except for chloroplastic NADP-MDH (chMDH), which is required for the transfer of reducing equivalents from chloroplast stroma to cytosol (Hara et al., 2006).

There are various NAD(P)(H)-dependent oxidoreductases with a Rossmann fold ($\beta\beta\text{-}\alpha\text{C}$) for dinucleotide binding. The coenzyme-binding domains in the dehydrogenase enzyme family have been thoroughly investigated, revealing a conserved $\beta\beta\text{-}\alpha\text{C}$ motif of the Rossmann fold (Rossmann et al., 1974; Lesk, 1995). The ability of the dehydrogenases to discriminate against NADP(H) lies in the amino acid sequence of this $\beta\beta\text{-}\alpha\text{C}$ motif, which has been predicted to be a principal determinant for coenzyme specificity. By replacing the corresponding region of NAD(H)-MDH from *Thermus flavus* (*Tf*MDH) in the Rossmann fold with those of NADP(H)-MDH, tMDH was successfully altered from an NAD-dependent enzyme to NADP-dependent enzyme, showing that the preference of mutant *Tf*MDH for NADPH over NADH is mainly due to the combined recognition of the 2'-phosphate group of NADPH by four amino acid residues (Gly41, Ser42, Arg44, and Ser45) from NADP(H)-MDH in the Rossmann fold (Tomita et al., 2006b).

Several attempts to alter the coenzyme specificity have been made for several dehydrogenases, and complete reversal was accomplished in xylitol dehydrogenase from *Pichia stipitis* and *Saccharomyces cerevisiae* (Watanabe et al., 2005; Krahulec et al., 2012), alcohol dehydrogenase from *Rana perezi* (Rosell et al., 2003) and lactate dehydrogenases from *T. flavus* (Tomita et al., 2006a). However, the information on alteration of coenzyme specificity in bacterial MDHs is scarce, except for *Tf*MDH.

Streptomyces (multicellular prokaryotes) are Gram-positive soil bacteria that carry out complex morphological differentiation in the formation of filamentous mycelium-like fungi (eukaryotes), an example of convergent evolution caused by adaptation to similar ecological niches. Also, they are model prokaryotes for the study of evolution and secondary metabolism. Previously, MDH from *Streptomyces coelicolor* A3(2) (*Sc*MDH) was overexpressed in *Escherichia coli* and its biochemical properties were identified in detail (Ge et al., 2010). *Sc*MDH is an NAD(H)-dependent thermostable enzyme composed of two identical subunits. However, the information on the coenzyme specificity evolution of *Sc*MDH is very limited.

To get a preliminary understanding of the molecular mechanism of coenzyme evolution relationship between NADP-MDH and NAD-MDH, in this study, we introduced four point mutations (Glu42Gly, Ile43Ser, Pro45Arg, and Ala46Ser) by site-directed mutagenesis into *Sc*MDH and examined the coenzyme specificity of the resulting enzyme. The amino acid substitution in the Rossmann fold markedly shifted the coenzyme specificity of *Sc*MDH toward NADPH. To our knowledge, this is the first report on the coenzyme specificity of MDH from *Streptomyces*.

MATERIAL AND METHODS

Strains and reagents

E. coli DH5 α was used as the host for genetic cloning. *E. coli* Rosetta (DE3) and plasmid pET-28b(+) were used for protein expression. PureYield™ Plasmid Miniprep System kit and Wizard® SV Gel and PCR Clean-Up System kit were from Promega (Madison, WI, USA). Restriction enzymes were from Sangon Biotech (Shanghai, China). All PCR reactions were performed with PrimeSTAR® HS DNA Polymerase (TaKaRa, Japan). Proteins were purified using BD TALON® xTractor Buffer and TALON Metal Affinity Resins (Clontech, Beijing, China). Oxaloacetate (OAA) was from Sigma-Aldrich (Shanghai, China). NAD(P)⁺ and NAD(P)H were from Bio Basic Inc. (Markham, Canada). Primers were synthesized by GenScript Co., Ltd. (Nanjing, China). The *ScMDH* gene was cloned from the recombinant plasmid pScMDH (Ge et al., 2010).

Primer design

The synthetic oligonucleotide primers were designed with the aid of the Primer Premier 5.0 software (Premier, Canada) and were based on the recombinant plasmid pScMDH. Four oligonucleotides were used: two flanking primers were positioned upstream (U) and downstream (D) of the mutation site, and two mutagenic primers, forward mutagenic (FM) and reverse mutagenic (RM) with a 20-bp overlap between adjacent fragments. The mutation site was located in the middle of the mutagenic primers. T4 FM and T4 RM were used as mutagenic primers for the construction of mutant enzyme having the Glu42Gly, Ile43Ser, Pro45Arg and Ala46Ser exchanges; the mutant was named *ScMDH*-T4. The primer sequences used in this study are shown in Table 1.

Table 1. Mutagenic and flanking primers.

Primers	Sequence (5' to 3') ^a
U	GAGACACCGGCATACTCTGCGACAT
D	CGCCCC <u>TCGAG</u> TCAGATGAGGCCGAGACCGCGC
T4 FM	CTTGAGactCcGGGTGcTCcCCAGGAGGCCGAGCTTGACC
T4 RM	gGAgCACCCgGagtCTCAAGGCCGCCGAGGGCACCGCGATGGA

^aNucleotide substitution is shown in boldface and lower case; underlined nucleotides (CTCGAG) represent the restriction site for *Xho*I enzyme.

Site-directed mutagenesis

Purification of recombinant plasmid pScMDH DNA was performed using the Wizard® purification kit. Site-directed mutations were introduced into the *ScMDH* gene by Simionatto's method (Simionatto et al., 2009). It was carried out according to a two-step PCR procedure to replace the mutated codons using an overlap extension-PCR method. Briefly, in the first step, two simultaneous PCRs were performed. One reaction was performed with a primer pair that included the U primer and the RM primer; the other reaction contained the D primer and the FM primer. PCRs were carried out using 1 ng recombinant plasmid pScMDH DNA, 40 nM

dNTPs, 10 pM of each primer, 1.3 U PrimeSTAR® HS DNA Polymerase, and 1X reaction buffer in a 50- μ L reaction volume. The amplification conditions were 3 min at 98°C followed by 30 cycles of 20 s at 98°C, 25 s at 65°C and 60 s at 72°C and a final extension of 5 min at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis and gel-purified using the Wizard® SV Gel and PCR Clean-Up System kit, according to manufacturer instructions. To obtain full-length mutated fragments, the two products from the first PCR were mixed at equimolar concentrations (approximately 1.5 pM) and used as templates during the second PCR, with the U and D primers. The reaction was performed using the same conditions as the first PCR except that the annealing temperature and extension time were 55°C and 100 s, respectively. This overlap extension-PCR yielded a full-length DNA fragment, which was gel-purified before cloning. The PCR product was digested with *Nde*I and *Xho*I and ligated into pET-28b(+)His-tag expression vector. The mutated *ScMDH* gene was confirmed by DNA sequencing. After confirming the presence of the mutated codons, the mutated plasmids were transformed into *E. coli* Rosetta cells (DE3). The recombinant mutant *ScMDH*-T4 was expressed and purified as previously described for the wild-type *ScMDH* (Ge et al., 2010).

Kinetic assays and data processing

Wild-type *ScMDH* and mutant *ScMDH*-T4 activity studies were performed in 100 mM Tris-HCl, pH 8.5, buffer according to the methods previously reported (Ge et al., 2010). The reaction was monitored by measuring the decrease in absorbance of NAD(P)H at 340 nm with a thermostated Cary 300 UV-Vis spectrophotometer (Varian, Shanghai, China) using a molar extinction coefficient of 6220 M⁻¹·cm⁻¹. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μ mol NAD(P)H/min under the assay conditions (30°C). All kinetic parameters were obtained from at least triplicate measurements. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin as standard.

Molecular simulation of wild-type *ScMDH* complex with NADH

Molecular simulation was carried out with the PyMOL-molecular visualization system and the SWISS-MODEL Workspace web tool (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1). The crystal structure of *Tj*MDH complex with NADH (PDB accession No. 1BMD) was used as an initial model of molecular simulation. The predicted three-dimensional structure of wild-type *ScMDH* complex with NADH was modeled.

RESULTS

Coenzyme specificity of wild-type *ScMDH* and mutant *ScMDH*-T4

The steady-state kinetic parameters of wild-type *ScMDH* and mutant *ScMDH*-T4 were determined with both coenzymes (NADH and NADPH) in the presence of OAA. The mutant enzyme was assumed to have the same kinetic mechanism as the wild-type enzyme. As shown in Table 2, wild-type *ScMDH* showed a significant preference for NADH over NADPH with 13.4-fold decreased K_m value for NADH and a 15-fold larger k_{cat} value for the reaction. As a consequence, wild-type *ScMDH* showed 218.7-fold coenzyme specificity for NADH over NADPH

taking $k_{\text{cat}}/K_{\text{m}}^{\text{coenzyme}}$ as a measure. The four-amino-acid replacement in *ScMDH*-T4 caused 4.8-fold increase in the K_{m} value for NADH, but the k_{cat} value was not decreased too much. As for the NADPH-dependent reaction, the replacement decreased the K_{m} value for NADPH by 8.2-fold. As a consequence, the K_{m} value for NADH was about 3-fold larger than that for NADPH in *ScMDH*-T4. The most dramatic change in the parameters by the amino acid replacement was found in the k_{cat} values. As described above, the k_{cat} value in the NADH-dependent reaction decreased a little. However, the replacement increased the k_{cat} value for NADPH oxidation by 47.5-fold, resulting in a 3.5-fold larger k_{cat} value in the NADPH-dependent reaction as compared with that in the NADH-dependent reaction. As a result, when $k_{\text{cat}}/K_{\text{m}}^{\text{coenzyme}}$ value was again considered as a measure of the catalytic efficiency of the enzyme, *ScMDH*-T4 increased 423.7-fold for NADPH and decreased 5.3-fold for NADH, compared with the case of wild-type *ScMDH*. When a preference for NADH over NADPH was defined by $(k_{\text{cat}}/K_{\text{m}}^{\text{NADH}})/(k_{\text{cat}}/K_{\text{m}}^{\text{NADPH}})$ (Tomita et al., 2006b; Andreadeli et al., 2008), it showed 218.67 for wild-type *ScMDH* and 0.098 for *ScMDH*-T4 (Table 3). This means that the four-amino-acid replacement shifted the coenzyme specificity of *ScMDH* 2231.3-fold toward NADPH, which is significantly larger than the shift in *TfMDH* alteration of coenzyme specificity, namely 1562.6-fold (Tomita et al., 2006b), as shown in Table 3.

Table 2. Kinetic parameters of wild-type and mutant *ScMDH*.

Enzyme	Coenzyme	K_{m} (μM)	k_{cat} (s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1}\cdot\mu\text{M}^{-1}$)
Wild-type <i>ScMDH</i>	NADH	82.58	542.00	6.56
	NADPH	1106.48	36.07	0.03
<i>ScMDH</i> -T4	NADH	399.72	494.54	1.24
	NADPH	134.66	1711.54	12.71

Table 3. Comparison of the degree of alteration of coenzyme specificity toward NADPH in *ScMDH*-T4 with the mutant MDH from *Thermus flavus*.

Enzyme	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1}\cdot\mu\text{M}^{-1}$)		Preference (A)/(B)	Degree of alteration
	NADH (A)	NADPH (B)		
Wild-type <i>ScMDH</i>	6.56	0.03	218.67	2231.3
<i>ScMDH</i> -T4	1.24	12.71	0.098	
<i>TfMDH</i>	61.1	1.7	35.94	1562.6
<i>TfMDH</i> -EX4	1.1	48.3	0.023	

In silico simulation of wild-type *ScMDH* structure

To understand the reason for the shift of the coenzyme specificity of *ScMDH* in detail, structural information is necessary. Although the high resolution three-dimensional structure of *ScMDH* has not yet been determined, the amino acid sequence of *ScMDH* is very similar to that of *TfMDH*, with 65% identity, and there is only one gap, located at a position far from the active site and the coenzyme binding site, between the two MDHs. Furthermore, they have high similar amino acid sequence in the Rossmann fold ($\beta\text{B}-\alpha\text{C}$) for dinucleotide binding (Figure 1). Hence, we performed *in silico* modeling of the three-dimensional structure of wild-type *ScMDH* using the crystal structure of *TfMDH* complex with NADH (PDB entry 1BMD) as the initial template structure (Figure 2A). As can be seen in Figure 2B, simulated *ScMDH* structure almost superimposes with the *TfMDH* binary complex with NADH.

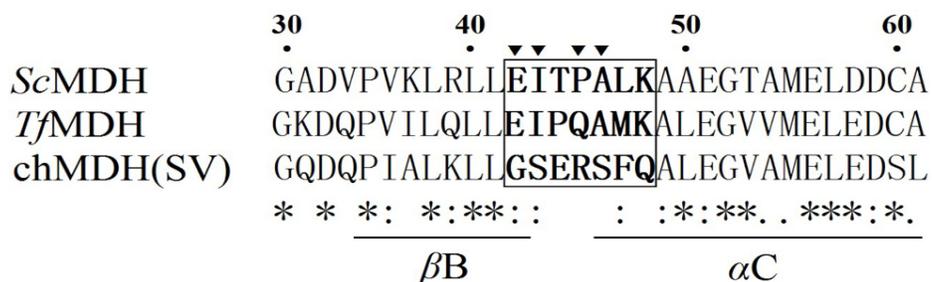


Figure 1. Alignment of the amino acid sequences in the region between β -strand B and α -helix C. *Sc*MDH, *Tf*MDH, and chMDH(SV) represent MDHs from *Streptomyces coelicolor* A3(2), *Thermus flavus* and *Sorghum vulgare* chloroplasts, respectively. The replaced amino acid residues in mutant *Sc*MDH was indicated by inverted dark triangle. The alignment was done using the CLUSTAL W 2.0. The residues in the β B- α C loop are boxed and the location of β -strand B and α -helix C are marked below the sequences. The amino acid sequence numbers above the sequences are those for *Sc*MDH.

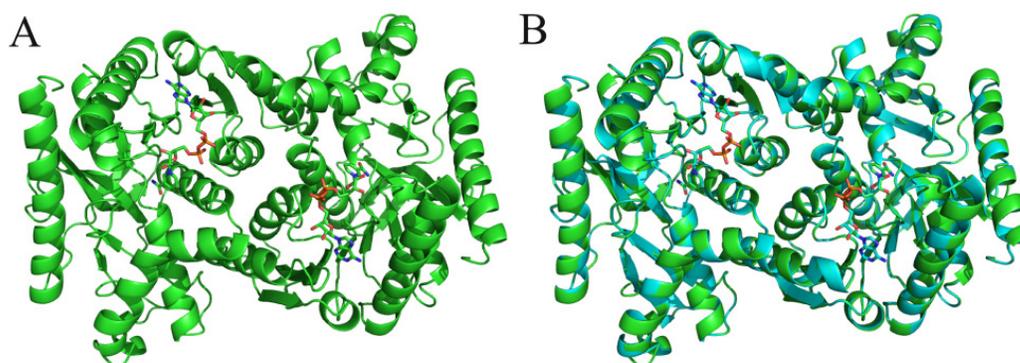


Figure 2. Comparison of the ribbon diagram between *Sc*MDH and *Tf*MDH. **A.** Simulated *Sc*MDH dimer three-dimensional structure binary complex with NADH; **B.** stereoview of a superposition of simulated *Sc*MDH three-dimensional structure (green) on *Tf*MDH (light blue) binary complex with NADH.

DISCUSSION

Previous studies had shown that the preference of mutant *Tf*MDH for NADPH over NADH is mainly determined by four amino acid residues (Gly41, Ser42, Arg44, and Ser45), which corresponded to the amino acid residues in NADPH-dependent chloroplast MDH's Rossmann fold (β B- α C) (Tomita et al., 2006b). The earlier investigations suggested that the hydrogen-bond interactions between two Ser residues (Ser42 and Ser45) and the 2'-phosphate of NADPH are the crucial factors in the high affinity of mutant *Tf*MDH for NADPH, where Gly41 allows the 2'-phosphate group of NADPH to enter the binding site (Tomita et al., 2006b). In addition, Arg44 was presumed to form an additional electrostatic interaction with the 2'-phosphate of NADPH to stabilize the binding in mutant *Tf*MDH (Tomita et al., 2006b).

In this study, we succeeded in altering the coenzyme specificity of MDH from *Streptomyces coelicolor* A3(2) by substituting the Rossmann fold (β B- α C) with the corresponding

four amino acids in NADPH-dependent chloroplast MDH: Glu42Gly, Ile43Ser, Pro45Arg, Ala46Ser. The replacement shifted the coenzyme specificity 2231.3-fold from NADH to NADPH in the *ScMDH-T4* mutant. This result suggested that the preference of *ScMDH-T4* for NADPH over NADH was mainly due to the four-amino-acid replacement from NADP(H)-MDH in the Rossmann fold ($\beta\beta$ - α C).

Structure simulation revealed that the predicted three-dimensional structure of wild-type *ScMDH* was essentially the same as that of *TfMDH* (Figure 2B). The high homology in three-dimensional structure coupled with the high similar amino acid sequence in the Rossmann fold suggested that *ScMDH* had a similar coenzyme catalytic mechanism as *TfMDH*.

In comparison to the results in the four-amino-acid mutant *TfMDH*, in *ScMDH-T4*, the reversal of coenzyme specificity from NADH to NADPH was also achieved with the concerted mutation of four residues (Glu42Gly, Ile43Ser, Pro45Arg, Ala46Ser). It was reported that the negatively charged residue is one of the most principal determinants for the preference for NADH over NADPH, and the reasons for the coenzyme specificity change of MDH lied in the side-chain differences (Tomita et al., 2006b). Thus, the high affinity of *ScMDH-T4* for NADPH over NADH could be speculated on the basis of these possible effects. First, due to electrostatic repulsion along with steric hindrance, the carboxyl group of Glu41 prevents the 2'-phosphate group of NADPH from binding. In *ScMDH-T4*, due to the Glu42Gly mutation, the hydrogen-bonds between the carboxyl group of Glu42 and the O2' and O3' atoms of the adenine ribose moiety of NADH are removed, and the 2'-phosphate group of the adenine ribose in NADPH can enter and occupy the position vacated by the mutation. Second, in *ScMDH-T4*, Ser43 and Ser46 are located too far from the O2' and O3' atoms of the adenine ribose moiety of NADH to form hydrogen-bonds according to the crystal structure of the mutant *TfMDH* complex with NADPH (Tomita et al., 2006b). On the other hand, OPIA of NADPH is hydrogen-bonded to the side-chain of the Ser residues replaced at positions 43 and 46. The hydrogen-bond interactions between these Ser residues, 43 and 46, and the 2'-phosphate of NADPH may be the crucial factors in the tight affinity of *ScMDH-T4* for NADPH; in addition, the importance of the corresponding Arg residues in NADPH specificity has been shown in mutant *TfMDH* and other NADPH-dependent dehydrogenases (Scrutton et al., 1990; Levy et al., 1996; Schepens et al., 2000). In this study, we speculated that the substitution of Pro45Arg in *ScMDH-T4* enabled the Arg45 to form an additional electrostatic interaction with the 2'-phosphate of NADPH to stabilize the binding between the enzyme and the coenzyme.

Notably, phylogenetic analysis indicates that the NADPH-dependent MDHs seem to have descended from NADH-dependent progenitors during MDH gene evolution (Ocheretina et al., 2000; Schnarrenberger and Martin, 2002). However, no conclusive experimental evidence has been provided to support this theory up to now. Site-directed mutagenesis experiments have shown that the coenzyme specificity of MDH is determined by a limited number of amino acid residues (Tomita et al., 2005, 2006b), and that the coenzyme specificity of some dehydrogenases could be shifted by alteration of a few specific amino acids in the enzyme to adapt to the environment during the enzyme's evolution (Zhu et al., 2005). In the present study, we altered the coenzyme specificity of MDH from a Gram-positive bacterium *S. coelicolor* A3(2). Together with the study of coenzyme specificity alteration in *TfMDH*, our investigation of *ScMDH* may give new experimental evidence for the coenzyme specificity evolution theory of MDH described above. That is to say, the coenzyme specificity of MDH can be altered just by a few amino acids substitution at crucial sites of the enzyme. Moreover,

amino acid substitution is not a difficult event during the long evolutionary history of MDH. Thus, it can be supposed that the coenzyme specificity of an NADPH-MDH ancestor was shifted from NADH toward NADPH by the mutation of just a few amino acids in response to the external pressures from living environment.

In summary, our study describes for the first time the alteration of coenzyme specificity of MDH from *Streptomyces*. The results showed that ScMDH was successfully altered from an NADH-dependent to an NADPH-dependent enzyme by a four-amino-acid substitution. This work provides the further insight into the universality of coenzyme conversion and the coenzyme specificity of ScMDH. The crystal structures of ScMDH and ScMDH-T4 require further investigation to support the speculated reasons for coenzyme specificity alternation in ScMDH.

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