Identification and Biochemical Characterization of a Thermostable Malate Dehydrogenase from the Mesophile Streptomyces coelicolor A3(2)

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We identified and characterized a malate dehydrogenase from Streptomyces coelicolor A3(2) (ScMDH). The molecular mass of ScMDH was 73,353.5 Da with two 36,675.0 Da subunits as analyzed by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS). The detailed kinetic parameters of recombinant ScMDH are reported here. Heat inactivation studies showed that ScMDH was more thermostable than most MDHs from other organisms, except for a few extremely thermophilic bacteria. Recombinant ScMDH was highly NAD$^{+}$-specific and displayed about 400-fold ($k_{\text{cat}}$) and 1,050-fold ($k_{\text{cat}}/K_{m}$) preferences for oxaloacetate reduction over malate oxidation. Substrate inhibition studies showed that ScMDH activity was inhibited by excess oxaloacetate ($K_i = 5.8 \text{ mM}$) and excess 1-malate ($K_i = 12.8 \text{ mM}$). Moreover, ScMDH activity was not affected by most metal ions, but was strongly inhibited by Fe$^{2+}$ and Zn$^{2+}$. Taken together, our findings indicate that ScMDH is significantly thermostable and presents a remarkably high catalytic efficiency for malate synthesis.

Key words: malate dehydrogenase; matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS); thermostability; catalytic efficiency; Streptomyces coelicolor

Malate dehydrogenase (MDH) belongs to the superfamily of 2-ketoadic NAD(P)$^{+}$-dependent dehydrogenases, and catalyzes the reversible conversion of malate to oxaloacetate utilizing the NAD$^{+}$ or the NADP$^{+}$ cofactor system.\(^1\)\(^2\) 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.\(^3\)\(^-\)\(^5\) For example, in the case of the malate-aspartate shuttle, involved in gluconeogenesis, the mitochondrial MDH (mMDH) and the cytosolic MDH (cMDH) are required for good balance of the NAD/NADH ratio between the mitochondrial and cytosolic pools.

According to preference for cofactors, 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).\(^5\) NAD-MDH is generally organized as dimers or tetramers with subunit molecular mass of 30–37 kDa, whereas NADP-MDH possesses a larger subunit, of about 42 kDa.\(^1\)\(^3\) Most bacterial and archaeal MDHs are NAD-MDH. Eukaryotic MDH isoforms are all NAD-MDH, including cMDH, mMDH, glyoxysomal MDH (gMDH), and chloroplastic NAD-MDH (cmMDH), except for chloroplastic NADP-MDH (chMDH), which is required for the transfer of reducing equivalents from chloroplast stroma to cytosol.\(^3\)

The crystal structures of the MDHs of many species have been determined at high resolution, including NAD-MDH from bacteria Escherichia coli and Thermus flavus,\(^6\)\(^7\) the archaeon Haloarcula marismortui, porcine cytoplasm, and mitochondria,\(^8\)\(^-\)\(^10\) and NADP-MDH from the chloroplasts of the C4 plants Flaveria bidentis and Sorghum vulgare.\(^1\)\(^1\)\(^2\) The structure of MDHs from Streptomyces has not been analyzed, except for preliminary diffraction studies of Streptomyces aureofaciens MDH.\(^13\)

Phylogenetic analysis indicates that the lineage of MDH isozymes shows three groups.\(^2\)\(^3\)\(^4\)\(^14\)\(^15\) The first clade includes most eubacterial (e.g., E. coli) MDHs, mMDHs, and cMDHs. The second clade comprises some eubacterial (e.g., T. flavus) MDHs, cMDHs, and chMDHs. The third clade contains all MDHs, which occupy intermediate phylogenetic positions and have high amino acid sequence identity and similar crystal structures (tetramer) to lactate dehydrogenase (LDH) subfamily instead of the other two dimeric MDH groups. Thus, LDHs appear to have evolved from LDH-like MDHs by very recent gene duplication,\(^2\)\(^1\)\(^6\) as confirmed by the conversion of

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Abbreviations: MDH, malate dehydrogenase; NAD-MDH, NAD-dependent MDH; NADP-MDH, NADP-dependent MDH; cMDH, cytosolic MDH; mMDH, mitochondrial MDH; gMDH, glyoxysomal MDH; cmMDH, chloroplastic NAD-MDH; LDH, lactate dehydrogenase; IPTG, isopropyl β-D-1-thiogalactopyranoside; MALDI-TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; SA, sinapinic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide

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E. coli MDH into an LDH and of Bacillus steatorrhophilus LDH into a highly specific MDH.\(^{17,18}\)

MDH is a widespread enzyme widely distributed throughout the three domains of life.\(^{1,5}\) Although it has been isolated and studied extensively from many diverse sources, information on bacterial MDHs is scarce, and biochemical analyses of them are not yet as well known as eukaryotic MDHs. 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 the most important industrial producers of antibiotics and other secondary metabolites. As they are model prokaryotes for the study of evolution and secondary metabolism, the complete genome sequences of several Streptomyces species have been done,\(^{19}\) but information on MDHs from Streptomyces is very limited.\(^{5,20}\)

Streptomyces coelicolor A3(2) was the first strain of Streptomyces to be genome sequenced.\(^{19}\) It can produce numerous antibiotics and bioactive substances, including actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, and methylenomycin. Hence this strain is a potential resource for drug discovery. The investigation of key enzymes in metabolism should contribute to metabolic engineering for the development and utilization of S. coelicolor A3(2). In this study, thermostable MDH from S. coelicolor A3(2) (ScMDH) was expressed in E. coli as a fusion protein. Then the protein was purified and its biochemical properties were determined in detail. To our knowledge, this is the first report on the kinetics (\(V_{\text{max}}\) and \(k_{\text{cat}}\)), the optimal temperature, the thermostability, and the metal ion dependence of MDH from Streptomyces. MDH is widely used in NAD(H) regeneration, antigen immunoassay, and bioreactors. Enzymatic analysis of ScMDH might provide basic information for its utilization.

Materials and Methods

**Strains and reagents.** Streptomyces coelicolor A3(2) was purchased from the China General Microbiological Culture Collection Center (Beijing, China). E. coli DH5\(\alpha\) was used as the host for genetic cloning. E. coli Rosetta (DE3) harboring pSCMDH was cultured in LB medium containing kanamycin (30 \(\mu\)g/ml) and chloramphenicol (30 \(\mu\)g/ml) at 37°C, and then inoculated into fresh LB medium until the density reached an \(OD_{600}\) of 0.5–0.6. Isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) was then added to the culture at a final concentrations of 0.1, 0.3, 0.5, 0.7, and 0.9 mM with subsequent cultivation for 4–18 h at 20°C and 37°C respectively. Cells were harvested and sonicated in a buffer containing 100 mM K\(_2\)HPO\(_4\) (pH 7.0), 100 mM NaCl, 2 mM MgCl\(_2\), 1 mM EDTA, and 2 mM dithiobiotyl (DTT). The debris was removed by centrifugation at 15,000 \(\times\) g for 10 min at 4°C, and the supernatant was analyzed by SDS-PAGE.

Overexpression of His\(_6\)-tagged ScMDH was carried out under optimal conditions (0.7 mM IPTG at 20°C for 18 h). Cells were harvested and resuspended in BD TALON\(\times\)Tractor Buffer with 0.75 mg/ml of lysozyme and 2 \(\mu\)l of DNase. Debris was removed by centrifugation at 15,000 \(\times\) g for 20 min at 4°C, and the His\(_6\)-tagged proteins were loaded on a Co\(_2\)-metal affinity column and purified using BD TALON Metal Affinity Resins following the manufacturer’s instructions.

**Purity measurement and Western blot assay.** Enzyme purity was measured by discontinuous SDS-PAGE with a 12% separating gel and a 5% stacking gel. The gels were stained with Comassie Brilliant Blue R-250. For Western blot analysis, protein samples (25 \(\mu\)g each) were separated by electrophoresis on 12% SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, New Jersey, USA). The membrane was probed with His\(_6\)-tagged polyclonal antibody (Cell Signaling Technology, Beverly, USA), and alkaline phosphatase conjugated anti-rabbit IgG (Promega, Madison, WI, USA) was used as secondary antibody. Peroxidase reaction products were detected using Lumi-Phos(TM) WB Chemiluminescent reagent (Pierce, Rockford, USA). X-Ray film was exposed to the blots for appropriate time periods, and the chemiluminescence signal corresponding to the specific antibody-antigen reaction was visualized.

**Molecular mass determination.** The molecular mass of the recombinant ScMDH was identified by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS). Peptide mass fingerprints were acquired using a MALDI-TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Foster, USA) (Beijing Proteome Research Center, Beijing, China). The matrix solution was prepared by dissolving sinapinic acid (SA) in acetonitrile (ACN) and H\(_2\)O (1:1 vol/vol) with 0.1% trifluoroacetic acid (TFA). For MALDI-TOF analysis: 0.5 \(\mu\)l of sample was mixed directly onto the target with the matrix solution (1:3 vol/vol), and this was air-dried. Mass spectra were recorded in linear positive mode with a nitrogen laser (wavelength, 337 nm). Positive ions were accelerated at 18.2 kV with 480 ns of pulsed ion extraction delay. In a working mass range of 10,000 to 150,000 \(m/z\), 2,000 laser shots per spot were accumulated. Bovine serum albumin (BSA) was used for external calibration. A mass accuracy of 0.092% for the parent ion and 0.1 Da for product ion masses was used as search parameter.

**Enzyme assay and kinetic analysis.** MDH activity was measured in 1-ml cuvettes (light path, 1 cm) in a standard assay mixture at 30°C. OAA reduction was assayed in a buffer containing 100 mM Tris–HCl (pH 8.5), 0.75 mM oxaloacetate, and 0.16 mM NADH. Malate oxidation was assayed in a buffer containing 100 mM Tris–HCl (pH 8.5), 5 mM L-malate, and 2 mM NAD\(_{+}\).\(^{20}\) The decrease in absorbance at 340 nm of NADP\(_{+}\) was monitored with a thermostated Cary 300 UV–vis spectrophotometer (Varian, Shanghai, China) using a molar extinction coefficient of 6.220 \(\text{mM}^{-1} \text{cm}^{-1}\). One unit (U) of activity was defined as 1 \(\mu\)mol NADP\(_{+}\) formed per min. The kinetic parameters for MDH were determined by measuring the enzyme activity at various substrate (OAA and malate) and co-substrate (NADH and NAD\(_{+}\)) concentrations. The profiles of substrate inhibition of the recombinant ScMDH were determined at 30°C in 100 mM Tris–HCl buffer (pH 8.5). Activity was measured by varying the OAA concentration with 0.32 mM NADH and by varying the L-malate concentration with 2 mM NAD\(_{+}\). All kinetic parameters were obtained from at least three independent measurements. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin as standard.
Optimum pH, optimum temperature, and thermostability. The optimum pH was determined in 100 mM Tris–HCl buffer at pH values ranging from 7.0 to 10.0. Between pH 5.7 and 8.0, 0.1 M sodium phosphate buffer was used instead. The optimal temperature was determined by standard activity assay at temperatures from 40 °C to 60 °C. The effect of temperature on ScMDH activity was determined by incubating the enzyme aliquots for 20 min in a water bath at 10–60 °C. Then aliquots were immediately cooled on ice and assayed for remaining enzyme activity. The thermostability of ScMDH at 50 °C was also determined.

Effects of metal ions and reagents. The effects of monovalent ions (Ag⁺, K⁺, Li⁺, Na⁺, and Rb⁺) and divalent ions (Ca²⁺, Mg²⁺, Cu²⁺, Fe³⁺, Mn³⁺, and Zn²⁺) on ScMDH were determined by measuring activity after incubating it at 30 °C in a buffer containing 100 mM Tris–HCl (pH 8.5), 0.75 mM oxaloacetate, 0.16 mM NADH, and 2 mM ions. The effects of the compounds (EDTA, DTT, DMSO, and Triton X-100) on enzymatic activity were determined by the same procedure, but using these compounds at various concentrations.

Structure-based amino acid sequence alignment. The X-ray structures of T. flavus MDH (18MD), Aquaspirillum arcticum MDH (1BSB), pig mMDH (1MLD), watermelon gMDH (1SEV), and F. bidentis cMDH (1CIV) were downloaded from the PDB web site (http://www.rcsb.org/pdb/). Structure-based amino acid sequence alignment was done using the ClustalX program (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) and the ESPript 2.2 web tool (http://esprit.ibcp.fr/ESPrйдет/ESPrйдет/21-23).

Results

Mass spectrum identification of recombinant ScMDH

Recombinant ScMDH was composed of 329 amino acids with a predicted molecular mass of 36 kDa detected by SDS–PAGE (Fig. 1A). Western blot analysis revealed one specific protein band with the anti-His antibody as probe (Fig. 1B). High-accuracy molecular mass determination of ScMDH was done by MALDI-TOF-MS. The results showed that MALDI-TOF-MS produced distinguishable spectral fingerprints, and that the molecular mass of ScMDH was 73,353.5 Da, with two 36,675.0 Da subunits (Fig. 1C).

Catalytic properties of recombinant malate dehydrogenase

The effects of changes in pH on ScMDH activity in the direction of the OAA reduction were determined in 0.1 M phosphate (pH 5.7–8.0) or 0.1 M Tris–HCl (pH 7.0–10.0). The optimal pH for ScMDH was about pH 6.8 at 50 °C in phosphate buffer and pH 8.5 at 30 °C in Tris–HCl buffer (Fig. 2A). Maximum activity of recombinant ScMDH in OAA reduction was observed at 50 °C (Fig. 2B). ScMDH maintained about 82% of initial activity after incubation for 20 min at 40 °C, and 70% of activity at 50 °C (Fig. 3A). A 90% loss of activity occurred at 55 °C, and ScMDH was totally inactivated after incubation at 60 °C. The thermal stability of ScMDH was investigated at 50 °C. It was found to be stable, and maintained about 71% of initial activity after 30 min, and about 60% after 90 min, showing a half-life (t₁/₂) of 120 min (Fig. 3B).

Kinetic constants were determined, and were summarized in Table 1. Recombinant ScMDH exhibited a high cofactor specificity for NAD(H), being almost inactive with NADP(H) (0.04% of the activity with NADH, data not shown). The V max values for OAA and NADH were much higher than that for malate and NAD⁺ (Table 1). These data provide strong evidence that the highest enzyme turnover (k cat) for ScMDH occurs in the direction of malate synthesis. The calculated k cat/K m ratio (catalytic efficiency) indicated that k cat/K m for OAA reduction was about 1,050-fold of that for malate oxidation. Evidently, ScMDH prefers OAA as a substrate in vitro. Substrate inhibition studies indicated that ScMDH activity was inhibited by excess OAA (50% at 6 mM, K i = 5.8 mM) (Fig. 4A) and excess l-malate (60% at 30 mM, K i = 12.8 mM) (Fig. 4B).

The effects of 12 metal ions and four chemical reagents on ScMDH activity were also examined (Table 2). Evidently, ScMDH activity was not affected by most of the metal ions tested in this study. Activity was inhibited by Zn²⁺ and Co²⁺, resulting in approximately 27% and 64% of the maximal activity (Table 2) respectively. No activity was detected in the presence of Fe³⁺. Neither EDTA nor DTT affected activity. ScMDH activity was reduced with increasing the concentrations of DMSO and Triton X-100 (Table 3).
A BLAST search of the available sequence databases suggested that the deduced amino acid sequence of ScMDH is highly homologous to other MDHs. This search showed that ScMDH shared high amino acid sequence identity to MDHs from the bacteria Thermus flavus (64%) (TfMDH) (GenBank accession no. CAA38008), Burkholderia pseudomallei (62%) (P80536), and A. arcticum (62%) (AAD13225). The calculated sequence identity was 54% for porcine cMDH (P11708), 47% for S. bicolor chMDH (P17606) and 46% for F. bidentis chMDH (P46489). ScMDH showed low sequence identity to E. coli MDH (26%) (ACI77195), and fungal mMDHs, such as Talaromyces emersonii (28%) (AAL40803) and yeast (27%) (AAA34759), and porcine mMDH (16%) (P00346) and tetrameric H. marismortui MDH (25%) (AA73368).

Further analysis of the ScMDH amino acid sequence indicated that each subunit had 14 /C12-sheet and 11/12 /C11-helix, under structure-based sequence alignment with MDHs from Thermus flavus (PDB 1BMD) and Aquaspirillum arcticum (PDB 1B8P) (Fig. 5A). A glycine motif involved in NAD\(^+\) binding, G-A-A-G-Q-I-X (G\(_{12}\)AAGQIG\(_{18}\)), was found in the N-terminal sequence (Fig. 5B), which is highly conserved within the MDH family. ScMDH also had some conserved sequence motifs homologous to other NAD-MDHs, such as NAD\(^+\) binding sites, an active site (proton acceptor), and substrate binding sites (Fig. 5A).
The N-terminal consensus sequence of MDHs is involved in the binding of the ADP portion of NAD\(^+\), and is considered to be the typical MDH signature.\textsuperscript{24,25} Structure-based sequence alignment indicated that the N-terminal amino acid sequence of \(S\)\textsubscript{c}MDH contains a consensus sequence (G\textsubscript{12}AAQQIG\textsuperscript{18}) like all other MDHs from bacteria, archaeans, animals, and plants (Fig. 5B). Residue Q\textsuperscript{16} in ScMDH is specific to all bacterial MDHs and eukaryotic cMDHs. The N-terminal consensus sequences are also conserved among all organelle-bound MDHs,\textsuperscript{24} e.g., G-A-X-G-I-G in mMDHs and gMDHs, and G-A-A-G-M-I-S in chMDHs (Fig. 5B). Furthermore, the N-terminus sequences of organellar MDHs (precursor) contains not only common coenzyme-binding sites but also their own set of motifs responsible for translocation into organelles (Fig. 5B).\textsuperscript{24} Bacterial MDH (\(S\)\textsubscript{c}MDH and \(T\) flavus MDH) appears to be closer to the cMDH group.

The pH optimum of OAA reduction by recombinant \(S\)\textsubscript{c}MDH was found to be pH 6.8 in phosphate buffer (pH buffering capacity within a range of pH 5.8–8.0) at 50 °C, and pH 8.5 in Tris–HCl buffer (pH buffering capacity within a range of pH 7.0–9.0) at 30 °C (Fig. 2A). As compared with other MDHs, the optimum pH of \(S\)\textsubscript{c}MDH at 30 °C was close to MDHs from \(S\) aureofaciens (pH 8.0),\textsuperscript{20} and the fungi Humicola lanuginosa (pH 8.5–9) and \(M\) pusillus (pH 7.8–8.5),\textsuperscript{21} but was much more alkaline than the mMDH from the fungus \(T\) emersonii (pH 7.5).\textsuperscript{20} The optimal temperature for \(S\)\textsubscript{c}MDH in OAA reduction was about 50 °C, although it was rapidly inactivated above this temperature (Fig. 2B), similar to the MDHs from three thermophilic fungi, \(H\) lanuginosa (50 °C), \(M\) pusillus (50 °C), and \(T\) emersonii (52 °C).\textsuperscript{25,26} Thermal inactivation studies have indicated that \(S\)\textsubscript{c}MDH is thermostable, with an estimated \(t\textsubscript{1/2}\) of 120 min at 50 °C (Fig. 3B), much more thermostable than MDHs from thermophilic fungi reported so far,\textsuperscript{27} in spite of the fact that it is less thermostable than MDHs from a few extremely thermophile bacteria. The \(t\textsubscript{1/2}\) values at 50 °C were <10 min for the MDHs from \(P\) duponti, \(S\) pororichum thermophile, and \(T\) aurantius; <30 min for those from \(H\) lanuginosa and \(C\) thermophile var. coprophile; and ≥30 min for those from \(T\) emersonii (\(t\textsubscript{1/2}\) = 30 min) and \(M\) pusillus (\(t\textsubscript{1/2}\) = 60 min).\textsuperscript{25–27} Therefore, \(S\)\textsubscript{c}MDH is a significantly thermostable enzyme from the mesophilic soil organism \(S\) coelicolor A3(2).


discussion

Here we report the cloning and expression of a malate dehydrogenase from \textit{Streptomyces coelicolor} A3(2) (\(S\)\textsubscript{c}MDH) and detailed biochemical characterization of the recombinant \(S\)\textsubscript{c}MDH. A BLAST search indicated that \(S\)\textsubscript{c}MDH has high sequence identity (above 60%) to homodimeric MDHs of the bacteria \(T\) flavus, \(B\) pseudomallei, and \(A\) arcticum, but low sequence identity (16–28%) to fungal mMDH, porcine mMDH, and tetrameric archaeal MDH. The molecular mass of the recombinant \(S\)\textsubscript{c}MDH was about 36 kDa as determined by SDS–PAGE (Fig. 1A). MALDI-TOF-MS experiments showed that the recombinant \(S\)\textsubscript{c}MDH was a dimer in solution, and that the molecular mass was about 73 kDa, with two subunits of 37 kDa each (Fig. 1C). A similar result has been obtained for \(S\) aureofaciens MDH by gel filtration chromatography: a 70 kDa homodimer with two 38 kDa subunits.\textsuperscript{13,20} Evidently, \(S\)\textsubscript{c}MDH is a dimeric enzyme homologous to known homodimeric MDHs from other sources.

\begin{table}[h]
\centering
\caption{Effects of Metal Ions on the Activity of the Recombinant \(S\)\textsubscript{c}MDH}
\begin{tabular}{|c|c|}
\hline
Metal ions & Relative activity (%) \\
\hline
Monovalent ions & \\
None & 100.00 \\
Na\textsuperscript{+} & 97.57 ± 1.148 \\
K\textsuperscript{+} & 96.49 ± 0.587 \\
Li\textsuperscript{+} & 95.29 ± 1.896 \\
Rb\textsuperscript{+} & 94.69 ± 1.115 \\
Ag\textsuperscript{+} & 92.89 ± 0.488 \\
\hline
Divalent ions & \\
None & 100.00 \\
Ca\textsuperscript{2+} & 101.32 ± 0.995 \\
Cu\textsuperscript{2+} & 99.92 ± 0.763 \\
Mn\textsuperscript{2+} & 94.32 ± 0.953 \\
Mg\textsuperscript{2+} & 93.70 ± 0.947 \\
Co\textsuperscript{2+} & 64.21 ± 1.325 \\
Zn\textsuperscript{2+} & 27.44 ± 1.440 \\
Fe\textsuperscript{2+} & 0 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Effects of Compounds on the Activity of the Recombinant \(S\)\textsubscript{c}MDH}
\begin{tabular}{|c|c|}
\hline
Compounds & Relative activity (%) \\
\hline
None & 100.00 \\
EDTA (2 mM) & 102.05 ± 0.279 \\
DTT (2 mM) & 99.10 ± 0.950 \\
DTT (5 mM) & 95.74 ± 0.909 \\
DMSO (2%, vol/vol) & 88.80 ± 0.390 \\
DMSO (4%, vol/vol) & 80.69 ± 0.639 \\
DMSO (8%, vol/vol) & 68.47 ± 0.394 \\
Triton X-100 (2%, vol/vol) & 80.04 ± 1.407 \\
Triton X-100 (4%, vol/vol) & 67.59 ± 1.505 \\
Triton X-100 (8%, vol/vol) & 57.96 ± 0.356 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}Values indicate the means of at least three independent measurements.

\textsuperscript{b}Values indicate the means of at least three independent measurements.

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space that the 2' phosphate of NADP(H) fills and prevents NADP(H) binding, making these proteins NAD(H)-specific, whereas the NADP-MDH protein has a Gly at the equivalent position, which allows either NAD(H) or NADP(H) to bind (Fig. 5B). The alteration in the coenzyme specificity of T. flavus MDH confirms the point.

The $K_m$ value of 0.189 mM for OAA by recombinant ScMDH was close to the values determined for MDHs of S. aureofaciens (0.1 mM) and the fungus H. lanuginosa (0.12 mM), but different from the reported $K_m$ values for mMDHs from other fungal sources, e.g., T. emersonii and Phytophthora infestans (0.022 mM). On the other hand, the $K_m$ value for ScMDH with L-malate as substrate (0.494 mM) was similar to the data for the fungus Cryptococcus neoforms (0.25 mM), but distinctly different from the $K_m$ values for MDHs from S. aureofaciens (9 mM) and

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**Fig. 5.** Structure-Based Protein Sequence Alignments.

A, Alignment based on the X-ray structures of T. flavus MDH (1BMD) (top) and A. arcticum MDH (1B8P) (bottom). The NAD$^+$-binding sites (●), substrate binding sites (○), and active site (●) are shown, and the completely conserved residues are shaded. B, Partial sequence alignments based on the X-ray structures of T. flavus MDH (1BMD), pig mMDH (1MLD), watermelon gMDH (1SEV), and F. bidentis chMDH (1CIV). The fingerprint sequences of MDH (●), essential residues for cofactor specificity (●), and two Cys for disulfide bond formation in the N-terminus of chMDH (●) are shown; completely conserved residues are shaded. MDHs compared are as follows: TfMDH, T. flavus MDH (GenBank accession no. CAA38008); ScMDH, S. coelicolor MDH (CA097430); AaMDH, A. arcticum MDH (AAD13225); Arabidopsis cMDH, Arabidopsis thaliana cMDH (AAEM65532); rice cMDH, Oryza sativa cMDH (AAC13573); mouse cMDH, Mus musculus cMDH (P14152); pig cMDH, Sus scrofa cMDH (P11708); pig mMDH, Sus scrofa mMDH (P00346); yeast mMDH, Saccharomyces cerevisiae mMDH (AAA34759); Arabidopsis nMDH, A. thaliana nMDH (AAEM65855); brassica mMDH, Brassica napus mMDH (Q43744); watermelon gMDH, Citrullus lanatus gMDH (P19446); Arabidopsis gMDH, A. thaliana gMDH (Q06205); soybean gMDH, Glycine max gMDH (P37228); rice gMDH, Oryza sativa gMDH (Q42972); Flaveria chMDH, F. bidentis chMDH (P46489); pea chMDH, Pisum sativum chMDH (P21528); sorghum chMDH, S. bicolor chMDH (P17056); maize chMDH, Zea mays chMDH (P15719); sugarcane chMDH, Saccharum officinarum chMDH (C4C87689).
fungi, such as \(H. lanuginosa (5.8 \text{ mm})\) and \(M. pusillus (4.44 \text{ mm})\), \(T. emersonii (1.0 \text{ mm})\), and \(P. infestans (0.64 \text{ mm})\). With NADH as co-substrate, the \(K_m\) value for \(S. \text{MDH}\) (0.083 mM) was very similar to the data for \(S. aureofaciens \text{ MDH} (0.085 \text{ mM})\) and \(P. \text{infestans} \text{ mMDH} (0.086 \text{ mM})\). Recombinant \(S. \text{MDH}\) displayed a similar \(K_m\) value for NAD\(^+\) (0.15 mM), corresponding to enzymes from \(S. aureofaciens (0.27 \text{ mM})\) and \(T. emersonii (0.13 \text{ mM})\, C. \text{ neoformans (0.11 \text{ mM})\, H. lanuginosa (0.1 \text{ mM})\, and M. pusillus (0.16 \text{ mM})}\).

Therefore, \(S. \text{MDH}\) has higher affinity towards OAA than malate in vitro, as is the case for mMDHs and cMDHs from numerous sources.

Like other MDHs, \(S. \text{MDH}\) had higher \(V_{\text{max}}\) and \(K_{\text{cat}}\) values towards OAA than towards malate. The \(V_{\text{max}}\) and \(K_{\text{cat}}\) values for OAA reduction were approximately 400-fold of the values for malate oxidation at 30°C and pH 8.5 respectively. Analysis of the \(K_{\text{cat}}/K_m\) ratios showed that \(S. \text{MDH}\) had about 1,050-fold preference for OAA reduction over L-malate oxidation. Therefore, \(S. \text{MDH}\) has a markedly high catalytic efficiency for malate production.

Similar results were obtained for \(T. \text{emersonii, P. \text{infestans, wheat, and pineapple}}\). Generally, the activity of eukaryotic mMDHs is inhibited by excess OAA, while cMDHs are often inhibited by excess malate.\(^{30,34}\) Substrate inhibition kinetics showed that \(S. \text{MDH}\) was strongly inhibited by excess OAA (\(K_i = 5.8 \text{ mM}\)), similarly to fungus \(P. \text{infestans} \text{ mMDH} (K_i = 2.25 \pm 0.3 \text{ mM})\), but was very different from \(S. aureofaciens \text{ MDH}\), which did not display substrate inhibition at high OAA concentrations.\(^{30}\) \(S. \text{MDH}\) activity was also inhibited by excess malate (\(K_i = 12.8 \text{ mM}\)) (Fig. 4B), which was identical to \(P. \text{infestans} \text{ cMDH} (K_i = 12.3 \pm 5.6 \text{ mM})\). Thus the results suggest that \(S. \text{MDH}\) exhibits the characteristics of substrate inhibition in OAA reduction as mMDHs and in malate oxidation as cMDHs. We also observed that recombinant \(S. \text{MDH}\) is an ion-independent enzyme (Table 2). Enzyme activity was strongly inhibited by Zn\(^{2+}\), in accordance with a report that the activities of many fungal MDHs were inhibited by Zn\(^{2+}\), such as mMDH from \(C. \text{neoformans}\).\(^{31}\) \(S. \text{MDH}\) activity was also repressed by Co\(^{2+}\) and inactivated by Fe\(^{2+}\) (Table 2). Triton X-100 is a common nonionic detergent used for protein solubilization, cell permeabilization, and lysis. It is also used to retain protein conformation during purification. However, high concentrations of Triton X-100 might cause the structure to become unstable by disrupting the hydrophilic surfaces of proteins. Thus, in this study, high concentrations of Triton X-100 destroyed protein stability, resulting in a reduction in \(S. \text{MDH}\) activity. We did not find that EDTA or DTT at 2 mM had any effect on the activity of the recombinant enzyme (Table 3).

In summary, the present work provides information on the biochemical properties of MDH from \(S. \text{coelicolor}\). Recombinant \(S. \text{MDH}\) is a significantly thermostable enzyme that shows high specificity for NADH as a cofactor. It exhibits remarkably higher catalytic efficiency for oxaloacetate reduction than for malate oxidation in vitro. For a better understanding of the coenzyme specificity in NAD\(^+\)--dependent MDHs and the phylogenetic relationships between MDHs and LDHs, further investigation by protein engineering is in progress.

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References

Malate Dehydrogenase from S. coelicolor


