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The first examples of (*S*)-2-hydroxyacid dehydrogenases catalyzing the transfer of the pro-4*S* hydrogen of NADH are found in the archaea

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Abstract

Reduction of 2-oxoacids to the corresponding (*S*)-2-hydroxyacids is an important transformation in biochemistry. To date all (*S*)-2-hydroxyacid dehydrogenases belonging to the L-lactate/L-malate dehydrogenase family have been found to transfer the pro-4*R* hydrogen of either NADH or NADPH to C-2 of the 2-oxoacid substrates during their reduction. Here, we report that recombinantly generated (*S*)-2-hydroxyacid dehydrogenases present in the methanoarchaea *Methanococcus jannaschii* and *Methanothermus fervidus* use the pro-4*S* hydrogen of NADH to reduce a series of 2-oxoacids to the corresponding (*S*)-2-hydroxyacids. This information as well as the low sequence identity between these archaeal enzymes and the L-lactate/L-malate family of enzymes indicate that these enzymes are not evolutionary related and therefore constitute a new class of (*S*)-2-hydroxyacid dehydrogenases.

Keywords: Coenzymes; Archaeal malate dehydrogenases; Pyridine nucleotide stereospecificity; Enzyme evolution

1. Introduction

As first formulated by Bentley, enzymes accepting the same substrates, regardless of their sources, generally have the same stereospecificities [1]. Despite some observed violations to this generalization, to date all examples of L-lactate/L-malate dehydrogenases – whether from Archaea, Bacteria, or Eukarya – have without exception been found to follow this rule and to transfer the pro-4*R* hydrogen of either NADH or NADPH to C-2 of the 2-oxoacid substrates during their enzymatic reduction [2–4]. Pro-*R* stereospecificity of hydrogen transfer occurs throughout the wide range of 2-oxoacids that can serve as substrates for these enzymes [5–7].

The genome of *Methanococcus jannaschii* (DSM 2661) contains two putatively assigned malate dehydrogenases encoded by the MJ1425 and MJ0490 genes [8]. Their substrate and coenzyme specificity has been recently investigated [9]. Based on coenzyme specificities and catalyzed reactions, homologous enzymes were originally assigned as Mdh I and Mdh II in *Methanothermobacter marburgensis* Marburg^T (DSM 2133^T) [10]. N-terminal sequence analysis proved that the Mdh II enzyme corresponds to the MTH0188 encoded enzyme in *Methanothermobacter thermoautotrophicus* ΔH^T (DSM 1053^T) [11] and to the MJ0490-encoded enzyme in *M. jannaschii*. The Mdh II enzymes from *M. thermoautotrophicus* and from *M. jannaschii* were found to use either NADH or NADPH as coenzymes in the

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reduction of oxaloacetate to (*S*)-malate. Both Mdh II enzymes are members of the L-lactate/L-malate family of dehydrogenases, a family of enzymes with rather low-sequence identity but invariant catalytic centers and 3-dimensional structures [3,10,12,13].

The Mdh I enzyme from *M. marburgensis* corresponds to the MTH1205 encoded enzyme in *M. thermoautotrophicus* and to the MJ1425-encoded enzyme in *M. jannaschii*. The Mdh I enzymes do not align with a single member of the L-lactate/L-malate dehydrogenase family and belong to a new type of (*S*)-2-hydroxyacid dehydrogenase, so called to address the broad substrate specificity of these enzymes. The MJ1425-encoded enzyme from *M. jannaschii* has 44% sequence identity with the malate dehydrogenases from *Methanothermus fervidus* [14]. Unlike the Mdh I enzymes from *M. marburgensis* and *M. jannaschii*, the enzyme from *M. fervidus* uses both NADH and NADPH as coenzymes. Similarly to the L-lactate/L-malate dehydrogenase family, members of the new (*S*)-2-hydroxyacid dehydrogenase family exhibit not only different NADH/NADPH specificities, but also broad substrate specificities.

Here we report that the Mdh I-like (*S*)-2-hydroxyacid dehydrogenases present in methanoarchaea use the pro-4*S* hydrogen of NADH to reduce a series of 2-oxoacids to the corresponding (*S*)-2-hydroxyacids.

2. Experimental procedures

2.1. Chemicals

NAD, oxaloacetate, 2-oxoglutarate, pyruvate, methyl (*R*)-lactate, methyl (*S*)-lactate, (*S*)-malate (L-malate), (*R*)-malate (D-malate), (*S*)-2-hydroxyglutarate, (*R*)-2-hydroxyglutarate and malate dehydrogenase from porcine heart were obtained from Sigma–Aldrich Chemical Co.

2.2. Preparation and purification of the stereospecifically deuterated coenzymes

The (4*R*)-[4-$^2H_1$]NADH [15] and (4*S*)-[4-$^2H_1$]-NADH [16] were prepared enzymatically and purified [17] by previously published methods. Their enantiomer enrichments were measured by ^{1}H-NMR [16]. From the measured areas of the signal intensities for the 4*S* hydrogen at 2.66 ppm and the 4*R* hydrogen at 2.78 ppm, the enantiomeric purity of the samples was obtained. Thus the (4*S*)-[4-$^2H_1$]-NADH was found to be 96.0% of the desired isomer and 4.0% of either unlabeled NADH and/or (4*R*)-[4-$^2H_1$]NADH. The (4*R*)-[4-$^2H_1$]NADH was found to be 96.2% of the desired isomer and 3.8% of either unlabeled NADH and/or (4*S*)-[4-$^2H_1$]NADH.

2.3. Incubation of enzymes with substrates and measurement of incorporation of deuterium into products

Solutions of the recombinant enzymes (5–20 μl, 1–5 mg/ml protein) prepared as previously described [9] were incubated with substrate (5 mM) and deuterated NADH (5 mM) contained in 200 μl of 50 mM TES/Na^+, 25 mM $MgCl_2$ buffer (pH 7.4), for 30 min at 60°C. The resulting solutions were placed on Dowex 50–8X (H^+) columns (0.5×0.8 cm), and the hydroxyacids eluted with water. After evaporation of the water from the column eluates, the compounds were converted into volatile derivatives suitable for gas chromatography–mass spectrometry (GC–MS) analysis. Malate and 2-hydroxyglutarate were converted into their methylesters [18], while lactate was converted into its ditrimethylsilyl $(TMS)_2$ derivative [19]. Incorporation of label into malate dimethyl ester was measured from the (M^+-59) ion at *m*/*z* 103. The methyl esters of 2-hydroxyglutarate gave two gas chromatographic peaks, one for the dimethylester and one for the monomethyl ester lactone. Incorporation of label was measured from the (M^+-59) ion at *m*/*z* 85 for the monomethyl ester lactone. In some cases the dimethyl esters of 2-hydroxyglutarate were additionally derivatized with TMS to improve the chromatography. Incorporation of label into 2-hydroxyglutarate methyl ester TMS was determined from the (M^+-15) ion at *m*/*z* 233. Incorporation of label into lactate-$(TMS)_2$ was determined from both the (M^+-15) ion at *m*/*z* 219 and the (M^+-117) ion at *m*/*z* 117.

2.4. Measurement of the stereospecificity of the reduction products

The (*S*)-stereochemistry of each 2-hydroxyacid reaction product was determined by GC–MS analysis

of the methyl ester derivative using a type G-TA Chiraldex column (0.25 mm by 40 m: Advanced Separation Technologies, Whippany, NJ) programmed from 75°C to 180°C at 3°C/min [9].

3. Results

Stereospecifically labeled NADH was used to follow transfer of pro-4*R* or pro-4*S* hydrogens from the reduced coenzyme to the 2-oxoacid substrate. As can be seen from the data reported in Table 1, a representative malate dehydrogenase from porcine heart was found to incorporate deuterium from the (4*R*)-[4-$^2H_1$]NADH into malate, as was expected for a pro-*R* specific enzyme. The MJ0490-encoded enzyme from *M. jannaschii* was found to have the same stereospecificity and thus to unambiguously belong to the same family as all the other known malate dehydrogenases [2]. In contrast, both Mdh I enzymes, the MJ1425-encoded enzyme from *M. jannaschii* and the enzyme from *M. fervidus* incorporated deuterium from the (4*S*)-[4-$^2H_1$]NADH into the products, thus establishing these enzymes as pro-*S* specific. The MJ1425-encoded enzyme catalyzed the dehydrogenation of several different 2-hydroxyacids with the same pro-*S* stereoselectivity for all the substrates tested. In each case the enzymatic product was shown to have the absolute (*S*)-stereochemistry by GC–MS on a chiral column resolving the stereoisomers.

4. Discussion

The MJ1425-encoded enzyme from *M. jannaschii* and the malate dehydrogenase from *M. fervidus* represent the first examples of malate dehydrogenases that catalyze the transfer of the pro-4*S* hydrogen from stereospecifically deuterated NADH to oxaloacetate with the formation of deuterated (*S*)-malate. The MJ1425 derived enzyme also produces intermediates or precursors involved in the biosynthesis of methanoarchaeal coenzymes [9]. Analysis of these additional substrates for coenzyme stereospecificity demonstrates the universal validity of the hydrogen transfer for multiple substrates and the absolute stereospecificity of the product. Following the rules of Bentley [1] the MJ1425-derived enzyme from *M. jannaschii* catalyzes the NADH-dependent reduction of oxaloacetate, 2-oxoglutarate, and pyruvate to the corresponding (*S*)-2-hydroxyacids with the same pro-*S* NADH-stereospecificity. Interestingly, despite the difference in the NADH-stereospecificity the products of both malate dehydrogenase families investigated are (*S*)-2-hydroxyacids.

Table 1
Stereospecificities of hydrogen transfer from stereospecifically labeled NADH catalyzed by different malate/lactate dehydrogenases[a]

Substrate	Mdh II family (L-lactate/L-malate dehydrogenase)		Mdh I family ((*S*)-2-hydroxyacid dehydrogenase)	
	MDH Porcine heart	Mdh II *M. jannaschii*	Mdh I *M. jannaschii*	Mdh I *M. fervidus*
Oxaloacetate	Pro-*R* (78% pro-*R*)[b] (13% pro-*S*)	Pro-*R* (90% pro-*R*) (23% pro-*S*)	Pro-*S* (11% pro-*R*) (86% pro-*S*)	Pro-*S* (15% pro-*R*) (84% pro-*S*)
2-Oxoglutarate	n.a.[c]	n.a.	Pro-*S* (18% pro-*R*) (79% pro-*S*)	n.a.
Pyruvate	n.a.	n.a.	Pro-*S* (3% pro-*R*) (75% pro-S)	n.a.

[a]MDH was the malate dehydrogenase from porcine heart, Mdh II from *M. jannaschii* was the MJ0490-derived enzyme, the Mdh I from *M. jannaschii* was the MJ1425-derived enzyme, and the Mdh I from *M. fervidus* was its malate dehydrogenase.
[b]Measured atom % excess ^2H incorporated into the product when the indicated stereospecifically labeled NADH was used in the incubation.
[c]No activity detectable.

Although non-stereospecific hydride transfer to and from C-4 of NAD by lactate dehydrogenase has been shown to be a very rare event [20], this is clearly not the case for these malate dehydrogenases. The presence of non-stereospecific hydride transfer is confirmed here by our measurements showing the incorporation of 10–20% of deuterium into the substrates from the oppositely labeled NADH. Earlier reported data from other investigators show similar results for this type of experiments [21–23]. In the literature no explanation for the non-specific hydrogen transfer has been published and the contamination of the product from the oppositely labeled NADH appears to have largely been ignored. A possible explanation is that the dehydrogenases have different affinities for the binding of each face of NADH. At high NADH concentrations, as used in these experiments, the affinity for binding the opposite conformation is sufficient to account for the observed discrepancies.

The very low sequence similarity of the MJ1425 encoded enzyme with the MJ0490 encoded enzyme (15% sequence identity) can now be explained by their differences in coenzyme stereospecificity. The Mdh II enzymes (pro-*R* specific) bind the NADH in the *anti* conformation in respect to the ribose [24–26] and transfer the pro-*R* hydrogen to the 2-oxoacid. To transfer the pro-*S* hydrogen the Mdh I enzymes (pro-*S* specific) need to bind the nicotinamide ring in the *syn* conformation with the substrate in a similar position as in the Mdh II enzymes. If the Mdh I enzymes evolved from the Mdh II enzymes, the reduced pyridine ring must have flipped around the glycosidic bond from an *anti* conformation to a *syn* conformation of the nicotinamide ring. Attempts to specifically engineer the binding of NADH in the opposite conformation failed to produce catalytically active enzymes [27]. Therefore we propose a convergent evolution of the L-lactate/L-malate dehydrogenases and the (*S*)-2-hydroxyacid dehydrogenases.

Members of the new (*S*)-2-hydroxyacid dehydrogenase family are not restricted to the methanoarchaea. Using the National Center for Biotechnology Information PSI BLAST program [28] significant alignments show protein coding sequences from other archaea (PAB1791 from *Pyrococcus abyssi*), bacteria (ybiC from *Escherichia coli*, yjmC from *Bacillus subtilis*) and eukarya (AE007329 from *Drosophila melanogaster*). Unfortunately the functionality of most of these enzymes is unknown except for the enzyme from *Ralstonia eutropha* which has been identified as a L-lactate dehydrogenase [29]. It is remarkable that members of pro-*S* dehydrogenases differ in their substrate specificities in a similar manner as the pro-*R* dehydrogenases.

It will be interesting to establish if the three-dimensional structure of the MJ1425-encoded enzyme as a representative of the (*S*)-2-hydroxyacid dehydrogenases is the same as, or different from, that found in established members of the L-lactate/L-malate dehydrogenase family [25].

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