Archaeobacterial malate dehydrogenases*

The enzymes from the thermoacidophilic organisms *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* show A-side stereospecificity for NAD⁺

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The stereoselective transfer of hydrogen from NADH to oxaloacetate catalysed by malate dehydrogenases (EC 1.1.1.37) from the thermoacidophilic archaeobacteria *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* was studied by the p.m.r. method described by Zhou & Wong [(1981) *J. Biochem. Biophys. Methods* 4, 329–338]. Both enzymes are A-side (*pro-R*) stereospecific for NADH.

Since the discovery that the two hydrogen atoms at the 4-position of the nicotinamide ring in NADH and NADPH are enzymically non-equivalent, about 150 pyridine-nucleotide-dependent enzymes have been studied and classified in two distinct groups (You, 1982). The A-side-stereospecific enzymes remove the hydrogen at the A-side (*pro-R*) of the dihydropyridine ring, and the B-side-stereospecific enzymes remove the hydrogen at the B-side (*pro-S*) (Cornforth *et al.*, 1966). It was noted by Bentley (1970) that the stereospecificity of a particular enzymic reaction is fixed and does not depend on the source of the enzyme. Malate dehydrogenases of a number of animals, plants and eubacteria have been studied and are found to be A-side-specific (You, 1982). To date there are no reports on the stereospecificity of nicotinamide-nucleotide-dependent dehydrogenases from archaeobacteria, organisms which, it is proposed, represent a third primary line of evolutionary descent (Woese & Fox, 1977). We report here that, like eukaryotes and eubacteria, the thermoacidophilic archaeobacteria *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* possess malate dehydrogenases which are A-side-specific.

Experimental

Alcohol dehydrogenase (yeast), glutamate dehydrogenase (ox liver), lactate dehydrogenase (rabbit muscle) NAD⁺, oxaloacetate and dithiothreitol were obtained from Boehringer, Mannheim, Ger-

Abbreviation used: SDS, sodium dodecyl sulphate.

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many, and lipoamide dehydrogenase (torula yeast) from Sigma, München, Germany. ²H₂O (99.75%) and (²H)ethanol (99%) were purchased from Merck, Darmstadt, Germany; and α -lipoamide from Fluka, Buchs, Switzerland. CM-Sephadex C-50, DEAE-Sephadex A-50, Sephacryl S-200 and Red Sepharose CL-6B were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical-reagent grade.

Sulfolobus acidocaldarius (DSM 7639) was grown aerobically at 70°C (pH 2–3) in the basal salt medium described by Brock *et al.* (1972), with the addition of yeast extract (2 g/l). *Thermoplasma acidophilum* (A.T.C.C. 27 658) was grown at 56°C in the medium described by Christiansen *et al.* (1975). The medium was slightly modified. It contained 0.2% yeast extract and the pH was adjusted to pH 1.1. *S. acidocaldarius* was kindly provided by Dr. W. Zillig, München, Germany, and *T. acidophilum* was a gift from Dr. E. A. Freundt, Aarhus, Denmark. The bacteria were harvested by centrifugation and stored at –20°C.

To purify malate dehydrogenase from *S. acidocaldarius*, 35 g of wet cell paste were thawed in 50 ml of 0.1 M-Tris/HCl buffer, pH 7, and the cells were disrupted with a French press. After removing cell debris by centrifugation, the crude extract was fractionated with (NH₄)₂SO₄. Malate dehydrogenase from *S. acidocaldarius* was found in the fractions corresponding to 48–95%-satd. (NH₄)₂SO₄. The (NH₄)₂SO₄ fraction was dialysed against 7.5 mM-citrate/7.5 mM-phosphate buffer adjusted to pH 6 with NaOH and applied to a column (8 cm × 30 cm) of CM-Sephadex C-50 equilibrated with the same buffer. The enzyme was

eluted with a linear gradient of NaCl (0–0.3M) in a total volume of 5000ml of dialysis buffer. The active fractions were combined, diluted with an equal volume of 0.1M-Tris/HCl buffer, pH7, and concentrated by membrane filtration. The preparation was applied to a column (3cm × 5cm) of Red Sepharose CL-6B. Unbound material was washed off and malate dehydrogenase was eluted with a linear gradient of NADH (0–2mM) in a total volume of 100ml of 0.1M-Tris/HCl, pH7. The active fractions were combined, concentrated and applied to a column (3cm × 80cm) of Sephacryl S-200 equilibrated with 0.1M-Tris/HCl, pH7, containing 0.1M-NaCl. Malate dehydrogenase from *S. acidocaldarius* eluted from the Sephacryl column was purified 240-fold with a resulting specific activity of 277 units/mg at 40°C. The enzyme was homogeneous when subjected to SDS/polyacrylamide-gel electrophoresis.

To purify malate dehydrogenase from *T. acidophilum*, 10g of wet frozen cells were thawed in 20ml of 1M-sodium/potassium phosphate buffer, pH7. This treatment lysed the cells. After removing cell debris by centrifugation, DNA was precipitated by the addition of protamine sulphate. After centrifugation the clear supernatant was submitted to (NH₄)₂SO₄ fractionation. Malate dehydrogenase was found in the fraction corresponding to 65–95%-satd.-(NH₄)₂SO₄. This fraction was dialysed against 10mM-Tris/HCl buffer, pH8.5, and applied to a column (5cm × 12cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.3M) in a total volume of 1000ml of 10mM-Tris/HCl buffer, pH8.5. The most active fractions were combined and concentrated, and after dialysis against 10mM-Tris/HCl buffer, pH7, the enzyme solution was loaded on a column (1.3cm × 6.5cm) of Red Sepharose CL-6B. Unbound material was washed off and malate dehydrogenase was eluted with a 5mM solution of NADH in 0.1M-Tris/HCl buffer, pH7. After dialysis against 50mM-Tris/HCl buffer, pH7, the solution was concentrated by ultrafiltration and subsequently applied to a column (2.6cm × 100cm) of Sephacryl S-200. Malate dehydrogenase was eluted with 0.1M-Tris/HCl buffer, pH7, containing 0.1M-NaCl. The resulting enzyme preparation was purified 50-fold with a resulting specific activity of 180units/mg at 40°C. The enzyme was homogeneous on the basis of SDS/polyacrylamide-gel electrophoresis.

Malate dehydrogenase was assayed at 40°C in 25mM-potassium phosphate buffer (pH7.4)/0.48mM-oxaloacetate/0.24mM-NADH. The reaction, in a final volume of 1050µl, was started with oxaloacetate and the oxidation of NADH was monitored with time at 340nm.

The stereospecificity of malate dehydrogenase for NAD was determined in 0.1M-sodium phosphate buffer in ²H₂O at p²H7.6. The total reaction volume was 1ml. When [4A-²H]NADH was generated, the reaction mixture contained 7.8mM-NAD⁺, 160mM-(²H)ethanol, 150units of alcohol dehydrogenase, 20mM-oxaloacetate and 7units of archaeobacterial malate dehydrogenase. When [4B-²H]NADH was generated, the reaction mixture contained 5mM-NAD⁺, 1mM-lipoamide, 20mM-dithiothreitol, 18units of lipoamide dehydrogenase, 20mM-oxaloacetate and 7units of archaeobacterial malate dehydrogenase. A solution of 7.8mM-NAD⁺ in 0.1M-sodium phosphate buffer in ²H₂O at p²H7.6 was used as reference. The reaction mixtures were stored at room temperature for 16h. The spectra were recorded on a Bruker WM250n.m.r. spectrometer interfaced to a Fourier-transform system. The H²HO resonance signal was suppressed. Chemical shifts, δ values, are expressed as p.p.m. relative to internal sodium trimethylsilyl [U-²H]propionate.

Results

Reduction of NAD⁺ by alcohol dehydrogenase (A-side-specific) with (²H)ethanol leads to the formation of [4A-²H]NADH, whereas reduction by lipoamide dehydrogenase (B-side-specific) in the presence of [²H₂]dihydro-α-lipoamide yields [4B-²H]NADH.

When [4A-²H]NADH is reoxidized by an A-side specific enzyme, the resulting NAD⁺ will contain no ²H, whereas oxidation by an B-side-specific enzyme will lead to NAD⁺ being ²H-labelled at the 4-position. The converse is true for [4B-²H]NADH: reoxidation by an A-side-specific dehydrogenase leads to NAD⁺ ²H-labelled in the 4-position, whereas oxidation by a B-side-specific enzyme will lead to NAD⁺ containing no ²H (Scheme 1).

The presence or absence of ²H at the nicotinamide 4-position can be analysed by p.m.r. For NAD⁺ with a proton at the pyridine 4-position, the p.m.r. spectrum shows a resonance at 8.83 p.p.m. from the trimethylsilyl [U-²H]propionate peak (Fig. 1a). For [4-²H]NAD⁺, where there is a ²H atom instead of a proton at the 4-position, there is no resonance.

The specifically ²H-labelled reduced coenzyme [4A-²H]NADH and [4B-²H]NADH were reoxidized *in situ* with oxaloacetate by the archaeobacterial malate dehydrogenases from *S. acidocaldarius* and *T. acidophilum* as described in the Experimental section. Figs. 1(a) and 1(b) and Figs. 2(a) and 2(b) show that both archaeobacterial malate dehydrogenases are A-side-specific, converting [4A-²H]NADH into NAD⁺ and [4B-²H]NADH

Fig. 1. Aromatic region of the p.m.r. spectra of NAD^+ after reoxidation of stereospecifically ^2H -labelled NADH by malate dehydrogenase from *Sulfolobus acidocaldarius* (a) Reoxidation of $[4\text{A-}^2\text{H}]\text{NADH}$ by malate dehydrogenase from *S. acidocaldarius* leads to NAD^+ showing a resonance signal at 8.83 p.p.m. The enzyme removes the ^2H atom from the A-side of the dihydronicotinamide ring. (b) Reoxidation of $[4\text{B-}^2\text{H}]\text{NADH}$ by malate dehydrogenase from *S. acidocaldarius* leads to NAD^+ showing no resonance signal at 8.83 p.p.m. The enzyme removes the proton from the A-side of the dihydronicotinamide ring.

into $[4\text{-}^2\text{H}]\text{NAD}^+$. In all experiments, the p.m.r. spectrum of NAD^+ ^2H -labelled at the 4-position showed less than 15% of the signal of the non- ^2H -labelled coenzyme. Control experiments were performed with A-side-specific lactate dehydro-

Fig. 2. Aromatic region of the p.m.r. spectra of NAD^+ after reoxidation of stereospecifically ^2H -labelled NADH by malate dehydrogenase from *Thermoplasma acidophilum* (a) Reoxidation of $[4\text{A-}^2\text{H}]\text{NADH}$ by malate dehydrogenase from *T. acidophilum* leads to NAD^+ showing a resonance signal at 8.83 p.p.m. The enzyme removes the ^2H atom from the A-side of the dihydronicotinamide ring. (b) Reoxidation of $[4\text{B-}^2\text{H}]\text{NADH}$ by malate dehydrogenase from *S. acidocaldarius* leads to NAD^+ showing no resonance signal at 8.83 p.p.m. The enzyme removes the proton from the A-side of the dihydronicotinamide ring.

genase and B-side-specific glutamate dehydrogenase, leading essentially to the same amount of labelling.

Discussion

About 150 nicotinamide-nucleotide-dependent dehydrogenases have been classified according to their stereospecificity in transferring either the hydrogen attached to the A-side (*pro-R*) or the

Scheme 1. Reoxidation of stereospecifically ^2H -labelled NADH by A-side or B-side-specific enzymes. The A-side (*pro-R*) or B-side (*pro-S*) of NADH is labelled with ^2H by specific enzymes. After reoxidation the resulting NAD^+ will be ^2H -labelled or not, depending on the specificity of the test enzyme. The p.m.r. spectrum of non- ^2H -labelled NAD^+ shows a resonance signal at 8.83 p.p.m., whereas ^2H -labelled NAD^+ does not.

B-side (*pro-S*) of the reduced coenzyme (You, 1982). A generalization formulated by Bentley (1970) states that the stereospecificity of a particular reaction is fixed and does not depend on the source of the enzyme. The malate dehydrogenases from a large number of animals, plants and eubacteria show A-side stereospecificity (You, 1982). The data provided here demonstrate that the malate dehydrogenases of the two thermoacidophilic archaeobacteria *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* are also A-side-specific. Archaeobacteria are supposed to represent a third primary line of evolutionary descent in addition to eukaryotes and eubacteria (Woese & Fox, 1977). So far there are no data in the literature on the stereospecificity of archaeobacterial dehydrogenases. The results reported here show that the malate dehydrogenases from the thermoacidophilic archaeobacteria are in accord with the generalization formulated by Bentley (1970). Recently Benner (1982) provided evidence that the stereoselectivity of nicotinamide-nucleotide-dependent alcohol dehydrogenases correlates with the thermodynamic stability of the substrates. He also put forward several arguments supporting the view that the stereospecificity of dehydrogenases reflects evolutionary pressure to produce enzymes that approach

catalytic 'perfection' (Knowles & Albery, 1977). In this context it is interesting to observe that archaeobacterial malate dehydrogenases show the same stereospecificity as the so-far-known enzymes from eukaryotic and eubacterial sources (You, 1982).

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