Re-face stereospecificity at C4 of NAD(P) for alcohol dehydrogenase from *Methanogenium organophilum* and for (*R*)-2-hydroxyglutarate dehydrogenase from *Acidaminococcus fermentans* as determined by ^{1}H-NMR spectroscopy

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Abstract The two diastereotopic protons at C4 of NAD(P)H are seen separately in ^{1}H-NMR spectra. This fact was used to determine the stereospecificity at C4 of NAD(P) for the NADP-dependent alcohol dehydrogenase from *Methanogenium organophilum* and for the NAD-dependent (*R*)-2-hydroxyglutarate dehydrogenase from *Acidaminococcus fermentans*. The reduction of NADP$^+$ with [$^2H_6$]ethanol was found to yield (4*R*)-[4-$^2H_1$]NADPH and the oxidation of (4*R*)-[4-$^2H_1$]NADH with 2-oxoglutarate to yield unlabelled [4-^{1}H]NAD$^+$. These results indicate that both enzymes are *Re*-face stereospecific at C4 of the pyridine nucleotides.

Key words: Stereospecificity; NAD(P)$^+$; ^{1}H-NMR spectroscopy; Dehydrogenase; Archaea

1. Introduction

Many NAD(P)-dependent dehydrogenases have been analyzed with respect to the stereospecificity at C4 of the pyridine nucleotides [1]. In the most recent compilation of 157 dehydrogenases 77 are *Re*-face specific and 80 are *Si*-face specific [2,3]. Interestingly, there is a correlation between the stereospecificity of these dehydrogenases and the redox potentials of the enzymes' natural substrates [4,5]. The enzymes catalyzing NAD(P)-dependent oxidations with $pK_{eq} > 11.2$ ($pK_{eq} = -\log K_{eq}$) are generally *Re*-face specific and those with $pK_{eq} < 11.2$ are *Si*-face specific [5]. Based on this finding it has been proposed that thermodynamic necessities [6–8] rather than historical chances [9] determine the stereospecificity of dehydrogenases.

We report here on the stereospecificity of an NADP-dependent alcohol dehydrogenase from a methanogenic Archaeon, a physiological function of which is to catalyze the oxidation of ethanol ($pK_{eq} = 11.4$; in the direction of NAD(P)H formation) [10,11]. We also analyzed the stereospecificity of the NAD-dependent (*R*)-2-hydroxyglutarate dehydrogenase from the anaerobic bacterium *Acidaminococcus fermentans*, the physiological function of which is to catalyze the reduction of 2-oxoglutarate ($pK_{eq} = 11.8$) in the pathway of glutamate fermentation [12,13]. On the basis of thermodynamic explanations both enzymes were expected to be *Re*-face stereospecific [6], although in the case of the archaeal alcohol dehydrogenase also a *Si*-face stereospecificity had to be envisaged for two reasons:

(i) In contrast to most alcohol dehydrogenases, which are *Re*-face specific, the alcohol dehydrogenase from *Drosophila melanogaster* is *Si*-face specific [6]. This finding was explained by the fact that the oxidation of ethanol ($pK_{eq} = 11.4$) is close to the point of 'stereochemical inversion' ($pK_{eq} = 11.2$), where other factors such as physiology might become important. While the in vivo function of *Re*-face specific alcohol dehydrogenases is considered to be the reduction of acetaldehyde, that of the *Si*-face specific *Drosophila* enzyme is the oxidation of ethanol. The archaeal alcohol dehydrogenase is involved in ethanol oxidation and could therefore also be *Si*-face stereospecific.

(ii) Although a physiological substrate of the analyzed archaeal alcohol dehydrogenase is ethanol ($K_m = 50$ mM, $V_{max} = 5$ U/mg), a much better physiological substrate is 2-propanol (propan-2-ol; $K_m = 0.78$ mM, $V_{max} = 10$ U/mg) [14]. According to Benner's rule, the oxidation of 2-propanol ($pK_{eq} = 8.2$) [10], which is far below the inversion point, should be *Si*-face specific with respect to C4 of NADP since the best substrate should govern the cryptic stereochemistry [6].

2. Materials and methods

[1-^{2}H]Glucose and ^{2}H-formate were from Aldrich Chemicals. [$^2H_6$]Ethanol was from Merck. ^2H_2O (99.9%) was from Sigma. [1-^{2}H]Glucose-6-phosphate was synthesized in ^2H_2O from [1-^{2}H]glucose and ATP in the presence of hexokinase, pyruvate kinase and phosphoenol pyruvate. After completion of the reaction the enzymes were removed by ultrafiltration.

(4*R*)-[4-$^2H_1$]NADH was obtained by reduction of NAD$^+$ with [^{2}H]formate in ^2H_2O in the presence of *Re*-face stereospecific formate dehydrogenase from *Xylaria digitata* (Boehringer Mannheim) [1]. (4*S*)-[4-$^2H_1$]NADPH was synthesized by reduction of NADP$^+$ in ^2H_2O with [1-^{2}H]glucose-6-phosphate in the presence of *Si*-face stereospecific glucose-6-phosphate dehydrogenase from yeast (Boehringer Mannheim) [2]. After completion of the reaction the enzymes were removed by ultrafiltration.

NADP specific alcohol dehydrogenase from *M. organophilum* [15] and NAD specific (*R*)-2-hydroxyglutarate dehydrogenase from *A. fermentans* [12] were purified as described.

The N-terminal amino acid sequence of the alcohol dehydrogenase was determined to be AKMIKGLAMKRIGEIG(W)IEKEAPK-GPLDALVKPLALAP. A comparison of the N-terminal amino acid sequence with the amino acid sequence of other ethanol dehydrogenases in the databases revealed that the enzyme from *M. organophilum* is phylogenetically most closely related to other NADP-de-

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pendent alcohol dehydrogenases, e.g. from *Thermoanaerobacter brockii* [16] or *Entamoeba histolytica* [17].

The stereospecificity of the alcohol dehydrogenase from *M. organophilum* with ethanol as substrate was determined at room temperature in a 3 ml serum bottle containing 1.5 ml assay mixture in ^2H_2O: 50 mM sodium bicarbonate/NaO^2H buffer p^2H 10.5, 100 mM semicarbazide (adjusted to p^2H 10.5), 5 mM $NADP^+$, 100 mM [$^2H_6$]ethanol and 7.5 U ethanol dehydrogenase. For determination of the stereospecificity of acetone reduction to 2-propanol, the assay mixture contained 50 mM potassium phosphate p^2H 7.0, 5 mM (4*S*)-[4-$^2H_1$]NADPH, 100 mM acetone and 3 U alcohol dehydrogenase. The glutamate dehydrogenase reaction as a control contained 20 mM 2-oxoglutarate, 20 mM $(NH_4)_2SO_4$ and 5 U *Si*-face specific glutamate dehydrogenase from beef liver. The reactions were followed by measuring the increase and decrease in absorbance at 340 nm, respectively. After completion of the reaction the enzymes were separated by ultrafiltration and the ultrafiltrates subjected to ^{1}H-NMR analysis. 2-Propanol and excessive acetone were removed by lyophilization.

The stereospecificity of the (*R*)-2-hydroxyglutarate dehydrogenase from *A. fermentans* was determined at room temperature in a 3 ml serum bottle containing 1.5 ml assay mixture in ^2H_2O: 100 mM potassium phosphate p^2H 7.0, 5 mM 2-oxoglutarate, sodium salt, 5 mM (4*R*)-[4-$^2H_1$]NADH and 5 U (*R*)-2-hydroxyglutarate dehydrogenase. The reaction was followed by measuring the decrease of absorbance at 340 nm. After completion of the reaction the enzyme was removed by ultrafiltration and the ultrafiltrate subjected to ^{1}H-NMR analysis.

All substrates, buffers and enzymes were lyophilized and redissolved in ^2H_2O prior to being used in the experiments. The ^{1}H-NMR spectra were recorded on a 300 MHz NMR spectrometer from Bruker.

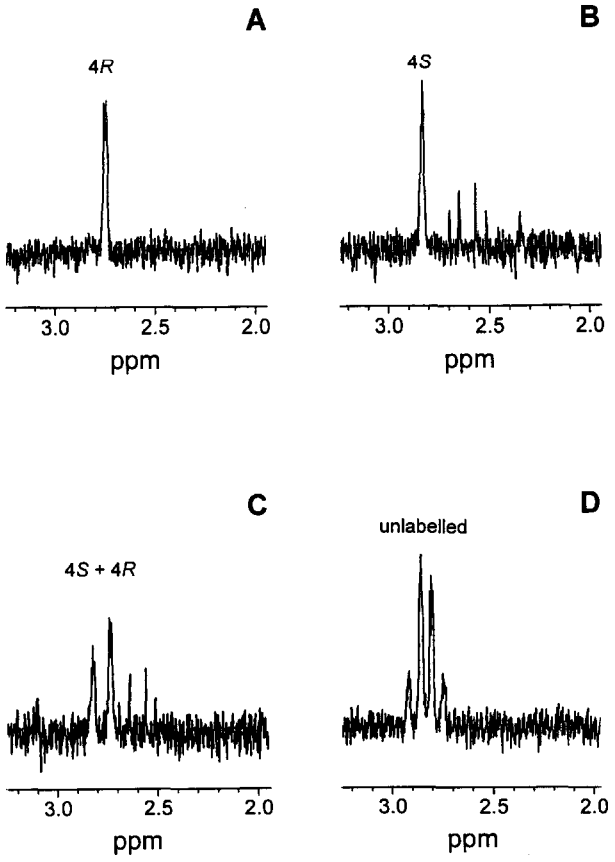

Fig. 1. ^{1}H-NMR spectra of the hydrogen at C4 of NADPH. (A) NADPH generated from $NADP^+$ by reduction with [$^2H_6$]ethanol as catalyzed by alcohol dehydrogenase from *M. organophilum*. (B) (4*S*)-[4-$^2H_1$]NADPH. (C) A 1:1 mixture of NADPH from A and B. (D) Commercially available NADPH.

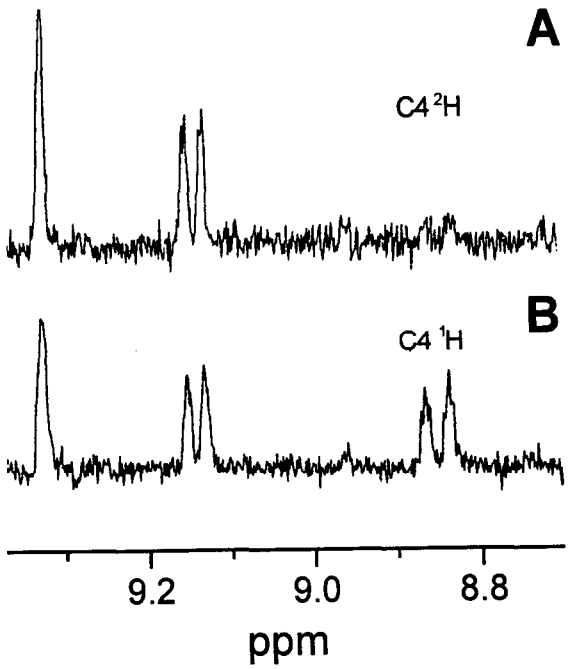

Fig. 2. ^{1}H-NMR spectra of the hydrogen at C4 of $NADP^+$. (A) $NADP^+$ generated from (4*S*)-[4-$^2H_1$]NADPH by oxidation with acetone as catalyzed by the NADP-dependent alcohol dehydrogenase from *M. organophilum*. (B) $NADP^+$ formed from (4*S*)-[4-$^2H_1$]NADPH by oxidation with 2-oxoglutarate and NH_4^+ as catalyzed by *Si*-face stereospecfic glutamate dehydrogenase from beef liver.

3. Results and discussion

The diastereotopic hydrogen atoms at C4 of NAD(P)H, (4*R*)-[4-$^2H_1$]NAD(P)H and (4*S*)-[4-$^2H_1$]NAD(P)H exhibit different ^{1}H-NMR spectra in the 2.8 ppm region [18,19]. $NAD(P)^+$ exhibits a proton resonance in the 8.8 ppm region which is not found with [4-^{2}H]$NAD(P)^+$ [20]. These spectroscopic differences were exploited to determine the stereospecificity at C4 of NAD(P) of the NADP-dependent alcohol dehydrogenase from *M. organophilum* and of the NAD-dependent (*R*)-2-hydroxyglutarate dehydrogenase from *A. fermentans*.

3.1. Alcohol dehydrogenase from M. organophilum

The stereospecificity of the alcohol dehydrogenase with ethanol as a substrate was deduced from a comparison of the ^{1}H-NMR spectrum of unlabelled NADPH (Fig. 1D) and of (4*S*)-[4-$^2H_1$]NADPH (Fig. 1B) with that of NADPH generated from $NADP^+$ by enzymatic reduction with [$^2H_6$]ethanol (Fig. 1A). The comparison revealed that the NADPH generated in the alcohol dehydrogenase reaction contained only one hydrogen at C4 and that the resonance of this hydrogen appeared at lower frequency (2.75 ppm) compared with that of the 4-hydrogen in (4*S*)-[4-$^2H_1$]NADPH (2.85 ppm). In a 1:1 mixture of the NADPH generated in the alcohol dehydrogenase reaction and of (4*S*)-[4-$^2H_1$]NADPH two resonances with a difference in chemical shift of 0.1 ppm in the 2.8 ppm region were observed (Fig. 1C). These two resonances were not split by geminal coupling as in unlabelled NADPH which contains two ^{1}H at C4 (Fig. 1D). The results clearly indicated that the NADPH generated by $NADP^+$ reduction with [$^2H_6$]ethanol was (4*R*)-[4-$^2H_1$]NADPH and that

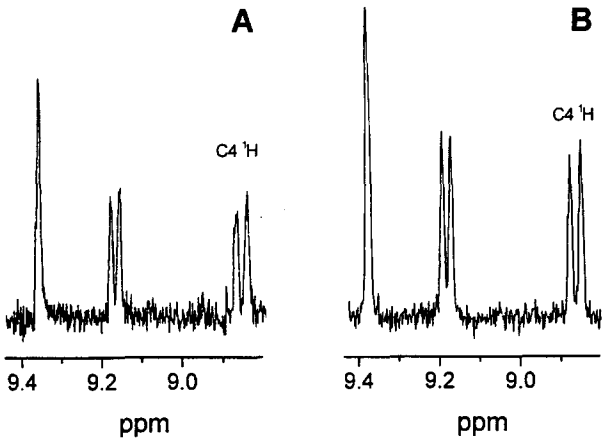

Fig. 3. ^{1}H-NMR spectra of the hydrogen at C4 of NAD^+. (A) NAD^+ generated from (4*R*)-[4-$^2H_1$]NADH by oxidation with 2-oxoglutarate as catalyzed by (*R*)-2-hydroxyglutarate dehydrogenase from *A. fermentans*. (B) Commercially available NAD^+.

the NADP-dependent alcohol dehydrogenase from *M. organophilum* catalyzed hydrogen transfer to the *Re*-face of NADP despite the physiological function of the enzyme in catalyzing the oxidation of ethanol as in the case of the *Si*-face specific alcohol dehydrogenase from *Drosophila*.

Fig. 2 shows a comparison of the ^{1}H-NMR spectra of $NADP^+$ generated by oxidation of (4*S*)-[4-$^2H_1$]NADPH (A) with acetone catalyzed by alcohol dehydrogenase from *M. organophilum* and (B) with 2-oxoglutarate and ammonia catalyzed by *Si*-face specific glutamate dehydrogenase. While after oxidation with acetone almost no resonances were detectable in the 8.8–8.9 ppm region of the ^{1}H-NMR spectrum, after oxidation with 2-oxoglutarate and NH_4^+ two distinct resonances were observed. These findings indicated that in the alcohol dehydrogenase reaction the ^{1}H was taken away from the *Re*-face of the nicotinamide moiety, leaving one ^{2}H at the C4 position of the oxidized coenzyme. In contrast, in the glutamate dehydrogenase reaction the ^{2}H was taken away from the *Si*-face leaving one ^{1}H at C4 which displayed the two resonances due to coupling with the hydrogen at C3. Thus, also with 2-propanol as substrate the alcohol dehydrogenase from *M. organophilum* exhibited *Re*-face stereospecificity despite the fact that the thermodynamics of the oxidation of 2-propanol ($pK_{eq} = 8.2$), which is a 50 times better substrate (k_{cat}/K_m) than ethanol, would require a *Si*-face specific enzyme according to Benner's rules [5,6].

3.2. (*R*)-2-Hydroxyglutarate dehydrogenase from *A. fermentans*

The stereospecificity of the enzyme was determined from a comparison of the ^{1}H-NMR spectrum of unlabelled NAD^+ (Fig. 3B) with that of NAD^+ generated from (4*R*)-[4-$^2H_1$]NADH by enzymatic oxidation with 2-oxoglutarate (Fig. 3A). Both spectra were found to be almost identical indicating that the deuterium rather than the hydrogen at C4 of (4*R*)-[4-$^2H_1$]NADH was removed upon oxidation with 2-oxoglutarate. The results showed that the (*R*)-2-hydroxyglutarate dehydrogenase from *A. fermentans* is *Re*-face stereospecific at C4 of the pyridine nucleotide.

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