Re-face stereospecificity of NADP dependent methylenetetrahydromethanopterin dehydrogenase from *Methylobacterium extorquens* AM1 as determined by NMR spectroscopy

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Abstract MtdA catalyzes the dehydrogenation of N^5,N^{10}-methylenetetrahydromethanopterin (methylene-H_4MPT) with $NADP^+$ as electron acceptor. In the reaction two prochiral centers are involved, C14a of methylene-H_4MPT and C4 of $NADP^+$, between which a hydride is transferred. The two diastereotopic protons at C14a of methylene-H_4MPT and at C4 of NADPH can be seen separately in ^{1}H-NMR spectra. This fact was used to determine the stereospecificity of the enzyme. With (14a*R*)-[14a-$^2H_1$]-[14a-^{13}C]methylene-H_4MPT as the substrate, it was found that the *pro-R* hydrogen of methylene-H_4MPT is transferred by MtdA into the *pro-R* position of NADPH. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Methylenetetrahydromethanopterin; NADPH; Stereospecificity; ^{1}H-Nuclear magnetic resonance; *Methylobacterium extorquens* AM1

1. Introduction

Recently in *Methylobacterium extorquens* AM1 a novel enzyme was found that catalyzes the dehydrogenation of N^5,N^{10}-methylenetetrahydromethanopterin (methylene-H_4MPT) to N^5,N^{10}-methenyltetrahydromethanopterin (methenyl-H_4MPT$^+$) with $NADP^+$ as electron acceptor ($\Delta G^{\circ\prime}$ = −13 kJ/mol) [1,2] (Fig. 1). The enzyme designated NADP dependent methylenetetrahydromethanopterin dehydrogenase (MtdA) also catalyzes the dehydrogenation of N^5,N^{10}-methylenetetrahydrofolate to N^5,N^{10}-methenyltetrahydrofolate albeit with a 20-fold lower catalytic efficiency. It is, however, strictly specific for NADP. The homotrimeric enzyme, which is devoid of a prosthetic group, exhibits a ternary complex catalytic mechanism [1].

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Abbreviations: DQF-COSY, double-quantum filter correlated spectroscopy; HSQC, heteronuclear single-quantum correlation spectroscopy; MtdA, $NADP^+$ dependent methylenetetrahydromethanopterin dehydrogenase; Hmd, hydrogen forming methylenetetrahydromethanopterin dehydrogenase; H_4MPT, tetrahydromethanopterin

In the MtdA catalyzed reaction, a hydride is transferred from C14a of methylene-H_4MPT to C4 of $NADP^+$, which are both prochiral centers (Fig. 1). The C14a containing imidazolidine ring and the C4 containing pyridine ring thus have a *Si*-face and a *Re*-face with which they can bind to an enzyme and interact with one another. For hydrogen transfer to occur, either the *pro-S* or the *pro-R* hydrogen of methylene-H_4MPT must be in van der Waals contact with C4 of the pyridine ring, either from the *Si*-face or from the *Re*-face. From the stereochemistry of the hydride transfer the relative position of the two substrates within the active site of MtdA can therefore be deduced.

2. Materials and methods

$^2H_2$ was from Messer Griesheim. ^2H_2O, [^{13}C]formaldehyde, NaB^2H_4 and [1-^{2}H]glucose were from Aldrich, and $NADP^+$ and NADPH were from Biomol. Tetrahydromethanopterin (H_4MPT) was isolated from *Methanothermobacter marburgensis* (DSMZ 2133, formerly *Methanobacterium thermoautotrophicum* strain Marburg [3]) [4]. Glucose-6-phosphate dehydrogenase from yeast was from Boehringer.

[14a-^{13}C]Methylene-H_4MPT was prepared by spontaneous reaction of H_4MPT with [^{13}C]formaldehyde [5]. Methenyl-H_4MPT$^+$ was generated from methylene-H_4MPT at pH 6.0 by dehydrogenation. The reaction was catalyzed by hydrogen forming methylene-H_4MPT dehydrogenase (Hmd) [6]. Hmd was purified from *M. marburgensis* [7]. $NADP^+$ dependent methylene-H_4MPT dehydrogenase from *M. extorquens* AM1 (DSMZ 1338) was heterologously overproduced in *Escherichia coli* and purified as described in [2].

2.1. Preparation of ^2H stereospecifically labelled methylene-H_4MPT and NADPH

(14a*R*)-[14a-$^2H_1$]-[14a-^{13}C]Methylene-H_4MPT was generated by reduction of methenyl-H_4MPT$^+$ with $^2H_2$ in ^2H_2O containing 100 mM potassium phosphate p^{2}H 7.5 at room temperature as catalyzed by *Re*-face specific Hmd [8,9]. After completion of the reaction the enzyme was removed by ultrafiltration using a 30 kDa microconcentrator (Millipore). Since Hmd and methylene-H_4MPT are oxygen sensitive, all steps were performed under strictly anaerobic conditions. (4*S*)-[4-$^2H_1$]NADPH was synthesized by reduction of $NADP^+$ with [1-^{2}H]glucose-6-phosphate as catalyzed by *Si*-face specific glucose-6-phosphate dehydrogenase from yeast [10]. After completion of the reaction the enzyme was removed by ultrafiltration. (14a*S*)-[14a-$^2H_1$]-[14a-^{13}C]Methylene-H_4MPT was generated by reduction of methenyl-H_4MPT$^+$ with NaB^2H_4 [9,11].

2.2. Assay for the determination of the stereospecificity of MtdA

The 1 ml assay mixture in ^2H_2O contained 100 mM potassium

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phosphate p^{2}H 7.5, 2 mM NADP$^+$, 4 mM (14a*R*)-[14a-^{2}H$_1$]-[14a-^{13}C]methylene-H$_4$MPT or 4 mM (14a*S*)-[14a-^{2}H$_1$]-[14a-^{13}C]-methylene-H$_4$MPT. The reaction was started with 5 U MtdA at room temperature and completed after 5 min. Before and after the reaction the assay was analyzed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy.

2.3. Substrate and product analysis via ^{1}H-NMR spectroscopy

NMR spectra of the assays were recorded at 279 K (6°C) and at a ^{1}H frequency of 600.13 MHz on a DRX600 spectrometer (Bruker) and processed with the program XWINNMR 2.6.

One-dimensional ^{1}H spectra were recorded with 16 384 complex points over a spectral width of 6009.6 Hz. After 16 dummy scans to allow for preequilibration, 128 scans were signal averaged. The recycle delay was 2 s and a low power presaturation pulse was applied during the recycle delay. An exponential window function with 0.5 Hz line broadening was applied and the spectra were referenced to the H_2O signal at 4.95 ppm and 279 K.

Two-dimensional ^{13}C,^{1}H-heteronuclear single-quantum correlation (HSQC) spectra were collected using the standard HSQC pulse sequence [8,12–14] with 2048 complex points in t_2 over a spectral width of 7788.2 Hz. For each spectrum 512 t_1 experiments with 32 scans were acquired with a recycle delay of 2 s (measurement time 22 h). Spectra were zero filled to 4096 points in ω_1 and 2048 points in ω_2 to obtain a resolution of 1.5 Hz in ω_1 and 1.0 Hz in ω_2. A 90° shifted squared sinebell window function was applied for apodization prior to Fourier transformation in both dimensions. Automated baseline correction was applied in both dimensions.

Two-dimensional double-quantum filter correlated spectroscopy (DQF-COSY) spectra in ^2H_2O were collected using the standard pulse sequences [15–17] with 2048 complex points in t_2 over a spectral width of 6009.6 Hz. A total of 512 t_1 experiments with 32 scans were acquired with a recycle delay of 2 s (measurement time 22 h). Spectra were zero filled to 4096 points in ω_1 and 2048 points in ω_2 resulting in a resolution of 2.9 Hz in ω_1 and 1.5 Hz in ω_2. A 90° shifted squared sinebell window function was applied for apodization prior to Fourier transformation in both dimensions. Automated baseline correction was applied in both dimensions.

3. Results

3.1. Stereospecificity of MtdA at C14a of methylene-H$_4$MPT

The diastereotopic protons at C14a of methylene-H$_4$MPT exhibit different ^{1}H-NMR resonances, the chemical shift of the *pro-S* proton being 3.4 ppm and of the *pro-R* proton being 4.8 ppm [8] (Fig. 2, trace A). The resonance of the C14a proton of methenyl-H$_4$MPT$^+$ is at 9.0 ppm (Fig. 2, trace B). In ^{13}C,^{1}H-HSQC spectra of [14a-^{13}C]methylene-H$_4$MPT only the resonances at 3.4 and 4.8 ppm are observed and of

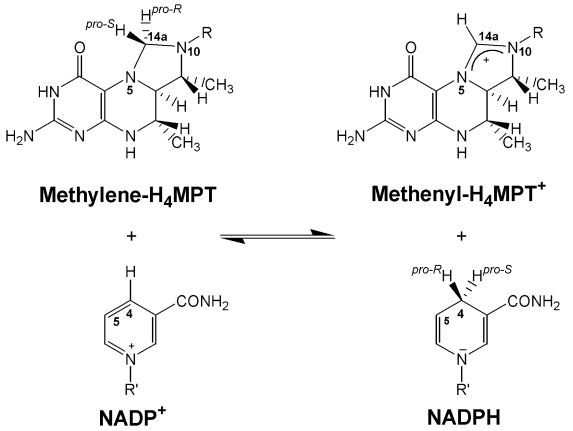

Fig. 1. Reaction catalyzed by NADP dependent MtdA. For the complete structure of tetrahydromethanopterin see [1]. H$_4$MPT is structurally and functionally analogous to tetrahydrofolate [25].

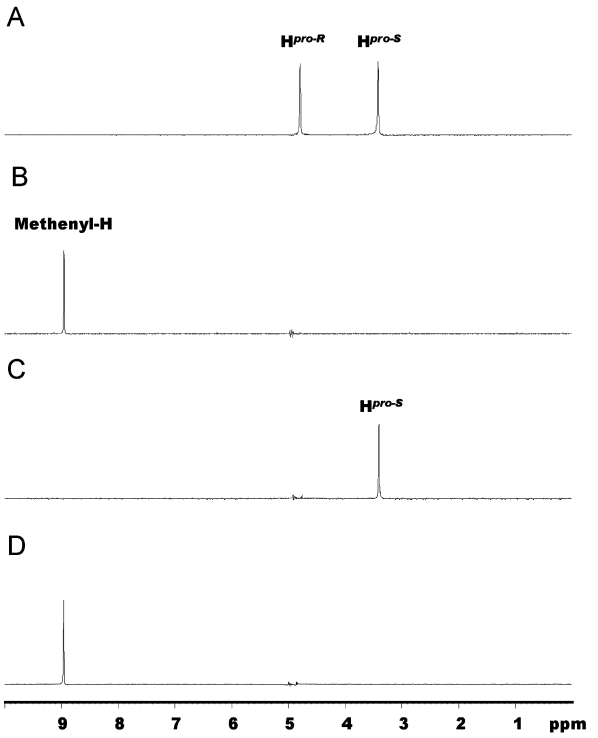

Fig. 2. Traces through ^{13}C,^{1}H-HSQC spectra along the ^{13}C resonance of C14a of [14a-^{13}C]methylene-H$_4$MPT and [14a-^{13}C]methenyl-H$_4$MPT$^+$ in ^2H_2O at pH 7.5 and 279 K. A: Methylene-H$_4$MPT (2 mM) showing the *pro-R* and *pro-S* protons at C14a. B: Methenyl-H$_4$MPT$^+$ (2 mM) showing the ^{1}H at C14a. C: (14a*R*)-[14a-^{2}H$_1$]-[14a-^{13}C]Methylene-H$_4$MPT (4 mM) showing the *pro-S* proton at C14a. The chemical shift of the *pro-S* proton changed slightly due to the geminal isotope effect of the C14a-^{2}H^{pro-R}. D: [C14-^{1}H]Methenyl-H$_4$MPT$^+$ obtained by oxidation of (14a*R*)-[14a-^{2}H$_1$]-[14a-^{13}C]methylene-H$_4$MPT with NADP$^+$ at pH 7.5 as catalyzed by MtdA from *M. extorquens*. The observation of the C14-^{1}H proton in the methenyl-H$_4$MPT$^+$ spectrum proves the *pro-R* specificity of the reaction.

[14a-^{13}C]methenyl-H$_4$MPT$^+$ only at 9.0 ppm. These differences in chemical shifts were exploited to determine the stereospecificity of MtdA at C14a of methylene-H$_4$MPT.

In Fig. 2, trace C, the ^{13}C,^{1}H-HSQC spectrum of (14a*R*)-[14a-^{2}H$_1$]-[14a-^{13}C]methylene-H$_4$MPT shows the resonance of the *pro-S* proton of C14a at 3.4 ppm, and Fig. 2, trace D, depicts the spectrum of the compound after oxidation to methenyl-H$_4$MPT$^+$ with NADP$^+$ in the presence of MtdA. The spectrum of the product (trace D) is identical to that of methenyl-H$_4$MPT$^+$ with a ^{1}H at C14a (trace B). The *pro-S* ^{1}H of (14a*R*)-[14a-^{2}H$_1$]-[14a-^{13}C]methylene-H$_4$MPT was thus retained in the product indicating that the ^{2}H$^-$ in the *pro-R* position rather than the ^{1}H$^-$ in the *pro-S* position of the labelled substrate was transferred to NADP$^+$. When (14a*S*)-[14a-^{2}H$_1$]-[14a-^{13}C]methylene-H$_4$MPT rather than (14a*R*)-[14a-^{2}H$_1$]-[14a^{13}C]methylene-H$_4$MPT was used to reduce NADP$^+$, methenyl-H$_4$MPT$^+$ with ^{2}H$_1$ at C14a was formed (not shown). The results indicate that MtdA is *Re*-face specific with respect to C14a of methylene-H$_4$MPT.

3.2. Stereospecificity of MtdA at C4 of NADP

The one-dimensional ^{1}H-NMR spectrum of the protons at C4 of NADPH shows two resonances which are both split by the geminal coupling between the two C4 protons (Fig. 3, trace A) [18]. These resonances were overlapped in the one-

dimensional spectrum by the resonances of other protons when, in addition to NADPH, methylene-H_4MPT and the enzyme MtdA were also present in the solution. In the two-dimensional ^{1}H DQF-COSY NMR spectrum (Fig. 3), however, the C4, C5 proton cross peak was well resolved.

In Fig. 3C the ^{1}H DQF-COSY NMR spectrum of (4*S*)-[4-$^2H_1$]NADPH is shown. The resonances of the *pro-R* hydrogen are shifted to lower ppm values due to the ^{2}H isotope effect exerted by the $^2H^{pro-S}$. The geminal proton deuterium coupling is too small to be observed. In Fig. 3D the spectrum of $NADP^+$ after reduction to NADPH with (14a*R*)-[14a-$^2H_1$]-[14a-^{13}C]methylene-H_4MPT in the presence of MtdA indicates that (4*R*)-[4-$^2H_1$]-[NADPH] was formed. The negative deuterium isotope effect and the removal of the splitting of the H^{pro-S} resonance is consistent with the presence of ^{2}H in the *pro-R* position. When (14a*S*)-[14a-$^2H_1$]-[14a-^{13}C]-methylene-H_4MPT rather than (14a*R*)-[14a-$^2H_1$]-[14a-^{13}C]methylene-H_4MPT was used to reduce $NADP^+$, NADPH containing two protons at C4 was formed (not shown). The results

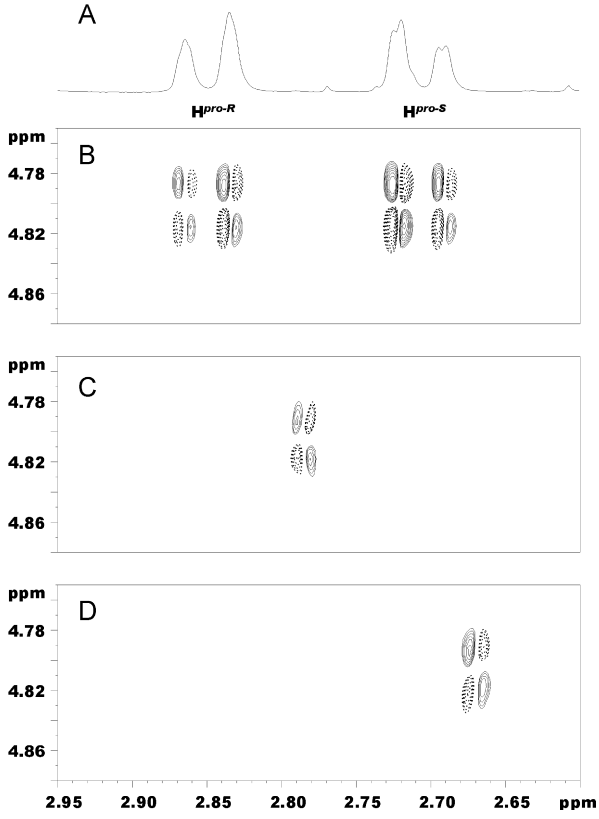

Fig. 3. ^{1}H-NMR spectra of the protons at C4 of NADPH in ^2H_2O at pH 7.5 and 279 K. One-dimensional ^{1}H-NMR spectrum of the protons at C4 (A) and cross peaks between the C4 and the C5 proton resonances in a ^{1}H DQF-COSY NMR spectrum of a sample containing 4 mM NADPH (B). C: Cross peak between the C4 and the C5 protons in a two-dimensional ^{1}H DQF-COSY NMR spectrum of 2 mM (4*S*)-[4-$^2H_1$]NADPH. The chemical shift of the C4 H^{pro-R} changed due to the deuterium isotope effect by C4 $^2H^{pro-S}$. D: Cross peak between the C4 and the C5 protons of a DQF-COSY spectrum of 2 mM NADPH generated by reduction of 2 mM $NADP^+$ with 4 mM (14a*R*)-[14a-$^2H_1$]-[14a-^{13}C]methylene-H_4MPT as catalyzed by MtdA from *M. extorquens* which was shown to be *Re*-face specific with respect to C14a of methenyl-H_4MPT^+ (see Fig. 2). The chemical shift of C4 $^1H^{pro-S}$ proton changed due to the deuterium isotope effect by the C4 $^2H^{pro-R}$. Negative contours are plotted in dashed lines.

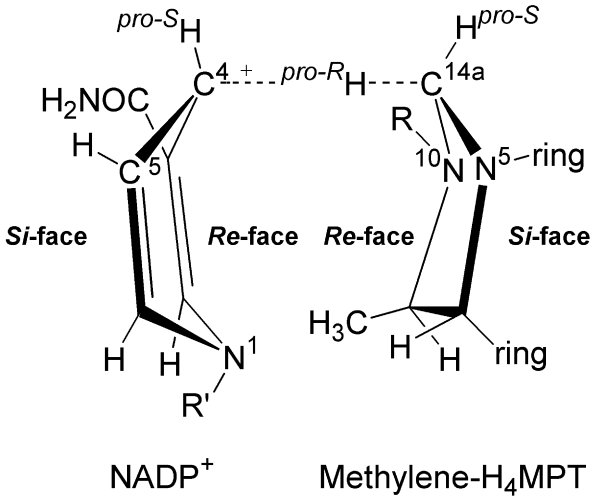

Fig. 4. Reduction of $NADP^+$ with methylene-H_4MPT as catalyzed by *Re*-face stereospecific MtdA (see also Fig. 1). The hydride transfer proceeds stereoselectively from the *pro-R* position of methylene-H_4MPT into the *pro-R* position of $NADP^+$. The conformations of $NADP^+$ and methylene-H_4MPT are shown for the transition state as proposed by [24,26].

thus indicate that the ^{2}H in the *pro-R* position of methylene-H_4MPT was transferred into the *pro-R* position of NADPH. MtdA is thus *Re*-face specific with respect to C4 of NADP.

4. Discussion

In Section 3 it was shown that MtdA is *Re*-face specific with respect to both C14a of methylene-H_4MPT and C4 of $NADP^+$. The *pro-R* hydrogen of methylene-H_4MPT is thus transferred into the *pro-R* position of $NADP^+$ as shown in Fig. 4.

The crystal structure of MtdA with $NADP^+$ bound has recently been determined to 1.9 Å resolution [19]. The pyridine nucleotide was located in a wide cleft with its *Si*-face bound to the protein. From the stereochemistry of hydride transfer we can now predict that methylene-H_4MPT has to bind on top of $NADP^+$ with its *Re*-face facing the *Re*-face of $NADP^+$, and that consecutive binding of the two substrates to the enzyme occurs with $NADP^+$ binding first.

MtdA from *M. extorquens* AM1 has the same stereospecificity as NAD(P) dependent methylenetetrahydrofolate dehydrogenase from Eucarya [11,20–23]. This was not per se predictable since MtdA and methylenetetrahydrofolate dehydrogenases do not show sequence similarities and are therefore considered to have evolved independently [1,24].

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