H_2-Forming N^5,N^{10}-Methylenetetrahydromethanopterin Dehydrogenase from *Methanobacterium thermoautotrophicum* Catalyzes a Stereoselective Hydride Transfer As Determined by Two-Dimensional NMR Spectroscopy†

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ABSTRACT: 5,6,7,8-Tetrahydromethanopterin is a coenzyme playing a key role in the energy metabolism of methanogenic archaea. In *Methanobacterium thermoautotrophicum*, the reduction of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin at C(14a) with H_2 to N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin can be catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase, a new hydrogenase present in most methanogenic archaea, which is unique because it does not contain nickel or iron/sulfur clusters. In this work, the stereochemistry of this enzymatic hydride-transfer reaction is elucidated by means of a series of heteronuclear two-dimensional NMR experiments. It is found that the hydride from H_2 is transferred by the enzyme into the *rel-(pro-R)* position of the C(14a) methylene group of the reaction product N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin. NMR experiments are described that show that the hydrogen nucleus of the hydride transferred to the oxidized coenzyme partially originates from water. The stereochemical course of this reaction is the same as that for direct hydride transfer. It is demonstrated that the diastereotopic atoms at C(14a) of the reaction product epimerize in an uncatalyzed reaction under the conditions of operation of the enzyme (k = 0.01 s^{-1} at 58 °C and pH 6.5). On the basis of the known relative configuration of the pterin moiety of 5,6,7,8-tetrahydromethanopterin [Schleucher, J., Schwörer, B., Zirngibl, C., Koch, U., Weber, W., Egert, E., Thauer, R. K., & Griesinger, C. (1992) *FEBS Lett. 314,* 440–444], the absolute configuration of this moiety is tentatively assigned to be (6*S*,7*S*, 11*R*) on the basis of a comparison of the CD spectra of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin and its analog N^5,N^{10}-methenyl-5,6,7,8-tetrahydrofolate. Given this absolute configuration of the pterin moiety, the *rel-(pro-R)* stereochemistry of the C(14a) methylene proton corresponds to the absolute (*pro-R*) stereochemistry.

5,6,7,8-Tetrahydromethanopterin (H_4MPT)[1] (Figure 1) serves as a carrier of C_1 fragments in the metabolism of methanogenic archaea (Keltjens & Vogels, 1988; DiMarco et al., 1990), being an analog of tetrahydrofolate, which is utilized by higher organisms as a C_1 carrier (Blakley & Benkovic, 1984). In the course of methanogenesis, a C_1-fragment is transferred to H_4MPT at the oxidation level of formic acid and is subsequently reduced to the methyl oxidation level in a stepwise manner. In the course of this reaction sequence, N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin ($CH{\equiv}H_4MPT^+$) and N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin ($CH_2{=}H_4MPT$) (Figure 1) are observed as intermediates.

In a previous paper (Schleucher et al., 1992) we described the relative stereospecific assignment of the diastereotopic methylene protons at C(14a) of $CH_2{=}H_4MPT$ and the determination of the relative configuration of the pterin moiety of H_4MPT. We found an anti/anti arrangement of the protons C(7a)H/C(6a)H and C(6a)H/C(11a)H. Recently, Keltjens et al. (1993) proposed a syn/syn configuration. Their assignment of the relative configuration is based on a coupling constant $^3J_{C(6a)H,C(7a)H} \approx 0$ Hz instead of 9.5 Hz (our work) as well as on an insufficient set of coupling constants: e.g. $^3J_{C(6a)H,C(11a)H}$ = 6.4 Hz (8.3 Hz our work). Keltjens et al. (1993) interpret this latter coupling by a dihedral angle of 0°. This implies a dihedral angle of 120° for C(6a)H–C(12a), which cannot be reconciled with the heteronuclear coupling constant $^3J_{C(6a)H,C(12a)}$ = 7.2 Hz (our work). In contrast, the $^3J_{C(6a)H,C(11a)H}$ = 8.3 Hz from our work and the $^3J_{C(6a)H,C(12a)}$ = 7.2 Hz are in agreement with dihedral angles of $\approx -150°$ and $\approx -20°$, respectively. Furthermore, the syn/syn arrangement of the protons at C(6a), C(7a), and C(11a) as proposed by Keltjens et al. (1993) contradicts our observation of an ROE between C(7a)H and C(11a)H that is stronger than the vicinal ROE between C(6a)H and C(11a)H.

H_2-forming methylenetetrahydromethanopterin dehydrogenase catalyzes the reversible reduction of $CH{\equiv}H_4MPT^+$ with H_2 to $CH_2{=}H_4MPT$ and H^+. The novel hydrogenase is found in most methanogenic archaea (Schwörer & Thauer, 1991; Rospert et al., 1991). The enzyme has been purified from *Methanobacterium thermoautotrophicum* (Zirngibl et al., 1990), *Methanobacterium wolfei* (Zirngibl et al., 1992),

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[1] Abbreviations: H_4MPT, 5,6,7,8-tetrahydromethanopterin; $CH_2{=}H_4$MPT, N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin; $CH{\equiv}H_4MPT^+$, N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin; H_4F, 5,6,7,8-tetrahydrofolate; $CH_2{=}H_4$F, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; $CH{\equiv}H_4F^+$, N^5,N^{10}-methenyl-5,6,7,8-tetrahydrofolate; CD, circular dichroism; CIP, Cahn–Ingold–Prelog (1956); HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum correlation; NADP, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROE, rotating frame Overhauser effect.

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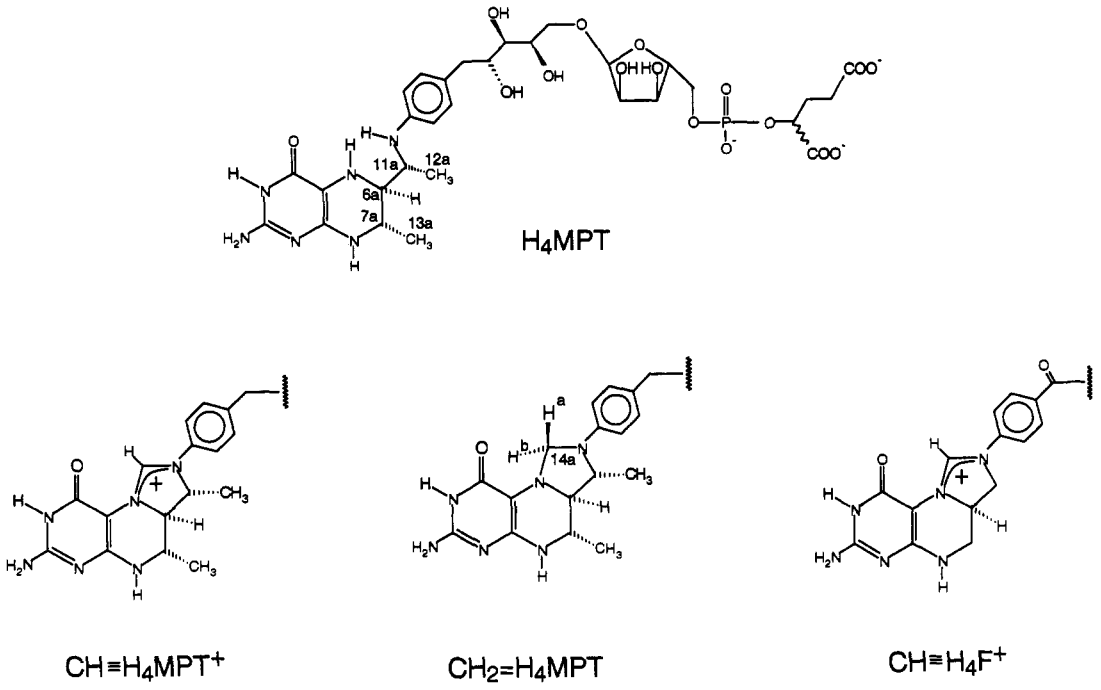

FIGURE 1: Formula of 5,6,7,8-tetrahydromethanopterin (H_4MPT) and relevant fragments of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin (CH≡H_4MPT$^+$), N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin (CH_2=H_4MPT), and N^5,N^{10}-methenyl-5,6,7,8-tetrahydrofolate (CH≡H_4F^+). The relative configuration of the pterin moiety of the H_4MPT derivatives and the relative stereospecific assignment of the protons of C(14a) of CH_2=H_4MPT [$\delta(H^a)$ = 4.8 ppm, $\delta(H^b)$ = 3.3 ppm] are indicated, as determined recently (Schleucher et al., 1992). The absolute configuration of the stereocenters of the pterin moiety of H_4MPT is indicated as proposed in this paper. The numbering scheme for the pterin moiety was adopted from van Beelen et al. (1984).

and *Methanopyrus kandleri* (Ma et al., 1991). The enzyme lacks chromophoric prosthetic groups, the primary sequences of the enzymes from *M. thermautotrophicum* and *M. kandleri* are devoid of characteristic binding motives for nickel or iron/sulfur clusters (von Bünau et al., 1991), and the only transition metal present in significant amounts appears to be zinc. Thus, no prosthetic group responsible for the activation of elemental hydrogen by this enzyme could be identified, in contrast to all hydrogenases known to date. Since the mechanism of H_2-activation by this novel enzyme is not yet understood, any information on the reaction mechanism is of importance.

The enzyme catalyzes the reduction of CH≡H_4MPT$^+$ with tritium-labeled hydrogen gas, generating CHT=H_4MPT. The specific activity of the labelled product after a 50-min incubation at 45 °C was 25% of that of the tritium-labelled H_2 or 50% of that of T (Zirngibl et al., 1992). This result can be interpreted to indicate that reduction of CH≡H_4MPT$^+$ by H_2 occurs via hydride transfer. An analysis of the isotopic composition of the hydrogen gas released during the oxidation of different isotopic combinations of methylene-H_4MPT and water indicates that the hydride transferred by the enzyme partially exchanges with another hydrogen atom from water before dihydrogen is released from the enzyme (Schwörer et al., 1993). Any mechanism to be proposed will have to take into account this exchange process.

Given the unique composition and properties of the novel enzyme, we have performed a series of hydrogenation experiments of methenyl-H_4MPT to methylene-H_4MPT in water, catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase from *M. thermoautotrophicum*. It proved to be necessary to permute the isotopic composition of methenyl-H_4MPT, hydrogen, and water, in order to distinguish between the direct hydride transfer and other reactions taking place during the hydrogenation reaction.

The absolute configuration of the pterin moiety of H_4MPT is tentatively obtained by comparison of the CD spectra of CH≡H_4MPT$^+$ and its analog N^5,N^{10}-methenyl-5,6,7,8-tetrahydrofolate (CH≡H_4F^+).

MATERIALS AND METHODS

[^{13}C]Formaldehyde (99% ^{13}C, 20% w/w in water) was from Cambridge Isotope Laboratories, CHDO (99.4%) was from MSD Isotopes (Montreal, Canada), [^{3}H]formaldehyde (1 mCi/mmol) and tritiated water (25 mCi/mL) were from New England Nuclear (Dreieich, Germany), [^{14}C]formaldehyde (0.27 mCi/mmol) was from Amersham Buchler (Braunschweig, Germany), and D_2O (99.96%) was from Sigma (Deisenhofen, Germany). Tritiated dihydrogen and D_2 were generated by reaction of tritiated water or D_2O with metallic lithium (Hallahan et al., 1986). 5,6,7,8-Tetrahydrofolate ((6*R*,*S*) diastereomeric mixture, 94% purity) was from Fluka (Neu-Ulm, Germany).

5,6,7,8-Tetrahydromethanopterin (H_4MPT) (DiMarco et al., 1990, Keltjens & Vogels, 1988) was isolated from *M. thermoautotrophicum* (Breitung et al., 1992) and further purified by reversed-phase HPLC on LiChrospher 100 RP-18 (4 mm × 125 mm column, Merck, Darmstadt, Germany) in 25 mM sodium formate buffer, pH 3.0 (Zirngibl et al., 1992). N^5,N^{10}-Methylene-5,6,7,8-tetrahydromethanopterin (CH_2=H_4-MPT) was generated from H_4MPT (3 μmol) and formaldehyde (3 μmol) in 0.5 mL of potassium phosphate buffer, pH 6.0, by spontaneous reaction (Escalante-Semerena et al., 1984). N^5,N^{10}-Methenyl-5,6,7,8-tetrahydromethanopterin was prepared by enzymatic dehydrogenation of N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin (Breitung et al., 1991). The compound was purified by HPLC as described for H_4MPT.

[*methenyl*-^{13}C,D]-N^5,N^{10}-Methenyl-5,6,7,8-tetrahydromethanopterin (^{13}CD≡H_4MPT$^+$) was generated from ^{13}CH≡H_4-

[2] Enzymes: N^5,N^{10}-methylene-tetrahydromethanopterin dehydrogenase (H_2-forming) (EC 1.12.99); N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate dehydrogenase (NADP-dependent) (EC 1.5.1.5).

Table 1: Summary of the Reactions Studied[a]

exp no.	reaction	products				fig. no.
		CH^aD^b=H_4MPT	CD^aH^b=H_4MPT	CH_2=H_4MPT	CD_2=H_4MPT	
1	^{13}CH≡H_4MPT$^+$ + D_2, D_2O	X	–	–	x[b,e]	5
2	^{13}CD≡H_4MPT$^+$ + H_2, H_2O	–	X	x[b]	–	6
3	^{13}CD≡H_4MPT$^+$ + H_2, D_2O	x[c]	X	–	x[d,e]	7
4	^{13}CH≡H_4MPT$^+$ + D_2, H_2O	X	x[c]	x[d]	–	8
5	^{13}CD≡H_4MPT$^+$ + D_2, H_2O	x[b,c]	x[d]	x[b,d]	X[e]	9
6	^{13}CH≡H_4MPT$^+$ + H_2, D_2O	x[d]	x[b,c]	X	x[b,d,e]	not shown

[a] In each case, the appropriate isotopomer of methenyl-H_4MPT$^+$ was incubated with H_2-forming methylenetetrahydromethanopterin dehydrogenase under H_2 or D_2 in H_2O or D_2O for 1 min at 40 °C. The composition of the reaction products as observed at low temperature is given. The product formed by the direct reaction of methenyl-H_4MPT with dihydrogen is marked "X". Other products formed are marked "x". Products not detected are marked "–". [b] Product formed due to exchange of the educt with the solvent. [c] Product formed by partial epimerization of the primary product. [d] Product formed via enzyme-mediated exchange of the hydrogen atom transferred with water. [e] Product cannot be observed in the spectra displayed but can be observed in ^{13}C-detected spectra.

MPT$^+$ by exchange of the methenyl hydrogen with D^+ in D_2O at pD 6.0 and 40 °C for 5 h (see the Results section).

Epimeric N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate (CH_2=H_4F) was generated from epimeric 5,6,7,8-tetrahydrofolate (H_4F)(1 μmol) and formaldehyde (3 μmol) in 0.5 mL of potassium phosphate buffer, pH 6.0, by spontaneous reaction (Kallen & Jencks, 1966). (6*R*)-N^5,N^{10}-Methenyl-5,6,7,8-tetrahydrofolate (CH≡H_4F^+) was prepared from CH_2=H_4F by dehydrogenation via NADP-dependent N^5,N^{10}-methylenetetrahydrofolate dehydrogenase from *Peptostreptococcus productus* (Wohlfarth et al., 1991). The dehydrogenation product was separated from the nonreacting epimer of methylenetetrahydrofolate by reversed-phase HPLC, as described for H_4MPT.

H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase was purified to apparent homogeneity from *M. thermoautotrophicum*, as described (Zirngibl et al., 1992). NADP-dependent N^5,N^{10}-methylenetetrahydrofolate dehydrogenase was purified from *P. streptococcus productus* (Wohlfarth et al., 1991).

pH measurements were performed using a glass electrode calibrated with buffer solutions in H_2O. The pD of reaction mixtures in D_2O was measured using the same calibrated electrode taking into account the experience that pD = pH + 0.41, where pH is the pH meter reading (Covington et al., 1968).

NMR Spectroscopy. Unless stated otherwise, NMR spectra were acquired on an AMX 600 spectrometer equipped with a broad-band inverse probe. Heteronuclear single quantum correlation spectra (HSQC) (Bodenhausen & Ruben, 1980) were typically recorded with 1024 real points in t_2 and 128 real points in t_1, covering a sweep width of 5000 and 1500 Hz, respectively. A spectral width of 750 Hz was chosen in ω_1 if the $^1J_{C,D}$ coupling was to be resolved. With the exception of the experiment display in Figure 9, samples were measured in D_2O and were lyophilized to remove H_2O if necessary. To suppress the signal of residual HDO, a spin-lock pulse without attenuation of 2-ms duration and/or z-spoil was included into the HSQC sequence (Messerle et al., 1989). To avoid any attenuation of the downfield resonance of the C(14a) methylene group of CH_2=H_4MPT close to the HDO resonance, no presaturation of the residual solvent line was applied. The spectrum displayed in Figure 9 was recorded in H_2O/D_2O, 9:1, on an AMX 600 spectrometer equipped with an inverse probe with an actively shielded gradient coil. Pulsed field gradients (Hurd & John, 1991) were used in this experiment both for water suppression and for coherence selection in t_1. The HSQC pulse sequence employed in this experiment (Kay et al., 1992) contained a heteronuclear gradient echo formed by the application of a gradient pulse during t_1 and prior to acquisition (Schleucher et al., 1993). ^{13}C GARP decoupling (Shaka et al., 1985) with a field strength of $\gamma B_1/2\pi$ = 2 kHz was employed during t_2. Spectra were Fourier-transformed after multiplication with a squared sine bell shifted by $\pi/3$ in both dimensions. Gaussian apodization was used in ω_1 to resolve the $^1J_{C,D}$ coupling. ^{13}C-filtered ^{1}H NMR spectra were recorded using a 1D X-filter (Wörgötter et al., 1986) pulse sequence. In all heteronuclear experiments, the carbon carrier frequency was placed at 70 ppm, close to the resonance frequency of the methylene carbon of CH_2=H_4MPT. Exchange spectra of samples of chemically synthesized CHD=H_4MPT were recorded on an AMX 600 spectrometer at 58 and 53 °C using a mixing time of 1.5 s. Chemical shifts are referenced relative to external 3-(trimethylsilyl)propionate-d_4 dissolved in the same buffer as was present in the samples to be referenced.

CD Spectroscopy. CD spectra were recorded at room temperature on a Cary 61 photometer (Applied Physics Corporation). The concentrations of the samples examined are given in the legend to Figure 10.

RESULTS

Evidence has been discussed that the transfer of a hydride from H_2 to CH≡H_4MPT$^+$ by H_2-forming methylenetetrahydromethanopterin dehydrogenase probably proceeds in a stereoselective manner (Schwörer et al., 1993). Reduction of CH≡H_4MPT$^+$ with D_2 should therefore yield only one of the two possible diastereomers of CHD=H_4MPT. The other isomer should be formed in the reaction of CD≡H_4MPT$^+$ with H_2. Besides these combinations of the isotopes present in the substrates, the solvent in which the reductions are carried out (H_2O or D_2O) can also be varied, resulting in four reactions to be studied. As will become obvious from the results of these four experiments, two more isotopic combinations can yield information on the mechanism of the hydride transfer. The reactions studied are summarized in Table 1.

Analysis of the products formed in such a reaction by NMR spectroscopy allows one to identify the protonated position by means of its proton chemical shift. On the basis of the relative stereospecific assignment of the diastereotopic methylene protons of CH_2=H_4MPT, the relative orientation of the transferred hydrogen atom in the pterin moiety of CH_2=H_4MPT can be inferred. Provided the absolute configuration of the pterin moiety of H_4MPT as derived by comparison of the CD spectra of CH≡H_4MPT$^+$ and CH≡H_4F^+ is correct, the absolute stereochemistry of the hydride transfer to CH≡H_4MPT$^+$ can be inferred.

To facilitate the analysis of the ^{1}H NMR spectrum of the reaction product methylene-H_4MPT, the educt methenyl-H_4-

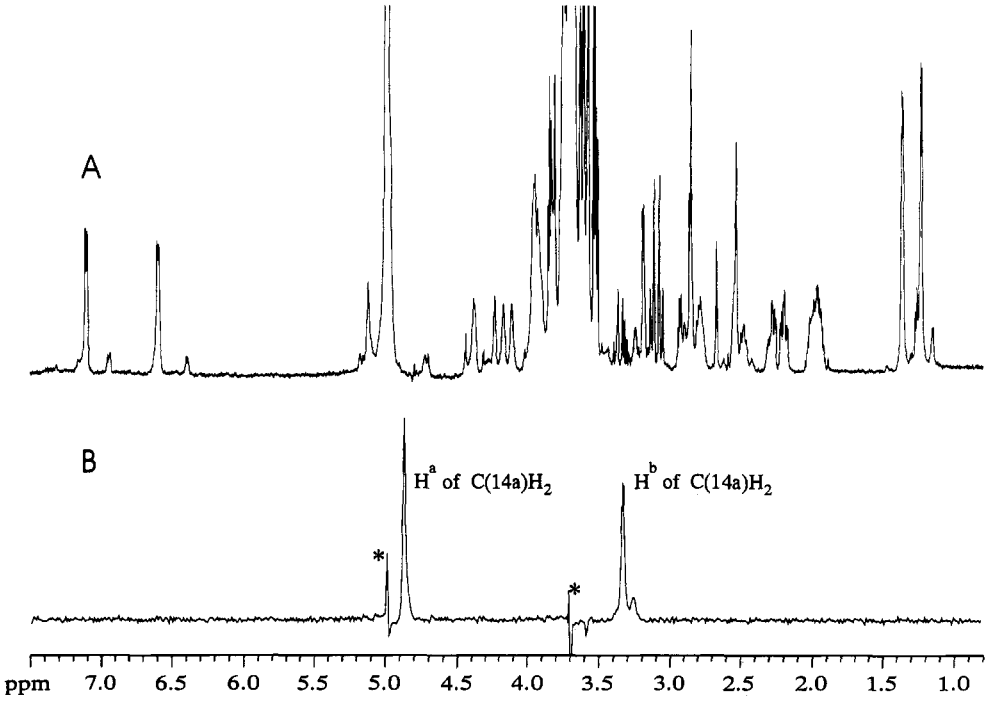

FIGURE 2: (A) ^1H NMR spectrum of the reaction product of experiment 2 at 8 °C. (B) Trace through the HSQC spectrum displayed in Figure 6A, showing the resonances of $^{13}CH_2$=H_4MPT formed in experiment 2. The signal-to-noise ratio in the two spectra differs because of different measurement times and because ^{13}C decoupling was applied in spectrum B. The asterisks mark artifacts remaining from the strong signals of residual HDO and buffer.

MPT was specifically labeled with ^{13}C in the methenyl position. This proved to be necessary given the overlap encountered in the one-dimensional ^1H spectrum (Figure 2A). ^{13}C-Filtering techniques avoid these problems completely, since only the interesting group is visible. Spontaneous reaction of H_4MPT with $^{13}CH_2O$ yields $^{13}CH_2$=H_4MPT, which is converted to ^{13}CH≡H_4MPT^+ by enzymatic dehydrogenation, which via exchange with D_2O forms ^{13}CD≡H_4MPT^+. Figure 2B, a trace taken from the spectrum displayed in Figure 6, shows the signals of the C(14a) methylene group of $^{13}CH_2$=H_4MPT, which resonate at 3.3 ppm (*rel-(pro-S)* position, H^a) and 4.9 ppm (*rel-(pro-R)* position, H^b). Analysis of the reaction products is performed by 2D ^1H, ^{13}C HSQC spectroscopy, since this methodology offers the unique possibility to observe the proton and the carbon chemical shifts and the carbon multiplicities with respect to deuterium simultaneously. The splitting of the carbon resonance by the $^1J_{C,D}$ coupling into a triplet provides an elegant proof for a CHD group. Since the measurement time is determined by the rather low sample concentration and not by the requirement to obtain adequate resolution in the indirectly sampled frequency domain, 2D experiments do not require substantially longer measurement times than 1D techniques. Experiment times for the 2D spectra did not exceed $^1/_2$ h.

Exchange of the Methenyl Hydrogen of CH≡*H_4MPT^+ with Protons of Water.* During recording of the proton NMR spectrum of CH≡H_4MPT^+ in D_2O it was observed that the signal of the methenyl hydrogen gradually disappeared, indicating that the methenyl hydrogen exchanged with D^+ of D_2O. It was already known that the methenyl hydrogen of N^5,N^{10}-methenyl-5,6,7,8-tetrahydrofolate (CH≡H_4F^+) slowly exchanges with protons of water (Poe & Benkovic, 1980).

For the interpretation of the results obtained from the enzymatic reduction of CH≡H_4MPT^+ with D_2 in D_2O, it was important to know the rate of CD≡H_4MPT^+ formation from CH≡H_4MPT^+ by exchange with D_2O under the experimental conditions. To estimate these rates, experiments

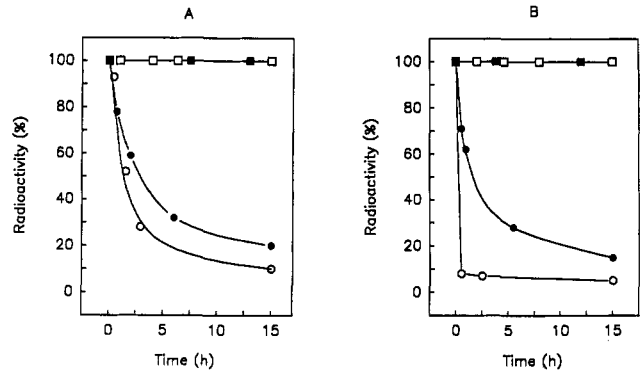

FIGURE 3: Exchange at 40 °C of the tritium in CT≡H_4MPT^+ and in CT≡H_4F^+ with protons of water: (A) time course of exchange at pH 6.0; (B) time course of exchange at pH 7.5. The anaerobic assay mixture (1 mL) contained 120 mM K_2HPO_4, pH 6.0 or pH 7.5, and 100 μM CT≡H_4MPT^+ or 100 μM CT≡H_4F^+ (58 Bq/nmol) or, as a control, 100 μM ^{14}CH≡H_4MPT^+ or 100 μM ^{14}CH≡H_4F^+ (6 Bq/nmol). The reaction was started at t = 0 by the addition of the labeled compound. At the times indicated 100-μL samples were withdrawn and subjected to HPLC for the analysis of the specific radioactivity (Zirngibl et al., 1992) of CT≡H_4MPT^+ or CT≡H_4F^+, respectively: □, ^{14}CH≡H_4MPT^+; ■, ^{14}CH≡H_4F^+; ○, CT≡H_4MPT^+; ●, CT≡H_4F^+.

with tritium-labeled methenyl-H_4MPT were performed.

The time course of the loss of tritium from CT≡H_4MPT^+ in H_2O at 40 °C was determined at pH 6.0 (Figure 3A) and pH 7.5 (Figure 3B). For a comparison the loss of tritium from CT≡H_4F^+ under the same conditions was measured. The findings indicate that exchange takes place on a time scale of hours, with the rate of exchange increasing with increasing pH. Exchange is more rapid for CH≡H_4MPT^+ compared to CH≡H_4F^+. The rate of exchange also showed a pronounced temperature dependence. At 60 °C the exchange rate was 12 times higher than that at 40 °C (not shown).

As a control we determined with ^{14}CH≡H_4MPT^+ and ^{14}CH≡H_4F^+ whether the methenyl group as such remained

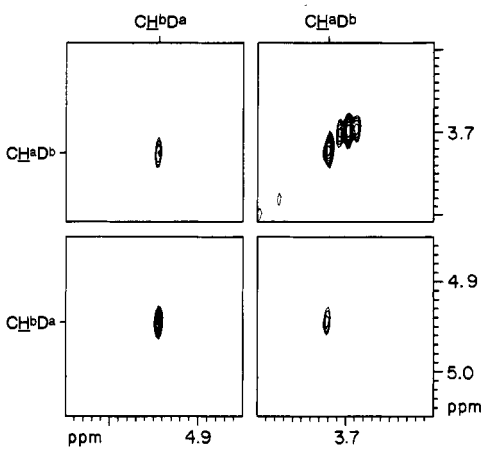

FIGURE 4: Exchange spectrum of chemically synthesized CHD=H_4-MPT recorded at 58 °C, pH 6.5. The exchange rate is found to be 0.01 s^{-1} under these conditions. Cross-peaks are plotted on a 50 times lower level than diagonal peaks.

intact under the experimental conditions. This was found to be the case. No radioactivity was lost from the ^{14}C-labelled compounds during the incubations (Figure 3).

We determined whether the H_2-forming methylenetetrahydromethanopterin dehydrogenase had an effect on the CT=H_4MPT^+/H^+ exchange rate. Such an effect was not observed. The exchange rates in the absence and presence of the enzyme (0.5 units) were essentially identical (not shown).

Epimerization of CHD=H_4MPT at C(14a). The reduction of CH=H_4MPT^+ with tritium-labeled dihydrogen in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase yielded CHT=H_4MPT. When this compound was immediately reoxidized by removal of the dihydrogen from the assay system, all the radioactivity was lost into the gas phase: The regenerated CH=H_4MPT^+ was not radioactive. This finding indicates that the enzymatic reduction and enzymatic oxidation are stereospecific with respect to the diastereotopic positions at C(14a) of methylene-H_4MPT. When the CHT=H4MPT was reoxidized after a period of 30 min at 40 °C, approximately 50% of the radioactivity was retained in the methenyl-H_4MPT^+. These findings suggest that stereospecifically tritium-labeled methylene-H_4MPT epimerized under the experimental conditions.

Epimerization could be demonstrated directly by exchange spectroscopy (Jeener et al., 1979) on samples of chemically synthesized CHD=H_4MPT (Figure 4). In this compound NOE contributions to the exchange cross-peaks between the methylene protons that would be observed in CH_2=H_4MPT are absent. The exchange rate was found to be 0.01 s^{-1} at 58 °C and pH 6.5, being strongly dependent on temperature and decreasing rapidly with increasing pH. No exchange of the methylene protons of CHD=H_4MPT with water was observed.

Enzymatic Reduction of Methenyl-H_4MPT with Dihydrogen. The epimerization of CHD=H_4MPT at elevated temperatures in the absence of enzyme, the exchange of the methenyl proton of CH=H_4MPT^+ with the solvent (see above), and the exchange of the methylene group of CH_2=H_4-MPT with free formaldehyde (Zirngibl et al., 1992) are side reactions that can interfere with the determination of the stereochemical course of the reduction under investigation. For the determination of the stereochemistry of the enzymatic reduction of methenyl-H_4MPT with dihydrogen to the isotopomers of methylene-H_4MPT, we therefore chose as reaction conditions the highest possible pH (8.5) and the lowest possible temperature (40 °C) and quenched the reaction after 1 min. Subsequent manipulations were performed at 4 °C,

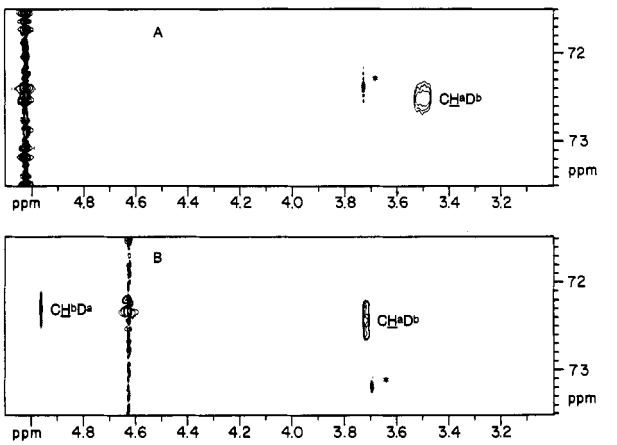

FIGURE 5: ^{13}C HSQC spectrum of methylene-H_4MPT generated by reduction of ^{13}CH=H_4MPT^+ with D_2 in D_2O in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase at pD 8.9. The enzyme-catalyzed reaction was performed in an 8-mL serum bottle filled with 100% D_2 and containing 0.5 mL of the following assay mixture: 120 mM K_2HPO_4, pD 8.9, 3.4 mM ^{13}CH=H_4MPT^+, and 3 units of purified dehydrogenase (activity in D_2O under reaction conditions). The reaction mixture was incubated at 40 °C for 1 min with continuous shaking at 200 rpm and then cooled on ice. After ultrafiltration (Centricon 30) the ultrafiltrate was frozen at −70 °C. After 2 h the sample was thawed and analyzed by NMR spectroscopy at 5 °C (A) and at 40 °C (B). The asterisks in the spectra mark signals originating from an impurity.

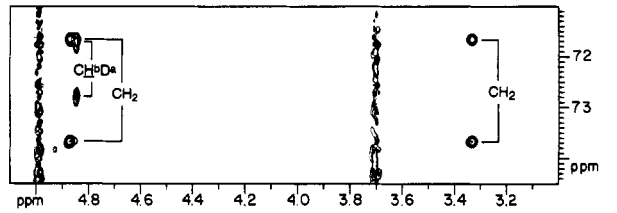

FIGURE 6: ^{13}C HSQC spectrum of methylene-H_4MPT generated by reduction of ^{13}CD=H_4MPT^+ with H_2 in H_2O in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase at pH 8.5. The enzyme-catalyzed reaction was performed in an 8-mL serum bottle filled with 100% H_2 and containing 0.5 mL of the following assay mixture: 120 mM K_2HPO_4, pH 8.5, 4 mM ^{13}CD=H_4MPT^+, and 2.5 units of purified dehydrogenase (activity in H_2O under reaction conditions). The reaction mixture was incubated for 1 min at 40 °C with continuous shaking (200 rpm) and then cooled on ice. After ultrafiltration (Centricon 30) the ultrafiltrate was frozen (−70 °C) and then lyophilized for 8 h. After storage for 12 h at −70 °C the lyophylisate was dissolved in 0.5 mL of D_2O at 5 °C directly before NMR spectroscopic analysis at 8 °C.

and NMR spectra were at first recorded at 5 °C. At pH values above 8.5 CH=H_4MPT^+ rapidly hydrolizes to N^{10}-formyl-H_4MPT in a spontaneous reaction (DiMarco et al., 1990). At temperatures below 40 °C H_2-forming methylene-H_4MPT dehydrogenase is no longer active, since the enzyme employed was from *M. thermoautotrophicum*, which is a thermophilic organism growing optimally at 65 °C.

Figure 5A shows a HSQC spectrum of the reaction product of the reduction of ^{13}CH=H_4MPT^+ with D_2 in D_2O (experiment 1 in Table 1) recorded at a temperature of 5 °C. Only the species $^{13}CH^aD^b$=H_4MPT, with H^a being the high-field methylene proton of the C(14a) methylene group, is observed. (That the signal of $^{13}CH^bD^a$=H_4MPT is not obscured by the water signal can be seen in Figure 6). Since D^- was transferred in this experiment, while the proton observed in this reaction product stems from ^{13}CH=H_4MPT^+, it is concluded that the hydride (D^- in this experiment) is transferred to the low-field site in the methylene group. At 40 °C (Figure 5B), partial epimerization has taken place, as is evident from the occurrence of $^{13}CH^bD^a$=H_4MPT. The

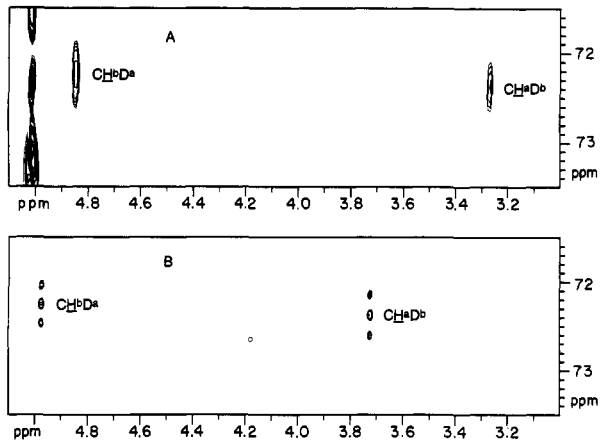

FIGURE 7: ^{13}C HSQC spectrum of methylene-H_4MPT generated by reduction of $^{13}CD{\equiv}H_4MPT^+$ (5 mM) with H_2 in D_2O in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase at pD 8.9. For experimental details see the legend to Figure 5. The sample was stored for 12 h at −70 °C before analysis by NMR spectroscopy at 5 °C (A) and at 58 °C (B).

$^1J_{C,D}$ coupling of the $^{13}CHD{=}H_4MPT$ species, which is not resolved in the spectrum recorded at low temperature, is visible along ω_1 in this spectrum, which proves unambiguously that the species observed at low temperature is $^{13}CH^aD^b{=}H_4$-MPT. In all experiments (Table 1), spectra were also recorded at elevated temperature and both the splitting due to $^1J_{C,D}$ and epimerization at C(14a) were observed. Spectra at elevated temperatures are displayed for experiments 1 (Figure 5B), 3 (Figure 7B), and 5 (Figure 9B) but not for the control experiments 2, 4, and 6.

The result of the reduction of $^{13}CD{\equiv}H_4MPT^+$ with H_2 in H_2O (experiment 2) is displayed in Figure 6. At 8 °C, $^{13}CH^bD^a{=}H_4MPT$ is observed as the only isomer of $^{13}CHD{=}H_4MPT$. Since the $^1J_{H,C}$ coupling was not refocused during t_1 in this experiment, the signal of $^{13}CH^bD^a{=}H_4MPT$ shows a splitting due to $^1J_{C,H} \approx 150$ Hz along ω_1. In addition, two doublets with a splitting of ≈300 Hz are visible. The observed multiplicity is expected for CH_2 groups. These signals originate from $^{13}CH_2{=}H_4MPT$, which is formed from $^{13}CH{\equiv}H_4MPT^+$ after exchange of $^{13}CD{\equiv}H_4MPT^+$ with the solvent as described above.

As can be seen in Figure 7A, the reaction of $^{13}CD{\equiv}H_4$-MPT^+ with H_2 in D_2O (experiment 3) results in predominant formation of $^{13}CH^bD^a{=}H_4MPT$. The observation of the other epimer in this experiment is probably due to partial epimerization of the primary product. At higher temperature, epimerization is complete and the $^1J_{C,D}$ coupling is resolved in ω_1 (Figure 7B).

The results of the fourth combination of isotopes, the reaction of $^{13}CH{\equiv}H_4MPT^+$ with D_2 in H_2O (experiment 4), are shown in Figure 8. Again, $^{13}CH^aD^b{=}H_4MPT$ dominates over $^{13}CH^bD^a{=}H_4MPT$, as expected from the outcome of the other reactions. The signals of $^{13}CH_2{=}H_4MPT$ appeared in this spectrum, too. The formation of $^{13}CH_2{=}H_4MPT$ in this reaction can be understood on the basis of the finding that during hydride transfer rapid stereospecific exchange between the hydrogen transferred and hydrogen atoms of water occurs (Schwörer et al., 1993). This reaction path can be demonstrated in experiments 5 and 6. Figure 9 shows the result of the reduction of $^{13}CD{\equiv}H_4MPT^+$ with D_2 in H_2O. The formation of protonated species of methylene-H_4MPT in this reaction unambiguously demonstrates the incorporation of hydrogen atoms from the solvent into the reaction product. The predominant formation of $^{13}CH^bD^a{=}H_4MPT$ shows that

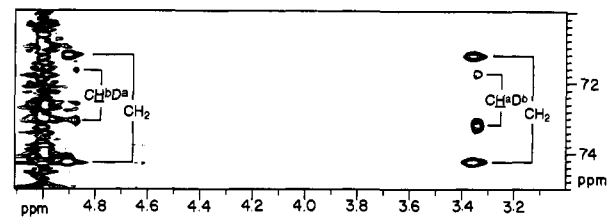

FIGURE 8: ^{13}C HSQC spectrum of methylene-H_4MPT generated by the reduction of $^{13}CH{\equiv}H_4MPT^+$ (4 mM) with D_2 in H_2O in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase at pH 8.5. For experimental details see the legend to Figure 6. The lyophylisate was stored for 2 h at −70 °C and dissolved in 0.5 mL of D_2O at 5 °C directly before NMR analysis at 6 °C. The spectrum was recorded on an AMX 400 spectrometer.

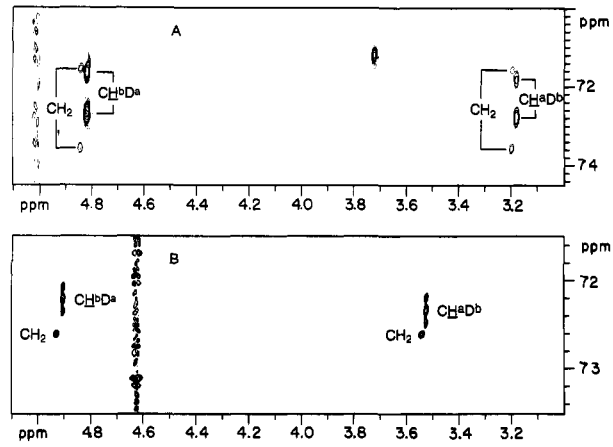

FIGURE 9: ^{13}C HSQC spectrum of methylene-H_4MPT generated by the reduction of $^{13}CD{\equiv}H_4MPT^+$ (4 mM) with D_2 in H_2O in the presence of H_2-forming methylenetetrahydromethanopterin dehydrogenase at pH 8.5. For experimental details see the legend to Figure 6. The assay mixture contained 120 mM K_2HPO_4, pH 8.5, 9 mM $^{13}CD{\equiv}H_4MPT^+$, and 9 units of purified methylenetetrahydromethanopterin dehydrogenase (activity in H_2O under reaction conditions). After a 1-min incubation at 40 °C followed by ultrafiltration at 4 °C, the sample was stored for 2 h at −70 °C and was then analyzed by NMR spectroscopy at 5 °C (A) and at 40 °C (B).

the hydride transferred via this solvent-mediated route is delivered to the same position as that for direct transfer from dihydrogen. Besides by epimerization of the primary product $^{13}CH^bD^a{=}H_4MPT$, the epimer $^{13}CH^aD^b{=}H_4MPT$ can also be formed by transfer of D^- to $^{13}CH{\equiv}H_4MPT^+$, which is formed by exchange of $^{13}CD{\equiv}H_4MPT^+$ with the solvent. (An analogous pathway exists for the formation of $^{13}CH^bD^a{=}H_4MPT$ in experiment 6). $^{13}CH_2{=}H_4MPT$ is observed as a byproduct formed due to exchange of both $^{13}CD{\equiv}H_4MPT^+$ and the D^- transferred with the solvent. As was to be expected from the results of the previous experiments, the reduction of $^{13}CH{\equiv}H_4MPT^+$ with H_2 in D_2O yielded mainly $^{13}CH^aD^b{=}H_4MPT$ in excess of $^{13}CH^bD^a{=}H_4MPT$ as well as $^{13}CH_2{=}H_4MPT$ formed by direct transfer of H^- (results not shown).

Determination of the Absolute Configuration of the Pterin Moiety of H_4MPT by Circular Dichroism. The relative configuration of the pterin moiety of H_4MPT has recently been established by heteronuclear NMR of $CH{\equiv}H_4MPT^+$ (Schleucher et al., 1992). Although centers of known absolute configuration are present in H_4MPT, they are unsuitable for the determination of the absolute configuration of the pterin moiety by NMR. However, the pterin moiety of $CH{\equiv}H_4$-MPT^+ is the only conjugated chromophore in this molecule, and its constitution is very similar to the constitution of the chromophoric part of $CH{\equiv}H_4F^+$ of known absolute config-

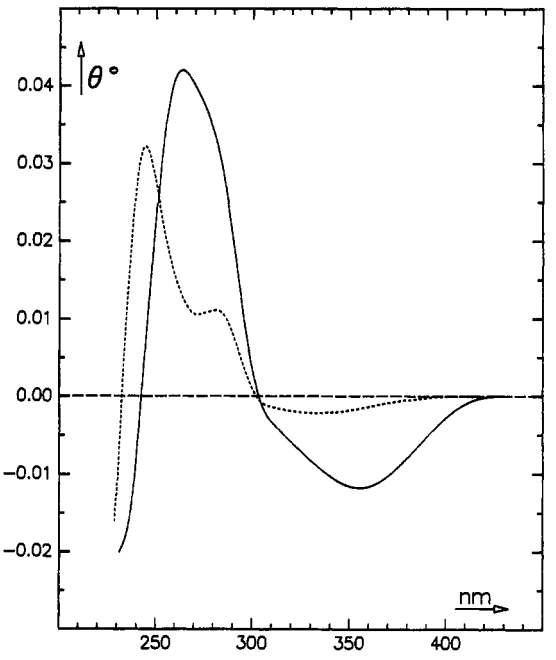

FIGURE 10: CD spectra of CH≡H_4MPT$^+$ (broken line) and of (6*R*)-CH≡H_4F$^+$ (solid line) in 25 mM potassium formate, pH 3.0, at room temperature. The concentrations of CH≡H_4MPT$^+$ and (6*R*)-CH≡H_4F$^+$ were 52 and 92 μM, respectively. The concentration of CH≡H_4MPT$^+$ was calculated from the absorbance difference (relative to buffer solution) at 334 nm (ϵ = 21.6 mM^{-1} cm^{-1} (Escalante-Semerena et al., 1984), and the concentration of CH≡H_4F$^+$, from the absorbance difference at 356 nm (ϵ = 25.1 mM^{-1} cm^{-1} (Rabinovic, 1963)).

uration ((6*R*) for CH≡H_4F$^+$, corresponding to (6*S*) for H_4F) (Fontecilla-Camps et al., 1979) (Figure 1). The conformations of the pterin moieties of CH≡H_4F$^+$ (Fontecilla-Camps et al., 1979; Khalifa et al., 1979) and CH≡H_4MPT$^+$ (Schleucher et al., 1992) are also very similar. It can be concluded that the conformation of the pterin moieties is governed by the asymmetric center C(6a), which is common to CH≡H_4MPT$^+$ and CH≡H_4F$^+$, while the introduction of the methyl substituents does not influence the conformation of this chromophore significantly. Therefore, a comparison of the CD spectra of CH≡H_4MPT$^+$ and CH≡H_4F$^+$ is a suitable way to deduce the absolute configuration of the pterin moiety of H_4MPT. CD spectra of CH≡H_4MPT$^+$ and CH≡H_4F$^+$ are shown in Figure 10.

The qualitative similarity of the CD spectra indicates that the absolute configuration of CH≡H_4MPT$^+$ at C(6a) is the same as the absolute configuration of CH≡H_4F$^+$ at this center. Therefore, the absolute configuration of the pterin moiety of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin is tentatively assigned to be (6*R*,7*S*,11*R*), which is the configuration we proposed in a previous paper (Schleucher et al., 1992). For free H_4MPT this translates into (6*S*,7*S*,11*R*). We did not recognize this change in the CIP descriptor for C(6a) of free H_4MPT, which was erroneously assigned to be (6*R*,7*S*,11*R*) in the abstract of the publication by Schleucher et al. (1992).

DISCUSSION

The results presented show that H_2-forming methylenetetrahydromethanopterin dehydrogenase catalyzes the stereospecific transfer of a hydride from dihydrogen into the low-field position of the C(14a) methylene group of methylene-H_4MPT. On the basis of the determination of the absolute configuration of H_4MPT as described above, the stereochemical designation of the low-field hydrogen, which is activated by H_2-forming methylenetetrahydromethanopterin dehydrogenase, is (*pro-R*). While this stereochemical course of the hydrogenation can be deduced from the outcome of experiments 1 and 2 alone, these experiments do not show whether the hydride transferred is derived from molecular hydrogen or from water. This question is addressed by experiments 3 and 4, which show that the hydride transferred indeed originates from molecular hydrogen. Whilst the observed stereoselectivity of this transfer is complete in experiments 1 and 2, only partial selectivity is observed in experiments 3 and 4. However, since transfer of both D^- and H^- is studied in experiments 1 and 2, the conclusion seems justified that the reduction is fully selective given the limits of sensitivity of the experiments performed. The smaller degrees of stereoselectivity observed in experiments 3 and 4 can be accounted for by varying degrees of spontaneous epimerization (as shown in Figure 4) of the respective primary products.

A complication regarding the mechanism of H_2-forming methylenetetrahydromethanopterin dehydrogenase arises from the result of experiment 4. CH_2=H_4MPT can only be formed in this experiment by transfer of a hydrogen atom originating from the solvent to CH≡H_4MPT$^+$. Therefore either the enzyme must catalyze a selective exchange between H^b of CH_2=H_4MPT (the H activated by the enzyme) and the solvent or an enzyme-catalyzed exchange between D_2 and H_2O without participation of any derivative of H_4MPT must have occurred. The second possibility, however, can be ruled out given the finding (Schwörer et al., 1993) that H_2-forming methylenetetrahydromethanopterin dehydrogenase does not catalyze an exchange of H_2 with D_2O in the absence of CH≡H_4MPT$^+$. Whilst it remains to be shown that H_2-forming methylenetetrahydromethanopterin dehydrogenase selectively catalyzes the exchange of H^b with the solvent, it can be concluded from the outcome of the experiments 5 (Figure 9) and 6 that the H^- transferred to CH≡H_4MPT$^+$ via this solvent-mediated route is delivered to the same diastereotopic position as is observed for direct transfer. Otherwise, the combination of isotopes of experiment 2 would also have yielded both epimers of CHD=H_4MPT. This conclusion is in accordance with the previous observation (Zirngibl et al., 1992) that one of the methylene hydrogens of CH_2=H_4MPT remains untouched by the enzyme.

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REFERENCES

Blakley, R. L., & Benkovic, S. J. (1984) in *Folates and Pterines, Vol. 1, Chemistry and Biochemistry of Folates*, J. Wiley and Sons, New York, Chichester.

Bodenhausen, G., & Ruben, D. J. (1980) *Chem. Phys. Lett. 69*, 185–189.

Breitung, J., Schmitz, R. A., Stetter, K. O., & Thauer, R. K. (1991) *Arch. Microbiol. 156*, 517–524.

Breitung, J., Börner, G., Scholz, S., Linder, D., Stetter, K. O., & Thauer, R. K. (1992) *Eur. J. Biochem. 210*, 971–981.

Cahn, R. S., Ingold, C. K., & Prelog, V. (1956) *Experientia 12*, 81.

Covington, A. K., Paabo, M., Robinson, R. A., & Bates, R. G. (1968) *Anal. Chem. 40*, 700–706.

Di Marco, A. A., Bobik, T. A., & Wolfe, R. S. (1990) *Annu. Rev. Biochem. 59*, 355–394.

Escalante-Semerena, J. C., Rinehart, K. L., Jr., & Wolfe, R. S. (1984) *J. Biol. Chem. 259*, 9447–9455.

Fontecilla-Camps, J. C., Bugg, C. E., Temple, C., Jr., Rose, J. D., Montgomery, J. A., & Kisliuk, R. L. (1979) *J. Am. Chem. Soc. 101*, 6114–6115.

Hallahan, D. L., Fernandez, V. M., Hatchikian, E. C., & Hall, D. O. (1986) *Biochimie 68*, 49–54.
Hurd, R. E., John, B. K. (1991) *J. Magn. Reson. 91*, 648–653.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–53.
Kallen, R. G., & Jencks, W. P. (1966) *J. Biol. Chem. 244*, 5851–5863.
Kay, L. E., Keifer, P., & Saarinen, T. (1992) *J. Am. Chem. Soc. 114*, 10663–10665.
Keltjens, J. T., & Vogels, G. D. (1988) *BioFactors 1*, 95–103.
Keltjens, J. T., Raemakers-Franken, P. C., & Vogels, G. D. (1993) in *Microbial Growth on C_1 Compounds* (Murrell, J. C., & Kelly, D. P., Eds.) pp 135–150, Intercept Publishers, Andover, U.K.
Khalifa, E., Bieri, J. H., & Viscontini, M. (1979) *Helv. Chim. Acta 62*, 1340–1344.
Ma, K., Zirngibl, C., Linder, D., Stetter, K. O., & Thauer, R. K. (1991) *Arch. Microbiol. 156*, 43–48.
Messerle, B. A., Wider, G., Otting, G., Weber, C., & Wüthrich, K. (1989) *J. Magn. Reson. 85*, 608–613.
Poe, M., & Benkovic, S. J. (1980) *Biochemistry 19*, 4576–4582.
Rabinovic, J. O. (1963) *Methods Enzymol. 6*, 814–815.
Rospert, S., Breitung, J., Ma, K., Schwörer, B., Zirngibl, C., Thauer, R. K., Linder, D., Huber, R., & Stetter, K. O. (1991) *Arch. Microbiol. 156*, 49–55.
Schleucher, J., Schwörer, B., Zirngibl, C., Koch, U., Weber, W., Egert, E., Thauer, R. K., & Griesinger, C. (1992) *FEBS Lett. 314*, 440–444.
Schleucher, J., Sattler, M., & Griesinger, C. (1993) *Angew. Chem. 105*, 1518–21; *Angew. Chem., Int. Ed. Engl. 32*, 1489–1491.
Schwörer, B., & Thauer, R. K. (1991) *Arch. Microbiol. 155*, 459–465.
Schwörer, B., Fernandez, V. M., Zirngibl, C., & Thauer, R. K. (1993) *Eur. J. Biochem. 212*, 255–261.
Shaka, A. J., Barker, P. B., & Freeman, R. (1985) *J. Magn. Reson. 64*, 547–552.
Van Beelen, P., Van Neck, J. W., de Cock, R. M., Vogels, G. D., Guijt, W., & Haasnoot, C. A. G. (1984) *Biochemistry 23*, 4448–4454.
Von Bünau, R., Zirngibl, C., Thauer, R. K., & Klein, A. (1991) *Eur. J. Biochem. 202*, 1205–1208.
Wohlfarth, G., Geerligs, G., & Diekert, G. (1991) *J. Bacteriol. 173*, 1414–1419.
Wörgötter, E., Wagner, G., & Wüthrich, K. (1986) *J. Am. Chem. Soc. 108*, 6162–6167.
Zirngibl, C., Hedderich, R., & Thauer, R. K. (1990) *FEBS Lett. 261*, 112–116.
Zirngibl, C., Van Dongen, W., Schwörer, B., Von Bünau, R., Richter, M., Klein, A., & Thauer, R. K. (1992) *Eur. J. Biochem. 208*, 511–520.