Reactions with Molecular Hydrogen in Microorganisms: Evidence for a Purely Organic Hydrogenation Catalyst

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I. Introduction

Molecular hydrogen is an important intermediate in the degradation of organic matter by microorganisms in anoxic habitats such as freshwater and marine sediments, wet land soils, and the gastrointestinal tract of animals. In these habitats H_2 is formed as a product in the fermentation of carbohydrates, lipids, nucleic acids and proteins by anaerobic and facultative bacteria. In some habitats anaerobic protozoa, anaerobic fungi, anaerobically adapted algae, and/or anaerobic archaea are also involved. The H_2 generated by these microorganisms is generally consumed by microorganisms living at the same site, the quantitatively most important H_2 consumers in anoxic habitats being methanogenic archaea, acetogenic bacteria, sulfate-reducing bacteria, and nitrate-reducing bacteria. Estimates are that in anoxic habitats more than 200 million tons of H_2 are globally formed and consumed per year. Despite this high rate the steady state concentration of H_2 in most anoxic habitats is only very low (1−10 Pa), indicating that H_2 formation rather than H_2 consumption is the rate-limiting step in the overall process.[1]

H_2 is also generated in oxic habitats (e.g. oxic soils and fresh water) by aerobic and microaerophilic microorganisms as side product of dinitrogen fixation. More than 10 million tons of H_2 are globally formed associated with this process. Microorganisms consuming H_2 in oxic habitats are "Knallgas" bacteria and most dinitrogen-fixing bacteria.

The formation and consumption of H_2 by microorganisms is catalyzed by enzymes named hydrogenases. Enzymes such as nitrogenase and carbon monoxide dehydrogenase catalyzing the formation of H_2 only as a side product are referred to as having hydrogenase activity. The latter enzymes are not considered in this article.

Until a few years ago all hydrogenases were considered to be iron−sulfur proteins, most containing additionally nickel. Recently, however, in methanogenic archaea a novel hydrogenase without nickel and iron−sulfur clusters was found.[2] The enzyme, which catalyzes the reversible reduction of N^5,N^{10}-methenyltetrahydromethanopterin with H_2 to N^5,N^{10}-methylenetetrahydromethanopterin and a proton (for structures see Figure 4), an intermediary step in CH_4 formation from CO_2 and $4H_2$,[3] appears to be a purely organic hydrogenation catalyst. Evidence was obtained that the reduction of $CH{\equiv}H_4MPT^+$ with H_2 could proceed via a mechanism very similar to that of the reversible formation of carbocations from alkanes in superacids.[4]

The article reviews the literature on the novel hydrogenase. It also includes results on this enzyme not published previously. The review begins, mainly for comparative purposes, with a short description of Fe-only hydrogenases and Ni-Fe hydrogenases. Only the most recent literature on these enzymes and a few historical papers will be quoted. For earlier literature the reader is referred to the excellent review on hydrogenases published 1994 by S. Albracht.[5] Also worth reading is a short article on the mechanism of splitting molecular hydrogen by Ni-Fe hydrogenases published 1995 by R. Cammack.[6]

II. Hydrogenases Containing Nickel and/or Iron–Sulfur Clusters

The Fe-only and the Ni-Fe hydrogenases have several catalytic properties in common which will be described first before summarizing what is known about the structure of these catalysts.[5−7]

A. Catalytic Properties in Common

Both, the Fe-only and the Ni-Fe hydrogenases catalyze the reversible conversion of H_2 to two protons and two electrons (reaction 1), the latter being acceptable by one-electron acceptors such as

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Rudolf K. Thauer was born in Frankfurt, Germany, in 1939. He studied biochemistry at the Universities of Frankfurt, Tübingen, and Freiburg, where he obtained his Ph.D. degree in 1968 in the laboratory of Prof. Dr. Karl Decker. In 1971 he was appointed Assistant Professor at the University of Freiburg and in 1972 Associate Professor for Biochemistry at the University of Bochum. Since 1976 he has been Professor for Microbiology at the Philipps-Universität Marburg and since 1991 has been additionally Director of the Department of Biochemistry of the Max-Planck-Institut für terrestrische Mikrobiologie. His studies are focused on the biochemistry of strictly anaerobic microorganisms. His group was involved in the discovery of three nickel-containing enzymes (carbon monoxide dehydrogenase, methyl-coenzyme M reductase, and hydrogenase) and of a tungsten-containing enzyme (formylmethanofuran dehydrogenase). In collaboration with A. Eschenmoser his group unravelled the structure of F430, a nickel porphinoid of unique structure and function.

Andreas A. Klein was born in Siegen, Germany, in 1966. He studied biology at the Philipps-University of Marburg and received his M.S. degree in 1992. Subsequently, he obtained his Ph.D. in 1995 at the Department of Microbiology. Since that time he has had a postdoctoral fellowship at the Max-Planck-Institut für terrestrische Mikrobiologie. His research interests are in the field of protein chemistry and enzymology.

viologen dyes. Both enzymes catalyze *per se* an exchange between H_2 and protons of water indicative of a ping-pong catalytic mechanism. Single exchanges (reaction 2) and double exchanges (reaction 3) are simultaneously observed.

$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (1)$$

$$H_2 + D^+ \rightarrow HD + H^+ \quad (2)$$

$$H_2 + 2D^+ \rightarrow D_2 + 2H^+ \quad (3)$$

Both enzymes also catalyze the conversion of para H_2 and ortho H_2 (reaction 4).[8]

$$\text{para } H_2 \rightleftharpoons \text{ortho } H_2 \quad (4)$$

Gudrun Hartmann was born in Mannheim, Germany, in 1970. She studied biology at the Philipps-University of Marburg where she concentrated her studies on microbiology, molecular genetics, and biochemistry. In 1994 she received her diploma under the supervision of Prof. Dr. R. K. Thauer for a study on the biochemistry of hydrogenases in methanogenic Archaea. Currently she is working as a Ph.D. student at the Max-Planck-Institut für terrestrische Mikrobiologie, elucidating an unusual hydrogen activation mechanism by enzymes, combining genetic and biochemical approaches.

The activity of both enzymes is inhibited by carbon monoxide, inhibition being competitive to H_2. Inhibition by carbon monoxide is revealed during illumination with near UV/visible light.[5,9] Competitive inhibition indicates that binding of carbon monoxide and H_2 to the enzyme is mutually exclusive and light reactivation shows that the binding site is a metal center. It is generally admitted that upon binding of H_2 with Fe-only or Ni-Fe hydrogenases the hydrogen molecule undergoes a reversible heterolytic cleavage resulting in a hydride bound to the metal center in the active site. Both enzymes contain at least one [4Fe-4S] cluster which is most probably not part of the active site but a site to which the electrons are transferred from H_2 and from which the electrons can be picked up by one-electron accepting dyes (Figure 1).

B. Structure of Fe-Only Hydrogenases

The first report on the possible structure of Fe-only hydrogenases is by O. Warburg in 1946.[10] He observed that H_2 formation in growing cultures of *Clostridium butyricum*, now known to contain an Fe-only hydrogenase,[11] is inhibited by carbon monoxide and that the inhibition can be reversed by light. From the activation spectrum and the extinction coefficient O. Warburg concluded that the active site of the clostridial hydrogenase harbors an iron complexed by sulfur ligands.[10]

Fe-only hydrogenases, in which no other metal than Fe can be detected, have been purified from *Acetobacterium woodii, Acetomicrobium flavidum, Clostridium pasteurianum, Desulfovibrio desulfuricans, Desulfovibrio vulgaris, Megasphaera elsdenii, Thermotoga maritima,* and *Trichomonas vaginalis*. The enzyme from *D. vulgaris* and from *C. pasteurianum* are the best characterized ones. The Fe-only hydrogenase from *Desulfovibrio vulgaris* contains 13−15 iron atoms and probably the same amounts of acid-labile sulfur per mole. Evidence is available

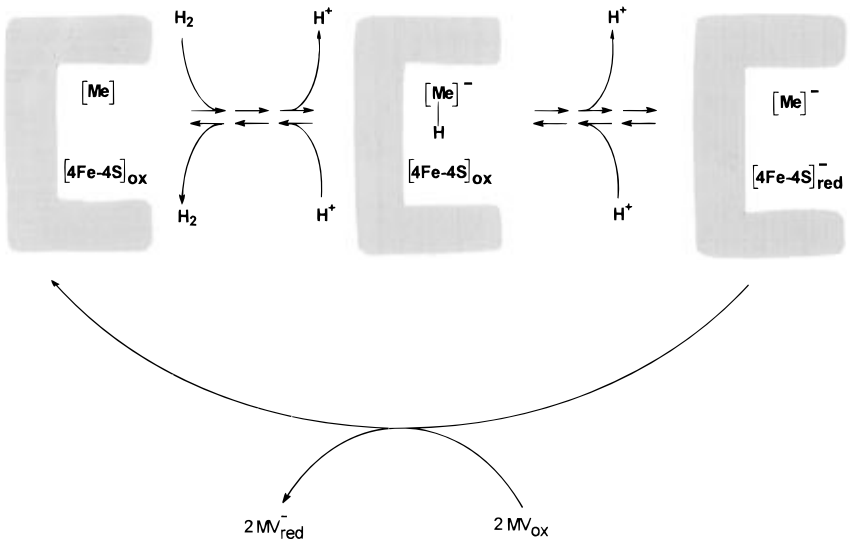

Figure 1. Catalytic mechanism proposed in principle for both Fe-only and Ni-Fe hydrogenases: Me, active site metal center; MV, methylviologen. Before being released into the solvent the protons are probably bound to basic functional groups of the enzyme. Note that the reoxidation of the two electron reduced enzyme with 2 MV proceeds successively and not, as simplified in the cartoon, simultaneously.

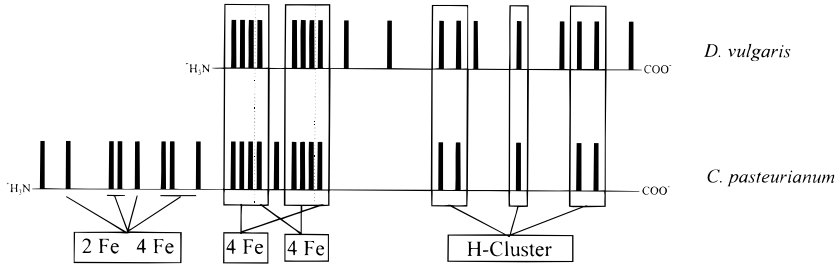

Figure 2. Representation of the amino acid sequences of the Fe-only hydrogenase from *Desulfovibrio vulgaris* and from *Clostridium pasteurianum* highlighting the conserved cysteines: 2Fe, [2Fe-2S] cluster; 4Fe, [4Fe-4S] cluster. The H cluster has been proposed to host six Fe atoms and to be involved in H_2 activation. (Modified from ref 5.)

that eight of the 13−15 iron atoms are organized in two classical cubane [4Fe-4S] clusters. These clusters are called the F clusters or ferredoxin clusters to distinguish them from the hydrogen-activating cluster proposed to host 6Fe atoms. The Fe-only hydrogenase from *Clostridium pasteurianum* appears to additionally harbor an atypical [2Fe-2S] cluster and an atypical [4Fe-4S] cluster.[5,7]

The Fe-only hydrogenase from *Clostridium pasteurianum* is composed of only one subunit of molecular mass of 60 kDa. The enzyme from *Desulfovibrio vulgaris* is composed of a 46 kDa and a 10 kDa subunit. The 60 kDa subunit of the *C. pasteurianum* enzyme and the 46 subunit of the *D. vulgaris* enzyme show sequence similarity. At least 13 cysteines in the primary structures of the two enzymes in both enzymes are conserved. The positions of these cysteines in the primary structures of the two enzymes are shown in Figure 2. Eight of the cysteines are arranged in two blocks of cysteines with the classic C-x-x-C-x-x-C-x-x-x-C- motif. The five conserved cysteine residues in the C-terminal part of the sequence are supposed to host the H cluster to which possibly also one or more of the four conserved methionine residues in this region contribute. The cysteine residues in the N-terminal part of the sequence of the enzyme from *C. pasteurianum* probably are involved in the formation of the atypical [2Fe-2S] cluster and atypical [4Fe-4S] cluster additionally found in this enzyme.[5,7]

C. Structure of Ni-Fe Hydrogenases

Hydrogenases from most bacteria are nickel proteins as first established by Graf and Thauer in 1981 for the enzyme from *Methanobacterium thermoautotrophicum*.[12] The purified enzyme from this methanogenic archaeon was found to harbor one nickel per mole and displays a simple rhombic EPR signal which could be ascribed to nickel by using ^{61}Ni as stable isotope with a nuclear magnetic moment of $^3/_2$. Moreover, the signal disappeared upon contact of the enzyme with H_2, indicating that the nickel in the enzyme was redox active.[13] Nickel hydrogenases have been characterized from various organisms. Besides one nickel they all appeared to contain several iron, most of the iron being organized in iron−sulfur clusters, at least one having cubane structure. There is spectroscopic evidence (Mössbauer spectroscopy and EXAFS) for the presence of one iron in close vicinity to the nickel, the redox state of the special iron shuttling between low-spin paramagnetic Fe(III) and low-spin diamagnetic Fe(II) at an $E^{\circ\prime}$ of +150 mV.[14]

All presently known Ni-Fe hydrogenases are composed of at least two different subunits, the larger

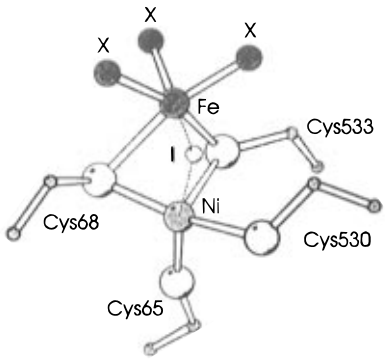

Figure 3. Proposed configuration of the active site of the Ni-Fe hydrogenase from *Desulfovibrio gigas*. The Ni coordination site labeled I is not occupied by any protein ligand. Three of the Fe coordination sites are presently discussed to be occupied by diatomic ligands. (Modified from ref 15.)

one having a molecular mass of 46−72 kDa and the smaller one a molecular mass of 23−38 kDa. Evidence is available that the larger subunit harbors the nickel-containing active site and the smaller subunit the iron−sulfur clusters.[5]

The primary structure derived from the structural genes encoding the two subunits are now known for more than 25 different Ni-Fe hydrogenases. For a year the crystal structure of one Ni-Fe hydrogenase has also been available, that of the enzyme from *Desulfovibrio gigas*.[15] The structure reveals that the large subunit indeed harbors the active site and the small subunit the iron−sulfur clusters. The active site, which contains besides nickel a second metal ion, most probably an iron atom,[14] is buried in a pocket that is thought to repel water and to attract H_2.[6] As shown in Figure 3 the nickel is coordinated by S γ of Cys 530 in an apical position and by three equatorial cysteine ligands (Cys 65, Cys 68, Cys 533). The iron, which sits less than 3 Å away from the Ni, is bound by Cys 68 S γ and Cys 533 S γ, which bridge the two metals, and by three putative diatomic molecules. The Ni coordination site labeled I, which is also a coordination position for the iron, is not occupied by any protein ligand. It could be a binding site for H_2. Current thinking is that the nickel strips off the two electrons, which are quickly whisked away, and the iron stabilizes the unusual oxidation state that the nickel must adopt to perform its function.[16,17]

It should be noted that the crystal structure of the Ni-Fe hydrogenase from *D. gigas* is that of an inactive protein obtained after aerobic purification. The nickel site might look quite differently when the enzyme is active.[18,19]

Interestingly, in some bacteria and archaea the Ni-Fe hydrogenases contain 1 mol of selenium in the subunit harboring the nickel active site. In these enzymes one of the four cysteines involved in nickel coordination (Cys 530 in Figure 3) is replaced by selenocysteine.[5]

D. Structural Properties in Common

Three novel infrared-detectable groups absorbing in the 2100−1800 cm^{-1} spectral region have recently been detected in Ni-Fe hydrogenases.[20] The position of the infrared band is dependent on the redox state of the hydrogenases. The three infrared-detectable groups in Ni-Fe hydrogenases are assigned to the three unidentified diatomic nonprotein ligands that coordinate the iron in the binuclear Ni/Fe active site as observed in the X-ray structure.[15] Unexpectedly, infrared bands in the 2100−1800 cm^{-1} spectral region were also recently found in all Fe-only hydrogenases.[21] On the basis of this finding it was proposed that the active sites of Ni-Fe hydrogenases and of Fe-only hydrogenases share a similar, unique architecture required for the activation of molecular hydrogen in nature. Interestingly, the novel hydrogenase without nickel and iron−sulfur clusters did not exhibit the infrared bands in the 2100−1800 cm^{-1} spectral region.[21]

III. A Novel Hydrogenase without Nickel and Iron−Sulfur Clusters

A novel hydrogenase, which appears to contain neither nickel nor iron−sulfur clusters, was recently found in methanogenic archaea.[2,22−24] It differs from the Fe-only and the Ni-Fe hydrogenases in the following catalytic properties: (i) The enzyme does not catalyze the reduction of one or two electron accepting artificial dyes (reaction 1);[25] (ii) it *per se* does not mediate an exchange between H_2 and D^+ (reaction 2) or between H_2 and 2 D^+ (reaction 3),[25−27] (iii) it *per se* does not catalyze the conversion of para H_2 and ortho H_2 (reaction 4);[28] and (iv) it is not inhibited by CO or other compounds known to inhibit Fe-only and/or Ni-Fe hydrogenases (acetylene, NO, NO_2^-).[25] As Fe-only and Ni-Fe hydrogenases, the novel hydrogenase is not inhibited by cyanide or azide.[25]

A. Reaction Catalyzed by the Novel Hydrogenase and Chemical Properties of the Reactants

The novel hydrogenase catalyzes the reduction of N^5,N^{10}-methenyl-5,6,7,8-tetrahydromethanopterin ($CH{\equiv}H_4MPT^+$) with H_2 to N^5,N^{10}-methylene-5,6,7,8-tetrahydromethanopterin ($CH_2{=}H_4MPT$) and a proton (reaction 5). The enzyme also catalyzes the

$$H_2 + CH{\equiv}H_4MPT^+ \rightleftharpoons CH_2{=}H_4MPT + H^+ \qquad \Delta G^{\circ\prime} = -5.5\ \text{kJ/mol} \quad (5)$$

reverse reaction, the formation of H_2 and $CH{\equiv}H_4MPT^+$ from $CH_2{=}H_4MPT$ and a proton, which is indicated by the systematic name H_2-forming methylenetetrahydromethanopterin dehydrogenase (EC 1.12.99.−).[2]

The equilibrium concentrations of the reactants are pH-dependent, alkaline pH (pH > 7) favoring the reduction of $CH{\equiv}H_4MPT^+$ with H_2 and acidic pH (pH <7) favoring the dehydrogenation of $CH_2{=}H_4MPT$.

The structures of $CH{\equiv}H_4MPT^+$ and of $CH_2{=}H_4MPT$ are shown in Figure 4.[29−32] The two compounds are derivatives of 5,6,7,8-tetrahydromethanopterin (H_4MPT), which is an analogue of tetrahydrofolate (H_4F) (Figure 4). Both H_4MPT and H_4F function as a carrier of one-carbon units, H_4MPT being the preferred carrier in anaerobic archaea and H_4F the one in aerobic archaea, bacteria, and eucarya.

H_4MPT and H_4F carry one-carbon units at the oxidation level of formate, formaldehyde, and metha-

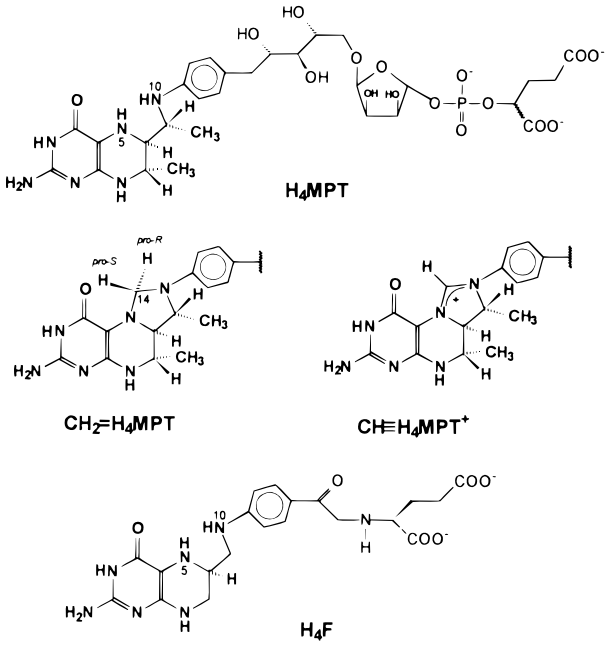

Figure 4. Structures of tetrahydromethanopterin (H_4-MPT) and of tetrahydrofolate (H_4F) and structures of the reactive moiety of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2{=}H_4MPT$) and of N^5,N^{10}-methenyltetrahydromethanopterin ($CH{\equiv}H_4MPT^+$).

nol. These one-carbon units are bound to N^5 (N^5-formyl-, N^5-methyl-), N^{10} (N^{10}-formyl-) or both N^5 and N^{10} (N^5,N^{10}-methenyl-, N^5,N^{10}-methylene-) of the two reduced pterins.

The functionally most important difference between H_4MPT and H_4F appears to be that H_4MPT has an electron-donating methylene group in conjugation to N^{10} via the aromatic ring, whereas H_4F has an electron-withdrawing carbonyl group in this position. The pK_a of the para amino group has been estimated to be +2.4 in H_4MPT and to be −1.2 in H_4F.[33] This difference in pH reflects different properties of the C_1 units bound to N^{10} of the two reduced pterins. For the present discussion the most important differences, which are summarized in Table 1, are as follows:

(i) The N^5,N^{10}-methenyl group in $CH{\equiv}H_4MPT^+$ has stronger carbocation character than in $CH{\equiv}H_4F^+$ as evidenced by a higher rate of exchange of the methenyl proton with protons of water.

(ii) The redox potential $E^{\circ\prime} = -390$ mV of the $CH{\equiv}H_4MPT^+/CH_2{=}H_4MPT$ couple is about 100 mV more negative than $E^{\circ\prime} = -300$ mV of the $CH{\equiv}H_4F/CH_2{=}H_4F$ couple. Note that $E^{\circ\prime}$ of the $CH{\equiv}H_4MPT^+/CH_2{=}H_4MPT$ couple is almost identical to that of the H_2 electrode at pH 7 which is −414 mV.

For the investigation of the reaction mechanism the property of H_4MPT to spontaneously react with formaldehyde to $CH_2{=}H_4MPT$ was of importance.[34] Thus, $^{13}CH_2{=}H_4MPT$, $CT_2{=}H_4MPT$, and $CD_2{=}H_4$-MPT were synthesized from H_4MPT and $^{13}CH_2O$, CT_2O, and CD_2O, respectively.

Properties of $CH{\equiv}H_4MPT^+$ and of $CH_2{=}H_4MPT$, which complicated the interpretation of the results, are (i) the spontaneous exchange of the methenyl proton with protons of water, (ii) the spontaneous epimerization of the prochiral methylene group, (iii) the spontaneous hydrolysis of $CH{\equiv}H_4MPT^+$ to N^{10}-formyl-H_4MPT at a pH above 7, and (iv) the spontaneous oxidation of $CH_2{=}H_4MPT$ with O_2 requiring all experiments to be performed under strictly anaerobic conditions.[32]

Table 1. Properties of Tetrahydromethanopterin (H_4MPT) and Tetrahydrofolate (H_4F) and of Their N^5,N^{10}-Methenyl, N^5,N^{10}-Methylene, and N^5-Methyl Derivatives

property	H_4MPT	H_4F
pK_a for N^5 of H_4F or H_4MPT	+4.8[a]	+4.8[b]
pK_a for N^{10} of H_4F or H_4MPT	+2.4[c]	−1.2[b]
$E^{\circ\prime}$ for the methenyl/methylene couple	−390 mV[29]	−300 mV[d]
$E^{\circ\prime}$ for the methylene/methyl couple	−320 mV[29]	−200 mV[e]
$T_{1/2}$ for exchange of the methenyl proton with H_2O[32]	1 h	3 h
$T_{1/2}$ for exchange of the methylene group with CH_2O[32]	1 h	<15 min
rate of epimerization of the methylene group[32]	0.01 s^{-1}	0.1 s^{-1}
stability in the presence of O_2[32]	low	very low

[a] pK_a for N^5 of H_4MPT and of H_4F assumed to be the same. [b] Kallen, R. G.; Jencks, W. P. *J. Biol. Chem.* **1966**, *241*, 5845. [c] The ΔpK for N^{10} of H_4F and H_4MPT was assumed to be identical to the ΔpK for N^{10} of folic acid (pK = 0.36)[a] and N^{10} of methanopterin (pK = 4.0)[33] [d] Wohlfarth, G.; Diekert, G. *Arch. Microbiol.* **1991**, *155*, 378. [e] Wohlfarth, G.; Geerligs, G.; Diekert, G. *Eur. J. Biochem.* **1991**, *192*, 411.

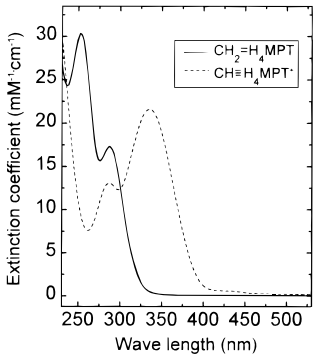

Figure 5. UV/vis spectra of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2{=}H_4MPT$) and of N^5,N^{10}-methenyltetrahydromethanopterin ($CH{\equiv}H_4MPT^+$).

$CH{\equiv}H_4MPT^+$ and $CH_2{=}H_4MPT$ differ significantly in the UV/visible spectrum, the largest difference being at 335 nm (Figure 5). The difference in extinction coefficient at this wavelength is 21.6 mM^{-1} cm^{-1}. The formation or consumption of $CH{\equiv}H_4MPT^+$ can therefore be followed with high sensitivity using a photometric assay. For the analysis of the mechanism it was also of importance that $CH{\equiv}H_4MPT^+$ and $CH_2{=}H_4MPT$ can be separated by reversed-phase HPLC.

B. Molecular Properties of the Enzyme

When testing for $CH_2{=}H_4MPT$ dehydrogenation to $CH{\equiv}H_4MPT^+$ and H_2 in cell extracts of *Methanobacterium thermoautotrophicum* it was found that the rate of the reaction increased proportionally to the amount of cell extract added to the assay: A plot of the rates versus the protein concentrations gave a straight line which passed through zero. This finding indicated that only one independent component in the cell extract was required for catalysis. Upon purification, indeed, it turned out that in the catalysis

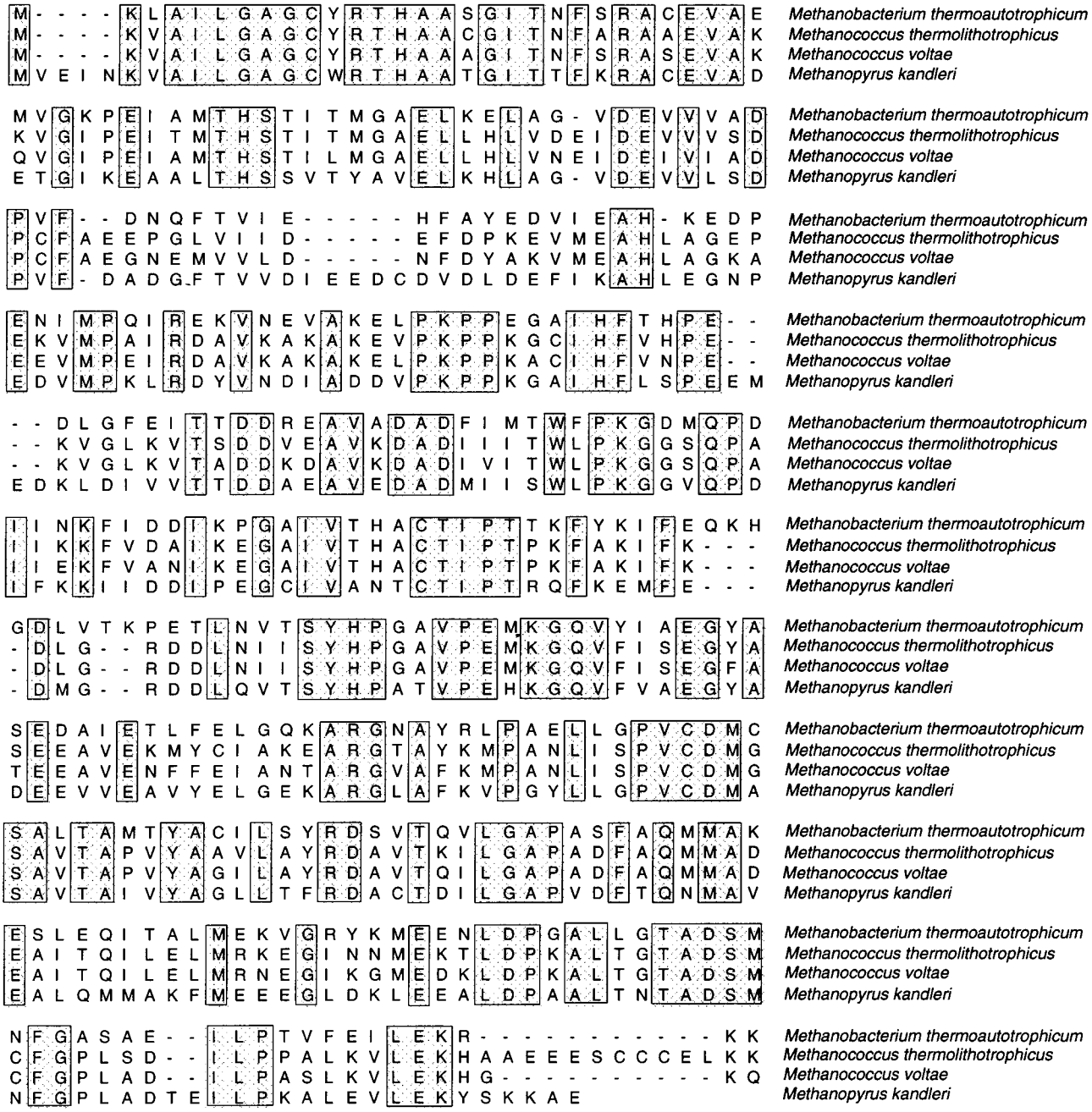

Figure 6. Alignment of the primary structures of the H_2-forming methylenetetrahydromethanopterin dehydrogenase from four different methanogens.[24,25,37] Primary structures are also available for the enzyme from *Methanobacterium thermoautotrophicum* strain ΔH (accession no. U19363)[35], from *Methanobacterium thermoautotrophicum* strain Winter (acccession no. U19364),[35] and for the enzyme from *Methanobacterium thermoformicicum* (Vaupel and Thauer unpublished). They were omitted from the figure because they show over 95% sequence identity to the enzyme from *M. thermoautotrophicum* (strain Marburg). The strains of *M. thermoautotrophicum* and *M. thermoformicicum* are phylogentically very closely related.[51]

of the reaction only one protein with an apparent molecular mass of 80 kDa was involved. SDS/PAGE of the protein revealed that the enzyme was composed of two identical subunits with an apparent molecular mass of 43 kDa. The H_2-forming methylenetetrahydromethanopterin dehydrogenase from *M. thermoautotrophicum* thus has a homodimeric structure.[2,25] This was substantiated by the finding that the gene encoding for the enzyme is monocistronically transcribed.[35]

The purified enzyme exhibited an UV/visible spectrum almost identical to that of serum albumin. The extinction coefficient at 280 nm was approximately that calculated from the amino acid composition (based on the tryptophane, tyrosine, and phenylalanine content).[25] These findings indicated that the purified enzyme is devoid of a chromophoric prosthetic group including iron−sulfur clusters, since these are known to efficiently absorb light in the UV/visible region, the extinction coefficient at the absorption maximum being approximately 10 mM^{-1} cm^{-1} for [2Fe-2S] clusters and 15 mM^{-1} cm^{-1} for [4Fe-4S] clusters.[36]

The presence of transition metals in the novel hydrogenase was sought by inductively coupled plasma mass spectroscopy (ICP-MS) and by atomic absorption spectroscopy (AAS). Neither nickel, iron, cobalt, nor molybdenum or tungsten were found in

Table 2. Molecular Properties of the H_2-Forming Methylenetetrahydromethanopterin Dehydrogenase from Different Methanogenic Archaea (nd, not determined)

enzyme from organism	apparent molecular mass (kDa) native	apparent molecular mass (kDa) denaturated	isoelectric point, p*I*	crossreactivity with antibody[a]	sequence similarity[b] (%)
Methanobacterium thermoautotrophicum	80	43	4.4	++	100
Methanobacterium thermoformicicum[c]	90	43	4.6	+	95
Methanothermus fervidus[d]	170	43	5.3[f]	+	nd
Methanopyrus kandleri	170	44	4.1	++	52
Methanococcus thermolithrotophicus	150	43	5.5	−	58
Methanococcus igneus[e]	170	43	5.7[f]	±	nd
Methanococcus voltae	nd	43	5.1	−	57

[a] The polyclonal antibody used was raised against the enzyme from *M. thermoautotrophicum*: (++) strong; (+) medium; (±) weak; (−) no reaction. [b] The similarity value (DNASTAR, Lasergene) is based on the amino acid sequence comparison deduced from the DNA sequence. [c] Vaupel and Thauer, unpublished results. [d] N-terminus: MEIKVAILGAGCYRTHAASGITNFAR. [e] N-terminus M−−KI−xxxAGxYVTHAAAGIT. [f] Isoelectric point determined experimentally rather than deduced from the amino acid sequence.

significant amounts. Some preparations contained 1−2 mol zinc, the zinc content, however, did not correlate with the specific activity. All preparations analyzed were purified with activity yields above 10% and exhibited specific activities of about 1000 U/mg protein which is higher than the specific activity of most purified Fe-only and Ni-Fe hydrogenases. From these findings it was concluded that the novel hydrogenase does not require the presence of a redox active transition metal for activity.

In Figure 6 the amino acid sequences of the novel hydrogenases from 4 different methanogenic Archaea are compared. The sequences, which show no similarity to any amino acid sequence of proteins in the data bank, were deduced from the nucleotide sequences of the encoding genes.[24,25,37] All of these amino acid sequences lack the two sequence motifs R-G-X-E-X_{16}-R-X-C-G-X-C-X-X-X-H and D-P-C-X-X-C-X-X-H, which in Ni-Fe hydrogenases[5] are highly conserved and which in these enzymes provide the four cysteines ligating the nickel in the active site as shown in Figure 3. The H_2-forming methylene-H_4MPT dehydrogenase also lack sequence motifs that could indicate the presence of iron−sulfur clusters.

Comparison of the amino acid sequence of the 4 novel hydrogenases (Figure 6) revealed a high degree of sequence identity which is noteworthy since the organisms, from which the enzymes were analyzed, are phylogenetically only distantly related. Also noteworthy is that only three cysteines in the sequence appear to be conserved.

From the amino acid sequence the molecular mass of the subunit was calculated to be 38 910 Da for the enzyme from *Methanopyrus kandleri*. The molecular mass determined by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) was 38 878 Da which is not considered to be significantly different from the calculated value. The N-terminal amino acid sequence determined by Edman degradation and the sequence deduced from the nucleotide sequence of the encoding gene were identical. Determination of the molecular mass of the peptides obtained after digestion of the enzyme from *M. kandleri* with cyanogen bromide revealed that the C-terminus of the enzyme had the same sequence as the one deduced from the nucleotide sequence of the encoding gene. All these findings together appear to exclude any post-translational modifications of the enzyme.

The gene encoding the novel hydrogenase in *Methanopyrus kandleri* has been expressed in *Escherichia coli*. SDS/PAGE of the protein revealed that it had the same apparent molecular mass as the enzyme purified from *M. kandleri*. It was, however, inactive probably due to incorrect folding as evidenced by differences in solubility in ammonium sulfate. Attempts to reactivate the recombinant protein by unfolding and refolding have failed until now. However, purified enzyme from this methanogen could not be reactivated after inactivation by unfolding in guanidinium hydrochloride or urea (Shima, Klein, and Thauer, unpublished results). Most likely a chaperon is required to facilitate the correct folding of the polypeptide chain *in vivo* and *in vitro*.

Also not understood is the property of the enzyme to become inactive in the presence of O_2. The oxygen sensitivity was found to be highest when the enzyme is still present in cell extracts under reducing conditions and to become less pronounced upon purification. An explanation for this phenomenon is that in cell extracts O_2 is reduced to $O_2^{\bullet-}$ which is a much more reactive oxidant than O_2.

Purified H_2-forming methylenetetrahydromethanopterin dehydrogenase was inactivated by O_2 in the presence and absence of H_2 at the same rate. This property is completely different from that of Fe-only hydrogenases and Ni-Fe hydrogenases which are much more rapidly inactivated by O_2 when reduced with H_2.

The molecular properties of the H_2-forming methylenetetrahydromethanopterin dehydrogenase from seven different methanogenic archaea have been determined. They are compared in Table 2. A difference is that the enzyme from some methanogens have a dimeric structure and from others have a tetrameric structure. It is interesting that the cross reactivity with polyclonal antibodies against the enzyme from *Methanobacterium thermoautotrophicum* did not correspond to the sequence similarities of the investigated enzymes (Figure 6).

C. Catalytic Properties of the Enzyme

The concentration dependence of $CH{\equiv}H_4MPT^+$ reduction with H_2 catalyzed by the novel hydrogenase was shown to follow Michaelis−Menten kinetics. Reciprocal plots of the rates versus the concentrations of the one substrate at different constant levels of

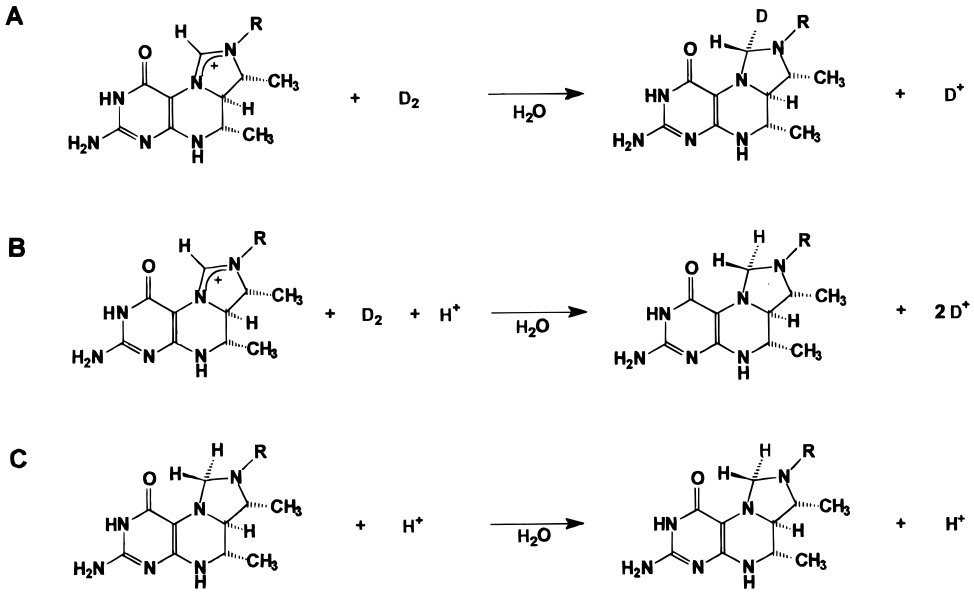

Figure 7. Stereochemical course of N^5,N^{10}-methenyltetrahydromethanopterin reduction with D_2 (A and B) and of the exchange of the methylene hydrogen of N^5,N^{10}-methylenetetrahydromethanopterin with protons of water.

the second substrate yielded straight lines which intersected in one point on the abscissa to the left of the ordinate. This finding and the inability of the enzyme to catalyze an H_2/H^+ exchange in the absence of $CH{\equiv}H_4MPT^+$ indicated that the novel hydrogenase exhibits a ternary complex rather than a ping-pong catalytic mechanism.[38,39]

The rates (V_{max}) of $CH{\equiv}H_4MPT^+$ reduction with H_2 and with D_2 and the K_m for H_2 and D_2 were found to be almost identical. The absence of a kinetic deuterium isotope effect indicates that the reaction of H_2 with $CH{\equiv}H_4MPT^+$ bound to the enzyme is not a rate-determining step in the catalytic mechanism of the reaction.[38] (The situation may, however, be more complex since it is, at least theoretically possible that a compensating inverse isotope effect precedes the rate-determining cleavage of the D−D (or H−H) bond.)

Reduction of $CH{\equiv}H_4MPT^+$ with tritium-labeled H_2 yielded tritium-labeled $CH_2{=}H_4MPT$ (reaction 6), the label being removed again upon reoxidation to $CH{\equiv}H_4MPT^+$.[25]

$$CH{\equiv}H_4MPT^+ + T_2 \rightleftharpoons CHT{=}H_4MPT + T^+ \quad (6)$$

The stereochemistry of the enzymatic hydride transfer reaction was elucidated in the laboratory of C. Griesinger (Frankfurt) by means of a series of heteronuclear two-dimensional NMR experiments. It was found that the hydride from H_2 is transferred by the enzyme into the (*pro-R*) position of the C(14) methylene group of the reaction product $CH_2{=}H_4$-MPT (Figure 7A), and it was shown that the hydrogen nucleus of the hydride transferred to $CH{\equiv}H_4$-MPT^+ partially originates from water, the stereochemical course of this reaction being the same as that for the hydride transfer from H_2 (Figure 7B).[32]

The incorporation of protons from water into the (*pro-R*) position of the methylene group of $CH_2{=}H_4$-MPT during $CH{\equiv}H_4MPT^+$ reduction with H_2 was not understood until it was found that the enzyme catalyzes a direct exchange of the (*pro-R*) hydrogen of the C(14) methylene group with protons of water (Figure 7C). Under equilibrium conditions this exchange rate was 2.4 times higher than the rate of $CH{\equiv}H_4MPT^+$ formation from $CH_2{=}H_4MPT$ which was determined via proton exchange spectroscopy (EXSY) by following the transfer of the magnetic label from the (*pro-R*) hydrogen of the methylene group into the proton pool of water and from the (*pro-S*) hydrogen into the methenyl group.[40]

The property of the enzyme to catalyze a direct exchange of the (*pro-R*) hydrogen of the methylene group of $CH_2{=}H_4MPT$ with protons of water (Figure 7C) can also explain the finding that dehydrogenation of $CH_2{=}H_4MPT$ in D_2O is associated with the simultaneous formation of HD (reaction 7) and D_2 (reaction 8) as determined by on-line mass spectroscopy in the laboratory of V. Fernández (Madrid).

$$CH_2{=}H_4MPT + D^+ \rightarrow CH{\equiv}H_4MPT^+ + HD \quad (7)$$

$$CH_2{=}H_4MPT + 2D^+ \rightarrow CH{\equiv}H_4MPT^+ + D_2 + H^+ \quad (8)$$

In the presence of $CH{\equiv}H_4MPT^+$ the enzyme was found to catalyze an H_2/D^+ and an $H_2/2D^+$ exchange (reactions 9 and 10) (Figure 8A)[26,27] and the conversion of ortho and para H_2 (reaction 11) (Figure 8B).[28] Note that catalysis of these reactions by the metal-free hydrogenase was strictly $CH{\equiv}H_4MPT^+$ dependent.

$$H_2 + D^+ \rightarrow HD + H^+ \quad (CH{\equiv}H_4MPT^+ \text{ dependent}) \quad (9)$$

$$H_2 + 2D^+ \rightarrow D_2 + 2H^+ \quad (CH{\equiv}H_4MPT^+ \text{ dependent}) \quad (10)$$

$$\text{para } H_2 \rightleftharpoons \text{ortho } H_2 \quad (CH{\equiv}H_4MPT^+ \text{ dependent}) \quad (11)$$

The catalytic properties determined for the H_2-forming methylenetetrahydromethanopterin dehy-

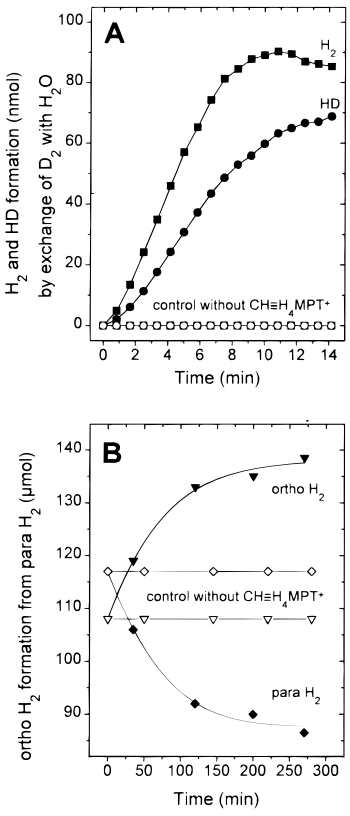

Figure 8. N^5,N^{10}-Methenyltetrahydromethanopterin (CH≡H_4MPT$^+$) dependent exchange of D_2 with H_2O (A) and CH≡H_4MPT$^+$-dependent para−ortho H_2 conversion (B) as catalyzed by purified H_2-forming methylenetetrahydromethanopterin dehydrogenase. For experimental reasons the para−ortho H_2 conversion assay contained approximately 500 times more enzymes than the D_2/H^+ exchange assay.

drogenase from seven different methanogens are summarized in Table 3. This comparison reveals that the enzymes all show an apparent K_m for H_2 of above 0.1 mM which is more than 10 times higher than the apparent K_m for H_2 of Ni-Fe hydrogenases and Fe-only hydrogenases. Apparently, the metal-containing hydrogenases have a higher catalytic affinity for H_2 than the metal-free ones.

D. Proposed Catalytic Mechanism

When discussing the results obtained for the H_2-forming methylenetetrahydromethanopterin dehydrogenase from methanogens with A. Berkessel[4] (Heidelberg), A. Berkessel has proposed a catalytic mechanism for CH_2=H_4MPT dehydrogenation to CH≡H_4MPT$^+$ and H_2 analogous to that elucidated by Olah[41,42] for the reversible formation of carbocations and H_2 from alkanes in superacids as exemplified for isobutane conversion to $(CH_3)_3C^+$ and H_2:[43]

$$(CH_3)_3CH + H^+ \rightleftharpoons \left[(CH_3)_3C\begin{matrix}H\\H\end{matrix}\right]^+ \rightleftharpoons (CH_3)_3C^+ + H_2 \quad (12)$$

A pentacoordinated carbonium ion has been shown to be the intermediate in reaction 12. It is formed by the reversible protonation of the tertiary C−H bond generating a transient 3-center, 2-electron bond. Formation of this intermediate can explain the following reactions proceeding in superacids containing isobutane:

$$(CH_3)_3CH + D^+ \rightarrow (CH_3)_3C{-}D + H^+ \quad (13)$$

$$(CH_3)_3CH + D^+ \rightarrow (CH_3)_3C^+ + HD \quad (14)$$

$$(CH_3)_3CH + 2\,D^+ \rightarrow (CH_3)_3C^+ + D_2 + H^+ \quad (15)$$

The similarities between these superacid born reactions 12−15 and the reactions catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase (reactions 5−11; Figure 6) become evident when considering that C14 of the methenyl group of CH≡H_4MPT$^+$ (Figure 4) has carbocation character and that C14 is the carbon atom which reacts with H_2 in the enzyme-catalyzed reaction. The similarity even extends to the stereochemistry and kinetics of these reactions: (i) The exchange of the hydrogen of optically active hydrocarbons with protons in superacids proceeds with retention of stereoconfiguration[43,44] as does the exchange of the (*pro-R*) hydrogen of the methylene group of CH_2=H_4MPT with protons in water;[40] and (ii) neither the reduction of $(CH_3)_3C^+$ with H_2[45] nor the reduction of CH≡H_4MPT$^+$ with H_2[38] exhibits a kinetic deuterium isotope effect.

There is, however, an important difference between the superacid born reactions and the enzyme-catalyzed reactions. The formation of $(CH_3)_3C^+$ and

Table 3. Catalytic Properties of Purified H_2-Forming Methylenetetrahydromethanopterin Dehydrogenases from Different Methanogenic Archaea[a]

enzyme from organism	apparent K_m (μM) for H_2	apparent K_m (μM) for CH≡H_4MPT$^+$	apparent K_m (μM) for CH_2=H_4MPT	V_{max}[b] (U/mg)	pH optimum[b]	temperature (°C) optimum[b]	stereochemistry at C14 of CH_2=H_4MPT
Methanobacterium thermoautotrophicum (65 °C)[c]	200	50	50	1360	6.5	60	*pro-R*[d]
Methanobacterium thermoformicicum (55 °C)[e]	140	20	35	410	5.5	70	*pro-R*[d]
Methanothermus fervidus (85 °C)[c]	100	180	45	860	5.0	75	*pro-R*[d]
Methanopyrus kandleri (>95 °C)[c]	nd	nd	50	2700	≤4.5	>90	*pro-R*[f]
Methanococcus thermolithotrophicus (65 °C)	200	60	85	760	5.0	80	nd
Methanococcus igneus (85 °C)[c]	150	250	120	500	6.0	80	*pro-R*[f]
Methanococcus voltae (37 °C)[g]	nd	nd	55	140	5.3	60	nd

[a] The temperatures in brackets indicate the growth temperature optimum of the respective organism. CH_2=H_4MPT, N^5,N^{10}-methylenetetrahydromethanopterin; CH≡H_4MPT$^+$, N^5,N^{10}-methenyltetrahydromethanopterin; n.d. not determined. [b] Determined with purified enzyme for the dehydrogenation of CH_2=H_4MPT. [c] CH≡H_4MPT$^+$ dependent H_2/D^+ exchange demonstrated for the enzyme from this organism. [d] Determined by proton-exchange spectroscopy.[40] [e] Vaupel and Thauer, unpublished results. [f] Determined as described: Klein, A.; Thauer, R. K. *Eur. J. Biochem.* **1995**, *227*, 169. [g] von Bünau and Klein, unpublished results.

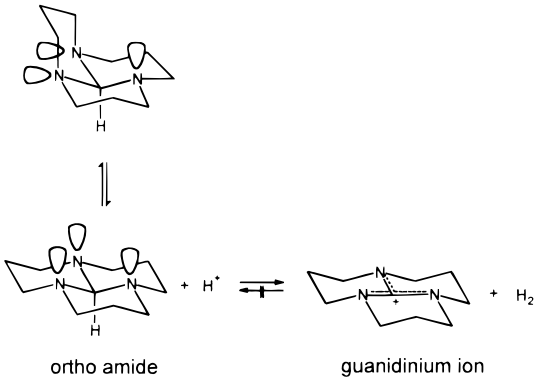

Figure 9. Transformation of perhydro-3a,6a,9a-triazaphenalene (ortho amide) to the guanidinium ion and H_2 in HCl.

H_2 from $(CH_3)_3CH$ and H^+ is thermodynamically feasible only at the very high H^+ concentrations prevailing in superacids. In contrast, the formation of $CH{\equiv}H_4MPT^+$ and H_2 from $CH_2{=}H_4MPT$ and H^+ is already exergonic at proton concentrations as low as 10^{-7} M (pH 7). This difference in thermodynamics reflects the difference in positive charge delocalization but also the difference in relative solvation energies of the proton in a superacid versus water. The positive charge in $(CH_3)_3C^+$ is almost completely carbon-centered, whereas that in $CH{\equiv}H_4MPT^+$ is delocalized among N^5, C14, and N^{10}, the cation being stabilized by conjugation.

Structurally related to $CH_2{=}H_4MPT$ is perhydro-3a,6a,9a-triazaphenalene (Figure 9). The ortho amide, after treatment with equimolar amounts of HCl or HBF_4, has been shown to be converted quantitatively to the guanidinium ion and H_2 by pyrolysis at 110 °C for 23 h.[46] In the guanidinium salt, which is structurally related to the formamidinium ion $CH{\equiv}H_4MPT^+$, the positive charge is even more delocalized than in $CH{\equiv}H_4MPT^+$. (Note that the guanidinium ion analogy is not an accurate one as $CH{\equiv}H_4MPT^+$ has a secondary carbocationic center with only two neighboring nitrogen atoms.) The free energy change associated with the conversion of the ortho amide to the guanidinium salt and H_2 is therefore predicted to be more negative than that of $CH_2{=}H_4MPT$ conversion to $CH{\equiv}H_4MPT^+$ and H_2. In agreement with this prediction is the finding that H_2 formation from the ortho amide proceeded irreversibly.[46]

Perhydro-3a,6a,9a-triazaphenalene has two preferred conformations (Figure 9).[47] The conformation with the three lone pair orbitals of the nitrogen atoms antiperiplanar to the σ^* orbital of the central C−H bond is the one which reacts with H^+. In the "antiperiplanar" conformation the central C−H bond is weakened as evidenced by a C−H stretch vibration with an unusually large wavelength (4.0 μm "Bohlmann band"). The electron density at the methine hydrogen is increased, facilitating protolysis of the C−H bond (stereoelectronic effect).[48] In analogy, it is proposed that $CH_2{=}H_4MPT$ is only reactive when bound to the enzyme in a conformation, in which the two lone pair orbitals of N^5 and N^{10} are positioned antiperiplanar to the $C{-}H_{pro-R}$ bond, which is the C−H bond protolyzed in the enzyme catalyzed reaction (Figure 10). An important function of the H_2-forming methylenetetrahydromethanopterin dehydrogenase is, therefore, to lock $CH_2{=}H_4MPT$ exactly in a conformation in which the $C{-}H_{pro-R}$ bond is maximally activated.[4]

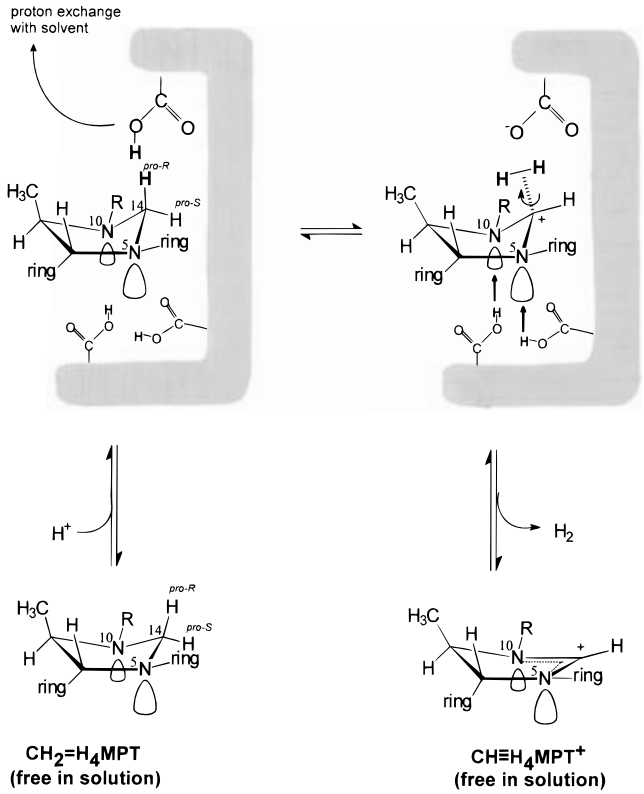

Figure 10. Catalytic mechanism proposed for the H_2-forming methylenetetrahydromethanopterin dehydrogenase: $CH_2{=}H_4MPT$, N^5,N^{10}-methylenetetrahydromethanopterin; $CH{\equiv}H_4MPT^+$, N^5,N^{10}-methenyltetrahydromethanopterin. (Modified from ref 4.)

A second function of the enzyme is to provide the proton in a proper position for protolysis of the $C{-}H_{pro-R}$ bond. In Figure 10 the proton is considered to be provided by a juxtapositioned carboxyl group of an aspartate or glutamate residue of the enzyme. Because in the dehydrogenation of $CH_2{=}H_4MPT$ a proton is consumed, a proton path between the acidic functional group in the enzyme's active site and the solvent must exist. It is proposed that the proton in the active site reacts with the $C{-}H_{pro-R}$ bond of $CH_2{=}H_4MPT$, yielding a 3-center, 2-electron bond and resulting in a "carbocation"-bound H_2 molecule which rotates (Figure 10). This hypothesis readily explains (i) the rapid isotope exchange of the (*pro-R*) hydrogen of $CH_2{=}H_4MPT$ with the solvent, (ii) the isotope exchange between H_2 and the solvent, and (iii) why catalysis of the H_2/H^+ reaction is dependent on the presence of either $CH{\equiv}H_4MPT^+$ or $CH_2{=}H_4MPT$.[4]

In $CH{\equiv}H_4MPT^+$ the positive charge at C14 is optimally stabilized in the flattened five-membered imidazolidine ring (Figure 10). In this conformation $CH{\equiv}H_4MPT^+$ cannot react with H_2. This is only possible after the stabilization of the cation center at C14 by the lone pair orbitals of the neighboring nitrogen atoms has been turned off. What is chemically involved is activation of a delocalized carbocationic center by cutting down neighboring nitrogen participation. A further function of the enzyme is, therefore, to distort and destabilize the planar cation

in $CH{\equiv}H_4MPT^+$. This is assumed to be achieved by binding of $CH{\equiv}H_4MPT^+$ to the enzyme upon which the lone pair orbitals at the nitrogen atoms could be protonated, again by acidic residues in the enzyme's active site, resulting in an envelope conformation of the imidazolidine ring. The electrophilicity of the cation center at C14 in this conformation should be dramatically increased relative to the planar conformation of $CH{\equiv}H_4MPT^+$ in solution. The reactivity of the resulting cation should more closely resemble that of carbocations generated in solution under superacidic conditions allowing it to abstract a hydride ion from molecular hydrogen, yielding a proton and $CH_2{=}H_4MPT$. Reduction of $CH{\equiv}H_4MPT^+$ with H_2 is thus made possible by activation of $CH{\equiv}H_4$-MPT^+ rather than by activation of H_2 which can explain why a redox-active transition metal is not required in the catalysis and why the reaction does not exhibit a kinetic deuterium isotope effect.[38] Note that Ni-Fe hydrogenases and Fe-only hydrogenases activate the H_2 molecule instead of the electron acceptor.[5]

In situ chemical studies, which were the basis of Olah's paper entitled "Superelectrophiles" [41] gave a good number of examples for protolytic (or electrophilic) activation. It is not just "superacidic chemistry" because the underlying principle of five-coordinate carbon involvement in the interaction is a general one and extends our knowledge of Kekulé's four-valent carbon to five or higher bounded new vistas.

At first glance the assumption of intermediates in an enzyme transformation that are otherwise found only under superacidic conditions may appear somewhat risky. However, reaction patterns characteristic of organic molecules under superacidic conditions, for example the isomerization of alkanes, are also observed in the presence of solid ion exchangers or zeolites carrying sulfonic acids as functional groups.[41,42,49] Consequently, "superacid reactions" are also possible without the typical "superacid solution chemistry", provided that an adequate nonbasic environment and properly positioned acidic functional groups are present, probably by multifunctional catalysis.[50] This is also proposed for $CH{\equiv}H_4MPT^+$ reduction with H_2 in Figure 10. In this respect it is interesting that the acidic functional groups in H_2-forming methylenetetrahydromethanopterin dehydrogenase by far outnumber the basic groups and that many of the acidic functional groups appear to be conserved as revealed by a comparison of the amino acid sequences (Figure 6). Olah was the first to suggest that activation by solid superacids may have relevance to enzymatic systems.[41,42]

One last argument: Proteases hydrolyze amide bonds at room temperature at pH 7 within minutes, whereas the analogous acid-catalyzed reaction in solution requires heating to 120 °C for at least 24 h in 6 M HCl. These latter conditions are very similar to those required for the protolysis of the central C−H bond of perhydro-3a,6a,9a-triazaphenalene, a reaction comparable to $CH_2{=}H_4MPT$ dehydrogenation to $CH{\equiv}H_4MPT^+$ and H_2 as discussed above.

But one caution: "One cannot prove a mechanism. One can only disprove it."

IV. Acknowledgments

We thank Prof. Dr. Albrecht Berkessel (Heidelberg), Prof. Dr. Christian Griesinger and Dr. Jürgen Schleucher (Frankfurt), and Prof. Dr. Victor Fernández (Madrid) for the excellent collaboration. Without their help and ideas this work would not have been possible.

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