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Carbon Monoxide as an Intrinsic Ligand to Iron in the Active Site of the Iron−Sulfur-Cluster-Free Hydrogenase H_2-Forming Methylenetetrahydromethanopterin Dehydrogenase As Revealed by Infrared Spectroscopy

Erica J. Lyon,[†] Seigo Shima,[†] Reinhard Boecher,[†] Rudolf K. Thauer,*[,†] Friedrich-Wilhelm Grevels,[‡] Eckhard Bill,[‡] Winfried Roseboom,[§] and Simon P. J. Albracht*[,§]

Contribution from the Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Strasse, D-35043 Marburg, Germany, Max Planck Institute for Bioinorganic Chemistry, Stift Strasse 34-36, D-45470 Mülheim an der Ruhr, Germany, and Biochemistry, Swammerdam Institute for Life Sciences, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands

Received May 28, 2004; E-mail: thauer@staff.uni-marburg.de (R.K.T.); asiem@science.uva.nl (S.P.J.A.)

Abstract: The iron−sulfur-cluster-free hydrogenase Hmd (H_2-forming methylenetetrahydromethanopterin dehydrogenase) from methanogenic archaea has recently been found to contain one iron associated tightly with an extractable cofactor of yet unknown structure. We report here that Hmd contains intrinsic CO bound to the Fe. Chemical analysis of Hmd revealed the presence of 2.4 ± 0.2 mol of CO/mol of iron. Fourier transform infrared spectra of the native enzyme showed two bands of almost equal intensity at 2011 and 1944 cm^{-1}, interpreted as the stretching frequencies of two CO molecules bound to the same iron in an angle of 90°. We also report on the effect of extrinsic ^{12}CO, ^{13}CO, $^{12}CN^-$, and $^{13}CN^-$ on the IR spectrum of Hmd.

Introduction

Ten years ago Bagley et al. made the astonishing discovery that the hydrogenase from *Allochromatium vinosum* absorbed infrared light in a window (2100−1900 cm^{-1}) in which pure proteins do not absorb.[1,2] The absorption bands were at wavelengths characteristic for the stretching frequencies of CO and cyanide ligands bound end-on to transition metals. The bands shifted according to changes in the redox state of the active-site nickel. Via crystal structure determination and chemical analysis it was subsequently shown that the active site harbors a heterodinuclear Ni−Fe center in which two cyanides and one CO are bound to the Fe atom.[3−5] The Ni is coordinated to the protein by two terminal cysteinethiols and two cysteinethiols which bridge between the nickel and an iron atom (Figure 1A). The oxidized enzyme is inactive and contains a third oxygen-containing bridging ligand. The diatomic ligands give infrared absorption bands at 1943 cm^{-1} for the CO and at 2090 and 2079 cm^{-1} for the CN^-. When active [NiFe]-hydrogenases are exposed to CO, which effectively inhibits these enzymes, a Ni−CO bond is formed[6] which can be seen in the infrared (IR) spectrum.[1] Via ^{13}CO labeling it was found that the extrinsic CO did not exchange with the intrinsic CO and that they were not bound to the same metal. The extrinsic CO bound to Ni can be flashed off reversibly upon irradiation of the enzyme with visible light. There are many enzymes that are reversibly inhibited by CO or cyanide. However, the [NiFe]-hydrogenases were the first enzymes shown to contain CO and cyanide as intrinsic ligands on an active-site transition metal.

A little later Van der Spek et al. reported that [FeFe]-hydrogenases (previously called [Fe]-hydrogenases or Fe-only hydrogenases) show an IR spectrum similar in many features to that of [NiFe]-hydrogenases, indicating similarities in the architecture of the active site.[7] This was confirmed by the crystal structure of [FeFe]-hydrogenase, which revealed an Fe−Fe dinuclear center to which three CO molecules and two cyanides are bound (Figure 1B).[8,9] The active center of [FeFe]-hydrogenase is covalently coordinated to the protein via a single cysteinethiol, which bridges between the dinuclear active center

[†] Max Planck Institute for Terrestrial Microbiology.
[‡] Max Planck Institute for Bioinorganic Chemistry.
[§] University of Amsterdam.

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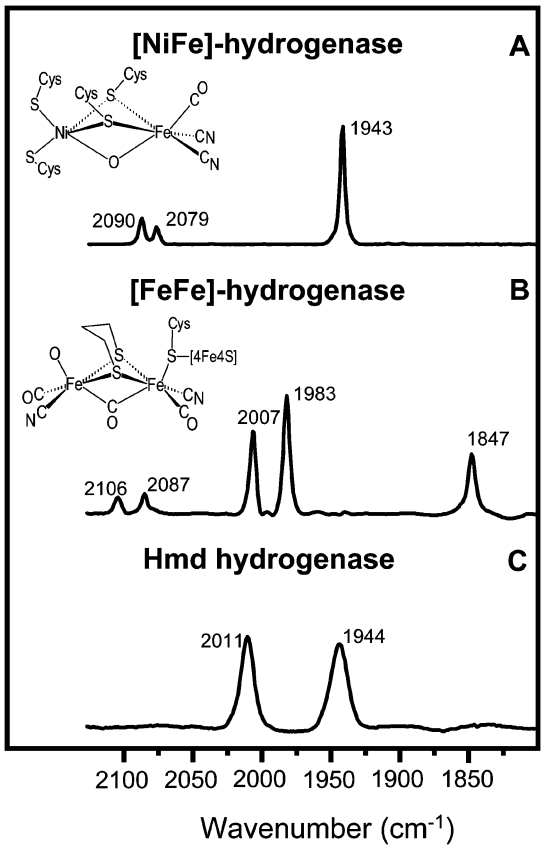

Figure 1. Infrared absorption in the region between 2100 and 1800 cm^{-1} of **(**A**)** [NiFe]-hydrogenase, **(**B**)** [FeFe]-hydrogenase, and **(**C**)** the Fe−S-cluster-free hydrogenase Hmd. The active-site structures of [NiFe]-hydrogenase and [FeFe]-hydrogenase are shown in their standard representations for the oxidized inactive states as revealed by X-ray crystallography.[3,8,9] In the structure of the [FeFe]-hydrogenase, the dithiolate bridge contains three nonprotein atoms and is speculated to be $NH(CH_2)_2$.[8,9] The structure of the Fe−S-cluster-free hydrogenase Hmd is not yet known. Shown are the spectra of the *A. vinosum* [NiFe]-hydrogenase in its oxidized ready state (A),[4] the *Desulfovibrio vulgaris* Hildenborough [FeFe]-hydrogenase as isolated in air (B),[10] and the Hmd as isolated at pH 6 (C). The absorption bands are characteristic of CO (strong bands) and CN^- (weak bands) ligands.

and a [4Fe−4S] cluster. There are two nonprotein sulfur atoms that bridge between the irons of the active center. They belong to a 1,3-propanedithiolate or the related bis(thiomethyl)amine unit.[8,9] Each of the iron centers contains one CO and one CN^-.[10] The CO that is between the two metals has bridging coordination in the oxidized form of the enzyme and terminal coordination when the enzyme is reduced. In its oxidized form, the infrared spectrum has one band at 1847 cm^{-1} for the bridging CO and two other intense bands at 2007 and 1983 cm^{-1}, which are attributed to terminal CO molecules. Two smaller bands at 2106 and 2087 cm^{-1}, due to the cyanide stretches, are also observed.[10] [FeFe]-hydrogenases are reversibly inhibited by CO, which was shown to bind to the iron distal from the [4Fe−4S] cluster (Figure 1B).[11,12] Again with ^{13}CO it was shown that the extrinsic CO does not exchange with the intrinsic CO molecules, at least not when the enzyme is kept in the dark.[13,14] When enzyme

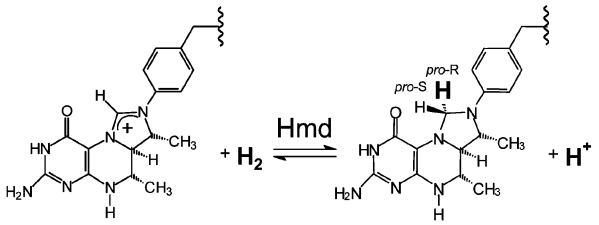

Figure 2. Reaction catalyzed by Hmd with the structures of methenyl-H_4MPT^+ and methylene-H_4MPT. A hydride, H^-, is reversibly transferred from H_2 into the *pro-R* position of methylene-H_4MPT.

inhibited by CO is irradiated with visible light, the extrinsic CO is flashed off first, only slowly followed by the intrinsic CO molecules.[14]

[NiFe]-hydrogenases and [FeFe]-hydrogenases have in common that they contain besides the dinuclear metal center, at least one [4Fe−4S] cluster. The Fe−S clusters are essential not only for electron transfer in these enzymes, but also for the redox reaction between H_2 and the enzyme.[15,16] These hydrogenases also catalyze the reduction of viologen dyes with H_2 and per se a H/D exchange reaction ($H_2 + D_2O \rightarrow HD + HDO$).[17−19] However, on the primary-structure level the two types of hydrogenases are not related.[18−20]

There is a third type of hydrogenase without Fe−S clusters, which was previously even considered to be "metal free".[21] This hydrogenase, which is composed of only one type of subunit with a molecular mass of 38 kDa, is phylogenetically not related to the [NiFe]-hydrogenases or [FeFe]-hydrogenases. Its systematic name is H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd or Fe−S-cluster-free hydrogenase). It catalyzes the transfer of a hydride (H^-) from H_2 to the substrate methenyl-H_4MPT^+, yielding methylene-H_4MPT, where H_4MPT is the abbreviation for tetrahydromethanopterin (Figure 2).[22−25] The enzyme does not catalyze the reduction of viologen dyes ($E^{\circ\prime} < -300$ mV) with H_2.[26−28] In addition, Hmd does not appear to catalyze a H/D exchange reaction unless either methenyl-H_4MPT^+ or methylene-H_4MPT is present.[27] In the absence of these substrates the exchange rate is less than 0.01% of the one observed in their presence.[29] Hmd has, until now, only been found in methanogenic archaea growing on H_2 and CO_2. In the hydrogenotrophic methanogens, the enzyme substitutes for [NiFe]-hydrogenases when the cells grow under conditions of nickel deprivation.[30,31]

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The metal-free hydrogenase from *Methanothermobacter marburgensis* has recently been shown to contain 1 mol of iron associated with a low molecular mass cofactor that can "reversibly" be extracted from the enzyme in the presence of mercaptoethanol.[32] Both the enzyme and the active cofactor are light sensitive.[33] The enzyme is inhibited by CO and by cyanide, albeit only at relatively high concentrations, and CO was shown to protect the enzyme from light inactivation.[33] Whereas inhibition by CO is a property shown by all three types of hydrogenases, inhibition by cyanide appears to be unique to the Fe−S-cluster-free hydrogenase. The structure of the cofactor light-inactivation product has been shown to be a 2-pyridone derivative.[34] The structure of the active cofactor is not yet known.

The light sensitivity and inhibition by CO and cyanide of the Fe−S-cluster-free hydrogenase Hmd prompted us to study the enzyme by infrared spectroscopy. The enzyme was found to exhibit two absorption bands at 2011 and 1944 cm^{-1} (Figure 1C). It will be shown that these are derived from two intrinsic CO molecules bound to the cofactor's iron.

Experimental Procedures

Enzyme, Cofactor, Substrates, and Inhibitors. Hmd was isolated and purified from *M. marburgensis* cells, grown at 65 °C under Ni-limited growth conditions, according to published procedures.[33] The final purification step was done on a gel-filtration column (HiPrep 26/10 desalting column, Amersham Biosciences, Uppsala, Sweden) equilibrated with water (pH 6). The enzyme was stored anaerobically in H_2O at −80 °C. Typical enzyme samples contained 12−18 mg/mL of the 38 kDa protein, which could be concentrated via ultrafiltration to yield 30−50 mg/mL (0.75−1.3 mM) samples for infrared measurements. The specific activity (see below) ranged from 400 to 600 U/mg depending on the sample.

The cofactor was extracted from the enzyme with 60% methanol, 140 mM β-mercaptoethanol, and 260 mM ammonia (final concentrations). The cofactor was isolated and purified as published.[33] Due to its thermal instability, sensitivity to low pH, and lability in the absence of thiols, the cofactor was kept in 10 mM ammonium carbonate (pH 9.0) and 10 mM mercaptoethanol at −80 °C.

H_4MPT and methenyl-H_4MPT^+ were purified from *M. marburgensis*.[35] The reduced substrate methylene-H_4MPT was made in situ by the spontaneous reaction of formaldehyde and H_4MPT, in a 10:1 ratio, under anaerobic conditions.[33,36]

^{13}CO (99% ^{13}C) was from Cambridge Isotope Laboratories and contained 10% ^{18}O. $K^{13}CN$ (99% ^{13}C) was from Sigma Chemical Co. (chemical assay 97%).

Measurement of Enzyme Activity. Enzyme activity was measured under N_2 at 40 °C in 1 mL quartz cuvettes closed with a rubber stopper. The 0.7 mL assay mixture contained 120 mM potassium phosphate (pH 6.0), 1 mM EDTA, 4 mM formaldehyde, and 40 μM H_4MPT. The assay was started by the addition of 0.05−0.1 μg of Hmd and monitored by following the increasing absorbance at 336 nm ($\epsilon_{336} = 21.6$ mM^{-1} cm^{-1}) due to the formation of methenyl-H_4MPT^+ from methylene-H_4-MPT. One unit of enzyme activity refers to 1 μmol of methenyl-H_4-MPT^+ formed per minute at 40 °C.

Infrared Measurements. Samples for infrared spectroscopy were prepared just prior to injection into the IR cell. The samples were manipulated in an anaerobic chamber (Coy) filled with 95% N_2/5% H_2 and containing a palladium catalyst for the continuous removal of O_2. Typically 5 μL of enzyme solution (approximately 1 mM) or 10 μL of cofactor solution (approximately 0.5 mM) was placed at the bottom of a 0.5 mL plastic cup contained inside an 8 mL brown glass vial. Then, in the case of the enzyme, an equal volume of 100 mM buffer was added. This was followed by the addition of substrate or inhibitor in volumes of 1−2 μL. Solutions of 10 mM KCN were prepared freshly for each sample. To exchange the gas phase, the vials were capped with a rubber stopper fastened with an aluminum cap. They were then removed from the anaerobic chamber and taken to a Schlenk line where they could be evacuated and refilled with CO, ^{13}CO, Ar, O_2, or H_2. Further additions of substrate, inhibitor, or gas could be made with a syringe, with which up to 10 μL of the sample was also withdrawn and injected into the IR cell. The gastight IR cell, constructed from two polished CaF_2 windows separated with a 50 μm Teflon spacer, was degassed in the anaerobic chamber or by blowing argon through the cell prior to injecting the sample.

FTIR spectroscopy measurements were made on a Bio-Rad 60A spectrometer equipped with an MCT detector cooled with liquid nitrogen. Routinely, each spectrum was the average of 762 scans recorded at a resolution of 2 cm^{-1}. The IR cell filled with the appropriate buffer was used as a reference. The spectra were baseline corrected using the multipoint method of the Bio-Rad spectrometer. Fitting of infrared spectra was performed with a minimal set of Gaussian functions using GRAMS software (Galactic Industries Corp.). The slight variations in water absorption due to humidity in the instrument were removed by subtraction of an appropriate water-vapor spectrum.

Light Inactivation. Light inactivation was carried out in the IR cell, and spectra were measured intermittently. The light source was a 450 W xenon arc lamp (Osram XBO-450 OFR) incorporated into an LAX 1450 lamp housing (Müller Elektronik, Moosinning, Germany). Light was filtered through a long-pass filter (filter 5146 with a 345 nm cutoff, Oriel, Stanford) and a water filter (Oriel liquid filter) and then focused via a total-reflection device (Oriel fiber bundle focusing assembly) into a light-guiding fiber and directed at the IR cell at an angle of about 30°.

Protein Determination. Protein concentration was estimated using the method of Bradford[37] and the reagents from Bio-Rad Laboratories. Bovine serum albumin was used as a standard.

CO Determination. Plastic cups (0.5 mL) containing 0.1 mL of 10% $HClO_4$ were placed in 12 mL glass vials closed with a rubber stopper and filled with 100% N_2 under atmospheric pressure. Enzyme (0.1 mL containing 1−2 mg of protein) was injected into the perchloric acid with a syringe, care being taken that the needle did not come in contact with the acid. The vials were heated for 30 min in a 60 °C

(29) The very low "rest" exchange rate (0.01%) could be due to contamination of the purified Hmd hydrogenase with small amounts of methenyl-H_4MPT^+ or methylene-H_4MPT or with small amounts of [NiFe]-hydrogenases, which are present in high concentrations in the cell extracts of *M. marburgensis*, from which the enzyme was purified. To test the possibility of contamination with the substrates, we determined whether the rest exchange activity decreased upon gel chromatography of purified Hmd on Superdex 75 (1 cm × 30 cm) equilibrated and eluted with potassium phosphate (pH 6.0) containing 200 mM NaCl and 1 mM EDTA. The rest exchange activity of Hmd did not change, arguing against a contamination of purified Hmd with methenyl-H_4MPT^+ or methylene-H_4MPT. To test the possibility of contamination with [NiFe]-hydrogenase, we determined the rest exchange activity under air using the purified [NiFe]-hydrogenase Mvh from *M. marburgensis* as a control. Whereas the rest exchange activity of Hmd was only slightly decreased (10%) in the presence of molecular oxygen, the exchange activity of the [NiFe]-hydrogenase was completely blocked under these conditions. Furthermore, we showed that the rest exchange activity of Hmd was light sensitive, a property not exhibited by [NiFe]-hydrogenases. It therefore has to be seriously considered that the rest exchange activity of Hmd is an intrinsic property of the enzyme, which would be of great importance for mechanistic considerations.

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water bath and then allowed to cool to room temperature for at least 30 min. The CO content was measured by the removal of 0.1 mL of the gas phase with a gastight syringe equipped with a Teflon valve. The gas was injected into an SRI 8610C gas chromatograph equipped with a 1/8 in. × 1 m stainless steel column hand-packed with molecular sieves (5 Å, 80/100 mesh, from Supelco) on which CH_4, CO, and CO_2 were separated. The oven temperature was 80 °C, and the carrier gas was H_2 (99.999%) at 12 psi (12.5 mL/min). After the column the gas passed a methanizer, in which CO and, when present, CO_2 were converted to methane (Varian Chromopack CP11952, at 350 °C). Methane was subsequently detected using a flame ionization detector (150 °C; the flow rate of pressurized air at 9 psi was 250 mL/min). Calibration of the instrument was done using standard gases (79.2 and 1006 ppm CO in N_2, accuracy of standard ±2%, from Messer-Griesheim) and dilutions of the standard gases in 100% N_2. Retention times for CH_4, CO, and CO_2 were 1.1, 1.3, and 4 min, respectively.

Iron Determination. Iron was determined in the samples used for the CO determination. In principle, the procedure published by Fish was employed.[38] To each of the 0.5 mL plastic cups with 0.1 mL of enzyme solution and 0.1 mL of 10% $HClO_4$ (see above) was added 0.1 mL of 4.5% $KMnO_4$. The samples were then incubated for 2 h at 60 °C. After the samples were cooled to room temperature, 0.3 mL of H_2O was added as well as 0.04 mL of a solution containing 6.5 mM ferrospectral (disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-tiazin), 13.1 mM neocuproin (2,9-dimethyl-1,10-phenanthroline), 2 M ascorbic acid, and 5 M ammonium acetate. The samples were mixed and left to stand for 30 min at room temperature before they were centrifuged for 1 min at 8000 rpm (5400*g*). Then the absorbance at 562 nm was measured in reduced-volume cuvettes with a path length of 0.3 cm and related to a standard curve. An Fe atomic absorption standard solution from Aldrich containing 1005 μg/mL Fe in 1% HCl was used as a standard.

Results and Discussion

The following results were obtained with Hmd from *M. marburgensis* purified to apparent homogeneity as described in the Experimental Procedures. The enzyme showed a UV/vis spectrum with a shoulder at 360 nm with an ϵ_{360} near 5 mM^{-1} cm^{-1} and contained approximately one iron per mole (mass 38 kDa).

Finding Intrinsically Bound CO. Infrared spectra of Hmd, as shown below, revealed absorption bands at 2011 and 1944 cm^{-1}, suggesting that this hydrogenase might also contain CO bound to iron. Chemical analysis of the enzyme for the presence of carbon monoxide consisted of CO liberation from the enzyme upon acidification followed by gas chromatography. At the end of the column CO was passed through a methanizer and converted into methane. The methane was then detected with flame ionization. The retention time on the column and its ability to burn in the flame detector conclusively identified the acid-liberated gas as CO. Per mole of Hmd protein approximately 2.4 ± 0.2 mol of CO was found (Table 1). Since the protein concentration was determined via the Bradford method, this stoichiometry may not be accurate. In control experiments, Hmd apoenzyme did not release CO.

After the CO content was determined, the amount of iron in each sample was measured. The ratio of CO to Fe varied, ranging from 2.0 to 2.7. The average CO/Fe ratio was 2.4 with a standard deviation of ±0.2 (Table 1). On the basis of the iron determination, the number of CO molecules in Hmd could be two or three.

Table 1. Number of Moles of CO and Fe per Mole of Hmd[a]

sample	Hmd amt (nmol)	CO amt (nmol)	Fe amt (nmol)	CO/Fe
1	23.0	52.8	19.3	2.4 ± 0.2
	46.0	91.2	37.9	
2	20.8	51.5	19.6	
	41.5	95.2	36.4	
3	30.0	63.1	25.2	
	60.0	110.1	47.8	
4	15.0	33.2	13.6	
	30.0	55.3	27.1	

[a] Samples 1−4 are from four separate enzyme preparations. The Hmd concentration was determined using the method of Bradford. CO was liberated from the enzyme by acidification and quantified via gas chromatography. Chemical determination of iron was carried out using a slight modification to the method of Fish.[38] The specific activity of the enzyme in each of the samples was around 500 U/mg. See the Experimental Procedures for details.

IR Spectrum of Hmd and the Effect of pH, O_2, and H_2. When measured by IR, Hmd was found to have two absorption bands between 2100 and 1900 cm^{-1}, indicating the presence of at least two diatomic ligands assuming homogeneous preparations. Cyanide tends to absorb at 2200−2000 cm^{-1}, CO at 2100−1800 cm^{-1}, and NO at 1900−1500 cm^{-1}. Also triatomic molecules with two adjacent double bonds absorb in this spectral region.[39] However, in light of the chemical analysis, the two absorption bands are most likely due to the stretching vibrations of CO molecules bound in an end-on fashion to iron.

The two absorption bands were located at 2011 and 1944 cm^{-1} and were of nearly equal intensity both at pH 9.5 and at pH 6.0 (Figure 3A,B). The appearance of the spectrum suggests the presence of an $Fe(CO)_2$ unit with an angle of about 90° between the two CO groups bound to one iron. In detail, the high-frequency band is commonly assigned to the in-phase or symmetric vibration of the two CO groups, whereas the low-frequency band represents the out-of-phase or asymmetric vibration. Assuming that only one kind of CO group is present, i.e., that the two CO molecules have equal force constants, the intensity ratio is determined by the bond angle 2θ between the two CO groups according to the relation $I_{sym}/I_{asym} = \text{cotan}^2\,\theta$.[40] For Hmd, with two ν(CO) bands of nearly equal intensities, it is obvious that 2θ is close to 90°. Since the actual ratio of the symmetric and asymmetric bands from Hmd at different pH values ranges from 1.14 to 0.86 (Figure 10), the calculated angle of the species varies between 86° and 94°. On the basis of the same assumption and using the energy-factored CO force field approximation,[41,42] one obtains from the observed frequencies the force and interaction constants $k_{CO} = 1580.2$ N·m^{-1} and $k_{CO,CO} = 53.6$ N·m^{-1}, respectively. For underpinning the assumption of chemically equivalent CO groups, additional frequency data from a ^{13}CO-enriched sample of Hmd would be necessary, but unfortunately, such a sample is not available.

Although not immediately apparent, both the 2011 and 1944 cm^{-1} absorption bands slightly tailed to the high-frequency side at pH 6.0 (Figure 3B). This tailing was more pronounced at pH 5.5, at which additional peaks at 2021 and 1952 cm^{-1}

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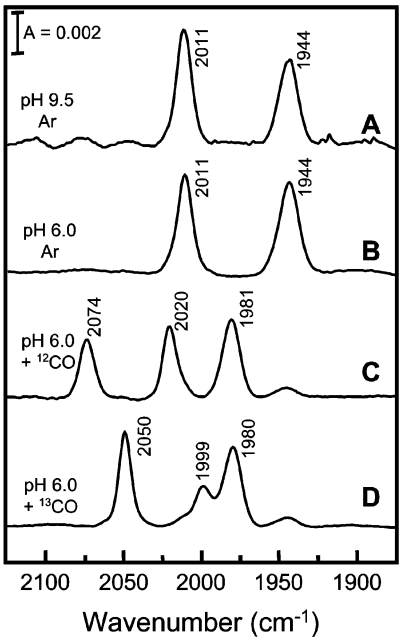

Figure 3. IR spectra of native Hmd in the absence and presence of ^{12}CO or ^{13}CO: **(**A) Hmd (pH 9.5) under 10^5 Pa of Ar; (B) enzyme (pH 6.0) under 10^5 Pa of Ar; (C) enzyme (pH 6.0) under 10^5 Pa of ^{12}CO; (D**)** enzyme (pH 6.0) under 10^5 Pa of ^{13}CO. Hmd (0.5 mM) was dissolved in 50 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0) or 50 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (Capso)/HCl (pH 9.5). Spectra were obtained at 20 °C. These spectra and those in the following figures were baseline corrected, and the enzyme concentrations were normalized for easier comparison. Scaling of the absorbances is the same for all traces within each figure. Half-maximal inhibition (K_i) of Hmd by CO is obtained with partial CO pressures of approximately 10^3 Pa (ca. 0.01 mM dissolved in aqueous solution) (see the text).

contributed 13% to the IR spectrum as deduced from a Gaussian analysis (not shown). At pH 6 this contribution was only 4%. Changing the pH to 8.0 or even to 9.5 removed the minor bands completely. The results suggest an acidic group, with a p*K* near 5, close enough to the iron so that the change in protonation affects the electron density of the transition metal.

The spectra shown in Figure 3A,B were recorded with Hmd under argon. When the gas phase was changed to N_2, O_2, or H_2, no change in the spectrum was observed. The fact that O_2 had no effect is consistent with the finding that the purified enzyme is not sensitive to oxygen and that the H/D exchange assay can be carried out under air.[33] The fact that H_2 did not change the IR spectrum is consistent with three observations: (i) Hmd showed the same UV/vis spectrum under argon and under H_2; (ii) the enzyme was EPR silent under argon and H_2; (iii) the enzyme in the absence of methenyl-H_4MPT^+ or methylene-H_4MPT catalyzed a H/D exchange at a rate of only 0.01%.[29]

The line widths (full width at half-maximum or fwhm) of both the 2011 and 1944 cm^{-1} bands were approximately 14 cm^{-1}, which is relatively large: e.g., the widths of ν(CO) bands in IR spectra of the [NiFe]-hydrogenase from *A. vinosum* and of the [FeFe]-hydrogenase from *D. desulfuricans* are 4−5 and 5−6 cm^{-1}, respectively, when measured under the same instrumental conditions (Figure 1; Albracht, S. P. J., unpublished data).

IR Spectrum of Hmd in the Presence of ^{12}CO or ^{13}CO. Hmd is reversibly inhibited by CO with an estimated apparent K_i of 0.01 mM (1% CO in the gas phase at a total pressure of approximately 10^5 Pa).[43] The spectral changes described below were pH independent, having been observed at pH 6.0 (50 mM 2-morpholinoethanesulfonic acid/KOH) as well as at pH 8.0 (50 mM tris(hydroxymethyl)methylamine (Tris)/HCl).

Incubation of the enzyme under CO resulted in a spectrum with three bands at 2074, 2020, and 1981 cm^{-1} of comparable intensities (Figure 3C). When Hmd was incubated under 10^5 Pa ^{13}CO, its IR spectrum showed bands at 2050, 1999, and 1980 cm^{-1} (Figure 3D). The application of the Teller−Redlich product rule,[42] $\Pi(\tilde{\nu}^*/\tilde{\nu}) = (\mu/\mu^*)^{n/2}$, shows that the tricarbonyliron unit formed with $^{13}C^{16}O$ contains one isotopically labeled carbon monoxide. The experimental data yield (2050 × 1999 × 1980)/(2074 × 2020 × 1981) = 0.97766, which compares very well with the value 0.97771 expected for the presence of one $^{13}C^{16}O$ group ($n = 1$). The fact that just one of the ν(CO) bands, that at 1980 cm^{-1}, is not affected by the isotopic labeling immediately indicates that the labeled carbon monoxide resides in the mirror plane that bisects the two Fe−CO bond vectors associated with the intrinsic CO ligands. Again using the energy-factored CO force field approximation,[41,42] one obtains from the observed frequencies the force and interaction constants $k_u = 1714.7$ N·m^{-1}, $k_e = 1628.2$ N·m^{-1}, $k_{eu} = 27.7$ N·m^{-1}, and $k_{ee} = 43.8$ N·m^{-1} (the subscripts "u" and "e" designate the CO groups in the unique and in the two equivalent positions, respectively).

The effect of CO on the IR spectrum of Hmd was completely reversed upon changing the gas phase back to Ar, H_2, N_2, or air (not shown). The reversibility was also seen in the case of ^{13}CO. The observation that extrinsic CO did not exchange with the intrinsically bound CO was irrespective of incubation times and conditions. This is remarkable insofar as many organocarbonylmetal complexes are more or less fluxional, i.e., undergo $^{12}CO/^{13}CO$ positional exchange sufficiently fast to show coalescence of the carbonyl signals in the ^{13}C NMR spectrum.[44] With $Fe(CO)_3(\eta^4$-diene) complexes, for example, rate constants at ambient temperature were reported to be on the order of 10^3 to 10^4 s^{-1}.[45] There are some extreme cases where the CO positional exchange is so rapid ($>10^{12}$ s^{-1} at ambient temperature) that even the ν(CO) IR bands show dynamic line broadening and coalescence.[46] While this rigidity of the iron carbonyls in Hmd is indeed remarkable, it has been shown that the intrinsic CO ligands in [NiFe]- and [FeFe]-hydrogenases do not exchange with extrinsic CO.[2,4,13,14]

The CO force and interaction constants given above show that the force constant of the two CO groups in the $Fe(CO)_2$ moiety of Hmd increased from 1580.2 to 1628.2 N·m^{-1} upon binding of the third CO. This suggests that the metal (dπ) → CO (π^*) back-donation to the two intrinsic CO groups is substantially diminished. This is to be expected, taking into account that the incoming CO, owing to its electron-withdrawing capacity, will diminish the metal (dπ) electron density. The force

(43) The K_i of Hmd for CO increases in the presence of methenyl-H_4MPT^+. At 3 μM methenyl-H_4MPT^+ the apparent K_i for CO was determined to be 20% CO [0.2 mM CO], at 10 μM 40% CO [0.4 mM CO], and at 30 μM 150% CO [1.5 mM CO] as determined in the H/D exchange assay. Since in the absence of methenyl-H_4MPT^+ the enzyme does not catalyze a H/D exchange, the K_i for CO in the absence of methenyl-H_4MPT^+ was extrapolated to be approximately 1% CO [0.01 mM CO] at room temperature.

(44) *Dynamic NMR Spectroscopy*; Jackman, L. M., Cotton, F. A., Eds.; Academic Press: New York, 1975.

(45) See, for example: Kruczynski, L.; Takats, J. *Inorg. Chem.* **1976**, *15*, 3140.

(46) Grevels, F. W.; Kerpen, K.; Klotzbucher, W. E.; McClung, R. E. D.; Russell, G.; Viotte, M.; Schaffner, K. *J. Am. Chem. Soc.* **1998**, *120*, 10423.

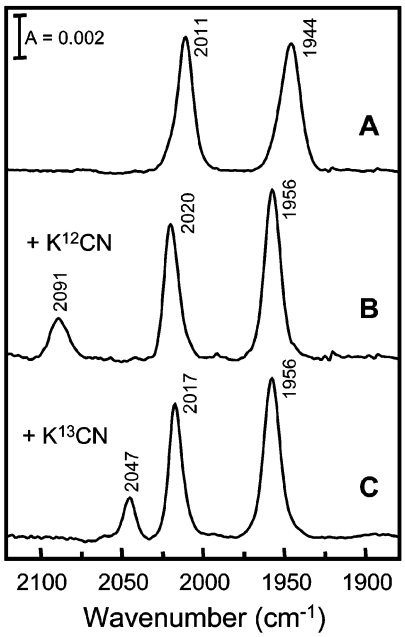

Figure 4. IR spectra of native Hmd in the absence and presence of $^{12}CN^-$ or $^{13}CN^-$: (A) no cyanide present; (B) 5 mM $K^{12}CN$; (C) 2 mM $K^{13}CN$. The gas phase was 10^5 Pa of Ar. The enzyme concentration was 0.5 mM in 50 mM Tris/HCl (pH 8.0). Spectra were measured at 20 °C. The K_i of Hmd for cyanide is approximately 0.1 mM.

constant of the extrinsic CO, 1714.7 N·m^{-1}, was much larger than that of the intrinsic CO molecules, indicating that the metal (dπ) → CO (π^*) back-donation to this CO is much smaller, i.e., the extrinsic CO is less strongly bound to the metal. This appears to be also indicated by the finding that the extrinsic CO is readily released upon removal of CO from the gas phase whereas the intrinsic CO molecules are not. However, the latter finding may also be due to the possibility that the intrinsic CO molecules of the cofactor are confined by the protein and are thus less readily released as in [NiFe]- and [FeFe]-hydrogenases.[3,8,9]

The reversible binding and relatively low affinity for CO made manipulations of the IR samples difficult. Even when the work was done carefully and quickly, some of the native Hmd would be present in the sample that was incubated under CO (note the remaining band at 1994 cm^{-1} in trace C of Figure 3).

IR Spectrum of Hmd in the Presence of $^{12}CN^-$ and $^{13}CN^-$. Hmd is reversibly inhibited by cyanide with an estimated apparent K_i of 0.1 mM.[33] The infrared spectra for the $^{12}CN^-$- and $^{13}CN^-$-inhibited enzyme were measured and are shown in Figure 4. Upon addition of $^{12}CN^-$ the IR spectrum with two CO absorption bands at 2011 and 1944 cm^{-1} changed to one with three bands at 2091, 2020, and 1956 cm^{-1}. When $^{13}CN^-$ was added to the enzyme, its IR spectrum also showed three bands, which were at 2047, 2017, and 1956 cm^{-1}. Two of these bands, those at 2017 and 1956 cm^{-1}, were almost at the same position and had the same intensity as in the spectrum of the $^{12}CN^-$-inhibited enzyme. The third band at 2047 cm^{-1} was, however, at a significantly lower wavenumber than the third band at 2091 cm^{-1} of the $^{12}CN^-$-inhibited enzyme. Also the intensities of these bands were lower.

These results strongly suggest that the bands at 2020 (2017) and 1956 cm^{-1} are derived from CO stretches and those at 2091 and 2047 cm^{-1} from cyanide stretches, which are at very best only weakly coupled to the CO stretches. A very weak, and often nonobservable, coupling is characteristic for cyanide and CO interactions.[47] Therefore, the cyanide stretches can generally be treated separately from the CO stretches. That this is indeed possible is indicated by the finding that the wavenumber ratio 2047/2091 was 0.97895. This value is close to 0.97908 which is calculated from the square root of the ratio of the reduced masses of $^{12}CN^-$ and $^{13}CN^-$.[39]

The equal intensities of the ν(CO) bands of the Fe(CO)$_2$(CN$^-$) species again suggest an angle of about 90° between the two CO groups (see above). Since binding of cyanide and that of extrinsic CO are mutually exclusive (see below), it is reasonable to assume that the Fe(CO)$_2$(CN$^-$) and Fe(CO)$_3$ units have similar geometries. However, given the fact that the IR stretches of cyanide groups tend not to couple strongly with adjacent CO moieties, it is tenuous to make structural comparisons between the proposed Fe(CO)$_2$(CN)$^-$ and Fe(CO)$_3$ units on the basis of IR alone.

For the purpose of comparison with the Fe(CO)$_2$ moiety of Hmd, the CO force and interaction constants of this unit in the CN$^-$ adduct were calculated.[42] With 2018.5 cm^{-1} (the average of the high-frequency ν(CO) data of the $^{12}CN^-$ and the $^{13}CN^-$ adducts) and 1956 cm^{-1}, and assuming that the two CO groups are in chemically equivalent positions, one obtains k_{CO} = 1595.7 N·m^{-1} and $k_{CO,CO}$ = 50.2 N·m^{-1}. Comparison with Hmd (k_{CO} = 1580.2 N·m^{-1} and $k_{CO,CO}$ = 53.6 N·m^{-1}) and its CO adduct (k_e = 1628.2 N·m^{-1} and $k_{e,e}$ = 43.8 N·m^{-1}) shows that CN$^-$ expectedly functions as a π acceptor, though less efficiently than CO.

The samples were measured at both pH 6.0 using 2-morpholinoethanesulfonic acid/KOH and pH 8.0 Tris/HCl, and the results were the same. Since the pK_a of cyanide is 9.2, fresh dilutions of 10 mM KCN in buffer were made for each sample from a 1 M KCN in 1 mM KOH stock solution just prior to mixing and injecting into the infrared cell.

Competition of CO and CN$^-$ for Binding to Hmd. As seen from the IR spectra in Figure 5, the addition of KCN to enzyme that was incubated under 10^5 Pa of CO (ca. 1 mM)[48] led to the formation of the CN$^-$-inhibited enzyme. Already at a concentration of 0.5 mM KCN, the majority of the enzyme was in the CN-inhibited form (Figure 5B). At 2 mM cyanide only the IR spectrum of the cyanide-inhibited enzyme was observable (Figure 5D). The results indicate that the binding of CO and that of CN$^-$ are mutually exclusive and that Hmd has a higher affinity for cyanide than for CO. There was no spectroscopic indication the enzyme can bind both cyanide and extrinsic CO simultaneously.

Formation of the CO-Inhibited Form of Hmd upon Exposure to Light. Hmd is sensitive to UV-A/blue light.[33] This process could be followed using IR. The sample within the infrared cell was illuminated for a short time (starting with 15 s; see the legend to Figure 6) and then a spectrum recorded, which was followed by further illumination (Figure 6). Within seconds absorption bands of the CO-inhibited form of the enzyme became visible. This indicates that CO released by illumination from some enzyme molecules binds to the active center of other, still intact enzyme molecules, resulting in a CO-inhibited form. As can be seen in Figure 6, the rate of loss of

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(48) Liquide, L. A. *Gas Encyclopaedia*; Elsevier: Amsterdam, The Netherlands, 1976.

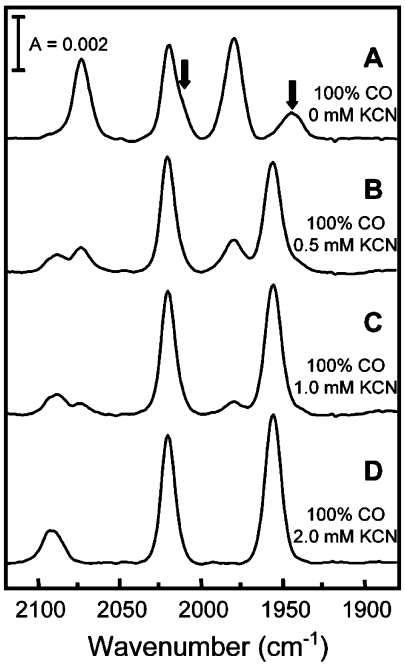

Figure 5. IR spectra of ^{12}CO-inhibited Hmd in the presence of increasing concentrations of cyanide: (A) no cyanide present (gray arrows indicate Hmd without CO bound); (B) 0.5 mM KCN; (C) 1.0 mM KCN; (D) 2.0 mM KCN. The gas phase was 10^5 Pa of ^{12}CO. The Hmd concentration was 0.5 mM in 50 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0). Spectra were measured at 20 °C.

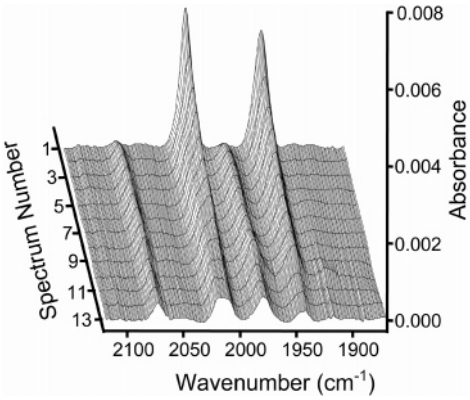

Figure 6. Light inactivation of native Hmd as followed by IR spectroscopy. The gas phase was 10^5 Pa of Ar. The infrared cell was filled with Hmd (0.25 mM) in 50 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0). A spectrum was recorded, after which the sample was illuminated in the FTIR spectrometer at 20 °C. Accumulation times for the successive spectra 1−13 (from back to front): 0 (spectrum 1), 15, 30, 45, 60, 90, 120, 150, 200, 300, 600, 1200, and 1800 (spectrum 13) s. Illumination eventually led to a loss of all absorption bands and thus of all CO ligands.

absorption bands was slowed more than expected for a zeroth-order decay due to the generation of the CO-inhibited form of the enzyme. This is consistent with the observation that the light sensitivity of the enzyme is diminished in the presence of CO, and that the rate of light inactivation at high protein concentrations was not constant and decreased with time.[33] Prolonged illumination resulted in the disappearance of all IR bands, indicating that the enzyme eventually loses all CO molecules that were once bound. The finding that extrinsic CO protects the enzyme from light inactivation indicates that in the CO-inhibited enzyme the extrinsic CO is flashed off first and that this CO can bind again. The finding that light inactivation was not reversible in the absence of CO indicates that in the non-CO-inhibited form the intrinsic CO is flashed off irreversibly.

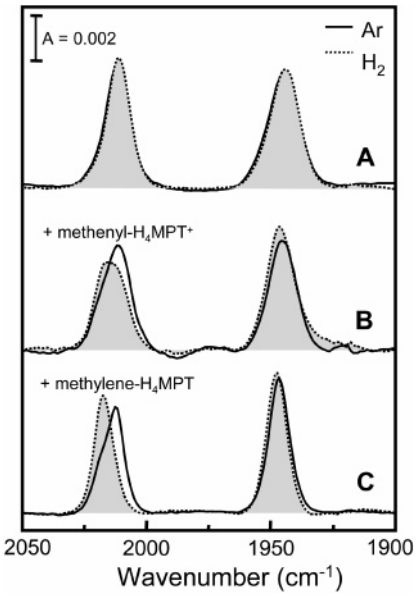

Figure 7. IR spectra of native Hmd under Ar (—) or H_2 (···) as gas phase and in the absence and presence of methenyl-H_4MPT^+ or methylene-H_4MPT: (A) Hmd alone; (B) Hmd + 2 mM methenyl-H_4MPT^+; (C) Hmd + 1 mM methylene-H_4MPT. The enzyme concentration was 0.5 mM in 50 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0). Spectra were measured at 20 °C. The spectra under H_2 are shaded.

IR Spectrum of Hmd in the Presence of Methenyl-H^4MPT^+ or Methylene-H_4MPT. As has already been indicated above, the IR spectra of Hmd under argon and under H_2 were identical (Figure 7A). However, the spectra of the enzyme under argon and H_2 differed when methenyl-H_4MPT^+ (Figure 7B) or methylene-H_4MPT (Figure 7C) was present. The experiments were performed at pH 6.0.

Under Ar the addition of methenyl-H_4MPT^+ (Figure 7B, solid line) caused little change in the 1944 cm^{-1} band, at most shifting it by 1 cm^{-1}. The 2012 cm^{-1} band became more asymmetric: a high-frequency shoulder around 2020 cm^{-1} increased from 4% of the peak area to 16%. Under H_2 the addition of methenyl-H_4MPT^+ (Figure 7B, dotted line) induced clearer changes. The 1944 cm^{-1} band slightly moved to higher frequency (to 1945 cm^{-1}), while the 2011 cm^{-1} band shifted to 2015 cm^{-1} and could not be fitted as one Gaussian. Addition of two Gaussians at 2018 (47%) and 2011 (53%) cm^{-1} gave a good fit; the width of these bands was about 70% of that of the 1945 cm^{-1} band.

The changes were even more pronounced when methylene-H_4MPT was added. Under Ar (Figure 7C, solid line) this resulted in a clear shift of the 1944 cm^{-1} band to 1946 cm^{-1} and a concomitant narrowing of the band (a change in the full width at half-maximum from 14 to 10 cm^{-1}). Again the asymmetry of the 2012 cm^{-1} band increased; the shoulder around 2020 cm^{-1} increased from 4% to 29% of the peak area. Under H_2, addition of methylene-H_4MPT caused the largest changes (Figure 7C, dotted line). The 1944 cm^{-1} band shifted to 1947 cm^{-1} and sharpened (from 14 to 10 cm^{-1}). The 2011 cm^{-1} band shifted to 2017 cm^{-1}, while sharpening from 11 to 9 cm^{-1}. The 2017 cm^{-1} band could be fitted well by only one Gaussian. Its area was 79% of the area of the 1947 cm^{-1} band.

In the samples with methylene-H_4MPT under Ar the enzyme catalyzed the conversion of methylene-H_4MPT to methenyl-

H_4MPT^+ and H_2 ($\Delta G^{\circ\prime} = -5.5$ kJ/mol).[24,25] The formation of H_2 in the sample at pH 6.0 under argon is consistent with the presence of the shoulder around 2020 cm^{-1} (Figure 7C, solid line).

The small but real shifts and the noticeable sharpening of the absorption bands of the enzyme in the presence of methenyl-H_4MPT^+ or methylene-H_4MPT suggest that these substrates bind close to or at the Fe site, resulting in a slight upward frequency shift of the CO bands. The 20−30% decrease of the widths of the bands in the 2050−1900 cm^{-1} spectral region is consistent with a decrease in the flexibility of the active-site pocket, i.e., less variation of the CO positions in space. These observations suggest a change in the Fe center's conformation or flexibility upon binding of methenyl-H_4MPT^+ or methylene-H_4MPT. In this respect it is noteworthy that in previous NMR studies a conformational change in methylene-H_4MPT was observed upon binding to the enzyme.[49,50]

It should be noted that the binding of extrinsic diatomic ligands such as CN^- and CO (Figures 3 and 4) also caused a sharpening of the two infrared bands of the enzyme, although the effect (13−15%) was noticeably smaller than the effect of methylene-H_4MPT in enzyme under H_2.

It is worthwhile to note that the effects of methenyl-H_4MPT^+ and of methylene-H_4MPT on the IR spectrum were amplified when H_2 was simultaneously present. Dihydrogen alone had no effect at all. This is consistent with the finding that the enzyme catalyzed a significant H/D exchange only in the presence of methenyl-H_4MPT^+ or methylene-H_4MPT and is the first indication that H_2, in the presence of methenyl-H_4MPT^+ or methylene-H_4MPT, might directly interact with the iron in the active site of the enzyme.[29] H−Fe or D−Fe stretches were looked for in the IR spectrum but not found.

Competition of Cyanide and Methenyl-H_4MPT^+ for Binding to Hmd. Activity measurements have shown that the apparent K_i of CN^- increases as the concentration of methenyl-H_4MPT^+ increases.[33] A clear effect was also seen in the IR spectra (Figure 8). The addition of methenyl-H_4MPT^+ at concentrations of 0.5 mM (Figure 8, shaded) and 1.6 mM (Figure 8, dotted line) to the CN-inhibited enzyme under Ar resulted in a change of the IR spectrum of the cyanide-inhibited form (Figure 8, solid line). The resulting spectrum was not identical to that of Hmd under Ar in the presence of methenyl-H_4MPT^+ alone (Figure 7B, solid line). Instead, when the spectrum was corrected for the contribution of the CN-inhibited enzyme, two bands were detected with exactly the same frequencies as those observed in the spectrum of the enzyme with methylene-H_4MPT in the presence of H_2, but with line widths that were noticeably (25%) smaller (not shown). When the spectrum, corrected for the contribution of the CN-inhibited enzyme, was compared to the spectrum of the native enzyme, a 51% change in line width was observed (Figure 8, inset). Apparently, the binding of cyanide and that of methenyl-H_4MPT^+ were not mutually exclusive, and binding of both compounds at the same time cannot be ruled out. Such remarkable changes in line widths have not been observed in other hydrogenases.

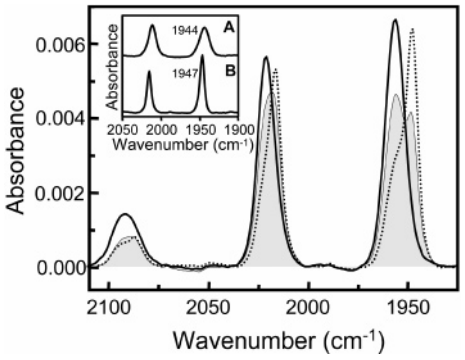

Figure 8. IR spectra of $^{12}CN^-$-inhibited Hmd under Ar in the absence and presence of methenyl-H_4MPT^+. The Hmd concentration was 0.5 mM in 50 mM 2-morpholinoethanesulfonic acid/KOH (pH 6.0) supplemented with 1.5 mM cyanide. Concentration of methenyl-H_4MPT^+ added: 0 mM, solid line; 0.55 mM, shaded; 1.6 mM, dotted line. The inset shows IR spectra of (A) Hmd at pH 6 and (B) the enzyme with 1.5 mM cyanide and 1.6 mM methenyl-H_4MPT^+, corrected for the contribution from the CN-inhibited enzyme (obtained by subtracting the properly scaled spectrum of CN-inhibited Hmd from the dotted line spectrum in Figure 8). The area of the low-frequency band is held constant; its full width at half-maximum is 14.3 and 7.3 cm^{-1} for (A) and (B), respectively.

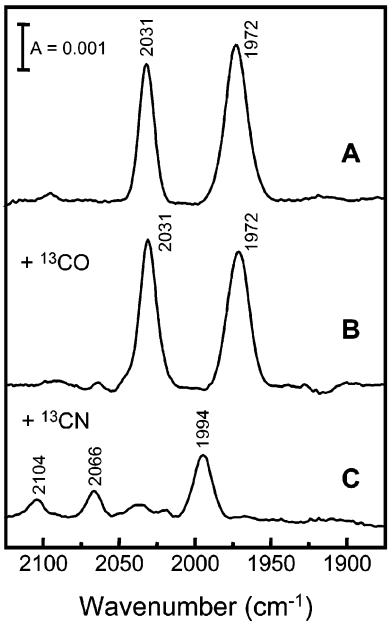

Figure 9. IR spectra of the extracted Hmd cofactor as purified and measured in 10 mM ammonium carbonate (pH 9.0) and 10 mM mercaptoethanol: (A) 0.45 mM cofactor measured at 5.7 °C under 100% Ar; (B) 0.4 mM cofactor measured at 20 °C under 100% ^{13}CO; (C) 0.45 mM cofactor after the addition of 50 mM $K^{13}CN$ under 100% Ar.

IR Spectrum of the Isolated Active Hmd Cofactor. The iron-containing cofactor of Hmd can be extracted from the enzyme upon denaturation in the presence of thiols. Infrared spectroscopy revealed that the two bands previously observed for the enzyme were shifted from 2011 and 1944 cm^{-1} to 2031 and 1972 cm^{-1} and that the two bands were the only ones observable (Figure 9A). A shift in this direction indicates that the metal has a decreased electron density available for metal ($d\pi$) → CO (π^*) back-donation. This can be the result of ligand exchange, from a ligand that donates electron density (e.g., a thiol) to a ligand that donates less or even accepts electron density (e.g., a hydroxide), or a change in the total number of ligands, i.e., going from 6-coordinate to 5-coordinate. This will

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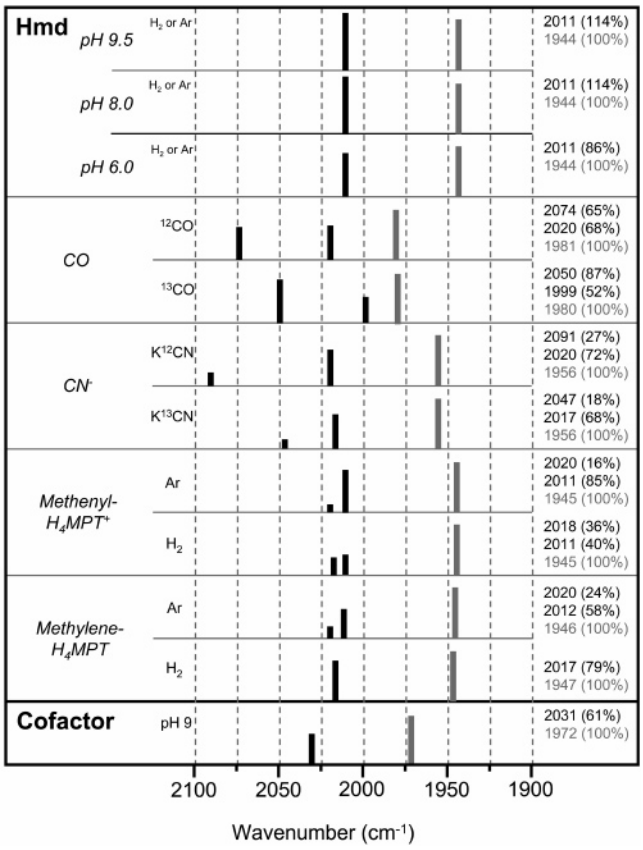

Figure 10. Infrared bands of active Hmd and of its cofactor in the absence and presence of substrate and inhibitors. The results of the infrared experiments are summarized in a line representation, showing the effects of pH, CO, KCN, and substrate. The frequencies are given on the right with a relative intensity in percent based on integrated band areas from Gaussian fits. The lowest frequency band is arbitrarily set at 100%, and shoulders contributing less than 15% are omitted.

in the future be analyzed by X-ray absorption spectroscopy (XAS).

Surprisingly, the cofactor did not coordinate exogenous CO, and like the enzyme, its intrinsic CO did not exchange with the exogenous CO. Incubation of the cofactor under ^{13}CO for 24 h showed no incorporation of the isotopically labeled CO, which is consistent with the finding that CO from the outside did not bind (Figure 9B).

Also cyanide (<2 mM) did not affect the infrared spectrum of the active cofactor (not shown). At high concentrations of cyanide (50 mM) at least one of the bound CO molecules was lost and the cofactor inactivated (Figure 9C). The band at 2066 cm^{-1} can be attributed to free cyanide in buffer. Concomitant with the loss of CO stretches, an absorption band at 1994 cm^{-1} increased in intensity. The identity of this new product is not known.

Like the enzyme, the cofactor is also light sensitive. Inactivation could be followed using IR. Similar to the enzyme, inactivation of the cofactor resulted in a loss of the infrared absorption at 2031 and 1972 cm^{-1}. However, unlike the enzyme, no intermediates were detected (not shown).

Conclusion

Figure 10 summarizes the IR spectra exhibited by the Fe−S-cluster-free hydrogenase Hmd under various conditions. What can be concluded from the spectra with respect to the structure of the active-site iron?

The spectra are most consistent with two CO molecules coordinated to one iron in the active site of Hmd. This is indicated not only by the native enzyme exhibiting two absorption bands at 2011 and 1944 cm^{-1} of nearly identical intensity but also by the positions and intensities of the absorption bands of the enzyme inhibited by ^{12}CO, ^{13}CO, $^{12}CN^-$, and $^{13}CN^-$. Chemical analysis yielded a CO to Fe ratio of 2.4 $\pm$ 0.2, which unfortunately is not conclusive, since it is halfway between 2 and 3. However, in view of the clear IR spectroscopic results we assume that systematic errors in the determination of CO and iron resulted in an overestimation of the ratio. To the best of our knowledge, there is no information in the literature of a tricarbonyliron complex that displays an IR spectrum with two absorption bands of equal intensity.[51]

In the case of the CO-inhibited form of the enzyme, assuming an $Fe(CO)_3$ moiety, the symmetry about the metal can be predicted from the IR data. The comparative experiments with ^{12}CO (Figure 3C) and ^{13}CO (Figure 3D) indicate (i) that C_s symmetry is applicable and (ii) that the ^{13}CO group resides in the unique position rather than in one of the two equivalent positions.

The finding that the iron in Hmd is able to coordinate three CO molecules (two intrinsic and one extrinsic) strongly suggests that the iron is more reduced than Fe(III) since tricarbonyliron(III) complexes are not reported in the literature. This leaves Fe(II), Fe(I), and Fe(0), which are known to form stable CO complexes. The fact that the iron can bind cyanide argues against Fe(0), since iron(0) cyanide compounds such as $[Fe(CO)_4(CN)]^-$ are rare.[52] Hmd was found to be EPR silent (5 K, 9.4 GHz, also measured in parallel mode), which appears to exclude Fe(I). The absence of an EPR signal could reflect a large zero-field splitting parameter. Thus, most probably, the Fe in the active site is an iron(II).

Fe(II) complexes with CO are generally low spin and 6-coordinated.[40] Of the ligands to iron in the active site of Hmd only two have been identified. Whether the binding site for the extrinsic CO or cyanide is open or whether ligand replacement occurs remains unknown. The finding that neither CO nor cyanide coordinates to the iron of the extracted cofactor appears to indicate that the Fe in the cofactor loses its open (or weakly coordinated) coordination site for these extrinsic ligands when it is released from the enzyme. However, the shift of the CO absorption bands upon extraction suggests that the coordination number in the isolated cofactor is actually lower than in the holoenzyme. It can be speculated that upon extraction anionic or strong donor ligands to the Fe center are replaced by neutral,

(51) An iron tricarbonyl complex can show a two-line IR spectrum only, if it has C_{3v} symmetry. The intensity ratio of the resulting bands with A_1 and E symmetry, however, would require very narrow CO−Fe−CO bond angles θ of about 72−86° to match the observed values that range from I_{A_1}/I_E = 1.14 at pH 9.5 to I_{A_1}/I_E = 0.86 at pH 6. This is derived from the relation $I_{A_1}/I_E = [3\cot^2(\theta/2) - 1]/4$ (ref 42). Furthermore, the CO adduct of such a putative tricarbonyl complex would be an $Fe(CO)_4$ species. That species may show three CO stretching bands as observed, but only if again the molecular symmetry is C_{3v}, giving rise to two A_1 bands and one E band. In this case, the intensity ratios of the observed bands (Figures 3 and 10) would require a pyramidal CO coordination pattern with three CO groups in or very close to the equatorial plane, and one in an apical position. We feel that such a species would hardly have sufficient open coordination sites to bind to the cofactor and the protein, as the biochemical experiments indicate. The very high symmetry of both species, native $Fe(CO)_3$ and its CO adduct, is also difficult to imagine in such an environment.

(52) Goldfield, S. A.; Raymond, K. N. *Inorg. Chem.* **1974**, *13*, 770.

weak donor ligands such as water. Another possibility is that a conformational change prevents other molecules, such as CO and cyanide, from binding by obstructing the open coordination site.

Finally, it is interesting that the iron in the active site of Hmd is apparently lacking an intrinsic cyanide, which is present in both the [NiFe]- and [FeFe]- hydrogenases. This may be reflected in the finding that of the three types of hydrogenases only Hmd is inhibited by cyanide.

We are presently pursuing the analysis of Hmd by Mössbauer spectroscopy and X-ray absorption spectroscopy to obtain further insight into the ligands of the active-site iron, which will remain elusive, however, until a crystal structure of the active enzyme at high resolution has been obtained.

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