FTIR Characterization of the Active Site of the Fe-hydrogenase from *Desulfovibrio desulfuricans*

Antonio L. De Lacey,*,† Christian Stadler,† Christine Cavazza,‡ E. Claude Hatchikian,‡ and Victor M. Fernandez†

Instituto de Catalisis, CSIC
Campus Universidad Autonoma
28049 Madrid, Spain
Institute de Biologie Structurale et
Microbienne CNRS 31
chemin Joseph Aiguier 13402
Marseille, Cedex 20, France

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High quality FTIR spectra of the active oxidized state ($\mathbf{H_{ox}}$) and of the carbon monoxide-inhibited state ($\mathbf{H_{ox}}$**-CO)** of *Desulfovibrio desulfuricans* hydrogenase are presented in this work. We demonstrate that the extrinsic CO binds to only one of the active site Fe atoms, and that it is vibrationally coupled to the intrinsic CO ligand at this Fe atom. Comparison of our results with the reported X-ray crystal structures for Fe-hydrogenases and with data obtained from other spectroscopic studies permits us to assign the measured FTIR bands and to discuss aspects of the electronic structure of the active site important for the catalytic mechanism of Fe-hydrogenases.

Hydrogenases are enzymes that catalyze the reversible oxidation of molecular hydrogen in several microorganisms. Nearly all of them are metalloproteins that can be classified in two groups: NiFe-hydrogenases[1] and Fe-hydrogenases.[2] The first X-ray crystal structure of a hydrogenase to be published was that of the NiFe-hydrogenase from *Desulfovibrio gigas*.[3] Recently, the crystallographic structures of two Fe-hydrogenases have also been published.[4] In both structures the active site of the enzyme, named in the literature as the H-cluster, is composed of a [4Fe4S] cluster bridged through a cysteine residue to an unusual 2Fe-unit. There are only small differences between the models proposed for the H-cluster of the *Clostridium pasteuranium* hydrogenase[4a] and the *D. desulfuricans* hydrogenase.[4b] The main difference resides in the unit that connects the two sulfur atoms which bridge the di-iron center. Despite this, it can be supposed that all Fe-hydrogenases have very similar structure in their H-cluster. In fact, the same rhombic "$g = 2.10$" EPR signal, attributed to the H-cluster, has been detected for several Fe-hydrogenases in the active oxidized state ($\mathbf{H_{ox}}$), whereas the active reduced state ($\mathbf{H_{red}}$) is EPR-silent.[2,5]

Fe-hydrogenases are strongly inhibited by carbon monoxide in a competitive manner.[5c,6] Binding of CO to the enzyme causes a change of the EPR signal of the H-cluster.[5c,6b,7] Recently, the X-ray crystal structure of the CO-inhibited form of *Clostridium pasteurianum* hydrogenase I was reported. The exogenous CO was bound to the Fe2 of the H-cluster at the site which had a terminally bound H_2O molecule in the crystallized native state of that enzyme.[8] This is a vacant site in the crystal structure reported for native *D. desulfuricans* hydrogenase and it is proposed to be the place were hydrogen binds to the H-cluster during the catalytic cycle. Figure 1 shows the model for the H-cluster inhibited by CO as characterized by Lemon and Peters.[8]

The presence of CO and CN^- ligands in the H-cluster was detected previous to the publication of the X-ray crystal structures by FTIR spectroscopy of *Desulfovibrio vulgaris* (Hildenborough)-hydrogenase.[9] In the present work we report the FTIR spectra of *D. desulfuricans* hydrogenase obtained in the presence and absence of exogenous CO in a FTIR-spectroelectrochemical cell.[10] Figure 2A shows the spectrum obtained by anaerobic reoxidation of active hydrogenase at a redox potential at which the $\mathbf{H_{ox}}$ state

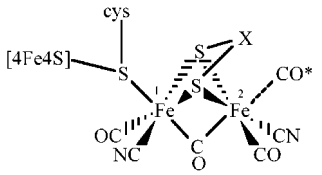

Figure 1. Representation of the H-cluster of the CO-inhibited form of the Fe-hydrogenase of *C. pasteuranium* according to a recent X-ray crystal structure.[8] CO* is the extrinsic carbon monoxide ligand. X represents an unknown bridging moiety.

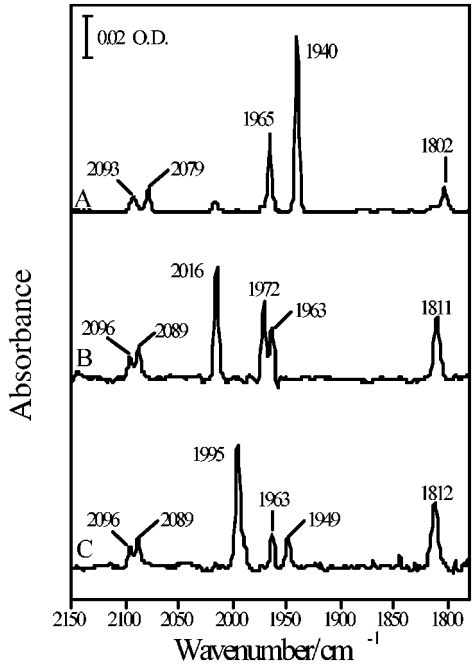

Figure 2. FTIR spectra of 1 mM *D. desulfuricans* hydrogenase in 50 mM Hepes buffer, pH 8.0, 100 mM KCl in the presence of a mixture of redox mediators at −300 mV vs NHE: (A) noninhibited form; (B) CO-inhibited form; (C) ^{13}CO-inhibited form. The temperature was 25 °C.

* To whom correspondence should be adressed. Telephone: 34-91-5854813. FAX: 34-91-5854760. E-mail: alopez@icp.csic.es.
† Instituto de Catalisis.
‡ Laboratoire de Bioenergetique et Ingenierie des Proteins.

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is detected by EPR.[5a,5b] Five bands are detected: the 2093 and 2079 cm^{-1} bands are typical for terminal CN^- stretching vibrational modes, the 1965 and 1940 cm^{-1} bands are in the range of terminal CO stretching modes, and the 1802 cm^{-1} band is typical of a bridging CO stretching modes.[11] Spectrum B was measured at the same redox potential as spectrum A but the sample had been previously saturated with CO at low temperature (in an ice bath). An additional CO-terminal band is detected and a shift to positive frequencies for all bands is observed relative to the noninhibited form. This spectrum is very similar to the one obtained for *D. vulgaris* (Hildenborough) hydrogenase under similar conditions.[9] Pierik and co-workers suggested that the exogenous CO ligand could be vibrationally coupled to one of the intrinsic CO terminal ligands of the *D. vulgaris* (Hildenborough) hydrogenase.[9] To check this possibility we saturated the sample with ^{13}CO and recorded the spectrum at −300 mV vs NHE (spectrum C). The bands corresponding to the two terminal CN^- (2096, 2089 cm^{-1}), one terminal CO (1963 cm^{-1}) and the bridging CO (1812 cm^{-1}) did not shift, whereas the other two bands corresponding to terminal CO ligands did. This suggests that the extrinsic CO ligand is vibrationally coupled to one of the intrinsic terminal ligands of the H-cluster in spectrum B. Otherwise, only the band corresponding to the extrinsic CO would have moved in spectrum C, due to isotopic shift, as observed with NiFe-hydrogenases.[12] The theoretical shift is −44 cm^{-1}, but due to the vibrational coupling we observe −21 and −23 cm^{-1}. Without doubt the 1949 cm^{-1} band corresponds to the extrinsic ^{13}CO, whereas the 1995 band, located exactly intermediate between the 2016 and 1972 cm^{-1} bands of spectrum B, corresponds to the now uncoupled intrinsic CO. In the absence of coupling the extrinsic CO should be observed at about 1993 cm^{-1} which is very close to the frequency of the uncoupled intrinsic CO. Strong coupling of the two vibrations then results in the two bands observed in spectrum B. They are separated by 44 cm^{-1}, a value that compares well to the values found for Fe model complexes with two strongly coupled terminal CO vibrators.[13]

By comparison of our spectra with the model of the CO-inhibited H-cluster shown in Figure 1, we can assign the CO bands of the $\mathbf{H_{ox}}$**-CO** and **H-**^{13}**CO** states as presented in Table 1. Spectrum A corresponds to the hydrogenase in the $\mathbf{H_{ox}}$ state, and according to the Mössbauer experiments reported by Popescu and Münck for *C. pasteuranium* hydrogenase, its Fe atoms are isoelectronic to $\mathbf{H_{ox}}$**-CO**.[14] Therefore, the binding of exogenous CO to Fe2 should cause significant positive shifts of the bands of the CO ligands bound to Fe2 due to a decrease of the π-back-bonding capability of this atom, whereas this effect is expected to be much smaller in the CO bound to Fe1. A similar trend, although of less magnitude, would be expected for the CN^- ligands.[11] Taking this into account, and assuming that the intensity ratio of the two bands of the intrinsic terminal CO ligands is not substantially changed by the binding of the extrinsic CO, the 1940 cm^{-1} band of the $\mathbf{H_{ox}}$ state corresponds to the ν(CO) vibrational mode of the terminal CO of Fe2, and the 1965 cm^{-1} band corresponds to the terminal CO of Fe1. Thus, the latter hardly shifts upon binding of extrinsic CO to Fe2, whereas the former shifts 55 cm^{-1} to higher frequencies. Following the same arguments, the bands due to CN^- ligands can be assigned accordingly (Table 1).

Table 1. Assignment of the FTIR Bands (in cm^{-1}) of Figure 3 to the Diatomic Ligands of the H-cluster Represented in Figure 1[a]

form	$\nu(CO)_b$	$\nu(CO)_{t1}$	$\nu(CO)_{t2}$	$\nu(CN^-)_{t1}$	$\nu(CN^-)_{t2}$
H_{ox}	1802	1965	1940	2093	2079
H_{ox}-CO	1811	1963	2016 (sym.) 1972 (asym.)	2096	2089
H_{ox}-^{13}CO	1812	1963	1995 (CO) 1949 (^{13}CO)	2096	2089

[a] Subscripts denote bridging (b) or terminal (t) binding modes, the number identifying the binding site.

Mössbauer spectroscopy of *C. pasteuranium* hydrogenase indicates that in the $\mathbf{H_{ox}}$ state the spin resides on the Fe atom placed distal to the [4Fe4S] center (Fe2).[14] In that work an $Fe^{II}-Fe^{III}/Fe^{II}-Fe^{II}$ redox transition for the Fe1−Fe2 site from $\mathbf{H_{ox}}$ to $\mathbf{H_{red}}$ during the catalytic cycle of the hydrogenase was proposed.[14] However, the authors did not exclude the possibility of an $Fe^{II}-Fe^{I}/Fe^{I}-Fe^{I}$ transition, as $Fe^{I}-Fe^{I}$ model complexes show similarities to the di-iron site of the H-cluster of Fe-hydrogenases.[15] According to our FTIR assignments, an $Fe^{II}-Fe^{I}$ state for the Fe1−Fe2 site of $\mathbf{H_{ox}}$ is more probable than $Fe^{II}-Fe^{III}$, as the bands corresponding to the terminal ligands of Fe2 are at lower frequencies than those of Fe1. If Fe2 had a higher oxidation state than Fe1, it would donate less π-electron density to its terminal ligands. Therefore, it would be expected that the bands due to the Fe2 ligands were at higher frequencies than those of Fe1, which is the contrary of what we have deduced from our FTIR experiments. In fact, it is more probable that Fe^{I} ligates an additional CO molecule than Fe^{III}, as CO is a strong π-electron-accepting ligand.[11]

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Supporting Information Available: Detailed description of the experimental procedure for the FTIR measurements (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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