Infrared-Spectroelectrochemical Characterization of the [NiFe] Hydrogenase of *Desulfovibrio gigas*[⊥]

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Abstract: Electrochemically controlled titrations of the different redox states detected by IR spectroscopy of *Desulfovibrio gigas* hydrogenase are reported. This enzyme has in common with other metal-containing hydrogenases the existence of three intense bands at exceptionally high frequencies that in *Chromatium vinosum* hydrogenase have been found to arise from two CN^- and one CO (Happe, R. P.; Roseboom, W.; Pierik, A. J.; Albracht, S. P. J.; Bagley, K. A. *Nature* **1997**, *385*, 126). Here we propose a tentative assignment of these special groups to the three diatomic active site Fe ligands observed in the crystal structure of *Desulfovibrio gigas* hydrogenase (Volbeda, A.; Garcin, E.; Piras, C.; de Lacey, A. L.; Fernandez, V. M.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *J. Am. Chem. Soc.* **1996**, *118*, 12989−12996), based on their interaction with the protein environment. IR states equivalent to each one of the Ni-EPR detectable states and their corresponding one-electron reduced EPR-silent forms have been identified by *in situ* redox titration. The similarity between the IR titration and the reported stoichiometric reductive titrations of EPR nickel signals of *D. gigas* enzyme (Roberts, L. M.; Lindahl, P. A. *J. Am. Chem. Soc.* **1995**, *117*, 2565−2572) suggest that there is a strong electronic interaction between the two metal centers in the active site. The IR-spectroelectrochemical technique used here allows for further insight into the activation, inactivation, and catalytic cycles of the enzyme. The path of activation and the identity of the active states have been characterized.

Introduction

Hydrogenases are enzymes that catalyze the reversible activation of H_2. The [NiFe] hydrogenase of *Desulfovibrio gigas* is a heterodimeric periplasmic protein consisting of 28 and 60 kD subunits. It contains 12 atoms of iron, 12 acid-labile sulfides, and 1 atom of nickel per molecule.[1−3] Eleven atoms of iron and the 12 sulfides are arranged as two [4Fe-4S]$^{2+/1+}$ and one [3Fe-4S]$^{1+/0}$ clusters.[4] The X-ray structure of the as-purified enzyme at 2.85 Å resolution has been published recently.[5] The structure shows that the three Fe−S clusters are distributed almost along a straight line in the small subunit, with the [3Fe-4S] cluster halfway between the two [4Fe-4S] clusters. This spatial arrangement of the three Fe-S clusters in *D. gigas* hydrogenase could provide an electron channel from the active site to the molecular surface.[5] The crystallographic analysis of the hydrogenase has also shown that the active site is buried in the large subunit and contains two metal ions, one of which is Ni. Later crystallographic data confirmed unambiguously that the second metal in the active site is an iron atom.[6] Moreover, a new crystal form solved at 2.5 Å resolution shows that the active site Fe binds three non-protein diatomic ligands and a putative oxygen species that also binds to the Ni center.[6]

The catalytic behavior of *D. gigas* hydrogenase can be explained in terms of interconversion between different forms of the enzyme: the *active* state, which is active in all assays, the *unready* state, which is totally inactive and requires long incubation under H_2 (or a reductant) for activation, and the *ready* state, which does not catalyze the activation of H_2, but which is rapidly converted to the active state by reductants.[7] *D. gigas* hydrogenase preparations isolated under aerobic conditions are characterized by various ratios of two Ni-EPR signals called Ni-**A** and Ni-**B**. Usually, a significant fraction of EPR-silent material is present. The proportion of *unready* state correlates with the Ni-**A** signal, whereas the proportion of *ready* state correlates with the Ni-**B** signal.[8] A third signal, named Ni-**C**, characterizes an activated state of the enzyme.[8−10] All three

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[⊥] Abbreviations: IR, infrared; XAS, X-ray absorption spectroscopy; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

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EPR signals have been assigned to Ni by isotopic labeling using ^{61}Ni.[10] Other redox states of the hydrogenase are EPR-silent, such as the fully reduced active state (form **R**),[10] and an intermediate state, named **SI**, obtained by oxidation of active enzyme[11] or by partial reduction of the form **B**.[12] Reduction of the enzyme in the **A** form gives an intermediate state that is EPR-silent.[2,13] These states have now been found in many nickel hydrogenases (see ref 14 for a review).

Fourier transform infrared studies (FTIR) of [NiFe] hydrogenase from *Chromatium vinosum* that reveal the presence of three absorption bands in the 2100−1900 cm^{-1} spectral region have been published recently.[15,16] The frequency shifts of these bands correlate with changes in the redox state of the enzyme generated by different pressures of hydrogen or by slow exposure of reduced samples to atmospheric oxygen.[16] Bagley *et al*.[16] concluded that these bands were due to intrinsic, non-protein groups situated very close to the nickel center. Following the same experimental procedure as Bagley *et al*.,[16] sets of three IR bands of similar frequencies were observed for the different redox states of *D. gigas* hydrogenase.[6] Very recently Happe *et al*.[17] have identified the groups responsible for the IR bands in *C. vinosum* hydrogenase, both chemically and by isotopic labeling of the enzyme, as two CN^- and one CO. These groups are most likely the three diatomic ligands of the active site Fe observed in the crystallographic analysis of the *D. gigas* enzyme.[6,17]

The detailed study of the structure of the active center of nickel hydrogenases and its catalytic function has great interest for the synthesis of biomimetic model compounds that catalyze the activation of hydrogen. Several catalytic mechanisms have been proposed for [NiFe] hydrogenases.[9,11,18−20] These proposals have been based mainly on EPR measurements with important contributions of other techniques such as Mössbauer, XAS, ENDOR, and visible spectroscopy. Thanks to the discovery of high frequency infrared-active groups in the active center of [NiFe] hydrogenases, the active center and its catalytic activity can now be studied from a different perspective. The comparison of FTIR results with those obtained by EPR and other techniques should be instrumental for the understanding of hydrogen biocatalysis.

In this paper we report the results obtained with *Desulfovibrio gigas* hydrogenase using an IR-spectroelectrochemical cell. This technique has several advantages by comparison to EPR redox titrations: (1) Besides Ni-EPR detectable states, Ni-EPR silent redox states of the hydrogenase active site are detected. (2) The redox potential of the sample is controlled *in situ*. (3) Redox titrations are performed at the same temperature as the IR spectroscopic measurements, whereas EPR spectra of Ni are recorded at low temperatures.[2,3] We have carried out redox titrations of the different IR states of the hydrogenase as well as kinetic studies of the activation and inactivation processes. Additionally, correlations between the different IR states and the catalytic properties of the enzyme have been established.

Experimental Section

Desulfovibrio gigas hydrogenase was purified as described.[21] The activity of the preparation used in most of the experiments reported in the present work was 500 μmol × min^{-1} × mg^{-1} (H_2-uptake assay, 1 mM methyl viologen, pH 8.5, 25 °C). The EPR signals of the [3Fe-4S] cluster and Ni-**A** accounted for 0.87 and 0.68 spin/molecule, respectively (no Ni-**B** was detected). Another preparation, which was used in the experiments run at pH 5.7, had an activity of 480 mmol × min^{-1} × mg^{-1}, 0.94 spin/molecule of [3Fe-4S] signal, and 0.55 spin/molecule of Ni-**A**/**B** of which 6% corresponded to Ni-**B**. Samples of the hydrogenase at different pH values were prepared as follows: aliquots of the stock sample were diluted × 100 with the chosen buffer followed by concentration on Centricon-30 (Amicon); the dilution and concentration steps were repeated twice. The pH of the buffers were corrected taking into account of their temperature dependence.

Activity Measurements. H_2-uptake and exchange activities were measured as described.[21] For the D_2/H^+ exchange assay, 1 mM glucose in 20 mM Tris buffer, pH 7.8, 7 μg/mL glucose oxidase, 7 μg/mL catalase, and 0.2 mM ethanol were used to scavenge oxygen. When appropriate, sodium dithionite was added to the reaction mixture through a septum with a microsyringe.

Infrared-Spectroelectrochemical Measurements. The infrared spectra were recorded in a Nicolet 5ZDX Fourier-transform spectrometer, equipped with a MCT detector and a purge gas system for removal of CO_2 and H_2O (Peak Scientific). The IR-spectroelectrochemical cell used was as described by Moss et al.[22] Redox equilibrium in the cell was reached 2−3 min after a potential was applied (checked by monitoring *in situ* the visible spectra of reduced methyl viologen). The cell pathlength was measured by visible absorption spectroscopy of 8 mM cytochrome *c*. An average value of 7.5 μm was measured from six different experiments. Enzyme solution (1.0−1.7 mM, 10 μL) was used for each FTIR experiment in the presence of 100 mM buffer (Tris, phosphate or MES), 100 mM KCl, and a mixture of redox mediators, 0.5 mM each. The redox mediators used were as follows: methylene blue (E'_o = +11 mV, Merck), indigo-tetrasulfonate (E'_o = −46 mV, Merck), 2-hydroxy-1,4-naphthoquinone (E'_o = −139 mV, ICN Pharmaceuticals), anthraquinone-1,5-disulfonic acid (E'_o = −170 mV, ICN Pharmaceuticals), anthraquinone-2-sulfonate (E'_o = −225 mV, Serva), phenosafranin (E'_o = −252 mV, Merck), neutral red (E'_o = −329 V, Serva), benzyl viologen (E'_o = −358 V, Sigma), and methyl viologen (E'_o = −446 mV, Aldrich). The redox potential was controlled with a BAS CV-27 potentiostat and measured with a Fluke 77 multimeter. All redox potentials are given against the standard hydrogen electrode. The temperature of the cell was controlled by a HETO C7 CV10-thermostat. The IR spectra were averaged from 1024 scans (unless otherwise stated), and the spectral resolution was 2 cm^{-1}. All spectra were blank-substracted and baseline-corrected using a parabolically shaped curve. The areas of overlapping bands were calculated by Fourier deconvolution with the program OMNIC from Nicolet.

Results

Redox Titration of Unready States. By controlling the redox potential of the IR-spectroelectrochemical cell, two different spectra of as isolated *D. gigas* hydrogenase were obtained at 15 °C in the presence of redox mediators, each spectrum consisting of three bands. At +20 mV the infrared spectrum showed bands at 1947, 2083, and 2093 cm^{-1}, whereas at −300 mV bands at 1950, 2089, and 2099 cm^{-1} were observed

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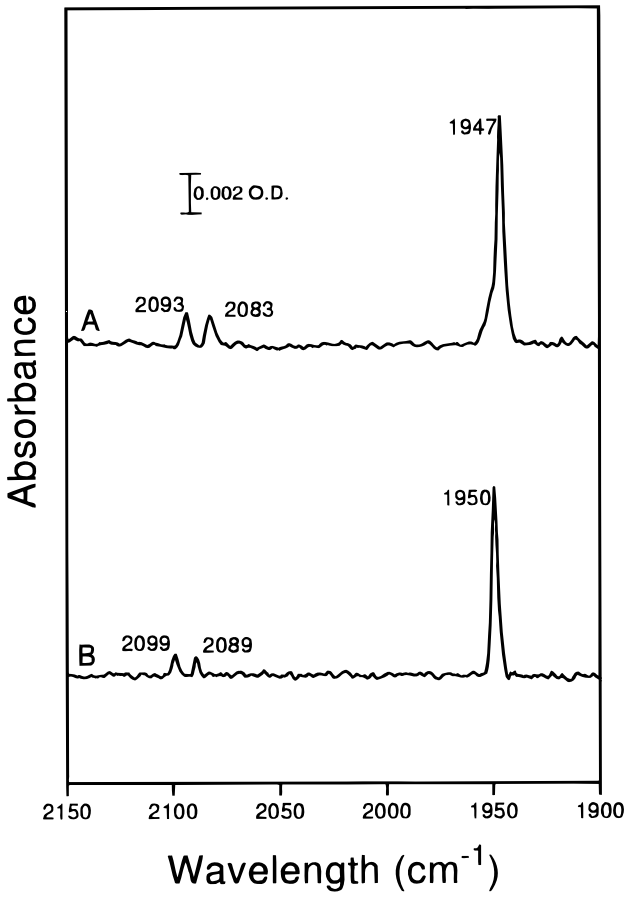

Figure 1. Absolute infrared spectra of 1.7 mM *Desulfovibrio gigas* hydrogenase in 100 mM Tris buffer, pH 8.3, 100 mM KCl, at 15 °C in the presence of redox mediators with an applied potential of (A) +20 mV and (B) −300 mV.

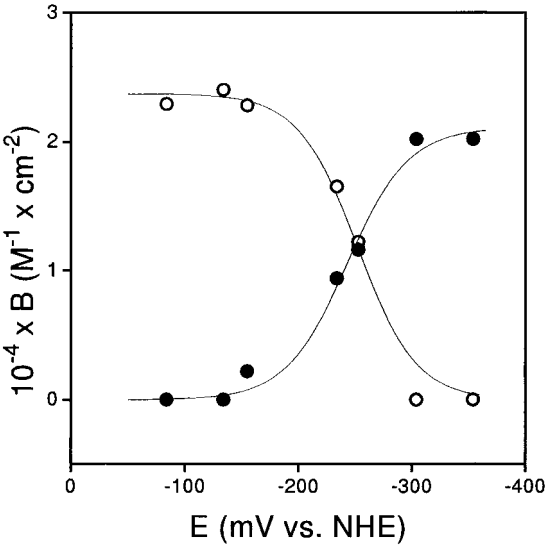

Figure 2. Potentiometric titration of the **A/SU** couple: (filled circles) apparent integrated absorption intensity of the 1950 cm^{-1} band; (open circles) apparent integrated absorption intensity of the 1947 cm^{-1} band. Solid lines are the best-fit curves for one-electron Nernstian processes. The same results were obtained for oxidative and reductive titrations. Same conditions as in Figure 1.

(Figure 1). It is known that activation of the hydrogenase proceeds very slowly at this temperature.[7] Therefore, these two spectra can be attributed to an *unready* oxidized state (form **A**) and to an *unready* reduced state (form **SU**). The conversion of one redox state to the other can be titrated with complete

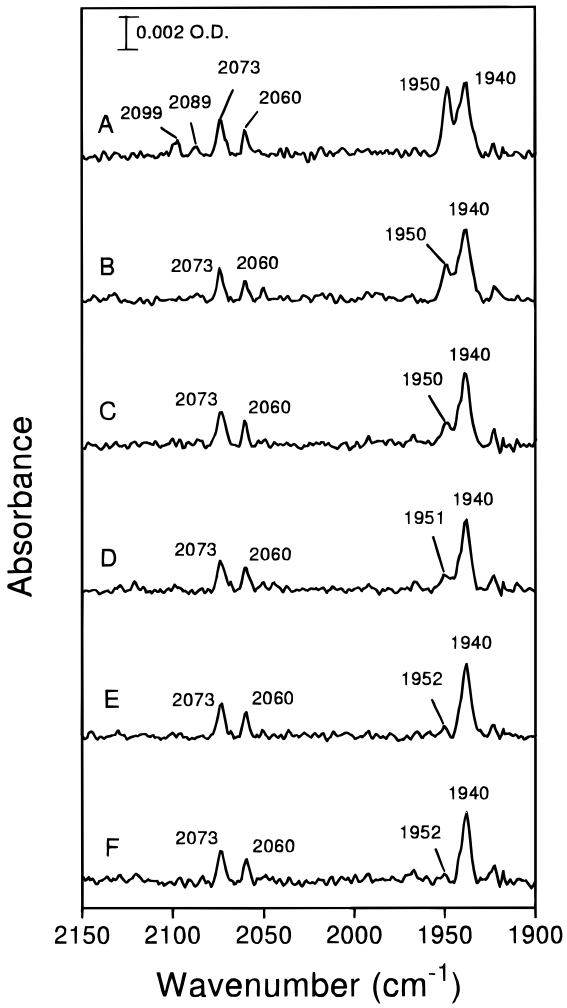

Figure 3. Absolute infrared spectra of 1.3 mM *Desulfovibrio gigas* hydrogenase in 100 mM Tris buffer, pH 7.7, 100 mM KCl, at 40 °C in the presence of redox mediators with an applied potential of −500 mV at different times: (A) 15 min, (B) 30 min, (C) 45 min, (D) 60 min, (E) 77 min, and (F) 105 min. Spectra were averaged from 124 scans.

reversibility (Figure 2). The experimental data of the plot of the integrated intensity of the main band of each of the states (at 1947 and 1950 cm^{-1}, respectively) *versus* the redox potential can be fitted to the theoretical Nernstian equation for a one-electron redox process. A midpoint potential (E_m') of −245 ± 5 mV was obtained for this redox process at pH 8.3 by calculating the mean value of the two curves. Redox titrations made at different pH's showed that E_m' decreased from pH 5.5 to 8.5 (not shown). E_m' changed −55 mV/pH unit, which is in agreement with a redox process in which one proton is involved.

Activation. In order to investigate the possibility of activating the hydrogenase in the IR-spectroelectrochemical cell, a redox potential of −500 mV was applied in the presence of redox mediators as indicated above at 40 °C. The IR spectra measured at different times are shown in Figure 3. After 77 min the spectra did not change further at this temperature. The final spectrum obtained was identical to the spectrum of hydrogenase of *D. gigas* which had been under H_2 for 2 h at 40 °C.[6] We conclude then that the hydrogenase can be activated inside the IR-spectroelectrochemical cell. As shown in Figure 4, the plot of the logarithm of the integrated intensity of the band at 1950 cm^{-1} band *vs* the activation time is linear. Thus, activation of the hydrogenase can be explained as a first-order reaction of the **SU** state. From the slope of this plot a first-order rate constant and the half activation time can be calculated (Table 1). The influence of pH and redox potential on the

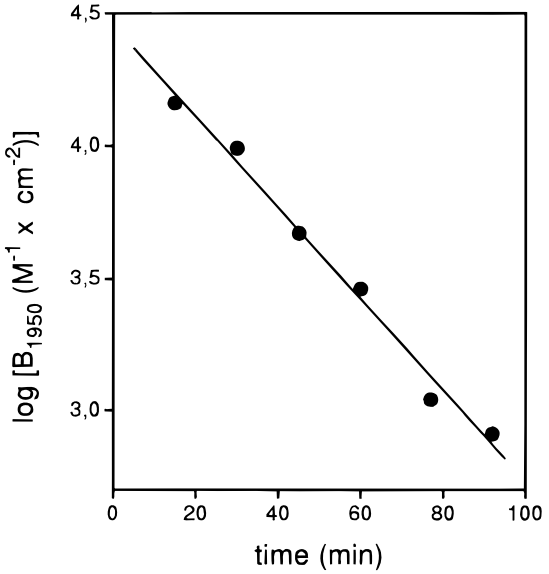

Figure 4. Semilogarithmic plot of activation kinetics of *Desulfovibrio gigas* hydrogenase. Experimental data are taken from Figure 3.

Table 1. Kinetic Constants of Activation

E (mV)	pH	k^a (h^{-1})	$t_{1/2}$(min)
−500	7.7	2.3	18
−400	7.7	1.1	38
−325	7.7	1.3	32
−400	8.6	1.2	35
−500	8.1	1.9	22
−450	7.1	0.9	46
−350	5.5	1.9	22

[a] Measured at 40 °C.

kinetics of activation was studied. In all cases the experimental data could be fitted to a first-order process. The calculated constants were of similar magnitude and no trend could be observed when the redox potential was more negative than −325 mV (Table 1). An average value of 1.5 h^{-1} for the rate constant was obtained. Although the rate constant was nearly independent of redox potential at values lower than −325 mV, the final spectrum after the activation process varied with the applied redox potential (Figure 5). An interesting result was that at −210 mV a significant proportion of the enzyme remained in the unready states (**A** and **SU**) after 6 h. At 0 mV no activation took place (not shown).

Redox Titration of Activated Enzyme. After the activation process four different redox states could be detected by IR at selected redox potentials (Figure 6). These spectra were exactly the same as those obtained in a normal IR cell by activation of *D. gigas* hydrogenase under H_2 and slow reoxidation with air.[6] The spectra were attributed to forms **B**, **SI**, **C**, and **R**. Each redox state has three IR bands, one intense band and two less intense bands at higher frequencies. An exception is the so-called **SI** form, where two bands at lower frequencies and four bands at higher frequencies were observed, that is, two sets of three bands that titrated simultaneously. The redox titration of the main band of each state at pH 7.7 is shown in Figure 7.[23] The titrations were completely reversible at 40 °C. The experimental data could be fitted to one-electron Nernstian curves and midpoint potentials could be calculated for each of the redox processes (Table 2). At redox potentials lower than −500 mV the experimental data deviated from the theoretical

(23) Although all three bands of each redox state titrate simultaneously, only the one with lowest frequency is taken into acount as it is much more intense than the other two and because some states have in common the frequency of one of the less intense bands.

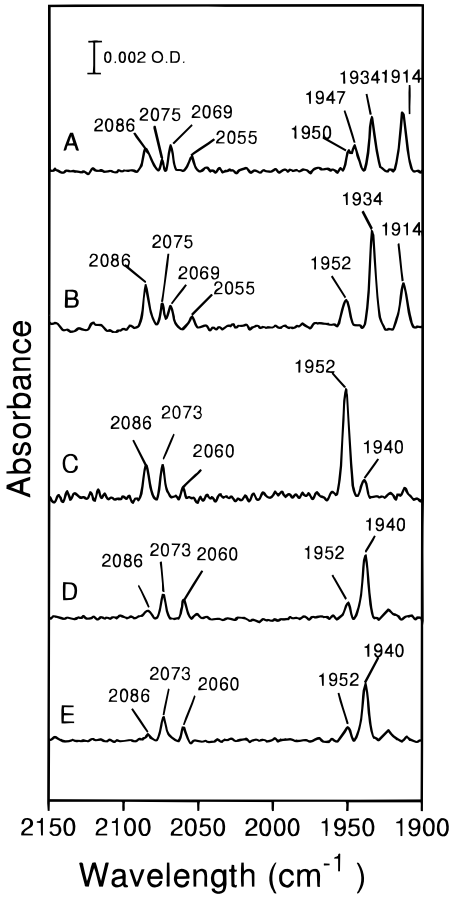

Figure 5. Spectra of 1.7 mM *Desulfovibrio gigas* hydrogenase in 100 mM Tris buffer, pH 7.7, 100 mM KCl activated at 40 °C in the presence of redox mediators with different applied redox potentials: (A) 6 h at −210 mV, (B) 2 h at −300 mV, (C) 2 h at −400 mV, (D) 2 h at −500 mV, and (E) 2 h at −600 mV.

Table 2. Midpoint Potentials

redox equilibrium	E_m,[a] (mV)
A/SU	−210 ± 5
B/SI	−135 ± 5
SI/C	−365 ± 5
C/R	−430 ± 5

[a] Measured at pH 7.7 and 40 °C.

curves, probably due to equilibrium of forms **C** and **R** with H_2 produced by the catalytic activity of the hydrogenase.[12] The midpoint potential increased with the pH for the three redox processes in the range 5.5−8.0, but the variation is smaller than the theoretical value of −62 mV/pH unit for a one proton-redox process at 40 °C: −36, −47, and −43 mV/pH unit were measured for the **B/SI**, **SI/C**, and **C/R** couples, respectively.

Two SI Species. Redox titrations of enzyme activated *in situ* at different pH values showed that the ratio of absorption intensities between the two sets of bands of the **SI** spectra was pH-dependent (Figure 8). Because of this, we were able to establish that the bands at 2075 and 2085 cm^{-1} were related to the 1934 cm^{-1} band (form $\mathbf{SI_{1934}}$), whereas the bands at 2055 and 2069 cm^{-1} were related to the 1914 cm^{-1} band (form $\mathbf{SI_{1914}}$). At pH lower than 7 only $\mathbf{SI_{1934}}$ is detected, whereas at pH higher than 8, $\mathbf{SI_{1914}}$ starts to predominate.[24] This result suggests that there is an acid−base equilibrium between the two **SI** species with $\mathbf{SI_{1934}}$ being the protonated species and $\mathbf{SI_{1914}}$ the unprotonated one. From the *y*-axis intercept of the

(24) A spectrum with only Ni−SI_{1914} could not be obtained because the enzyme was not stable at pH 10.5.

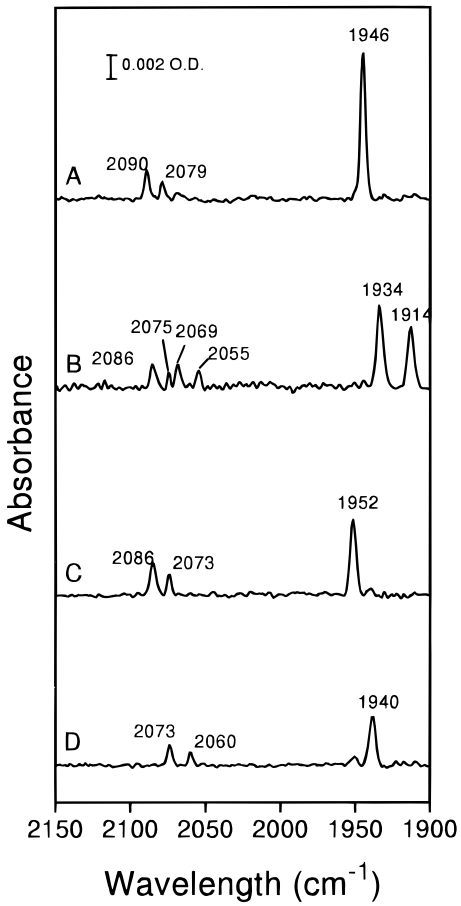

Figure 6. Spectra of 1.7 mM *Desulfovibrio gigas* hydrogenase in 100 mM Tris buffer, pH 7.7, 100 mM KCl at 40 °C and at different redox potentials after being activated as in Figure 5C: (A) −50 mV, (B) −225 mV, (C) −395 mV, and (D) −475 mV.

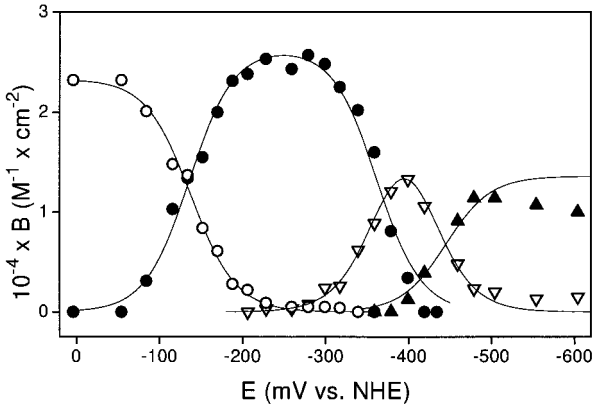

Figure 7. Potentiometric titration of the redox states of activated enzyme: (open circles) apparent integrated absorption intensity of IR bands at 1946 cm^{-1}; (filled circles) 1914 + 1934 cm^{-1} bands (it is assumed that both bands have the same extinction coefficient); (open triangles) 1952 cm^{-1}; (filled triangles) 1940 cm^{-1} band. Solid lines are the best-fit curves for one-electron Nernstian processes. Same conditions as in Figure 6.

plot of log($\mathbf{B_{1914}/B_{1934}}$) versus pH, an apparent pK_a around 8 can be estimated for the acid−base equilibrium (not shown).

Inactivation Kinetics. When redox titrations were attempted at 25 °C all redox conversions were reversible with the exception of the step between **SI** and **B** (not shown). Reduction of form **B** to **SI** state was a quick reaction, but oxidation of **SI** state to **B** was very slow. Therefore redox equilibrium was not reached when tuning the potential in the oxidative direction. In order to ascertain if there was some kinetics barrier for the oxidation

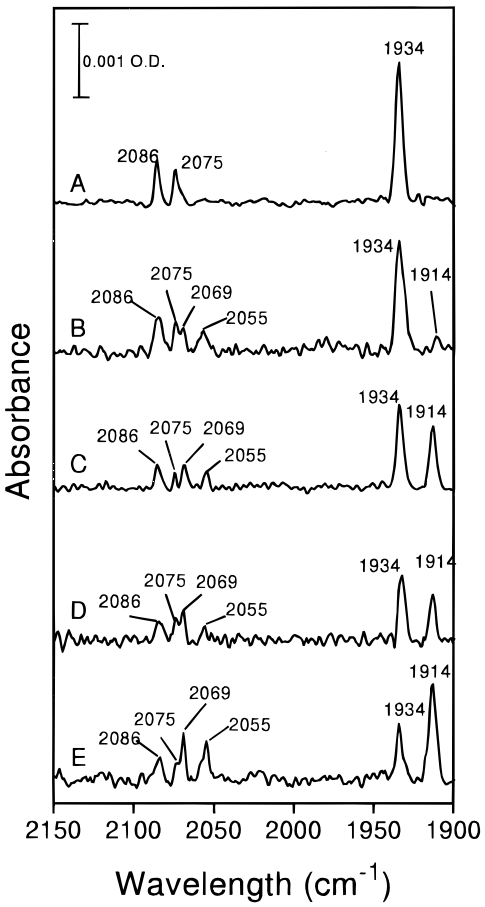

Figure 8. Spectra of 1 mM *Desulfovibrio gigas* hydrogenase in the SI state at 40 °C and at different pHs: (A) 100 mM MES buffer, pH 5.7, (B) 100 mM phosphate buffer, pH 7.1, (C) 100 mM Tris buffer, pH 7.7, (D) 100 mM Tris buffer, pH 8.1, and (E) 100 mM Tris buffer, pH 8.6.

of **SI**, the rate of disappearance of the IR bands associated to form **SI** was measured at different temperatures when a potential of 0 mV was applied. The data could be fitted to first-order kinetics and the rate constants calculated (Figure 9). This constant was highly temperature-dependent. An activation energy of 79 kJ/mol for the process was calculated from the Arrhenius plot (inset, Figure 9). The same experiment was done at pH 5.7, in which only $\mathbf{SI_{1934}}$ is detected (not shown). The results were very similar to the ones obtained at pH 8.1 with an activation energy of 88 kJ/mol.

Correlation between Redox State and D_2/H^+ Activity. Two samples of 0.1 mM hydrogenase in 100 mM Tris buffer, pH 8.1, 100 mM KCl, were incubated under H_2 for 3 h at 40 °C. IR spectra in a normal transmission IR cell and the D_2/H^+ exchange activity were measured after activation. Next, one of the samples was put in a 0 °C bath and the other one left at 40 °C. Both samples were bubbled with Ar in order to eliminate H_2 and oxidized with 1 mM 2,6-dichlorophenol-indophenol (DCIP) for 15 min under anaerobic conditions at 0 and 40 °C, respectively. Subsequently the IR spectra and the D_2/H^+ exchange activity were measured for each sample. Table 3 contains the specific activities of each of the samples and the main bands observed in the IR spectra as well as the corresponding redox states. The enzyme incubated under H_2 had full exchange activity and there was no increase of activity, in the presence of sodium dithionite. The IR spectra corresponded to forms **R** and **C**. When oxidized with DCIP at 40 °C the IR spectra corresponded to form **B**. The exchange activity was almost completely lost, and therefore enzyme in this state is

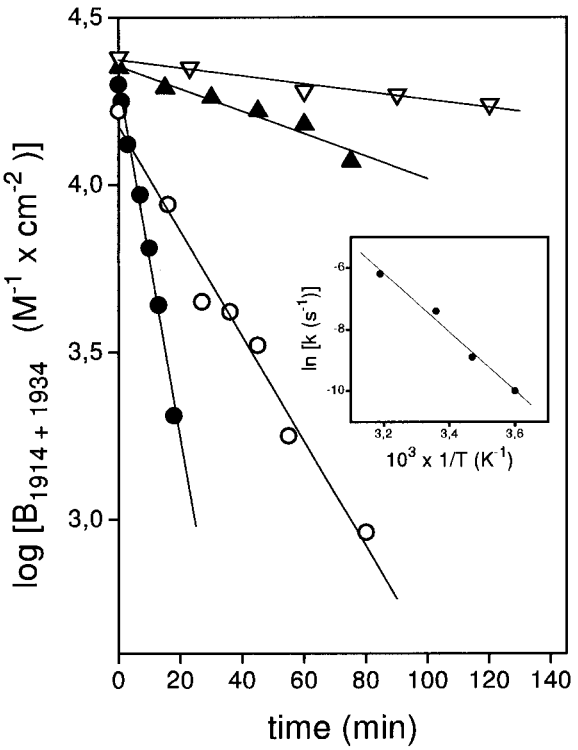

Figure 9. Linear plots of oxidation kinetics of the **SI** state of 1.3 mM *Desulfovibrio gigas* hydrogenase in Tris buffer, pH 8.1, 100 mM KCl, at different temperatures: (open triangles) 5 °C; (filled triangles) 15 °C; (open circles) 25 °C; (filled circles) 40 °C. Applied potential was 0 mV. Inset, Arrhenius plot of the temperature dependence of the first-order constant.

Table 3. Correlation between Hydrogenase D_2/H^+ Exchange Activity (μmol HD min^{-1} mg^{-1}) and IR Spectra

sample[d]	activity[a]	activity[b]	IR bands[c] (cm^{-1})	redox states
AH	107	107	1940; 1952	**R**; **C**
AH + DCIP, 40 °C	3	103	1946	**B**
AH + DCIP, 0 °C	54	54	1914, 1934; 1947	**SI**; **A**

[a] Exchange activity measured before addition of sodium dithionite. [b] Exchange activity measured in the presence of 1 mM sodium dithionite. [c] Only the lowest frequency band of each IR state is given. [d] All samples were 0.1 mM solutions of *D. gigas* hydrogenase, pH 8.1, incubated under H_2 for 3 h at 40 °C (AH, first row) and subsequently bubbled with Ar and oxidized with 1 mM DCIP at 40 °C (second row) or at 0 °C (third row).

unable to catalyze the heterolytic cleavage of H_2. Activity was quickly recovered upon addition of sodium dithionite to the reaction vessel. When the addition of DCIP was done at 0 °C, 50% of the activity remained, and no increase of activity was measured in the presence of sodium dithionite. The IR spectra corresponded to a mixture of $\mathbf{SI_{1914}}$, $\mathbf{SI_{1934}}$, and **A** forms. This indicates that the enzyme in at least one of the two **SI** forms catalyzes the D_2/H^+ exchange. However, since the sample obtained at 0 °C could not regain full activity after exposure to dithionite, it is likely that some oxygen entered the sample during manipulation and partially oxidized it to **A** (see the Scheme 1). No oxidation to form **B** took place as this process is very slow at low temperatures. Accordingly, no increase of the activity was detected in the presence of sodium dithionite. A similar experiment was run at pH 5.7. At this pH, the sample anaerobically oxidized with DCIP at 0 °C displayed a mixture of the IR bands corresponding to forms $\mathbf{SI_{1934}}$ and **A**, with no increase in exchange activity upon dithionite addition. Oxidation at 40 °C produced form **B**, and now the exchange activity greatly increased in the presence of dithionite (not shown). This indicates that the hydrogenase in form $\mathbf{SI_{1934}}$ state is catalytically active and can be oxidized to form **B** or **A**.

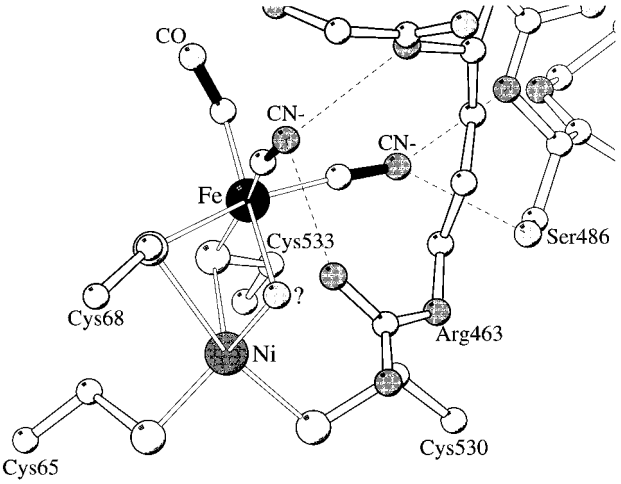

Figure 10. Coordination of the active site metals in the crystal structure of as-purified *Desulfovibrio gigas* hydrogenase. The nature of the protein environment of the putative CO group (labeled L3 in ref 6) is entirely hydrophobic (not shown). The much more hydrophilic CN^- groups (previously labeled L1 and L2) are tentatively assigned to two diatomic ligands that may be involved in hydrogen bonding interactions with the protein, as indicated by the dashed lines. The question mark labels a metal-bridging coordination site that in the "as purified" *unready* state of the enzyme is occupied by an oxygen species. This figure was adapted from Figure 4 in ref 6 and made with the program MOLSCRIPT (ref 41).

Discussion

Structural Assignment of the IR-Active Groups. IR bands in the 2100−1900 cm^{-1} spectral region have been observed in many metal-containing-H_2-activating hydrogenases but not in metal-free hydrogenases or in other proteins with Fe−S clusters or Ni atoms.[25] In previous works it has been argued that the three observed IR bands are due to the three diatomic ligands of the active site Fe detected in the X-ray structure of *Desulfovibrio gigas* hydrogenase.[6,16,17] Such non-protein groups are probably common to the active site of all [NiFe] hydrogenases and [Fe] hydrogenases.[25] Very recently, Happe *et al.*[17] have identified the three ligands as two CN^- and one CO using ^{15}N and ^{13}C substituted *C. vinosum* hydrogenase in FTIR experiments. Taking into account the nearly identical IR features of *C. vinosum*[16] and *D. gigas*[6] enzymes and using the X-ray structure of the latter,[6] the more hydrophobic CO ligand may be assigned to what we previously called ligand L3, as it is entirely surrounded by hydrophobic residues. The more hydrophillic CN^- ligands may be assigned to ligands L1 and L2 (Figure 4 in ref 6), since these are in an environment that allows hydrogen bonding interactions (Figure 10).

The IR spectrum of the enzyme is rather sensitive to changes in the redox state of the active site (see Figures 1, 3, 5, and 6). If the assumption of the diatomic ligands of the active site Fe as the IR-active groups is correct, the shifts of the IR bands are likely due to a change in the electron density of the active site Fe center, either by a change of its oxidation state or by changes in its coordination sphere. An increase of the electron density on the Fe should cause an increase of the π-donating effect to the $2p\pi^*$ orbital of a CO ligand or a decrease of the σ-donation from the 5σ orbital of a CN ligand and therefore a shift of ν(CO) or ν(CN) to lower frequencies.[26] A decrease of the electron

(25) Van der Spek, T. M.; Arendsen, A. F.; Happe, R. P.; Yun, S.; Bagley, K. A.; Stufkens, D. J.; Hagen, W. R.; Albracht, S. P. J. *Eur. J. Biochem.* **1996**, *237*, 629−634.

density on the Fe would cause the opposite effect. Thus, the magnitude and direction of the shifts observed in the IR spectra of *D. gigas* hydrogenase at different redox states reflect the changes that are taking place at the active site Fe center during the processes of activation, catalytic cycle, and inactivation of the enzyme. However, other factors like hydrogen bond formation with protein residues (Figure 10) could induce shifts of the IR band frequencies as has been clearly demonstrated to occur in coordination compounds of well defined structure.[27]

Activation. It has been generally thought that the reduction of either form **A** or form **B** generates the same EPR-silent state.[14] However, in this work we have shown that the one electron reduction of forms **A** and **B** gives rise to different states as observed by IR spectroscopy (compare spectrum A of Figures 1 and 6, respectively). We have maintained the **SI** designation for one-electron reduced form of **B**, whereas we have named the one-electron reduced form of **A** as **SU**. There is considerable difference between the **SI** and **SU** forms. Not only do they have different IR band frequencies and midpoint potentials but they also differ in their functionality: **SI** can catalyze D_2/H^+ exchange (Table 3), whereas **SU** cannot. Therefore, we can consider **SU** as an *unready* reduced state[8] which can be oxidized reversibly to form **A** at low temperatures, 5−15 °C (Figure 2). The **A/SU** midpoint redox potential of −245 mV measured in this work (Table 2) is similar to the values obtained when measuring the disappearance of the Ni-**A** EPR signal.[2,13] In addition, we have observed by IR spectroscopy that one proton and one electron are involved in the reduction of the form **A** (not shown), in accordance with earlier EPR results.[2]

The frequency shifts of the IR bands in going from **A** to **SU** are small when compared to those observed for other redox processes of the enzyme with the bands moving to slightly higher frequencies. This shift is in the opposite direction to what it should be expected for an increase in the electron density at the active site Fe center[26] and suggests that the electron goes to another redox center at the active site. Interestingly enough, the only significant shift of the Ni−K absorption edge spectra of *D. gigas* hydrogenase (−1.3 eV) is the one observed when comparing the **A** and **SI** forms (the **SU**, **B**, and **C** forms of *D. gigas hydrogenase* have not been well characterized by XAS as yet).[28,29] Consequently from the IR, EPR, and XAS data we can conclude that in the reduction of form **A** to form **SU** the Fe is not involved and that the electron goes to the Ni center, although it cannot be excluded the reduction of a sulfur ligands at this step.[20]

As the enzyme in the **SU** form has no catalytic activity and the midpoint redox potential of the **A/SU** equilibrium (−210 mV at pH 7.7 and 40 °C, Table 2) is much more positive than the corresponding potential of the H^+/H_2 couple (−478 mV), what is the nature of this state? From the results presented in Figures 3−5 it is clear that reduction of **A** to **SU** is an obligatory step in the activation process. This is in agreement with the results previously obtained[8] when studying the activation process of *D. gigas* hydrogenase where it was observed that the Ni-**A** EPR signal disappeared upon reduction with H_2 or other reductants before activation took place. Lissolo *et al*.[30] reported that hydrogenase activation under H_2 followed first-order kinetics and that the rate constants were independent of the partial pressure of hydrogen during activation. The results presented here suggest that the rate-determining step of activation is a slow process in which the **SU** form is converted into an active state. This step has first-order kinetics relative to **SU** concentration (Figure 4). The rate constant of the reaction is not significantly dependent on the pH or the applied potential once form **A** has been completely reduced to form **SU** (Table 1).

It has been reported that the rate of activation was strongly temperature-dependent and that no mechanisms of intermolecular electron exchange were involved.[7] The rate constants for the activation process[7] and the disappearance of **SU** (Table 1) are very similar (1.3 and 1.5 h^{-1} at 40 °C, respectively). The activation experiments reported here at different redox potentials show that complete activation (measured by D_2/H^+ exchange) of hydrogenase does not require reduction to form **C** or **R**, since complete conversion of **SU** to **SI** took place at −300 mV (Figure 5, spectrum B). We suggest that the **SU/SI** transition involves a slow change in the coordination in the active site metal coordination. In the X-ray structure of as-prepared *D. gigas* hydrogenase a putative oxygen has been detected bridging the Ni and the Fe centers.[6] Thus it is possible that the slow change in the coordination of the active site metals centers during activation consists in the loss of this oxygen species (see also Figure 10) after a one-electron reduction of the Ni center in form **A** to form **SU**.

Two SI Species. An interesting result of this work is the discovery of two isoelectronic species that correspond to the **SI** state. The IR spectra of **SI** at different pH values show that the two species are in an acid−base equilibrium with a pK_a about 8. This value is within the pK_a range of cysteine thiol groups[31] suggesting that one of the cysteine residues of the active site could become protonated in the form $\mathbf{SI_{1934}}$. The observed 20 cm^{-1} increases of the IR bands frequencies are consistent with a decrease of electron density at the active site Fe in the protonated form due to the higher electron-donor ability of a thiolate when compared to a thiol. However alternative sites of protonation cannot be excluded at present.

Inactivation. Hydrogenases are quickly inactivated when exposed to air.[7,30] Under anaerobic conditions, inactivation of the *D. gigas* hydrogenase can be achieved by addition of oxidants[7,32] or by electrochemical oxidation.[33] We reported that exposure of the enzyme in the **SI** form to air caused oxidation mainly to form **A**, as observed by IR spectroscopy.[6] By contrast, oxidation of **SI** by slow diffusion of oxygen produced mainly **B**. Once the enzyme is oxidized to **B** it is stable in the presence of air and does not convert to **A**.[6] It is noteworthy that these results are almost identical to those found with *C. vinosum* hydrogenase. Other reports have shown that anaerobic oxidation of active *D. gigas* hydrogenase results in the appearance of the EPR signal of Ni-**B**.[8,32] As shown here, the IR-spectrum of form **B** can be obtained by electrochemically-controlled oxidation of **SI**.[34] Enzyme in the **SI** form is capable to activate H_2, whereas form **B** is not, as indicated by the D_2/H^+ exchange activity of the two states (Table 3). Therefore two ways of

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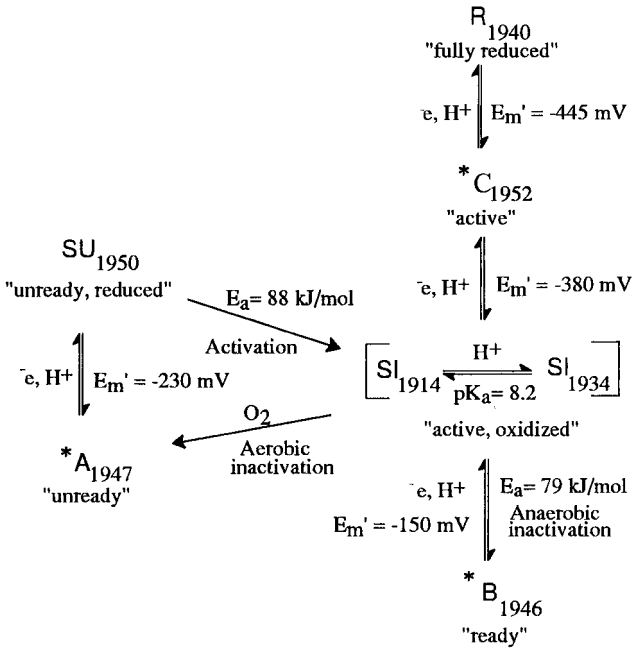
(34) Although the IR-spectroelectrochemical cell is not completely gas tight, the oxygen that diffuses into the cell is quickly reduced by the redox mediators present in the sample, therefore anaerobic conditions are maintained.

inactivating the enzyme are possible. The first one is exposure to air, a quick process that leads to form **A** which probably has a tightly bound oxygenated ligand in the coordination sphere of the active center.[6] Subsequent reactivation is then very slow. The second mechanism is a slow reaction which leads to **B** under anaerobic conditions or under low oxygen tension (with oxygen behaving as an oxidant rather than a coordination ligand). Form **B** can be reduced quickly back to **SI**, **C**, or **R** at low redox potentials. The midpoint redox potential measured in this work at pH 8.0 for the **SI/B** couple was −150 mV, which is very similar to the corresponding redox potential of −140 mV measured by Roberts and Lindahl[12] for the redox titration of the Ni-**B** EPR signal at the same pH. Mege and Bourdillon[33] reported that the midpoint redox potential for anaerobic inactivation, followed by the decrease of the H_2-uptake activity of the *D. gigas* enzyme, was −190 mV at pH 8.0. This in agreement with our statement that form **SI** is an *active* state, whereas form **B** is a *ready* state and therefore inactive as long as the enzyme stays oxidized. Coremans *et al.*[35] found that the reversibility of the **B/SI** transition in *Chromatium vinosum*, measured by EPR, was strongly dependent on pH and temperature. At pH 6.0 and 2 °C reduction of **B** was completely irreversible. Here we have found that oxidation of form **SI** to **B** in *D. gigas* hydrogenase is also highly temperature-dependent with activation energies of 79 and 88 kJ/mol for forms $\mathbf{SI_{1914}}$ and $\mathbf{SI_{1934}}$, respectively. Such a high kinetic barrier could account for the fact that the [3Fe-4S] cluster is reoxidized before the appearance of the EPR Ni-B signal upon anaerobic oxidation of form **C**, although the [3Fe-4S] cluster has a more positive redox potential than the **B/SI** couple.[8] On the other hand, the activation energy for the activation process of *unready* enzyme is 88 kJ/mol.[7] Although anaerobic activation (**SU** → **SI**) and inactivation (**SI** → **B**) have similar kinetic barriers, both processes have different mechanisms. The coordination of the active center in the **A** and **B** forms should be different, as reflected by their different EPR and IR spectra. The quick reactivation of the form **B** with a reductant may reflect either the absence or a different binding mode of the putative oxygen ligand present in form **A** (Figure 10).

Redox States of the Active Site and Catalytic Cycle. In Scheme 1 we propose reactions that correlate the different redox states detected by IR spectroscopy.[36] Forms **R**, **C**, and **SI** participate in the catalytic cycle of the hydrogenase, whereas **A**, **SU**, and **B** are involved in the activation and inactivation processes, as discussed above. The same redox transitions are observed by IR spectroscopy and EPR spectroscopy. Furthermore, the midpoint redox potentials reported here are very similar to the ones based on EPR titrations and have similar pH-dependence in most of the cases.[2,9,12,18]

Reductive stoichiometric titrations of EPR signals of *D. gigas* hydrogenase fit best to a model that assumes one-electron differences between the identified redox states of the active site.[12] Our experimental data (Figure 7) also fit best to one-electron transitions.[37] On the one hand, it has been shown that the EPR signals arise from a paramagnetic Ni in the active site,[10] and, on the other, it is assumed that the IR-active groups are the diatomic ligands of the active site Fe. As EPR and IR

Scheme 1



titrations have a very similar behavior, it can be concluded that there is a strong electronic interaction between the two metal atoms of the active site. The addition of 1 equiv of reductant produces a change of the EPR signal and concomitantly the conversion of one FTIR state into another. As discussed above, the **A** → **SU** transition involves most probably a one electron reduction of the Ni site. On the other hand, the **B** → **SI** transition involves most probably a changes of redox state of the Fe site, as there is a great shift to lower frequencies of the IR bands.

Several mechanisms have been proposed for the catalytic cycle of [NiFe] hydrogenases that considered the Ni atom as the only metal in the active site.[9,11,14,18,20,38] Not all of them contemplate changes of the valence state of the Ni atom during the catalytic cycle. Thus, Teixeira *et al.*[38] proposed that the disappearance of EPR-Ni signals associated to the activation of the enzyme was attained through antiferromagnetic coupling of the Ni(III) center ($S = 1/2$) and a reduced $[4Fe\text{-}4S]^{1+}$ cluster ($S = 1/2$). Later, Bagyinka *et al.*[20] suggested that the redox chemistry occurred at a sulfur ligand rather on the Ni. The discovery of a Fe atom as the second active site metal in the crystal structure of *D. gigas* hydrogenase strongly suggests that it has a role in the catalytic mechanism. Based on the crystallographic results, mechanisms for the catalytic cycle considering a binuclear active center have been proposed.[6,39] In one of these,[39] the oxidation state of Ni does not change through the catalytic cycle. Ni would have the role of being the transient binding site for H^- in the base-assisted heterolytic cleavage of H_2. Subsequent one-electron transfer steps would involve the Fe. This latter assumption is justified by the differences observed in the IR bands when comparing the spectra of the **R**, **C**, and **SI** forms and by recent XAS results on several [Ni-Fe] hydrogenases[29] which suggest that reduction of form **SI** to **C** or **R** does not involve a change in the electron density at the nickel. In this mechanism, the two metal atoms of the active site are formally monovalent in the **SI** form (Ni^+--Fe^+).[39] Accordingly, the conversion of **SI** to form **B** would involve a

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(36) For the sake of clarity, only the lowest frequency value of the IR spectrum of each one of the redox states is given in Scheme 1. The values of E_m at pH = 8.0 shown in this scheme have been calculated from those of Table 2 by taking into account their dependence of the pH, as determined in this work. The states for which Ni is EPR-detectable are marked with an asterisk.

(37) The electron balance of **SU** conversion to **SI** form has not yet been quantified.

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one-electron oxidation to Ni^{+}--Fe^{2+} and the conversion of **A** to **SU** would consist in the transition Ni^{3+}--$Fe^{2+} \rightarrow Ni^{2+}$--$Fe^{2+}$. Activation, in turn, would involve the loss of the oxygenated ligand with a change of the formal oxidation states of the metals, in order to obtain **SI** (Ni^{+}--Fe^{+}).

We have also proposed an alternative mechanism for the catalytic cycle assuming that in form **SI** there is a bridging hydride between the two metals ($Ni^{+}H^{-}Fe^{3+}$ or $Ni^{2+}H^{-}Fe^{2+}$).[6] This mechanism is also compatible with the results obtained in this work. The slow anaerobic inactivation of **SI** to **B** could be explained as an one-electron oxidation through the Fe−S clusters and subsequent replacement of the bridging hydride, presumably tightly bound, by a water molecule or a hydroxide. Proton and deuteron ENDOR measurements have detected an exchangeable proton in form **B** consistent with a bound H_2O.[40] Aerobic inactivation of enzyme in the **SI** form, on the other hand, would be a fast process because oxygen would react directly with the putative bridging hydride ligand to give enzyme in the **A** form.

Concluding Remarks

We can conclude that electrochemically controlled redox titrations of the different IR spectra of *Desulfovibrio gigas* hydrogenase have given valuable information about the redox reactions that take place in the activation, inactivation, and catalytic cycle of [NiFe] hydrogenases. The remarkable coincidence between EPR and IR spectroscopies concerning the potentials of the various redox states implies that the active site Ni and Fe centers are strongly coupled. Next challenges will be the determination of the nature of the bridging ligand between the Ni and Fe centers and the clarification of the role of the two **SI** species. High resolution crystallographic analysis of the enzyme in the *ready* and *active* states should be of great help in elucidating the catalytic mechanism of [NiFe] hydrogenases.

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