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Infrared-Detectable Groups Sense Changes in Charge Density on the Nickel Center in Hydrogenase from *Chromatium vinosum*¹

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ABSTRACT: Fourier transform infrared studies of nickel hydrogenase from *Chromatium vinosum* reveal the presence of a set of three absorption bands in the 2100–1900 cm⁻¹ spectral region. These bands, which do not arise from carbon monoxide, have line widths and intensities rivaling those of a band arising from the carbon monoxide stretching frequency (ν(CO)) in the Ni(II)CO species of this enzyme [Bagley, K. A., Van Garderen, C. J., Chen, M., Duin, E. C., Albracht, S. P. J., & Woodruff, W. H. (1994) *Biochemistry* 33, 9229–9236]. The positions of each of these three infrared absorption bands respond in a consistent way to changes in the redox state and in the presence of carbon monoxide. In seven of these states, the three IR absorption bands in the set have unique frequency positions. It is concluded that the set is due to intrinsic, non-protein groups in the enzyme, whose identities are presently unknown, and that these groups are situated very close to the nickel center and sense the charge density at the Ni site.

Hydrogenases are enzymes functioning in the metabolism of a great variety of microorganisms, where they catalyze the reversible oxidation of H₂ to protons. Two classes of hydrogenases are known at present: (i) Ni-hydrogenases, containing Ni and Fe, and (ii) Fe-hydrogenases, containing Fe as the sole metal. Recently, a H₂-forming enzyme was described, in which no metals could be detected at all: methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* (Zirngibl et al., 1992). Different aspects of hydrogenases have been reviewed over the past 6 years (Fauque et al., 1988; Lancaster, 1988; Adams, 1990; Albracht, 1990, 1994; Przybyla et al., 1992; Voordouw, 1992; Wu & Mandrand, 1993).

At present, the amino acid sequences of some 24 different nickel hydrogenases have been published. These sequences have been compared and discussed in some recent publications (Przybyla et al., 1992; Voordouw, 1992; Wu & Mandrand, 1993; Albracht, 1994). All enzymes contain a conserved amino acid sequence motif DPCxxCxxH at the carboxy terminus of the large subunit. The first Cys residue in this motif is coordinated to nickel. In some enzymes this residue is replaced by selenocysteine. EPR spectra of these enzymes show hyperfine interaction from ⁷⁷Se (I = 1/2), which is direct evidence for a Ni–Se bond.

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(He et al., 1989; Sorgenfrei et al., 1993). The amino-terminal part of the large subunit contains two additional strictly conserved Cys residues in an RgxEx₆Rx COx CxxH motif. It is not known whether any of these residues is involved in nickel coordination.

The small subunits of 17 nickel hydrogenases each contain 10 conserved Cys residues (Voordouw, 1992; Wu & Mandrand, 1993; Albracht, 1993) and were termed standard nickel hydrogenases (Albracht, 1994). The well-studied enzyme from *Desulfovibrio gigas* (Fernandez et al., 1986; Teixeira et al., 1989) belongs to this category. Although the amino acid sequence of the *Chromatium vinosum* enzyme is not yet known, its properties are very similar to those of the *D. gigas* enzyme. Hence, it is considered to belong to the standard nickel hydrogenases as well. Although no Cys patterns typical for Fe-S clusters can be recognized in the small subunit of these standard nickel hydrogenases, it is presently assumed that the small subunit hosts all Fe-S clusters of the enzyme. It is not known whether the two N-terminal Cys residues of the large subunit are also involved in the coordination of Fe-S clusters.

Only the first four Cys residues in the small subunit are strictly conserved in all nickel hydrogenases. In some enzymes, the last six Cys residues are replaced by eight Cys residues in two CxxCxxCxxx motifs (Alex et al., 1990;...
Hydrogenase from the purple sulfur bacterium Chromatium vinosum (strain DSM 185) is a water-soluble protein consisting of two subunits of 62 and 32 kDa (Van der Zwaan et al., 1987). In routine preparations, about 1 mol of nickel, 11–12 mol of iron, and 9 mol of acid-labile sulfur are detected per 94 kDa of molecular mass. In preparations of the enzyme, as isolated, the nickel ion is in the +3 valence state and EPR spectroscopy detects two distinct rhombic Ni(III) species (Albracht et al., 1983, 1984; Van der Zwaan et al., 1990). Fernandez et al. (1984, 1985) demonstrated, for the D. gigas enzyme, that these species correlated with two states of the oxidized enzyme, which differed in their ability to react with H₂. The form with \( g_{ex} = 2.33, 2.16, 2.02 \) reacted readily with H₂ and was called the ready enzyme. We will refer to nickel in this enzyme as Niᵢᵢ. The other form, with \( g_{ex} = 2.31, 2.23, 2.02 \) could react with H₂ only after prolonged (hours) contact with H₂ and was termed unready. We refer to the corresponding nickel ion as Niᵢᵤ. The same situation holds for the C. vinosum enzyme (Van der Zwaan et al., 1990), although the \( g \) values of the two nickel forms are slightly different (Niᵢᵢ, \( g_{ex} = 2.34, 2.16, 2.01; \) Niᵢᵤ, \( g_{ex} = 2.32, 2.24, 2.02 \)). The ratio of ready to unready Ni(III) can be changed by controlling the conditions of reoxidation of the reduced enzyme (Van der Zwaan, 1987; Chen, 1992).

After reductive activation of nickel hydrogenases (Lissolo et al., 1984; Fernandez et al., 1986), an active form of the enzyme is obtained showing a third EPR signal due to nickel, which is often referred to as the Ni-C signal. It was first demonstrated by Van der Zwaan et al. (1985) that the \( S = 1/2 \) nickel ion in this state binds hydrogen in a light-sensitive way. We will refer to nickel in this state as Niᵢᵢ(\( \text{H}_2 \)), for reasons outlined by Van der Zwaan et al. (1990), or as Ni-C. Upon complete reduction with H₂, a state is reached where nickel is EPR-silent again. Being the most reduced of the four different redox states of the nickel center in the enzyme, we will refer to this state as Niᵢᵤ(0).

From recent Mössbauer studies on the enzyme (Surerus et al., 1994), it is clear that the C. vinosum enzyme contains one [3Fe-4S]\(^{1+} \) cluster and two [4Fe-4S]\(^{2+} \) clusters. There are also some indications of the presence of an extra Fe site in the vicinity of both the nickel center and the 3Fe cluster. MCD studies on the enzyme provided evidence that the nickel site shows no magnetism whenever the nickel is unreactive. Being the most reduced of the four different redox states of the nickel center in the enzyme, we will refer to this state as Niᵢᵤ(0).

In a previous report (Bagley et al., 1994), we studied, for the first time, a nickel hydrogenase using FTIR, with the aim to monitor CO binding to divalent low-spin nickel. Because the nickel center in this form of the enzyme is diamagnetic, no information on the nickel center can be obtained by EPR or MCD. Accordingly, we turned to FTIR where, as anticipated, the stretching vibration of carbon monoxide (\( \nu(CO) \)) bound to nickel could be observed at 2060 cm\(^{-1} \) (Bagley et al., 1994). The Ni(II)CO bond was shown to be light-sensitive, and at temperatures below 80 K, this bond could be irreversibly photodissociated. Upon annealing at 200 K in the dark, the Ni–CO bond was seen to reform thermally (Bagley et al., 1994). During that study, we encountered several additional bands in the 2100–1900 cm\(^{-1} \) spectral region with similar line widths and intensities comparable or greater to those of \( \nu(CO) \). Some of these bands exhibited frequency shifts upon photolysis of the Ni(II)CO bond. In order to obtain more insight into the possible origin of these bands, we have extended our FTIR studies to cover up to eight different states of the nickel center in hydrogenase from C. vinosum.² It appears that a set of three bands is present in all of these states. The possible origin of these bands is discussed, as well as the possible consequences on our knowledge of the enzyme. In order to facilitate discussion, we have summarized our present understanding of the several states of the enzyme in Figure 1. We note that since valence state changes exceeding 1 in model compounds of Ni usually occur only for widely different redox potentials, it is unlikely that the actual charge density on the nickel ion in hydrogenase is in agreement with the formal valence states indicated in Figure 1. Still, for a discussion of the several redox states of the nickel center in the enzyme, it is useful to adhere to this notation.

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² These results were reported at the Fourth Annual Conference on the Molecular Biology of Hydrogenases, held in August, 1994, at Noordwijkerhout, The Netherlands.
MATERIALS AND METHODS

C. vinosum (strain DSM 185) was grown in a 700-L batch culture (Van Heerikhuizen et al., 1981) in a medium (Albracht et al., 1983) essentially as previously described (Hendry, 1955). Cells were harvested, and the enzyme was isolated and purified as previously described (Coremans et al., 1992b) and dissolved in 50 mM Tris-HCl buffer (pH 8.0). Enzyme activity was measured amperometrically at 30 °C as described previously (Coremans et al., 1989). The enzyme was concentrated to 1.5–2.5 mM using a Centricon-30 filter. Hydrogenase was converted into a state consisting of either predominantly the Ni,(III) form or predominantly the Ni,(III) form, as previously described (Van der Zwaan et al., 1990; Chen, 1992), and stored at or below -80 °C prior to sample manipulations.

For the infrared experiments, the samples were pretreated as described in the Results and Discussion section and then loaded into an anaerobic infrared transmittance cell (total sample volume of approximately 10 μL), which, for anaerobic experiments, had been flushed with Ar gas for at least 15 min prior to loading the cell. Both ZnS and AlO₃ were used as window materials in the infrared cell, and there were no significant differences in the infrared spectral features described in this report. The infrared cell was loaded inside an argon-flushed glove bag using a gas-tight syringe that had been flushed with Ar. Residual oxygen was removed from the carbon monoxide and argon used in these experiments by passing the gases over OxiClear cartridges (DGP-250-R1). Oxygen was removed from the hydrogen gas by passage over a palladium catalyst (Degussa, type E236P). When required, the enzyme was exchanged into D₂O by concentration in a Centricon-30 concentrator and redilution into 50 mM Tris-DCl (pD 8.0) a total of three times (dilution factor of >40 each time). Low-temperature (20–250 K) infrared spectra were collected in a Bio-Rad FTS-40 FTIR spectrometer equipped with a low-temperature optical cryostat (ADP Cryogenics Heli-tran LT-3-110, NaCl windows). The temperature of the sample was controlled via a LakeShore Cryotronics temperature controller (DRC-80C). The sample cell was aligned at 45° to both the infrared beam and the photolysis beam so as to allow photolysis of the CO from the sample during data collection. Photolysis at 78 and 20 K was achieved using white projector light passed through a heat-absorbing filter. The infrared spectra presented are an average of 1024 scans.

EPR measurements at X-band were obtained with a Bruker ECS 106 EPR spectrometer at a field modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG magnetic field meter. The microwave frequency was measured with an HP 5350B frequency meter.

RESULTS AND DISCUSSION

Oxidized Aerobic Enzyme. Like the D. gigas enzyme (Fernandez et al., 1986), the oxidized hydrogenase from C. vinosum can occur in the ready or unready state (Van der Zwaan et al., 1990). Transition of one form into the other can be obtained by complete reduction and appropriate reoxidation, as reported by Van der Zwaan et al. (1990) and as further worked out by Chen (1992). An FTIR spectrum at 20 K of enzyme containing 85% Ni,(III) and 15% Ni,(III) has been reported by us previously (Bagley et al., 1994) and is reproduced in Figure 2.

We have also measured the IR spectrum for a state containing 26% Ni,(III) and 74% Ni,(III) (spectrum not shown). There were no detectable differences in frequency for IR bands in the 2100–1800 cm⁻¹ spectral region for the two enzymes. In addition, we have reduced the 74% Ni,(III)/26% Ni,(III) enzyme and reoxidized it so as to optimize the Ni,(III) form. No spectral changes were observed (not shown), and the intensities of the 1944 cm⁻¹ bands were also the same, when the absorbances of both samples at 220 nm were matched. We have previously recorded the spectra of samples in D₂O completely reduced by D₂, treated with CO to obtain the Ni,(II)CO form, and subsequently reoxidized by air. From EPR studies, we know that in this case Ni,(III) is produced exclusively (Chen, 1992). The IR spectrum of the sample was indistinguishable from that shown in Figure 2. We conclude that Ni,(III) and Ni,(III) have the same FTIR spectra at 23 K in the 2100–1900 cm⁻¹ region and that the bands do not arise from vibrations involving exchangeable protons.

With the exception of ν(CO) in metalloproteins, bands in the 2100–1900 cm⁻¹ region have, to our knowledge, never before been observed in proteins. Such bands are absent from the low-temperature spectra of myoglobin (Alben & Caughey, 1968), cytochrome oxidase (Fiamingo et al., 1981), rhodopsin (Bagley et al., 1985, 1989), and bacteriorhodopsin (Bagley et al., 1982). Such bands are also absent from the infrared spectra of another Ni,Fe enzyme, CO dehydrogenase (Kumar & Ragsdale, 1992). We have therefore performed a number of control experiments to investigate whether these bands are due to artifacts introduced during the handling of the samples. FTIR spectra in the temperature range 20–300 K were recorded for (i) 50 mM Tris-HCl in H₂O (pH 8.0) in air, (ii) 50 mM Tris-DCl and D₂O (pD 8.0) in air, (iii) 50 mM Tris-HCl in H₂O (pH 8.0) that was evacuated and flushed with Ar several times and subsequently evacuated and flushed with CO several times, and (iv) 50 mM Tris-HCl in H₂O (pH 8.0) that was evacuated and flushed with H₂ 10 times, followed by incubation at 50 °C for 30 min, cooling in ice, evacuation and flushing with Ar eight times, and finally exposure to air. None of these samples
The possibility of interactions between the Tris buffer and the enzyme as possible sources of these bands was investigated as well. An aliquot of 100 μL of concentrated enzyme in 50 mM Tris-HCl (pH 8.0) was diluted in 1.9 mL of MOPS buffer (pH 7.9). The sample was concentrated again to 100 μL in a Centricon device. The dilution and concentration steps were repeated another two times. The final sample showed exactly the same FTIR spectrum as the untreated one, and the intensity of the 1943 cm⁻¹ band did not change, taking the absorbance at 220 nm as a reference. This excludes exchangeable interactions with the Tris buffer. It also excludes the effect of possible pH changes upon freezing, as the pKₐ value of MOPS is considerably less sensitive to temperature changes than that of Tris. In addition, a solution of 0.5 mM bovine serum albumin in 50 mM Tris-HCl (pH 8.0), concentrated six times in a Centricon device, gave no FTIR bands in the 2100-1900 cm⁻¹ region. This rules out possible specific Tris-protein interactions or contaminants from the Centricon cells as origins for the bands observed in hydrogenase. We also detected no changes if the 74% Ni₉(III)/26% Ni₉(II) sample was evacuated and flushed with CO six times. This eliminates bound N₂ as potentially responsible for bands in the spectrum. It also shows that CO does not bind to the metals in the enzyme in this state. Oxygen, or a partly reduced oxygen species, can bind close to Ni(III) in a very tight way (Van der Zwaan et al., 1990). It is not removed by evacuation and flushing with Ar or He, but it is fully eliminated upon reduction of the enzyme (Van der Zwaan et al., 1990). As will be shown in the following, reduction of the enzyme shifts the bands, but does not remove them. Hence, the effects on the enzyme from oxygen exposure can be ruled out as the source of these bands as well. We conclude that the bands are intrinsic to the enzyme.

The Ni₉(1)H₂ or Ni-C State. When enzyme is completely reduced and activated by incubation with H₂ at 50 °C for 30 min and then equilibrated with 1% H₂, the EPR signal of Ni₉(1)H₂ (Ni-C) is maximal (Coremans et al., 1992a). At temperatures below 80 K, the bond between hydrogen and nickel cannot be completely reduced under 1 bar of H₂ (Coremans et al., 1992a). Apparently, there is also a slight light-induced increase in the EPR spectra. The changes were irreversible when induced at either 23 or 75 K, but were completely reversed at 200 K in the dark (Figure 3C). A difference spectrum (light minus dark; Figure 3D) clearly shows the changes. The three bands at 2088, 2076, and 1950 cm⁻¹ from the Ni₉(1)H₂ state disappeared upon illumination, and another set of three bands appeared at 2058, 2044, and 1898 cm⁻¹.

Enzyme under 1 Bar of H₂: The Ni₉(0) State. In Figure 4, a set of spectra is shown of enzyme completely reduced with H₂. Figure 4A is the sample before illumination. Major bands at 2075, 2060, 1936, 1922, and 1910 cm⁻¹ are observed. Light induced only minor changes (Figure 4B; see also the difference spectrum in Figure 4D). The weak band at 1950 cm⁻¹ disappeared, concomitant with the appearance of a weak band at 1898 cm⁻¹. We conclude that this is due to the Ni₉(0)H₂ species (see Figure 3), which cannot be completely reduced under 1 bar of H₂ (Coremans et al., 1992a). Apparently, there is also a slight light-induced

The changes in the EPR spectra. The changes were irreversible when induced at either 23 or 75 K, but were completely reversed at 200 K in the dark (Figure 3C). A difference spectrum (light minus dark; Figure 3D) clearly shows the changes. The three bands at 2088, 2076, and 1950 cm⁻¹ from the Ni₉(1)H₂ state disappeared upon illumination, and another set of three bands appeared at 2058, 2044, and 1898 cm⁻¹.

In Figure 3, infrared spectra of C. vinosum hydrogenase that was repeatedly evacuated and flushed with 1 bar of H₂, incubated for 30 min at 50 °C, cooled on ice, and finally evacuated and flushed repeatedly with 1% H₂. (A) Absolute IR absorbance spectrum of enzyme in the dark at 23 K. (B) IR spectrum of enzyme that was illuminated with white light for 10 min at 23 K. The spectrum was accumulated while illuminating the sample with white light from a slide projector. (C) Absolute IR absorbance spectrum at 23 K in the dark, after the photolyzed enzyme (see trace B) was warmed 200 K for 15 min and then cooled in the dark to 23 K. (D) IR difference spectrum between the light-induced spectrum shown in trace B and the dark spectrum shown in trace A. Spectral resolution was 1 cm⁻¹. The reference spectrum for the absolute IR spectra shown in (A–C) was an empty infrared cell at room temperature. The absolute infrared difference spectra were baseline corrected using the same parabolically shaped curve for traces A–C. The difference spectrum shown in trace D was calculated using the raw data for traces A and B.

The Nia(1)H₂ or Ni-C State. When enzyme is completely reduced and activated by incubation with H₂ at 50 °C for 30 min and then equilibrated with 1% H₂, the EPR signal of Ni₉(1)H₂ (Ni-C) is maximal (Coremans et al., 1992a). At temperatures below 80 K, the bond between hydrogen and nickel cannot be photolyzed, and reannealing takes place only at 200 K (Van der Zwaan et al., 1985, 1990). We have studied these states with FTIR both in a H₂O/H₂ environment and a D₂O/D₂ environment. The spectra of comparable states were indistinguishable in H₂O/H₂ and D₂O/D₂, showing that these bands do not arise from vibrations involving contributions from a bond to an exchangeable proton. The H₂O/H₂ spectra are shown in Figure 3.

In the spectrum of the sample under 1% H₂, bands were observed at 2088, 2076, 2060, 1950, 1937, 1921, and 1911 cm⁻¹. Upon illumination for 10 min at 20 K, the bands at 2088, 2076, and 1950 cm⁻¹ diminished in intensity, whereas the band at 2060 cm⁻¹ increased in intensity (Figure 3B). New bands appeared at 2044 and 1898 cm⁻¹. The bands at 1937, 1921, and 1911 cm⁻¹ were not affected. As with the EPR experiments (Van der Zwaan et al., 1985), we noticed that the light-induced changes took a considerably longer time to complete when the experiment was performed in D₂O/D₂ instead of H₂O/H₂, suggesting that the events causing the changes in the FTIR spectra are the same as those causing the changes in the EPR spectra. The changes were irreversible when induced at either 23 or 75 K, but were completely reversed at 200 K in the dark (Figure 3C). A difference spectrum (light minus dark; Figure 3D) clearly shows the changes. The three bands at 2088, 2076, and 1950 cm⁻¹ from the Ni₉(1)H₂ state disappeared upon illumination, and another set of three bands appeared at 2058, 2044, and 1898 cm⁻¹.

FIGURE 3: Infrared spectra of C. vinosum hydrogenase that was repeatedly evacuated and flushed with 1 bar of H₂, incubated for 30 min at 50 °C, cooled on ice, and finally evacuated and flushed repeatedly with 1% H₂. (A) Absolute IR absorbance spectrum of enzyme in the dark at 23 K. (B) IR spectrum of enzyme that was illuminated with white light for 10 min at 23 K. The spectrum was accumulated while illuminating the sample with white light from a slide projector. (C) Absolute IR absorbance spectrum at 23 K in the dark, after the photolyzed enzyme (see trace B) was warmed 200 K for 15 min and then cooled in the dark to 23 K. (D) IR difference spectrum between the light-induced spectrum shown in trace B and the dark spectrum shown in trace A. Spectral resolution was 1 cm⁻¹. The reference spectrum for the absolute IR spectra shown in (A–C) was an empty infrared cell at room temperature. The absolute infrared difference spectra were baseline corrected using the same parabolically shaped curve for traces A–C. The difference spectrum shown in trace D was calculated using the raw data for traces A and B.
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Figure 4: Infrared spectra of *C. vinosum* hydrogenase that was repeatedly evacuated and flushed with 1 bar of H₂, incubated for 30 min at 50 °C, and then cooled on ice. (A) Absolute IR absorbance spectrum after illumination with white light for 10 min at 23 K. The spectrum was accumulated while illuminating the sample with white light from a slide projector. (C) IR spectrum in the dark at 23 K, after the photolyzed enzyme (see trace B) was warmed to 200 K for 15 min and then cooled in the dark to 23 K. (D) IR difference spectrum between the light-induced spectrum at 23 K in the dark, after the photolyzed enzyme (see trace A) and the dark spectrum shown in trace B. Spectral resolution was 1 cm⁻¹. The reference spectrum for the absolute IR spectra shown in (A–C) was an empty infrared cell at room temperature. The absolute infrared difference spectra were baseline corrected using the same parabolically shaped curve for traces A–C. The difference spectrum shown in trace D was calculated using the raw data for traces A and B.

The shift of the 1936 cm⁻¹ band to lower frequencies, but we could not always observe this particular change with other preparations. Hence, we conclude that the bands at 2075, 2060, and 1936 cm⁻¹, and possibly the band at 1921 cm⁻¹, are due to the Ni₄(0) state and that the IR spectra are not perturbed by the illumination of enzyme in this state. At the same time, we conclude that the light-insensitive bands at these positions in the spectra of the enzyme in the Ni-C state (Figure 3) are due to this Ni₄(0) species. Apparently, the transition from Ni₄(0) to Ni₄(I)H₂ upon going from 1 bar of H₂ (D₂) to 1% H₂ (D₂) was not complete. This would agree with the fact that, at pH 8, one can maximally detect about 55% of the nickel in the EPR-detectable Ni₄(I)H₂ state (Coremans et al., 1992a).

The Ni₄(II) State. Handling of the very minute and concentrated samples required for these studies (10 μL, 1.5–2.5 mM) is quite difficult. The preparation of the enzyme in stable states, like the ones discussed earlier, is relatively straightforward. It was more difficult, however, to obtain an IR spectrum of the reduced, inactive form of the enzyme, the Ni₄(II) state, in the absence of redox mediators, given its extreme oxygen sensitivity. Attempts therefore, were made to slowly reoxidize reduced samples in the IR cell directly by exposing the enzyme to air via the removal of the filling port screws. An example of the results is shown in Figure 5.

After spectra of the Ni-C form were measured in a H₂O/H₂ environment (conditions as in Figure 3, reproduced in Figure 5A for comparison), the sample was thawed in the sample cell holder and the filling port screws were removed. After 13 h in the sample holder at room temperature, visual inspection clearly showed differing optical density across the cell cross section, indicating that the infrared spectra of this sample represented a mixture of states. The spectrum at 20 K is shown in Figure 5B. The band at 1944 cm⁻¹ is presumably from the Ni₄(III) state, as observed earlier in Figure 3. The set of bands at 2067, 2051, and 1910 cm⁻¹ has not been observed before in any of the other redox states.

We tentatively conclude that these bands are due to divalent nickel in inactive enzyme, the Ni₄(II) state. Illumination of the sample in this state had no effect whatsoever. A spectrum taken at 200 K was identical. Subsequently, the sample was thawed, the filling port screws
were removed again, and the enzyme was left in the sample holder for another 19 h at room temperature. The sample then looked uniformly colored. The FTIR spectrum (Figure 5C) now showed a clearly increased 1944 cm⁻¹ band, accompanied by bands at 2094 and 2083 cm⁻¹, whereas the bands at 2067, 2051, and 1910 cm⁻¹ had completely disappeared. As before, the spectrum did not show any changes upon illumination. The sample was then thawed and stored at 4 °C for 12 days, while the filling port screws were removed. There were no further changes in the spectrum. A similar set of changes was observed upon opening the sample cell in the Ni(II).D₂/D₂O experiments.

Summary of Observations. We have summarized our findings of the FTIR spectra of hydrogenase from C. vinosum inspected thus far in Figure 6. The results suggest that each of the observed states of the enzyme can be characterized by a unique set of three FTIR bands, two weak bands and one strong band. Within each set, the two weaker bands are always observed within the region 2100–2040 cm⁻¹, and their mutual distance varies only slightly between 12 and 16 cm⁻¹. The strong bands are present in the range 1950–1895 cm⁻¹. The frequency difference between this strong band and the center of the two weak bands is nearly invariant (143–149 cm⁻¹) in the inactive enzyme [Ni₆(III) and Ni₆(II)H₂(II) states] and the Ni₆(II)CO light and dark states. The separation is noticeably smaller (132 cm⁻¹) in the Ni₆(II)H₂ (dark) and Ni₆(0) states and larger (154 cm⁻¹) in the Ni₆(II)H₂ light-induced state. The three bands in each set shift in a similar way between the various states of the enzyme. The IR spectrum of the Ni₆(0) state of the enzyme displays weak bands at 2019 and 2013 cm⁻¹, in addition to the set of three. We note that it cannot be excluded that the 1910 cm⁻¹ band is due to enzyme molecules that have not yet been activated, the Ni₆(II) state, since the divalent state of nickel in the inactive enzyme is proposed to possess such a band (Figure 5B). Additionally, it may be that in this spectrum we are beginning to see evidence of yet another EPR-silent species exhibiting its strongest IR band at 1921 cm⁻¹. Experiments are currently underway to explore this. Additionally, bands detected in the IR spectrum for the Ni₆(0) state can also be detected in the IR spectrum for the Ni-C state. We suspect that these bands probably arise from a contribution from Ni₆(0). This suspicion is supported by the insensitivity of the bands to illumination at cryogenic temperatures. Under no conditions has any effect on the FTIR spectra been observed by exchanging H₂O/H₄ for D₂O/D₂.

We conclude that none of these bands involve vibrations for groups with significant contributions from ν(X−H), where H is an exchangeable hydrogen species.

To eliminate the possibility that our results represent a biologically irrelevant, spectroscopic peculiarity of the C. vinosum enzyme, it was highly desirable to inspect a nickel hydrogenase from another bacterium. To this end, after the completion of the first draft of the present manuscript, we contacted Dr. E. C. Hatchikian (Marseilles, France) for a nickel hydrogenase sample from D. gigas. We were informed that he and Dr. V. M. Fernandez (Madrid, Spain) had performed some room-temperature FTIR experiments on CO binding to the D. gigas enzyme that were never mentioned in publication. These spectra appear to contain several bands in the 1900–2100 cm⁻¹ spectral region that are similar to those detected in the C. vinosum enzyme. This strongly suggests that the FTIR bands given in the present paper are not unique to the C. vinosum enzyme, but are present in other nickel hydrogenases as well. This also strengthens the presumption that the structures responsible for these IR signatures are intrinsic to the enzyme and have functional relevance.

What Can We Learn from These Spectra? Even without knowing the precise origin of the bands discussed earlier, we can draw some conclusions from our observations. From the control experiments, the fact that infrared bands in the 2100–1900 cm⁻¹ region have not been observed before in a protein, the reproducibility of the experiments with various preparations of the enzyme, and the detection of these bands in both the C. vinosum enzyme (present study) and in the D. gigas enzyme (V. M. Fernandez and E. C. Hatchikian, personal communication), we conclude that the bands are due to an inherent component of these hydrogenases and are presumably present in other hydrogenases as well. As the basic pattern of the bands in each state of the enzyme is the same, two weak bands and one strong band at relatively constant mutual separations (in cm⁻¹), we conclude that this set of three bands represents one and the same group or set of groups in all states. We cannot, of course, exclude the possibility that other bands outside the 2100–1850 cm⁻¹ region belong to these sets. Spectra taken on many occasions suggest that the position of these bands is not temperature-dependent and that the intensity and width of these bands are not dramatically different for spectra taken at 20 K and room temperature.

As the sets are so consistent between the widely different redox states of the enzyme and yet have unique positions in each of them, it is unlikely that the bands belong to any of the three Fe-S clusters of the enzyme. It is suggested that the bands are due to a novel chemical structure or structures in close proximity to the nickel center, possibly forming part of the coordination of the nickel. The strongest argument in favor of this is the extreme sensitivity of the bands to the redox state of the nickel center. The position of the three bands is not sensitive to the changes in coordination between Ni₆(III) and Ni₆(III), changes that are observed clearly with EPR (Fernandez et al., 1986; Van der Zwaan et al., 1990) and MCD (M. R. Cheesman, J. W. Van der Zwaan, P. J. Albracht, and A. J. Thomson, unpublished observations). However, reduction to Ni(II) in inactive enzyme shifts all
bands by about 30 wavenumbers to lower frequencies (Figures 5B and 6).

We have previously reported that a light-sensitive, EPR-undetectable Ni(II)CO species can be formed by the reduction of inactive enzyme in the Ni(III) state with ascorbate plus PMS under a CO atmosphere in C. vinosum enzyme (Bagley et al., 1994). Exactly the same FTIR spectrum was obtained when H2-reduced active enzyme [Ni(0) state] was extensively evacuated and flushed with CO. It was concluded that the species presumed to be formed, the Ni(II)CO and the Ni(II)CO species, respectively, have indistinguishable FTIR spectra for the 2100–1900 cm⁻¹ spectral region. Since then we have tested the possibility that ready enzyme might become active upon incubation with ascorbate, PMS, and CO. Figure 7 shows that enzyme in a predominantly ready form, which was incubated with ascorbate and PMS under CO for 30 min at 45 °C, did not show a lag phase in a H₂ uptake assay with benzyl viologen, in which oxygen was removed by a glucose/glucose oxidase system. However, when the same incubation was performed under Ar, a clear lag phase (reductive activation of the enzyme) was noticeable. Together with the FTIR data, this strongly suggests that CO can bind to nickel only once activated. These observations might be explained by assuming that, in inactive enzyme, i.e., the Ni(III) and Ni(II) states, an extra ligand to nickel is present, which prevents the binding of CO. This would also explain why the FTIR spectra of Ni{(II)} formed after photolysis of CO from the Ni{(II)}CO state (Bagley et al., 1994) are different from those of Ni{(II)} obtained upon the oxidation of Ni{(I)}H₂ (Ni-C) by air. Active forms of the enzyme are extremely sensitive to oxygen (Albracht et al., 1985). Oxygen will immediately inactivate the enzyme; hence, only inactive Ni{(II)} and Ni{(III)} forms might be expected under these conditions.

Photolysis of CO shifted all bands 4–6 cm⁻¹ to higher frequencies. Photolysis of hydrogen in the Ni{(I)}H₂ state, however, shifted the two weaker bands some 29–31 cm⁻¹ to lower frequencies and the strong 1950 cm⁻¹ band to 1898 cm⁻¹. Reduction to the Ni{(I)} state shifted the bands of the Ni-C state each by about 14 cm⁻¹ to lower frequencies and made the nickel center light-insensitive.

Possible Origin of the Set of Three Bands. We speculate that the relative frequency position of the set of three bands depends on the charge density on the nickel center. We remind the reader that Maroney and co-workers (Bagyinka et al., 1993), when extensively studying the nickel hydrogenase of Thiothrix roseopersicina with X-ray absorption spectroscopy, did not notice any changes in the position of the K-edge of nickel in any of the possible redox states of that enzyme. Possible changes were within the accuracy of the method (0.2 eV). This means that the effective charge density on the nickel ion changed by at most 10% of that measured for a complete formal change of 1 for the valence state of nickel in model complexes (2–3 eV) (Eidsness et al., 1988; Bagyinka et al., 1993; Whitehead et al., 1991; Maroney et al., 1990). Ligands therefore were assumed to be actively involved in buffering the changes in charge accompanied by redox changes in the nickel center.

Examination of the FTIR spectra (Figure 6) shows that, in the transition from Ni{(III)} to Ni{(II)} in inactive enzyme, the bands all shift some 26–34 cm⁻¹ to lower frequencies. The charge density on the nickel ion in the Ni{(II)} center is expected to be higher than that on the nickel ion in the Ni{(III)} center. Upon photolysis of the Ni{(I)}H₂ bond, all three bands shift to a considerably lower frequency: the two weaker bands shifted some 29–31 cm⁻¹ and the strong band shifted even more, 52 cm⁻¹. Incidentally, Bagyinka et al. (1993) found a possible 0.2 eV shift of the K-edge to lower energy in X-ray absorption spectra upon illumination of the Ni-C state. This was the largest shift observed in their
Nir(I) to the Nir,(II) state was 34 cm⁻¹, while photolysis frequency that is detected for transitions between the other results in frequencies higher than the free CO stretching in fact, they are significantly smaller than the shift in shifts in frequency that these IR bands exhibit upon photolysis of CO from the Ni,(II)CO species. Upon photolysis of CO from the Ni, the IR bands all shift up in frequency by 4–6 cm⁻¹. On the basis of our previous suggestion, this would indicate that the charge density at the Ni site decreases upon photolysis of CO from the Ni. Carbon monoxide can bind to metals as either a σ-donor or a π-acceptor. The frequency of the ν(CO) band relative to the frequency of free CO (2155 cm⁻¹) can be used to determine the extent of σ-donor vs π-acceptor character for CO ligated to a metal; the binding of CO primarily as a π-acceptor to a metal results in a ν(CO) for metal-bound CO at frequencies lower than the free CO stretching frequency, while the binding of CO as a σ-donor results in frequencies higher than the free CO stretching frequency (Nakamoto, 1986). The ν(CO) for Ni₆(II)CO is detected at 2060 cm⁻¹, nearly 100 cm⁻¹ lower than the frequency for free CO. We therefore conclude that, in the Ni₆(II)CO species, CO binds to the Ni predominantly as a π-acceptor and that upon photolysis of CO from Ni₆(II)CO the charge density on the Ni should go up. This contradicts our hypothesis based on changes observed when the enzyme shuttles between other states. However, we note that the shifts in frequency that these IR bands exhibit upon photolyzing the CO from the Ni₆(II)CO species are quite small; in fact, they are significantly smaller than the shift in frequency that is detected for transitions between the other states that were studied. (The shift in frequency of the most intense IR band upon the reduction of the enzyme from the Ni₆(III) to the Ni₆(II) state was 34 cm⁻¹, while photolysis of H₂ from the Ni₆(I)H₂ species resulted in a shift in frequency for the lowest band of 52 cm⁻¹.) Additionally, if compensatory ligation (for example, rebinding of a ligand previously displaced by CO, or photolysis of another ligand from the Ni in addition to CO) occurs when the Ni(II)CO species is illuminated at cryogenic temperatures, then the effect on the effective charge density at the Ni ion upon photolysis of CO may be offset by the effects of the second ligand. Experiments are currently underway to assess the validity of this proposal.

In conclusion, we note that the FTIR spectra of the C. vinosum enzyme can be used to easily discriminate between the various redox and activity states of the enzyme (see Figure 6). Infrared studies can therefore provide a useful tool in the study of these various states. For instance, lines attributable to Ni₆(0) are still detectable in the IR spectrum of the enzyme under 1% H₂ at pH 8.0. This is in line with the finding (Coremans et al., 1992a) that maximally 55–60% of the nickel is detected in the EPR-detectable Ni₆(I)H₂ state. At pH 6.0, however, up to 90% of the nickel can be observed with EPR under an atmosphere of 1% H₂ (Coremans et al., 1992a). Relevant FTIR measurements have yet to be carried out. It is clear that infrared spectroscopy provides a most useful spectroscopic tool for further study of the behavior and properties of the active site in nickel hydrogenases.

**ADDED IN PROOF**

After this manuscript was proof-printed, an X-ray crystallographic structure of the nickel hydrogenase from D. gigas was published (Volbeda et al., 1995). The structure indicates that a nickel and iron ion may constitute the active site. This is in agreement with an earlier proposal based on spectroscopic data (Surerus et al., 1994; Albracht, 1994), indicating the presence of a lone low-spin Fe(III) ion close to the nickel. The structure also shows that four conservative Cys residues from the large subunit are involved in binding the two metal ions in the active site, while all of the Fe-S clusters are coordinated by the small subunit, confirming earlier proposals (Voordouw, 1992; Albracht, 1994). Interestingly, the crystal structure indicates the presence of three non-protein groups as ligands for one of the metal ions in the active site. Combining this information with our infrared results suggests that the set of three IR detectable groups reported herein are potential candidates for these non-protein ligands. Since the ions involved, nickel and iron, are both low spin, these groups must be strong ligands. The infrared frequencies typically suggest groups containing either triple bonds or adjacent double bonds, such as nitriles, cyanide, or azide groups. Additionally, the high intensities of the IR bands [which rival the intensity of the ν(CO) detected in the Ni₆(II)CO species] are most consistent with the possibility that these IR bands arise from relatively polar triply bonded species or adjacent double bonds. Other infrared absorbers in this spectral region are excluded on chemical or spectroscopic grounds. Groups involving hydrogen (e.g., metal hydrides or sulfhydryls should be subject to H/D exchange, and even if buried and therefore nonexchangeable would be weak IR absorbers compared to metal-coordinated CO. It remains to be verified whether the unknown groups are covalently bound to the protein or not. We note that our previous data (Bagley et al., 1994) on the Ni₆(II)CO species exclude the possibility that CO is acting as a bridging ligand between both metals since this would result in a much lower ν(CO) (1880–1700 cm⁻¹) (Horiwitz & Shriver, 1984) than is observed (2060 cm⁻¹). In addition, all spectroscopic data point to binding of CO to Ni (Albracht, 1994).

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Infrared Studies on Nickel Hydrogenase

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