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

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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 formal redox state of the nickel center and to the photodissociation of hydrogen bound to the nickel. Up to eight different states of the nickel center have been produced, depending on the redox state and/or the activity state of the enzyme and 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 H$_2$ 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 H$_2$-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, 1993, 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 ^{77}Se ($I = 1/2$), which is direct evidence for a Ni−Se bond

(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 RGxEx$_{16}$RxCGxCxxxH 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*[1] 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 CxxCxxCxxxC motifs (Alex et al., 1990;

[1] Abbreviations: *C. vinosum*, *Chromatium vinosum*; *D. gigas*, *Desulfovibrio gigas*; PMS, phenazine methosulfate; IR, infrared; FTIR, Fourier transform infrared; EPR, electron paramagnetic resonance; Tris·HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol adjusted to the desired pH with hydrochloric acid; MOPS, 3-morpholinopropanesulfonic acid; Ni$_r$, nickel center in hydrogenase in its ready form; Ni$_u$, nickel center in hydrogenase in its unready form; Ni$_a$, nickel center in hydrogenase in its active form.

Halboth & Klein, 1992), usually associated with two regular cubane clusters. In two enzymes, the six Cys residues are missing altogether, and the amino acid sequence of the small subunit terminates shortly after the first four conserved residues (Böhm et al., 1990; Tran-Betcke et al., 1990). This indicates that the clusters coordinated by these Cys residues are not essential for hydrogen activation.

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 H_2. The form with $g_{xyz} = 2.33$, 2.16, 2.02 reacted readily with H_2 and was called the ready enzyme. We will refer to nickel in this enzyme as Ni_r. The other form, with $g_{xyz} = 2.31$, 2.23, 2.02 could react with H_2 only after prolonged (hours) contact with H_2 and was termed unready. We refer to the corresponding nickel ion as Ni_u. 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 ($Ni_r(III)$, $g_{xyz} = 2.34$, 2.16, 2.01; $Ni_u(III)$, $g_{xyz} = 2.32$, 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 $Ni_a(I)\cdot H_2$, for reasons outlined by Van der Zwaan et al. (1990), or as Ni-C. Upon complete reduction with H_2, 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 $Ni_a(0)$.

From recent Mössbauer studies on the enzyme (Surerus et al., 1994), it is clear that the *C. vinosum* enzyme contains one $[3Fe-4S]^{1+(1+:0)}$ cluster and two $[4Fe-4S]^{2+(2+:1+)}$ 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 undetectable in EPR spectroscopy (M. R. Cheesman, J. W. Van der Zwaan, S. P. J. Albracht, and A. J. Thomson, unpublished observations).

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 (ν(CO)) bound to nickel could be observed at 2060
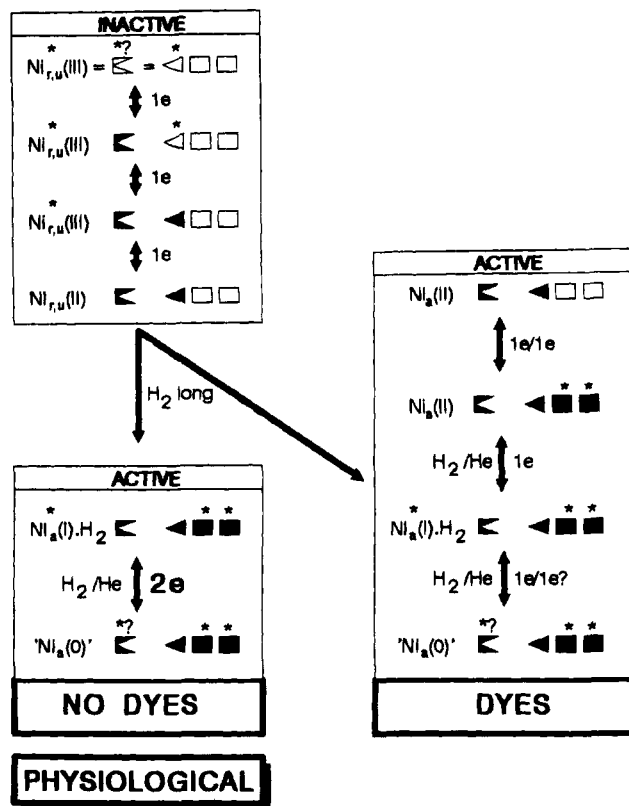

FIGURE 1: Redox states of *C. vinosum* hydrogenase. Squares represent [4Fe-4S] clusters; triangles represent the [3Fe-4S] cluster; an unknown group, possibly a lone Fe site, is indicated by the square with a missing triangle portion. Open symbols represent oxidized species, and black symbols represent reduced species. An asterisk indicates an $S = 1/2$ system; the = sign stands for spin coupling. H_2 long indicates a long incubation under hydrogen.

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 ν(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.

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 (Hendly, 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_r(III) form or predominantly the Ni_u-(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 AlO_3 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 D_2O 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 Cryotonics 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_r(III) and 15% Ni_u-
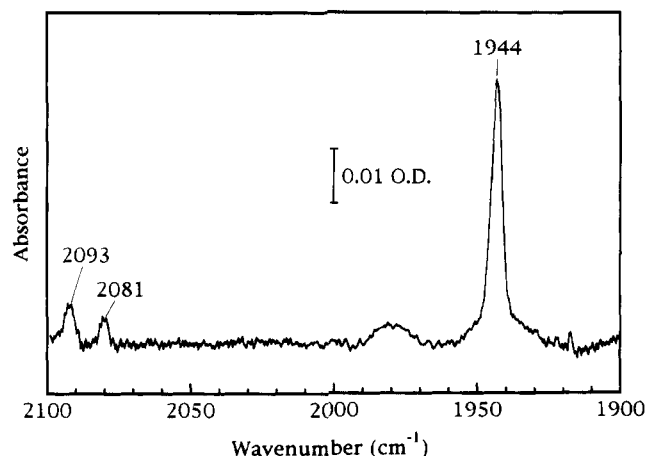
FIGURE 2: Absolute infrared absorbance spectrum of oxidized *C. vinosum* hydrogenase containing 85% Ni_r(III) and 15% Ni_u(III). Spectral resolution is 1 cm^{-1}. Reference spectrum was the empty IR cell at room temperature. The spectrum was baseline corrected using a parabolically shaped curve.

(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_r(III) and 74% Ni_u(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_u-(III)/26% Ni_r(III) enzyme and reoxidized it so as to optimize the Ni_r(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 D_2O completely reduced by D_2, 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_u(III) is produced exclusively (Chen, 1992). The IR spectrum of the sample was indistinguishable from that shown in Figure 2. We conclude that Ni_r(III) and Ni_u(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 H_2O (pH 8.0) in air, (ii) 50 mM Tris·DCl and D_2O (pD 8.0) in air, (iii) 50 mM Tris·HCl in H_2O (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 H_2O (pH 8.0) that was evacuated and flushed with H_2 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

gave any FTIR bands in the 2100–1900 cm^{-1} region. This excludes possible contamination from Tris·HCl, H_2O, D_2O, the vacuum line, Ar, CO, or H_2.

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 pK_a 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% Ni$_u$(III)/26% Ni$_r$(III) sample was evacuated and flushed with CO six times. This eliminates bound N_2 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 Ni$_a$(I)·H$_2$ or Ni-C State. When enzyme is completely reduced and activated by incubation with H_2 at 50 °C for 30 min and then equilibrated with 1% H_2, the EPR signal of Ni$_a$(I)·H$_2$ (Ni-C) is maximal (Coremans et al., 1992a). At temperatures below 80 K, the bond between hydrogen and nickel can 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 H_2O/H_2 environment and a D_2O/D_2 environment. The spectra of comparable states were indistinguishable in H_2O/H_2 and D_2O/D_2, showing that these bands do not arise from vibrations involving contributions from a bond to an exchangeable proton. The H_2O/H_2 spectra are shown in Figure 3.

In the spectrum of the sample under 1% H_2, 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 D_2O/D_2 instead of H_2O/H_2, suggesting that the events causing the changes in the FTIR spectra are the same as those causing
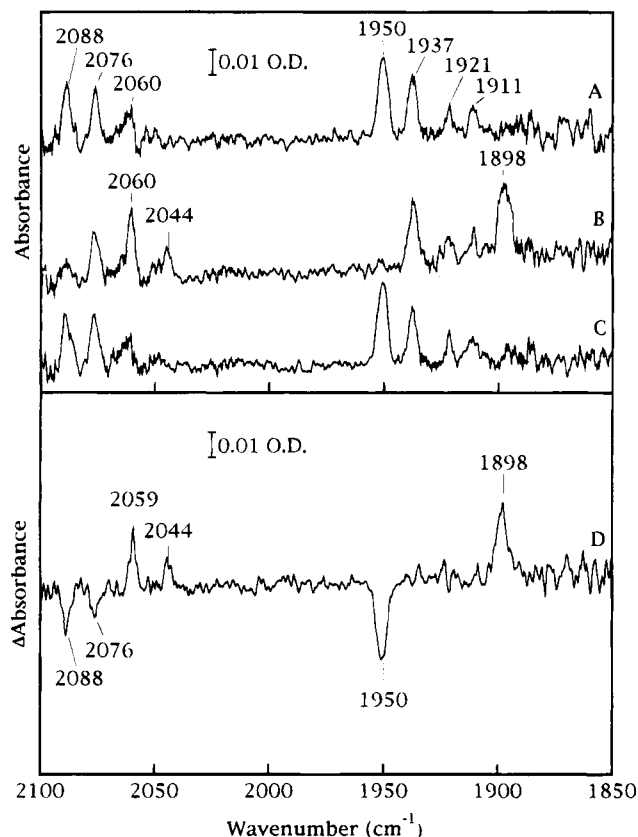

FIGURE 3: Infrared spectra of *C. vinosum* hydrogenase that was repeatedly evacuated and flushed with 1 bar of H_2, incubated for 30 min at 50 °C, cooled on ice, and finally evacuated and flushed repeatedly with 1% H_2. (A) Absolute IR absorbance spectrum of enzyme in the dark at 23 K. (B) IR spectrum 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) to 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 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 Ni$_a$(I)·H$_2$ state disappeared upon illumination, and another set of three bands appeared at 2058, 2044, and 1898 cm^{-1}.

Enzyme under 1 Bar of H_2: The Ni$_a$(0) State. In Figure 4, a set of spectra is shown of enzyme completely reduced with H_2. 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 Ni$_a$(I)·H$_2$ species (see Figure 3), which cannot be completely reduced under 1 bar of H_2 (Coremans et al., 1992a). Apparently, there is also a slight light-induced
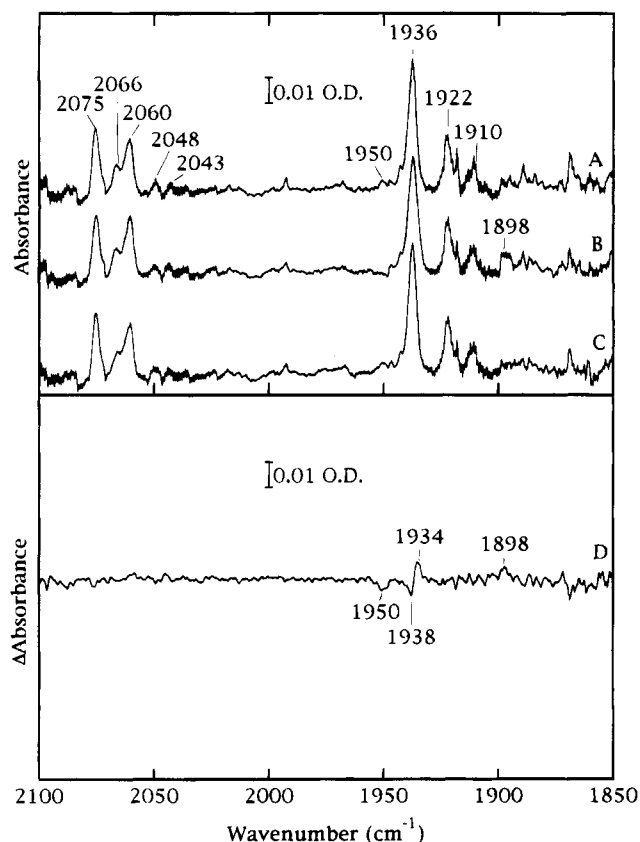
FIGURE 4: Infrared spectra of *C. vinosum* hydrogenase that was repeatedly evacuated and flushed with 1 bar of H_2, incubated for 30 min at 50 °C, and then cooled on ice. (A) Absolute IR absorbance spectrum in the dark at 23 K. (B) Absolute IR absorbance spectrum after illumination with white light for 10 min at 23 K. The spectrum was accumulated while illuminating the sample with white light from a slide projector. (C) IR absorbance spectrum at 23 K in the dark, 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 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.

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 $Ni_a(0)$ state[3] 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 $Ni_a(0)$ species. Apparently, the transition from $Ni_a(0)$ to $Ni_a(I)\cdot H_2$ upon going from 1 bar of H_2 (D_2) to 1% H_2 (D_2) 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 $Ni_a(I)\cdot H_2$ state (Coremans et al., 1992a).

The $Ni_{r,u}(II)$ State. Handling of the very minute and concentrated samples required for these studies (10 μL, 1.5−

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[3] As will be discussed later in the text, the band at 1910 cm^{-1} probably arises from enzyme molecules that are not completely reduced (perhaps in the Ni(II) state).
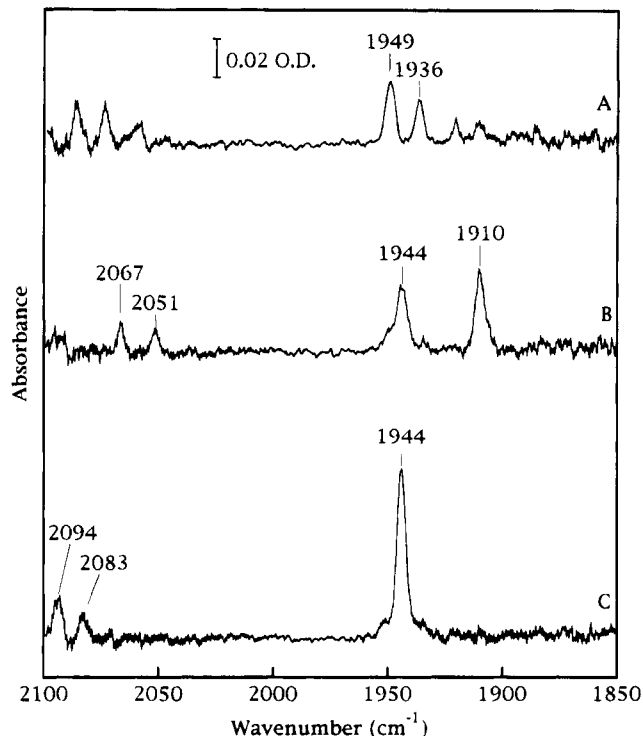

FIGURE 5: Absolute IR spectra of *C. vinosum* enzyme that had been repeatedly evacuated and flushed with 1 bar of H_2, incubated for 30 min under 1 bar of H_2, cooled on ice, and finally evacuated and flushed repeatedly with 1% H_2. (A) IR spectrum at 23 K in the dark. (B) IR spectrum in the dark at 23 K after the enzyme from trace A had been exposed to air through open filling ports in the IR cell at room temperature for 13 h. (C) IR spectrum in the dark at 23 K after the enzyme had been exposed to air via open filling ports in the IR cell for an additional 19 h at room temperature. Spectral resolution was 1 cm^{-1}. The reference spectrum was an empty infrared cell at room temperature. The absolute infrared difference spectra were baseline corrected using the same parabolically shaped curve for all three spectra.

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 $Ni_{r,u}(II)$ 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 srews. An example of the results is shown in Figure 5.

After spectra of the Ni-C form were measured in a H_2O/H_2 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 $Ni_{r,u}(III)$ 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 $Ni_{r,u}(II)$ 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
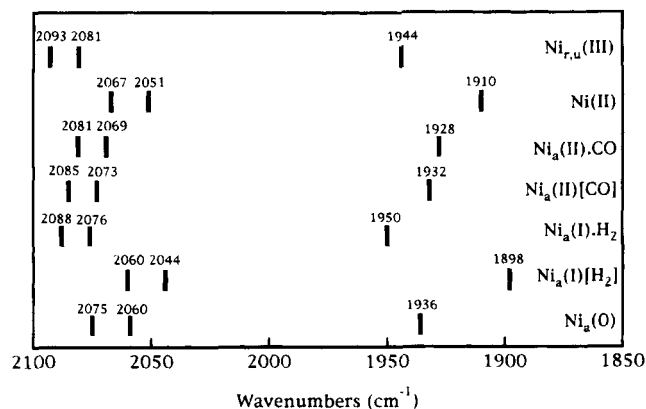
FIGURE 6: Behavior of the set of three IR bands in various redox forms of the *C. vinosum* enzyme. On the right-hand side of the figure we indicate the several states of the enzyme using the notation described earlier in the text. In addition, we use $Ni_a(II)[CO]$ and $Ni_a(I)[H_2]$ to indicate the states formed after illumination of the $Ni(II) \cdot CO$ and $Ni_a(I) \cdot H_2$ states, respectively.

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 $Ni_a(I) \cdot D_2/D_2O$ 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 [$Ni_{r,u}(III)$ and $Ni_{r,u}$(II) states] and the $Ni_a(II) \cdot CO$ light and dark states. The separation is noticeably smaller (132 cm^{-1}) in the $Ni_a(I) \cdot H_2$ (dark) and $Ni_a(0)$ states and larger (154 cm^{-1}) in the Ni_a-(I)$\cdot H_2$ 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 $Ni_a(0)$ state of the enzyme displays weak bands at 1921 and 1910 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 $Ni_u(II)$ 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 $Ni_a(0)$ 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 $Ni_a(0)$. 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 H_2O/H_2 for D_2O/ D_2. We conclude that none of these bands involve vibrations for groups with significant contributions from $\nu(X-H)$, 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 in 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 $Ni_r(III)$ and $Ni_u(III)$, 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, S. 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 $Ni_r(III)$ 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 H_2-reduced active enzyme [$Ni_a(0)$ state] was extensively evacuated and flushed with CO. It was concluded that the species presumed to be formed, the $Ni_r(II)$•CO and the $Ni_a(II)$•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 H_2 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 $Ni_{r,u}(III)$ and $Ni_{r,u}(II)$ states, an extra ligand to nickel is present, which prevents the binding of CO. This would also explain why the FTIR spectra of Ni_a-(II) formed after photolysis of CO from the $Ni_a(II)$•CO state (Bagley et al., 1994) are different from those of Ni(II) obtained upon the oxidation of $Ni_a(I)$•H_2 (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 $Ni_{r,u}(II)$ and $Ni_{r,u}$-(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 $Ni_a(I)$•H_2 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 $Ni_a(0)$ 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 *Thiocapsa 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-
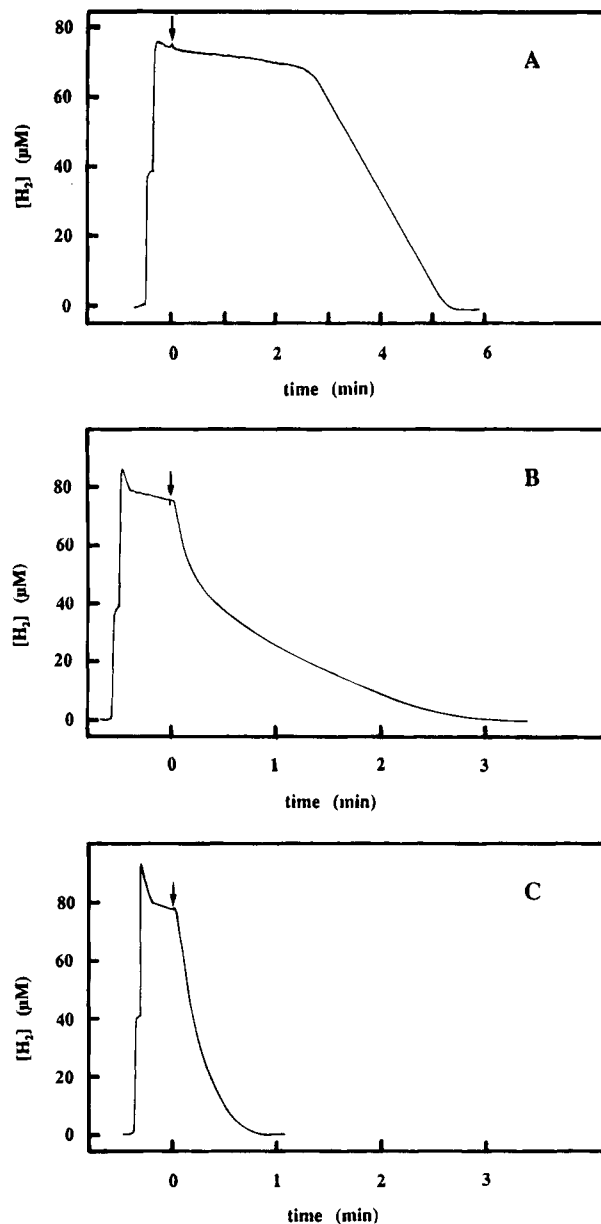

FIGURE 7: Hydrogen uptake activity of *C. vinosum* hydrogenase incubated under different conditions. Hydrogenase was dissolved in 100 mM Tris•HCl buffer (pH 8.0). The activity was assayed as described previously (Coremans et al., 1989). Two aliquots of hydrogen-saturated water were added to the reaction vessel. When the baseline had stabilized, enzyme was added (arrow) and the hydrogen concentration was followed in time. The traces differ solely in the way that the hydrogenase was treated prior to running the assay. (A) Hydrogenase was incubated with 10 mM ascorbate and 1 mM phenazine methosulfate (PMS) under Ar for 40 min at 45 °C. After repeated evacuation and flushing with Ar, the incubation was continued for another 30 min. (B) Enzyme was incubated with 10 mM ascorbate and 1 mM PMS under Ar for 40 min at 45 °C. After repeated evacuation and flushing with CO, the incubation was continued under CO for another 30 min. (C) Enzyme was incubated under H_2 for 40 min at 45 °C. After repeated evacuation and flushing with CO, the incubation was continued under CO for another 30 min.

(III) center. Upon photolysis of the $Ni_a(I)$•H_2 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

studies. It is conceivable that bound hydrogen in the Ni_a-$(I)\cdot H_2$ state can take up considerable charge density from the nickel; formally, a $Ni_a(III)\cdot(H^-)_2$ state is isoelectronic. If the nickel–hydrogen bond is broken, however, this charge-buffering capacity of hydrogen is no longer available, and the effective charge density on the nickel therefore might increase appreciably. In keeping with this possibility, we note that back donation of electrons from the metal to the antibonding orbitals of molecular hydrogen, when coordinated to the metal in an η^2 manner, is expected to facilitate the metal-assisted cleavage of H_2 (Kubas, 1988).

Upon reduction of the $Ni_a(I)\cdot H_2$ state to the $Ni_a(0)$ state, increased charge density on the nickel ion is expected again. This implies that the groups responsible for the set of three bands are situated very close to the nickel (presumably ligands) and respond to changes in the effective charge density on the nickel center, changes that are too small to be detected by X-ray absorption spectroscopy. The more charge density at the nickel site, the lower the frequencies of these groups.

One potential problem with this hypothesis is the behavior of this set of three bands upon photolysis of CO from the $Ni_a(II)\cdot CO$ 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 $Ni_a(II)\cdot CO$ is detected at 2060 cm^{-1}, nearly 100 cm^{-1} lower than the frequency for free CO. We therefore conclude that, in the $Ni_a(II)\cdot CO$ species, CO binds to the Ni predominantly as a π-acceptor and that upon photolysis of CO from $Ni_a(II)\cdot CO$ 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 $Ni_a(II)\cdot CO$ 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 $Ni_{r,u}(III)$ to the $Ni_{r,u}(II)$ state was 34 cm^{-1}, while photolysis of H_2 from the $Ni_a(I)\cdot H_2$ 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 $Ni(II)\cdot CO$ 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 $Ni_a(0)$ are still detectable in the IR spectrum of the enzyme under 1% H_2 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 $Ni_a(I)\cdot H_2$ state. At pH 6.0, however, up to 90% of the nickel can be observed with EPR under an atmosphere of 1% H_2 (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 $Ni_r(II)\cdot CO$ 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 $Ni_r(II)\cdot CO$ 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}) (Horwitz & 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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