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Infrared Studies on the Interaction of Carbon Monoxide with Divalent Nickel in Hydrogenase from *Chromatium vinosum*[†]

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ABSTRACT: Infrared spectra of a carbon monoxy-bound form of the EPR silent Ni(II) species of hydrogenase isolated from *Chromatium vinosum* are presented. These spectra show a band at 2060 cm^{-1} due to ν(CO) for a metal–CO complex. This absorbance shifts to 2017 cm^{-1} upon exposure of the enzyme to ^{13}CO. This band is attributed to ν(CO) from a Ni(II)-CO species. It is shown that the CO on this species is photolabile at cryogenic temperatures but rebinds to form the original carbon monoxy species at temperatures above 200 K. In addition to the ν(CO) band, infrared lines are detected at 2082, 2069, and 1929 cm^{-1}, which shift slightly higher in frequency upon photolysis of the CO from the Ni. These infrared bands do not arise from CO itself on the basis of the fact that the frequency of these bands is unaffected by exposure of the enzyme to ^{13}CO. Experiments in D_2O show that these bands do not arise from an exchangeable hydrogen species. It is concluded that these non-CO bands arise from species near or coordinated to the Ni active site. The possible nature of these bands is discussed.

Hydrogenases catalyze the simplest chemical reaction in nature: $H_2 \rightleftharpoons 2H^+ + 2e^-$. They occur in a wide variety of microorganisms. Presently two classes of hydrogenases can be distinguished on the basis of their metal content: (1) enzymes containing Fe as the only metal (Fe-hydrogenases) and (2) enzymes containing Ni as well as Fe (Ni-hydrogenases) (Adams, 1990; Albracht, 1990; Cammack, 1988; Fauque et al., 1988; Lancaster, 1988; Przybyla et al., 1992; Voordouw, 1992). Hydrogenase [the hydrogen-uptake enzyme (Coremans et al., 1992b)] from the purple-sulfur bacterium *Chromatium vinosum* (strain DSM 185) is a water soluble protein consisting of two subunits of 32 and 62 kDa. In routine preparations, approximately 1 mol of nickel, 11–12 mol of Fe, and 9 mol of acid-labile sulfur are detected per 94 kDa molecular mass. Like in the enzyme from *Desulfovibrio gigas* (Huynh et al., 1987; Teixeira et al., 1989), the *C. vinosum*[1] enzyme contains one [3Fe–4S] cluster and two [4Fe–4S] clusters as verified by Mössbauer spectroscopy (Surerus et al., 1994). As isolated, the enzyme is fully oxidized and usually shows two different $S = {}^1/_2$ EPR signals, both of which are generally believed to arise from Ni(III) (Albracht et al., 1983, 1984; Van der Zwaan et al., 1990). Fernandez et al. (Fernandez et al., 1985, 1986) demonstrated with the enzyme from *D. gigas* that the two forms of the oxidized enzyme are associated with differing abilities to activate H_2. One form of the enzyme (g_{xyz} = 2.33, 2.16, 2.02) was able to readily react with H_2 and was called the "ready" enzyme, while the second form (g_{xyz} = 2.31, 2.23, 2.02) could react only after prolonged contact (hours) with H_2 and was termed "unready". The conformations of nickel in "ready" and "unready" enzymes were indicated as Ni-B and Ni-A, respectively. In the past, the corresponding nickel conformations in the *C. vinosum* enzyme had been referred to as Ni-a (g_{xyz} = 2.34, 2.16, 2.01) and Ni-b (g_{xyz} = 2.32, 2.24, 2.01). In order to avoid confusion in nomenclature, we will refer to the two forms of the oxidized enzyme herein as Ni$_r$ for the ready enzyme and Ni$_u$ for the unready enzyme.

Even when the enzyme is in its ready form, it is not active right away. Nearly all nickel hydrogenases are transformed to the active state after a process generally referred to as "reductive activation". In this paper, we will refer to the Ni in "active" enzyme by adding the subscript a, i.e., Ni$_a$.

Redox titrations of nickel hydrogenases in the presence of mediating dyes have made it clear that in enzymes from a variety of sources [*D. gigas* (Teixeira et al., 1983, 1989), *C. vinosum* (Coremans et al., 1992a,b) and *Desulfovibrio vulgaris*, strain Miyazaki (Asso et al., 1992)] at least four different redox states with respect to the EPR properties of nickel can be observed. In both the inactive forms of hydrogenase from *C. vinosum*, Ni$_r$(III) and Ni$_u$(III), a n = 1 transition, as evidenced by the disappearance of the $S = {}^1/_2$ EPR signals associated with the Ni, could be observed in the presence of mediating dyes at pH = 8 and 30 °C at exactly the same midpoint potential (E_0' = –115 mV) (Coremans et al., 1992a). This transition was reversible and ascribed to a Ni(III)/Ni(II) transition for both the ready and unready forms of the enzyme. In the *D. gigas* enzyme, a similar transition has only been reported for the unready form of the enzyme (Cammack et al., 1987; Huynh et al., 1987; Teixeira et al., 1983, 1989). In reductive titrations with the enzyme from *D.*

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

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vulgaris Miyazaki, the $Ni_r(III)$ and $Ni_u(III)$ EPR signals disappeared with midpoint potentials of −230 and −310 mV, respectively. It was not reported, however, whether or not these titrations were reversible (Asso et al., 1992).

After reductive activation, nickel hydrogenases can be poised in three different redox states associated with the nickel site. Both at low potential (<−450 mV) and at high potential (>−100 mV), the Ni is EPR silent, while in the intermediate potential region, an $S = {}^1/_2$ EPR signal (g_{xyz} = 2.20, 2.15, 2.01) for Ni that is generally called Ni-C or form C is detected. This Ni-C signal has been observed in all enzymes investigated thus far.

To enable a clear and easy distinction of the various oxidation states of the enzyme as reflected by the EPR spectra of nickel, the states are indicated here as $Ni_r(III)/Ni_r(II)$, $Ni_u(III)/Ni_u(II)$, $Ni_a(II)$ (for the active enzyme which is EPR silent and formed at intermediate redox potentials), $Ni_a(I)$ (often called Ni-C), and $Ni_a(0)$ (the active enzyme formed at low potential, which is EPR silent). It must be stressed at this point that this terminology is by no means intended to indicate the true valence states of the nickel ion in these oxidation states of the enzyme. Rather, it is an indication of the redox states of the nickel site, including the metal and nearby redox-active species, and is used only for clear discussion. X-ray absorption measurements of the *D. gigas* enzyme (Eidsness et al., 1988) and the *Thiocapsa roseopersicina* enzyme (Whitehead et al., 1991, 1993) show no appreciable shifts of the K-edge energy of nickel in any of the redox states of the enzyme. This indicates that the effective charge density of the nickel ion does not change noticeably and that redox changes of the nickel site are probably buffered by its ligands and/or the Fe-S clusters.

The states indicated as $Ni_{r,u}(III)$ and $Ni_a(I)$ show $S = {}^1/_2$ EPR signals which are distinct from each other. The $Ni_a(II)$ and $Ni_a(0)$ states of the *C. vinosum* enzyme show no MCD signals for the nickel (Cheesman, 1989; M. R. Cheesman, J. W. Van der Zwaan, S. P. J. Albracht, and A. J. Thomson, unpublished results). The Ni(II) state of the *D. gigas* enzyme shows no magnetic susceptibility for the nickel (Wang et al., 1992). This probably means that in the EPR silent states, $Ni_a(II)$ and $Ni_a(0)$, the nickel is diamagnetic, most likely as low-spin divalent nickel. For hydrogenase from *C. vinosum* and *M. thermoautrophicum*, in the absence of mediating dyes, only the $Ni_a(I)/Ni_a(0)$ transition exists in a redox equilibrium with H_2 (Coremans et al., 1992b) and it shows a simple Nernst $n = 2$ behavior.

Van der Zwaan et al. (Van der Zwaan et al., 1985, 1986) have provided evidence that the $Ni_a(I)$ state in hydrogenase from *C. vinosum* has hydrogen, possibly in the form of dihydrogen, bound to Ni and that this bond is photolabile at temperatures below 77 K. We will refer to this state as $Ni_a(I)$-H_2. Carbon monoxide can replace hydrogen, resulting in a different EPR spectrum (called herein $Ni_a(I)$-CO) (Van der Zwaan et al., 1986). This CO complex is likewise photolabile at low temperatures. The experiments of Van der Zwaan et al. (Van der Zwaan et al., 1986) also showed that upon complete replacement of H_2 by CO in the gas phase of the active enzyme solution, the EPR signal of $Ni_a(I)$-CO disappeared. Reintroduction of H_2 led to the transient appearance of this signal and in the end to the reappearance of the original $Ni_a(I)$-H_2 state, thus showing that CO binding was completely reversible. Additionally, incubation of oxidized enzyme with CO for 5 h at room temperature has no effect on the EPR signals of the Ni(III) species or the Fe–S clusters (Van der Zwaan et al., 1986). These experiments point to the possibility that CO might also be bound to the $Ni_a(II)$ state and that in a mixture of CO and low H_2 concentrations the reversible reaction, $Ni_a(II)$-CO/$Ni_a(I)$-CO, might be observed.

In this paper, we present infrared studies on a carbon monoxy form of the EPR silent $Ni_r(II)$ and $Ni_a(II)$ species. These infrared spectra provide the first direct evidence that in the EPR silent Ni(II) species, carbon monoxide binds to the nickel and is photodissociable in a manner analogous to the behavior reported for the $Ni_a(I)$-CO species. We report here that the infrared spectra of $Ni_r(II)$-CO and $Ni_a(II)$-CO are indistinguishable and that the $Ni_u(II)$ species does not react with CO. The possible consequences of these findings for the active site of nickel hydrogenases are discussed. Finally, we show that upon photolysis of CO from Ni(II) a number of infrared lines between 2100 and 1900 cm^{-1} that do not arise from CO shift in frequency. The origin of these lines is also discussed.

MATERIALS AND METHODS

C. vinosum (strain DSM 185) was grown in a 700 L batch culture (Van Heerikhuizen, 1981) in a medium (Albracht et al., 1983) essentially described by Hendly (Hendly, 1955). Cells were harvested, and the enzyme was isolated from *C. vinosum* and purified as previously described (Coremans et al., 1992a). Hydrogenase, in 50 mM Tris-HCl, pH = 8.0, was concentrated to 1.3–2.6 mM, using a Centricon-30 filter. Hydrogenase was converted into the $Ni_r(III)$ form (85%) as previously described (Van der Zwaan et al., 1990) and stored at or below −80 °C prior to the sample manipulations described herein. For the infrared experiments, the Ni(II)-CO species was made in two different ways. (1) Via reduction with 1 bar of H_2: the enzyme (on ice) was alternately degassed and flushed with hydrogen gas for at least eight cycles. The sample was then incubated at 50 °C for 30 min. This leads to complete activation and maximal reduction of the enzyme. Finally, carbon monoxide was added to the ice-cooled sample by alternately degassing and flushing the sample with CO for six or more cycles. (2) Via reduction with ascorbate and phenazine methosulfate (PMS): the ice-cooled enzyme was alternately degassed and flushed with carbon monoxide for six cycles. Ascorbate (in 50 mM Tris-HCl, pH = 6.4) and PMS (in 50 mM Tris-HCl, pH = 8.0) were then added under anaerobic conditions to a final ascorbate concentration of 50 mM and a final PMS concentration of 200 μM. The enzyme was subsequently incubated at 50 °C for 40 min during which time the enzyme was shaken gently from time to time to maintain good contact with the carbon monoxide gas. The above procedures took place in septa-sealed cells. After these manipulations, the sample was loaded into an anaerobic infrared transmitting cell (total sample volume of approximately 10 μL, optical pathlength of approximately 50 μm) which had been flushed with Ar gas for at least 15 min prior to loading the cell. We note that 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 herein for experiments performed with these two window materials. The infrared cell was loaded inside an argon-flushed glovebag using a gas-tight syringe which had been flushed with argon. 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 by passage over a palladium catalyst (Degussa, type E236P). The enzyme was exchanged into D_2O for selected experiments by concentrating the enzyme in a Centricon-30 concentrator and rediluting the

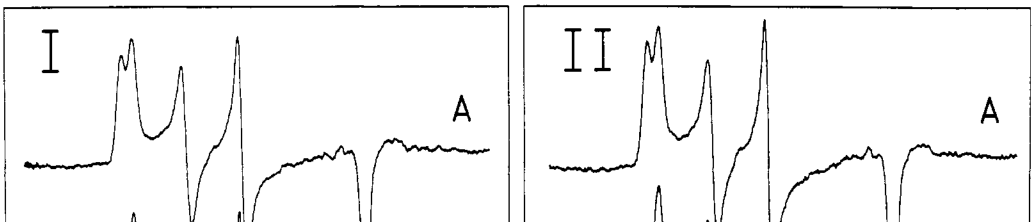

FIGURE 1: Specific reduction of Ni$_r$(III) induced by CO in a mixture of *C. vinosum* hydrogenase and ascorbate/PMS. I: (A) Purified hydrogenase (40 μM in Tris-HCl, pH = 7.4), under an Ar atmosphere in a Thunberg-type EPR tube, was mixed with ascorbate (pH = 6, final concentration 20 mM) and PMS (final concentration 200 μM) and incubated for 1 h at room temperature (22 °C). The enzyme solution was frozen in liquid nitrogen and inspected by EPR spectroscopy. (B) The sample was thawed and incubated for 3 min at 50 °C, after which the sample was again frozen and inspected by EPR spectroscopy. (C) EPR spectrum of frozen enzyme taken after an additional 22 min of incubation at 50 °C. (D) EPR spectrum of frozen sample after a total incubation time of 1 h at 50 °C. Enzyme activities are not impaired under these conditions. II: (A) EPR spectrum of enzyme as treated in IA. (B) After the solution had thawed, the gas phase was exchanged for CO by extensive evacuation and flushing with CO. Thereafter the sample was incubated for 3 min at 50 °C. (C) The sample was incubated for an additional 21 min at 50 °C. (D) The sample was incubated for a total time of 60 min at 50 °C. EPR conditions: microwave power incident to the cavity was 2 mW; modulation amplitude was 1.25 mT; modulation frequency was 100 kHz.

enzyme into 50 mM Tris-DCl, pD = 8.0, a total of three times (dilution factor of >40 each time). Infrared spectra of the Ni$_r$(III) species were taken on a sample which was not degassed prior to the measurement. Low-temperature (20–250 K) infrared spectra were collected on a BioRad FTS-40 FTIR spectrometer equipped with a HgCdTe detector. The sample holder was mounted in a low-temperature optical cryostat (APD Cryogenics Heli-tran LT-3-110) equipped with NaCl windows. The cryostat was mounted in the FTIR such that it was physically separated from the frame of the spectrometer, so as to minimize the possibility that vibrations of the cryostat could be translated to the spectrometer. 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 the 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 (9 GHz) were obtained with a standard Varian E-9 EPR spectrometer or a Bruker ECS 106 EPR spectrometer; in both cases, the field modulation frequency was 100 kHz. Cooling of the sample was performed by using the helium-flow system as described by Lundin and Aasa (Lundin & Aasa, 1972) (Varian machine) or with an Oxford Instruments ESR 900 cryostat with a ITC4 temperature controller (Bruker machine). The magnetic field was calibrated with an AEG magnetic field meter. The microwave frequency was measured with an HP 5244A frequency converter. The E-line spectrometer console was connected to a personal computer via a 12-bits A/D converter. Simulation was carried out as has been described elsewhere (Beinert & Albracht, 1982).

RESULTS

The midpoint potentials of the Ni(III)/Ni(II) for both the ready and unready enzymes in *C. vinosum* are the same (Coremans et al., 1992a). Its value, –115 mV at pH = 8.0, prevents reduction by ascorbate (E'_0 = 58 mV at pH = 7; Ball, 1937) in the presence of the redox mediator PMS. Under a CO atmosphere, we have found that incubation of hydrogenase from *C. vinosum* leads to the selective and complete reduction of Ni$_r$(III) as evidenced by the loss of the Ni$_r$(III) $S = {}^1/_2$ EPR signal, whereas the Ni$_u$(III) signal remained unchanged. This is demonstrated in Figure 1; incubation with ascorbate/PMS for 1 h at room temperature under an Ar atmosphere only affected a small part of the enzyme molecules, in which the Ni(III) species were spin coupled to the 3Fe-cluster. The coupling disappeared as a result of reduction (Coremans et al., 1992a), and a very slow reduction of the 3Fe-cluster set in (not shown). When monitored at 50 K,

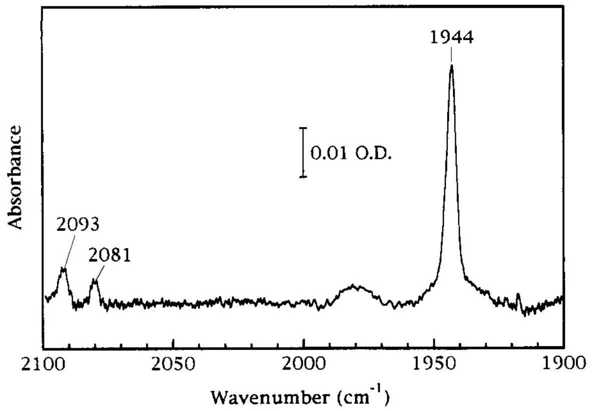

FIGURE 2: Infrared absorbance spectrum of untreated, oxidized hydrogenase from *C. vinosum* at 20 K. EPR measurements indicated that this untreated enzyme contained 85% Ni_r(III) and 15% Ni_u(III). Spectral resolution is 1 cm^{-1}. The reference spectrum for the absorbance calculation was an infrared spectrum of the empty infrared cell at room temperature.

both nickel signals increased somewhat in amplitude as a result of the breakage of the spin coupling (Albracht et al., 1984; Coremans et al., 1992a). The resulting nickel spectrum is shown in Figure 1IA. Even when the temperature was increased to 50 °C, no reduction of nickel occurred due to its redox potential. After 1 h at 50 °C, a slight conversion of Ni_u(III) to Ni_r(III) was noticed but there was no overall reduction of nickel. By that time, the 3Fe-cluser became reduced by more than 90% (not shown). In contrast, with CO as the gas phase, a selective disappearance of the Ni_r(III) signal was observed within 1 h at 50 °C (Figure 1II). The 3Fe-cluster reduction proceeded as in the Ar control experiment. This suggests that CO binds specifically to Ni_r(II), thereby increasing the apparent midpoint potential of the Ni_r(III)/Ni_r(II) couple to a value well above 0 mV. When CO was removed from the enzyme as treated in Figure 1IID and was replaced by air, an EPR signal due to Ni_u(III) was observed which represented a spin concentration comparable to the total spin concentration of Ni_r(III) plus Ni_u(III) which was present in Figure 1IIA (data not shown).

Since the Ni(II) species are EPR silent, we have turned to infrared spectroscopy to examine these Ni(II)-CO species further. Infrared spectroscopy can provide information on protein species that are otherwise spectroscopically silent and is particularly useful when applied in conjunction with π-acid ligands (CO, RCN, etc.) which have infrared absorbances near 2000 cm^{-1} where few intrinsic protein vibrations occur. For example, carbon monoxide complexed to metal ions displays an intense, sharp infrared absorbance between 1800 and 2100 cm^{-1} due to the C≡O stretch [ν(CO)] of a terminally bonded CO species (Horowitz & Shriver, 1984; Nakamoto, 1978), while uncomplexed CO absorbs at significantly higher frequencies (2155 cm^{-1}) (Nakamoto, 1978) and is generally too weak and broad in solution to be detected. The infrared spectra in the spectral region between 2100 and 1900 cm^{-1} are shown in Figures 2–6 for various forms of the enzyme.

The infrared spectrum of untreated, oxidized enzyme is shown in Figure 2. In this spectrum, a large band is detected at 1944 cm^{-1} along with two much smaller bands at 2093 and 2081 cm^{-1}. These bands are not affected by shining light on the sample at cryogenic temperatures (80 or 20 K) (spectra not shown). Figure 3A shows the infrared spectra for enzyme reduced with ascorbate/PMS under a CO atmosphere. The bands associated with oxidized enzyme at 1944, 2093, and 2081 cm^{-1} have become smaller, and bands at 1929, 2060, 2069, and 2082 cm^{-1} are now detected. Illumination of the sample in the infrared cell at 20 K leads to a noticeable change in the infrared spectrum as shown in Figure 3B. Namely, the 2060 cm^{-1} band disappears, whereas the bands at 2082, 2069, and 1929 cm^{-1} shift to 2085, 2073, and 1932 cm^{-1}, respectively. In agreement with the expectation that the 1944 cm^{-1} band arises from residual oxidized enzyme, it is not affected by illuminating the sample. The difference spectrum between the sample in the dark and the sample during illumination is shown in Figure 3D. We note that the light-induced changes at 20 K persist after turning off the photolysis light. After warming the sample to 200 K in the dark and equilibrating the temperature for 10 min or more, the infrared spectrum originally present in the dark is recovered (Figure 3C). Finally, the light-induced effects could be reproduced again at either 20 or 78 K, and the dark- and light-induced spectra taken at 78 K were identical to those obtained at 20 K.

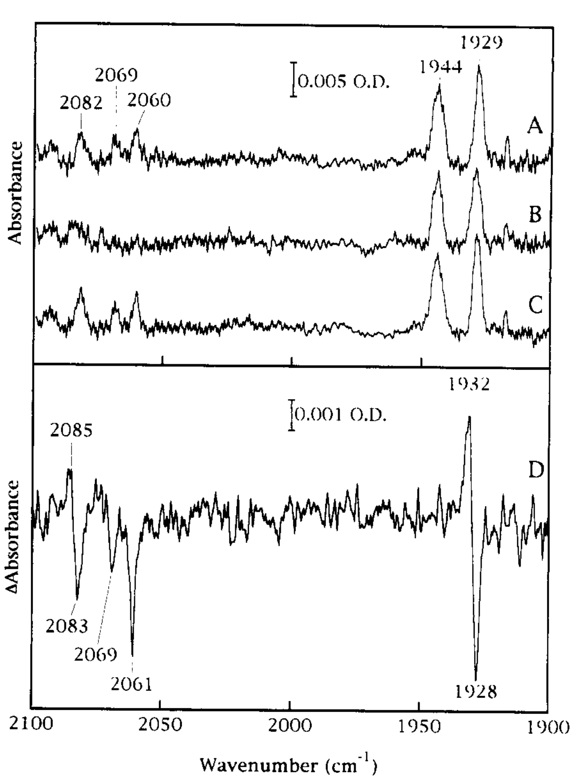

FIGURE 3: Infrared spectra of hydrogenase from *C. vinosum* poised in the Ni_r(II)-CO state by reducing the enzyme with ascorbate in the presence of PMS and CO. (A) Spectra collected in the dark at 20 K. (B) Spectra collected at 20 K while illuminating the sample with white light from a slide projector. (C) Spectra collected at 78 K in the dark after warming the illuminated sample to 200 K in the dark and allowing the sample to equilibrate for more than 10 min. (D) Light minus dark infrared difference spectra formed by subtracting the 20 K spectra of the sample in the dark from the light-induced spectra at 20 K. Spectral resolution was 1 cm^{-1}. The reference spectrum for the absorbance calculations in A–C was the infrared spectrum of an empty infrared cell at room temperature.

Infrared spectra, experimentally indistinguishable from those described above, were obtained by first fully activating and reducing the enzyme with H_2 and then replacing the H_2 in the gas phase by CO. The results are shown in Figure 4A–D. In this state, the enzyme showed the same light sensitivity changes as observed in the enzyme reduced with ascorbate/PMS under a CO atmosphere (compare Figure 3A to Figure 2A). These changes were also reversible upon warming the sample to 200 K. For convenience, the light minus dark infrared difference spectra for the Ni(II)-CO species formed by fully reducing the enzyme with H_2 and then

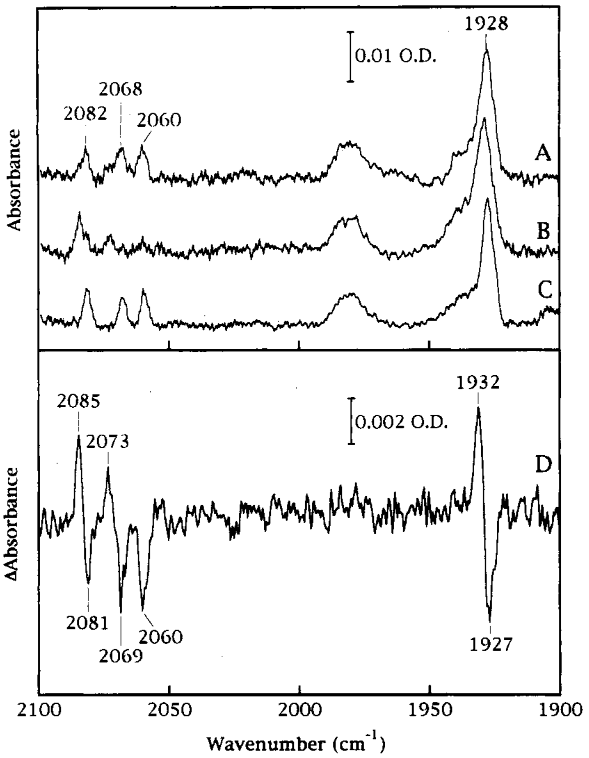

FIGURE 4: Infrared spectra of hydrogenase from *C. vinosum* poised in the $Ni_a(II)$-CO state by activating and fully reducing the enzyme under an H_2 atmosphere followed by replacing the H_2 atmosphere with CO. (A) Spectra collected in the dark at 20 K. (B) Spectra collected at 20 K while illuminating the sample with white light from a slide projection. (C) Spectra collected at 78 K in the dark after warming the illuminated sample to 200 K in the dark and allowing the sample to equilibrate for more than 10 min. (D) Light minus dark infrared difference spectra formed by subtracting the 20 K spectra of the sample in the dark from the light-induced spectra at 20 K. Spectral resolution was 1 cm^{-1}. The reference spectrum for the absorbance calculations in A–C was the infrared spectrum of an empty infrared cell at room temperature.

replacing the H_2 with CO and that for the enzyme reduced using ascorbate in the presence of PMS and CO are shown in Figure 5A,B, respectively. These light-induced differences are also experimentally indistinguishable from those described in the foregoing discussion.

Figure 5C shows the light minus dark infrared difference spectrum for hydrogenase reduced under 1 bar of H_2 and then treated with CO as in Figure 5B but in which the carbon monoxide is isotopically labeled with ^{13}C. Comparison of this spectra with the analogous spectrum for natural abundance CO shows that the downward going 2060 cm^{-1} band in the natural abundance CO spectrum (Figure 5B) shifts to 2017 cm^{-1} in the ^{13}CO spectrum (Figure 5C) while the differential features at 2085/2082, 2074/2069, and 1931/1928 cm^{-1} remain unchanged. From these spectra, it is clear that the 2060 cm^{-1} band in Figure 5B arises from the stretching frequency of CO bound to the enzyme; it is shifted to 2017 cm^{-1} when using ^{13}CO (Figure 5C), close to the shift calculated for ν(CO) using the diatomic approximation. The positions of the remaining bands are not affected by ^{13}CO and hence do not arise from CO itself.

Finally, we have also measured infrared spectra for the Ni(II)-CO species formed by fully activating and reducing the enzyme with D_2 in D_2O followed by replacing the D_2 with CO. No discernible differences in frequency between either the dark- or light-induced infrared spectra for enzyme reduced with H_2 in H_2O as compared to treating the enzyme with D_2 in D_2O were found (spectra not shown).

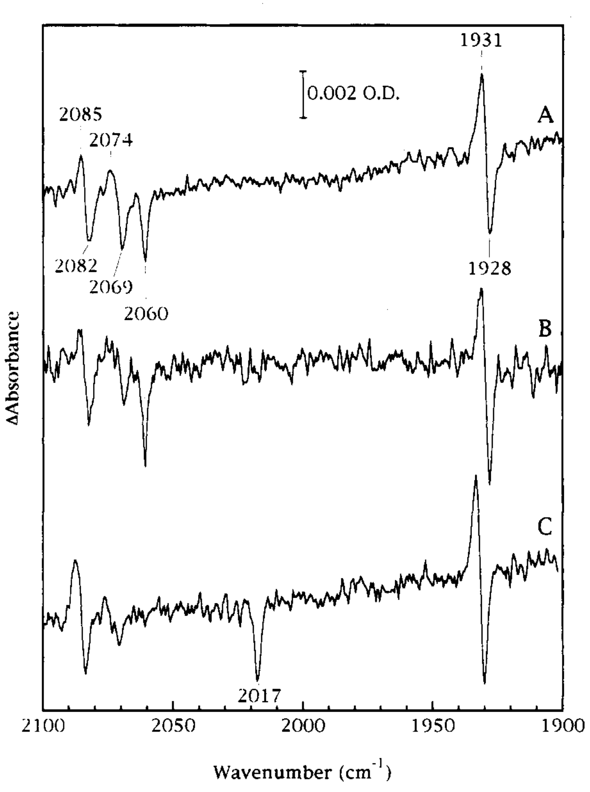

FIGURE 5: Comparison of the light minus dark infrared difference spectra for the CO-treated, EPR silent state of hydrogenase from *C. vinosum*. (A) The $Ni_a(II)$-CO state formed by activating and reducing the enzyme with H_2 and then replacing the H_2 with CO (isotopes at natural abundance), i.e., the $Ni_a(II)/Ni_a(II)$-CO difference spectrum. (B) The $Ni_r(II)$-CO state formed by reducing the hydrogenase with ascorbate in the presence of PMS and CO (isotopes at natural abundance), i.e., the $Ni_r(II)/Ni_r(II)$-CO difference spectrum. (C) The $Ni_a(II)$-^{13}CO state formed by reducing the hydrogenase with ascorbate in the presence of PMS and ^{13}CO (>90% ^{13}C), i.e., the $Ni_a(II)/Ni_a(II)$-^{13}CO difference spectrum. Spectral resolution is 1 cm^{-1}. All spectra were taken at 78 K.

DISCUSSION

In EPR experiments, when enzyme containing a mixture of $Ni_r(III)$ and $Ni_u(III)$ species was treated with ascorbate plus PMS under a CO atmosphere at 50 °C, the intensity of the $Ni_r(III)$ signal decreased with time. Ascorbate does not react with the enzyme, but reduced PMS, functioning as a mediator, is able to react slowly. With 200 μM PMS, in the presence of CO, full reduction of the $Ni_r(III)$ species could be accomplished at 50 °C within 1 h. With Ar as the gas phase, there was hardly any effect. The intensity of the $Ni_u(III)$ signal was not significantly affected in either case. These observations can be explained by assuming that CO can bind specifically to $Ni_r(II)$, thereby increasing its apparent midpoint potential [−115 mV at pH = 8.0 (Coremans et al., 1992a)] to such a value that excess ascorbate can reduce it and resulting in an EPR silent Ni(II)-CO species. The present experiments suggest that the apparent midpoint potential of the $Ni_r(III)/Ni_r(II)$ couple under 1 bar of CO is well above 0 mV [in fact, other experiments (J. W. Van der Zwaan, M. Chen, and S. P. J. Albracht, unpublished observations) indicated a potential higher than 350 mV]. When CO is removed, the EPR signal of $Ni_r(III)$ slowly reappeared. These experiments indicate that CO can bind to $Ni_r(II)$ but not to $Ni_u(II)$. This raises the interesting question as to whether H_2 can bind to $Ni_r(II)$ in the enzyme as well.

With the exception of the light insensitive band at 1944 cm^{-1} in the absolute infrared spectra of untreated enzyme, the absolute infrared spectra and the light minus dark infrared difference spectra formed at cryogenic temperatures for carbon monoxy enzyme are identical for reduced carbon monoxy enzyme formed via reduction with 1 bar of H_2 or via reduction with ascorbate and PMS. This observation suggests that in both cases we are forming predominantly the same species. The additional 1944 cm^{-1} line in the reduced carbon monoxy enzyme formed using ascorbate and PMS is attributed to a residual Ni(III) state that was not converted to the Ni(II)-CO species. The infrared spectrum of the untreated enzyme, which was 85% in the Ni_r(III) state and 15% in the Ni_u(III) state as observed in its EPR spectrum, also shows a band at 1944 cm^{-1}. Whether or not Ni_r(III) and Ni_u(III) both show this band is not at present clear. We note that ascorbate does not eliminate the Ni_u(III) EPR signal in the presence of PMS and CO.

For conditions under which the IR experiments on the enzyme reduced with H_2 were performed, we would also expect to produce the Ni(II)-CO state. It is known from earlier experiments (Van der Zwaan et al., 1986) that an EPR detectable Ni(I)-CO species can only be obtained from H_2-reduced enzyme immediately after replacement of H_2 by CO. Prolonged incubation and repeated evacuation and flushing with CO, as was performed in the present study, led to the complete loss of the EPR signal associated with the Ni(I)-CO species. When H_2 was reintroduced at this stage (Van der Zwaan et al., 1986), a transient reappearance of the Ni(I)-CO signal was observed. Van der Zwaan et al. (Van der Zwaan et al., 1986) also stated that it was possible to stabilize the Ni(I)-CO species at various intensities by using mixtures of H_2, He, and CO. This suggests that an equilibrium between Ni_a(I)-CO and Ni_a(II)-CO might exist which can be shifted by changing the potential via the partial pressure of H_2. Complete removal of the H_2, as is done in the infrared sample preparation, therefore would lead to the formation of the EPR silent Ni(II)-CO state.

The observation that the band at 2060 cm^{-1} in the infrared spectrum of the Ni(II)-CO species disappears completely upon illumination of the sample provides evidence that this band arises from a photolyzable bond in the enzyme. In addition, observation of a complete shift in this band in the Ni(II)/Ni(II)-^{13}CO spectrum to 2017 cm^{-1} provides conclusive evidence that this band arises from carbon monoxide which is photolyzed from the enzyme. We note that the frequency of this band, as well as the magnitude of the shift in frequency that this band experiences in the ^{13}CO species, is consistent with the assumption that the band arises from a Ni(II)-CO species (Kermarec et al., 1989; Sweaney et al., 1989). EPR studies of Van der Zwaan et al. (Van der Zwaan et al., 1990), using ^{13}CO, provide conclusive evidence that CO is bound directly to the Ni site for the Ni_a(I) state in hydrogenase isolated from *C. vinosum.*

The data show that CO can be photolyzed from the Ni(II)-CO species at cryogenic temperatures and that rebinding does not occur at temperatures below 78 K. However, rebinding does occur quickly to the Ni(II) site at temperatures at or above 200 K. We note that these results are reminiscent of the EPR experiments of Van der Zwaan et al. (Van der Zwaan et al., 1986, 1990) in which a Ni_a(I)-CO species was detected at cryogenic temperatures. Photolysis of the CO from the nickel led to the formation of a new EPR spectrum which was stable at 77 K. The EPR detectable Ni_a(I)-CO spectra could be re-formed by warming the sample to 200 K. This analogy suggests that our infrared results for the carbon monoxy adduct of the Ni(II) species also arise from a Ni-CO species which contains a CO that is photolyzable at cryogenic temperatures. All these lines of evidence strongly suggest that in the infrared spectra obtained using either method, we are detecting a photolyzable Ni(II)-CO species.

In addition to the Ni(II)-CO band at 2060 cm^{-1} in the Ni(II) species, bands at 2082, 2069, and 1929 cm^{-1} shift upwards in frequency upon photolysis of the CO from the hydrogenase. The resulting derivative-shaped difference bands remain at the same positions in analogous experiments using ^{13}CO and are therefore not attributable to CO bound to the enzyme. The fact that they shift in frequency after illumination at cryogenic temperatures, but regain their original frequencies at temperatures where the CO rebinds to the hydrogenase, suggests that they arise from non-CO bonds in the enzyme which are perturbed by the photolysis of CO from the Ni(II) site. It is proposed that they arise from species near or coordinated to the Ni site. Given that these bands do not arise from CO, they are most remarkable features. Proteins in general have no vibrations in this frequency region (2100–1900 cm^{-1}). Only vibrations arising from extremely strong bonds (e.g., triply bonded species) or vibrations involving atoms of low mass (e.g., hydrogen) are expected in this region.

In view of the experiments performed in D_2O/H_2O, where no shifts whatsoever were observed, it is also concluded that none of these bands arise directly from a bond to an exchangeable proton nor do they arise from a hydrogen species that is formed during reductive activation of the enzyme.

We now critically examine several possibilities as to the nature of these non-CO bands. The first possibility is that one or more of these bands arise from ν(Ni-H) of a nickel hydride. Infrared studies of Ni-H model compounds are in reasonable agreement with this suggestion, exhibiting ν(Ni-H) between 1800 and 2000 cm^{-1} (Cecconi et al., 1984; Darensbourg et al., 1984; Green & Saito, 1969; Jonas & Wilke, 1969). In particular, square planar Ni(II)-H complexes containing a sulfur ligand exhibit ν(Ni-H) bands near 1930 cm^{-1} (Darensbourg et al., 1984), in good agreement with the possibility that the 1929 cm^{-1} band in the Ni(II)-CO spectra arises from a Ni-H bond. The bands between 2000 and 2100 cm^{-1}, on the other hand, seem somewhat high in frequency compared to the published model compounds. However, given the lack of infrared data on a wide range of nickel complexes with various valence states, we do not feel that it is impossible that the infrared bands we detect between 2000 and 2100 cm^{-1} also arise from Ni-H bonds. However, with regard to the possibility that some of the non-CO bands in the Ni(II)-CO infrared spectra may arise from a Ni(II)-H species, we note that these bands are present regardless of how the hydrogenase is reduced. In particular, the same bands are present in the infrared spectrum when the Ni(II)-CO species is formed with ascorbate/PMS in the absence of hydrogen. In addition, it must be stressed that the experiments in D_2O/D_2 show that no exchangeable hydrogen species gives rise directly to these bands. Therefore we must conclude that if in fact these bands do arise from one or more metal hydrides, they are not accessible to the solvent nor are they formed in the reductive activation of the enzyme. Mechanistically, this would seem to be a remarkable circumstance. Finally, we note that the infrared absorption intensities of M—H bands are generally much weaker than carbonyl (C≡O) absorbances arising from CO complexes to metals rather than nearly equal in intensity as in the data presented here.

Secondly, we consider the possibility that some of the lines in this region of the IR spectra arise from the ν(S-H) of sulfhydryl groups coordinated to either the Ni site or an Fe-S center near the Ni site of the enzyme. Typically ν(S-H) for sulfhydryl species are found between 2550 and 2600 cm^{-1} (Nakanishi & Solomon, 1977) rather than in the spectral region between 2100 and 1900 cm^{-1}. However, there are few known examples of suitable model compounds for a sulfhydryl species coordinated to either Ni or Fe, and in the known metal coordinated sulfhydryl compounds, very little has been reported concerning the infrared frequency of the resulting ν(S-H). Interestingly, an unusually low ν(S-H) at 2290 cm^{-1} has been reported for a ruthenium complex exhibiting strong intermolecular S—H···S bridges (Sellmann et al., 1991). Strong S—H···H bridges with weak ν(S-H) infrared absorbances at 2334 and 2375 cm^{-1} have also been detected in a thiolate salt (Boorman et al., 1992). Given these observations, we must acknowledge the possibility that the non-CO bands that we detect between 1900 and 2100 cm^{-1} in the IR spectra of Ni(II)-CO arise from sulfhydryls coordinated to either the Ni or an Fe-S center near the Ni site. Furthermore, we note that the uncharacteristically low frequencies that we detect may arise from ν(S-H) associated with unusually strong S—H···S bridges in the enzyme's active site. As in the discussion of the M—H possibility, we note that the frequencies of these bands are not affected by suspending the enzyme in D_2O or by reducing the enzyme with D_2. Accordingly we must conclude that they do not arise from sulfhydryls which are accessible to the solvent at any time during the reductive activation. Also, as in the M—H case, the S—H absorbances are expected to be very weak relative to the carbonyl absorbances (D. Sellmann, personal communication).

Finally, we note that coupling of the nickel center to at least two exchangeable protons was observed in hydrogenase from *D. gigas* in ESEEM studies (Chapman et al. 1988) and ENDOR studies (Fan et al., 1991). The protons giving the largest hyperfine coupling were no longer observed in ENDOR spectra after illumination of the Ni_a(I) state in *T. roseopersicina* hydrogenase (Whitehead et al., 1993) and are probably the same protons observed by Van der Zwaan et al. (Van der Zwaan et al., 1985) in EPR spectra of *C. vinosum* enzyme in this redox state. It appears that none of these protons are observed in the infrared spectra between 2100 and 1900 cm^{-1} in the present report of the Ni(II)-CO state of the enzyme.

In summary, these infrared studies provide the first evidence that a photolyzable CO is bound to the Ni site in the EPR silent Ni(II) species of hydrogenase. Our results suggest that the redox state and coordination environment of the Ni in the Ni(II)-CO states that are formed by either reducing the enzyme with ascorbate in the presence of PMS and carbon monoxide or reducing the enzyme with H_2 and then replacing the H_2 with CO are the same, i.e., the nickel sites in the states referred to herein as Ni_r(II)-CO and Ni_a(II)-CO are indistinguishable. Additionally, we have shown that in the Ni(II)-CO species of hydrogenase, several non-CO bonds are present that suggest remarkable structural features and which are perturbed by the photolysis of CO from the Ni(II). Currently, experiments are underway to understand more fully the nature of these non-CO lines. Finally, this report indicates that infrared spectroscopy is a powerful technique, which is uniquely able to provide structure-specific information concerning the redox and coordination environment of the Ni site in the EPR silent states of hydrogenase.

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REFERENCES

Adams, M. W. W. (1990) *Biochim. Biophys. Acta 1020*, 115–145.

Albracht, S. P. J. (1990) in *The Molecular Basis of Bacterial Metabolism* (Hauska, G., & Thauer, R., Eds.) pp 40–51, Springer-Verlag, New York.

Albracht, S. P. J., Kalkman, M. I., & Slater, E. C. (1983) *Biochim. Biophys. Acta 724*, 309–316.

Albracht, S. P. J., Van der Zwaan, J. W., & Fontijn, R. D. (1984) *Biochim. Biophys. Acta 766*, 245–258.

Asso, M., Guigliarelli, B., Yagl, T., & Bertrand, P. (1992) *Biochim. Biophys. Acta* 1122, 50–56.

Ball, E. G. (1937) *J. Biol. Chem. 118*, 219–239.

Beinert, H., & Albracht, S. P. J. (1982) *Biochim. Biophys. Acta 683*, 245–277.

Boorman, P. M., Gao, X., & Parvez, M. (1992) *J. Chem. Soc., Chem. Commun.* 1656–1658.

Cammack, R. (1988) *Adv. Inorg. Chem. 32*, 297–333.

Cecconi, F., Ghilardi, C. A., Innocenti, P., Mealli, C., Midollini, S., & Orlandi, A. (1984) *Inorg. Chem. 23*, 922–929.

Chapman, A., Cammack, R., Hatchikian, E. C., McCracken, J., & Peisach, J. (1988) *FEBS Lett. 242*, 134–138.

Cheesman, M. (1989) Ph.D. Thesis, University of East Anglia, Norwich, U.K.

Coremans, J. M. C. C., Van der Zwaan, J. W., & Albracht, S. P. J. (1989) *Biochim. Biophys. Acta 997*, 256–267.

Coremans, J. M. C. C., Van der Zwaan, J. W., & Albracht, S. P. J. (1992a) *Biochim. Biophys. Acta 1119*, 157–168.

Coremans, J. M. C. C., Van Garderen, C. J., & Albracht, S. P. J. (1992b) *Biochim. Biophys. Acta 1119*, 148–156.

Darensbourg, M. Y., Ludwig, M., & Riordan, C. G. (1984) *Inorg. Chem. 28*, 1630–1634.

Eidsness, M. K., Scott, R. A., Prickril, B. C., DerVartanian, D. V., LeGall, J., Moura, I., Moura, J. J. G., & Peck, H. D., Jr. (1988) *Proc. Natl. Acad. Sci. U.S.A. 86*, 147–151.

Fan, C., Teixeira, M., Moura, J., Moura, I., Huynh, B.-H., LeGall, J., Peck, H. D., Jr., & Hoffman, B. M. (1991) *J. Am. Chem. Soc. 113*, 20–24.

Fauque, G., Peck, H. D., Jr., Moura, J. J. G., Huynh, B. H., Berlier, Y., DerVartanian, D. V., Teixeira, M., Przybyla, A. E., & Lespinat, P. A. (1988) *FEMS Microbiol. Rev. 54*, 299–344.

Fernandez, V. M., Hatchikian, E. C., & Cammack, R. (1985) *Biochim. Biophys. Acta 832*, 69–79.

Fernandez, V. M., Hatchiakian, E. C., Patil, D. S., & Cammack, R. (1986) *Biochim. Biophys. Acta 883*, 145–154.

Green, M. L. H., & Saito, T. (1969) *Chem. Commun.* 208.

Hendly, D. D. (1955) *J. Bacteriol. 70*, 625–634.

Horowitz, C. P., & Shriver, D. F. (1984) *Adv. Organomet. Chem. 23*, 219–305.

Huynh, B. H., Patil, D. S., Moura, I., Teixeira, M., Moura, J. J. G., DerVartanian, D. V., Czechowski, M. H., Prickril, B. C., Peck, H. D., Jr., & Jean, L. (1987) *J. Biol. Chem. 262*, 795–800.

Jonas, K., & Wilke, G. (1969) *Angew. Chem., Int. Ed. Engl. 8*, 519–520.

Kermarec, M., Lepetit, C., Cai, F. X., & Olivier, D. (1989) *J. Chem. Soc., Faraday Trans. 1 85*, 1991–2000.

Lancaster, J. R., Jr. (1988) *The Bioinorganic Chemistry of Nickel*, VCH Publishers, Inc., New York.

Lundin, A., & Aasa, R. (1972) *J. Magn. Reson. 8*, 70–73.

Nakamoto, K. (1978) *Infrared and Raman Spectra of Inorganic and Coordination Compounds*, John Wiley and Sons, New York.

Nakanishi, K., & Solomon, P. H. (1977) *Infrared Absorption Spectroscopy*, Holden-Day, Oakland.

Przybyla, A. E., Robbins, J., Menon, N., & Peck, H. D., Jr. (1992) *FEMS Microbiol. Rev. 88*, 109–135.

Sellmann, D., Lechner, P., Knoch, F., & Moll, M. (1991) *Angew. Chem., Int. Ed. Engl. 30*, 552–553.

Surerus, K. K., Chen, M., van der Zwaan, J. W., Rusnak, F. M., Kolk, M., Duin, E. C., Albracht, S. P. J., & Münck, E. (1994) *Biochemistry 33*, 4980–4993.

Sweaney, R. L., Polito, M. A., & Moroz, A. (1989) *Organometallics 8*, 2305–2308.

Teixeira, M., Moura, I., Xavier, A. V., DerVartanian, D. V., LeGall, J., Peck, H. D., Jr., Huynh, B. H., & Moura, J. J. G. (1983) *Eur. J. Biochem. 130*, 481–484.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., LeGall, J., DerVartanian, D. V., Peck, H. D., Jr., & Huynh, B.-H. (1989) *J. Biol. Chem. 264*, 16435–16450.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D., & Slater, E. C. (1985) *FEBS Lett. 179*, 271–277.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D., & Roelofs, Y. B. M. (1986) *Biochim. Biophys. Acta 872*, 208–215.

Van der Zwaan, J. W., Coremans, J. M. C. C., Bouwens, E. C. M., & Albracht, S. P. J. (1990) *Biochim. Biophys. Acta 1041*, 101–110.

Van Heerikhuizen, H., Albracht, S. P. J., Slater, E. C., & Van Rheenen, P. S. (1981) *Biochim. Biophys. Acta 657*, 26–39.

Voordouw, G. (1992) in *Advances in Inorganic Chemistry* (Cammack, R., & Sykes, A. G., Eds.) pp 397–422, Academic Press, Inc., New York.

Wang, C. P., Franco, R., Moura, J. J. G., Moura, I., & Day, E. P. (1992) *J. Biol. Chem. 267*, 7378–7380.

Whitehead, J. P., Colpas, G. J., Bagyinka, C., & Maroney, M. J. (1991) *J. Am. Chem. Soc. 113*, 6288–6289.

Whitehead, J. P., Gurbiel, R. J., Bagyinka, C., Hoffman, B. M., & Maroney, M. J. (1993) *J. Am. Chem. Soc. 115*, 5629–5635.