Carbon Monoxide and Cyanide as Intrinsic Ligands to Iron in the Active Site of [NiFe]-Hydrogenases

NiFe(CN)$_2$CO, BIOLOGY’S WAY TO ACTIVATE H$_2$*

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Infrared-spectroscopic studies on the [NiFe]-hydrogenase of *Chromatium vinosum*-enriched in $^{15}$N or $^{13}$C, as well as chemical analyses, show that this enzyme contains three non-exchangeable, intrinsic, diatomic molecules as ligands to the active site, one carbon monoxide molecule and two cyanide groups. The results form an explanation for the three non-protein ligands to iron detected in the crystal structure of the *Desulfovibrio gigas* hydrogenase (Volbeda, A., Garcin, E., Piras, C., De Lacey, A. I., Fernandez, V. M., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1996) J. Am. Chem. Soc. 118, 12989–12996) and for the low spin character of the ferrous iron observed with Mössbauer spectroscopy (Surerus, K. K., Chen, M., Van der Zwaan, W., Rusnak, F. M., Kolk, M., Duin, E. C., Albracht, S. P. J., and Münck, E. (1994) Biochemistry 33, 4980–4993). The results do not support the notion, based upon studies of *Desulfovibrio vulgaris* [NiFe]-hydrogenase (Higuchi, Y., Yagi, T., and Noritake, Y. (1997) Structure 5, 1671–1680), that SO is a ligand to the active site. The occurrence of both cyanide and carbon monoxide as intrinsic constituents of a prosthetic group is unprecedented in biology.

Hydrogenases catalyze the reversible splitting of dihydrogen (H$_2$ $\leftrightarrow$ 2H$^+$ + 2e$^-$) and are common in many microorganisms. Their physiological role is either to acquire reducing equivalents from H$_2$ or to dispose of excess reducing equivalents from fermentation via the reduction of protons. Hydrogenases are often intimately complexed to modules containing other redox proteins. In this way the metabolism of dihydrogen is linked to redox chemistry with a wide variety of electron acceptors and donors like NAD(P)(H), b- and c-type cytochromes, factor F$_{420}$, S$_2^-$, and membrane-bound (mena)quinones.

With regard to the overall metal content three classes of hydrogenases can be discriminated. The majority of hydrogenases contain nickel in addition to iron and are termed [NiFe]-hydrogenases. The minimal protein unit required for activity contains two subunits, a large one (46–72 kDa) and a small one (23–38 kDa; for review see Ref. 1). The three-dimensional structure of the enzyme from *Desulfovibrio gigas* disclosed (2, 3) that the active site is a Ni-Fe dinuclear center attached to the large subunit via four thiolates from Cys residues. The iron atom has three non-protein ligands, with an electron density equivalent to diatomic molecules. The small subunit contains two [4Fe-4S] clusters and one [3Fe-4S] cluster. From a comparison of the amino acid sequences of [NiFe]-hydrogenases, it can be concluded that only the cubane cluster closest to the active site is conserved in all enzymes (1). FTIR studies (4–6) showed that [NiFe]-hydrogenases contain a set of three infrared absorption bands in the 2100 to 1850 cm$^{-1}$ spectral region, not found in any other proteins. As the frequency of these bands is very sensitive to the status of the active site, it was concluded that they are due to intrinsic ligands (diatomic molecules with a triple bond or triatomic molecules with two adjacent double bonds) of the active site. Also a unique lone low spin Fe(II) site was detected, in addition to the high spin iron sites of the Fe-S clusters, by Mössbauer spectroscopy (7).

A second class forms the [Fe]-hydrogenases (for review see Ref. 8); no other metal than iron is present in these enzymes. The prosthetic groups are located in only one subunit and minimally consist of two classical [4Fe-4S] clusters and a hydrogen-activating site, called the H-cluster. The latter active site was speculated to be an Fe-S cluster with 4–7 iron atoms (9, 10). It was recently discovered (6) that also [Fe]-hydrogenases show FTIR bands in the 2100 to 1850 cm$^{-1}$ spectral region, which strongly shift upon changes of the redox state of the enzyme. Hence a similar architecture was suggested for the active sites of [NiFe]- and [Fe]-hydrogenases.

The third class of hydrogenases does not contain any metal and occurs in methanogenic *Archaea* (11, 12). These enzymes, H$_2$-forming $N^\alpha,N^\beta$-methylenebetatetrahydromethanopterin dehydrogenases, can activate H$_2$ only in the presence of their second substrate.

In this paper we present spectroscopic as well as chemical evidence that the molecules observed in the FTIR spectra of [NiFe]-hydrogenases are one CO and two CN$^-$ groups bound to iron in the Ni-Fe active site. A preliminary report of this work has appeared elsewhere (13).

EXPERIMENTAL PROCEDURES

Enzyme Preparation

*C. vinosum* (strain DSM 185) was grown in a 700-liter batch culture as described (14). For $^{15}$N or $^{13}$C enrichment, cells were grown in 10-liter batch cultures with 20 mM $^{15}$NH$_4$Cl as nitrogen source or 58 mM NaH$^{13}$CO$_3$ as carbon source. Isotopes were purchased from Cambridge.

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Isotope Laboratories (Cambridge, UK). Three different cultures were prepared with final, calculated enrichments of 98% 13N, 49% 15N, or 99% 14C. The cultures were maintained at pH 7.5 by the addition of 1 M sodium phosphate buffer. Cells were harvested, and the enzyme was isolated and purified as described previously (14). Due to the limited amounts of isotope-enriched iron samples, it was decided to use two 14-Fe-3S

\[X_{\text{act}} = [3Fe-4S]\]

clusters. The protein concentrations determined by the Bradford method correlated well with the values based on the metal contents, using a molecular mass of 94 kDa (14).

**Sample Preparation**

Usually, hydrogenase of *C. vinosum* as isolated in air is a mixture of two forms, called ready and unready, with quite different FTIR spectra and slightly different FTIR spectra for the two forms. Therefore, enzyme was converted to more than 90% into the ready form as follows. A dilute solution (10 μM) was incubated under 1.2 bar of H2 at 50 °C for at least 30 min, cooled to 2 °C, evaporated, and finally dried with argon, after which argon was replaced by 10 μM Tris-HCl (pH 8.0). After stirring for 10 min at 2 °C and 60 min at room temperature, the sample was concentrated by means of a Bio-Rad FTS 60A spectrometer equipped with an MCT detector. The spectra were recorded at room temperature with a resolution of 2 cm⁻¹ and are averages of 762 or 1524 scans. Enzyme samples (10 μl, 0.4–1.5 mm) were loaded into a gas-tight IR-transmittance cell (4) with polished CaF2 windows kept at 20 °C and sealed in an anaerobic cuvette, filled with an assay mixture containing 10% argon and 90% CO (g), released from hydrogenase upon denaturation was performed by binding to ferrous hemoglobin, essentially as described earlier (17). Typically, hydrogenase of *C. vinosum* (2–5 nml) denatured by treatment with 5% SDS under an argon atmosphere in 700-μl vials with lined aluminum seals. The sample was incubated for 10 min at 95 °C. After cooling, the vial was incubated under a nitrogen atmosphere under continuous stirring and centrifugation to spin down any water droplets, the total gas phase in the head space was carefully withdrawn with a gas-tight Hamilton syringe, while at the same time water was injected with another syringe to balance the under-pressure. The removed gas was injected into an anerobic cuvette, filled with an assay mixture containing 0.9 mg/ml hemoglobin, 100 mM NaCl, 8 mM sodium dithionite, and 25 mM CAPSO buffer (pH 8.0) under an argon atmosphere (17). The assay mixture was equilibrated with the gas phase for 30 min at room temperature, by repeatedly turning the cuvette upside down on a rotating tilted plate. Then the absorption spectrum was recorded from 400 to 460 nm on a Hewlett-Packard 8452A Diode Array Spectrophotometer, using a cuvette without added CO as background. Binding of CO to reduced hemoglobin leads to a blue shift of the Soret band (increase in the extinction coefficient for Hb at 420 nm from 109.5 to 192 nm⁻¹cm⁻¹) and decrease of the extinction coefficient for Hb at 430 nm from 140 to 60 nm⁻¹cm⁻¹ (17), and hence a ΔΔε(420–430 nm) = 162.5 nm⁻¹cm⁻¹ was used for quantification of the formed Hb-CO species. The recovery of the overall procedure tested with samples of CO-saturated water was 91%.

**Determination of Protein-bound CO**

The direct demonstration of CO₃₀₀ released from hydrogenase upon denaturation was performed by binding to ferrous hemoglobin, essentially as described earlier (17). Typically, hydrogenase of *C. vinosum* (2.5–5 nml) was denatured by treatment with 5% SDS under an argon atmosphere in 700-μl vials with lined aluminum seals. The sample was incubated for 10 min at 95 °C. After cooling, the vial was incubated under a nitrogen atmosphere under continuous stirring and centrifugation to spin down any water droplets, the total gas phase in the head space was carefully withdrawn with a gas-tight Hamilton syringe, while at the same time water was injected with another syringe to balance the under-pressure. The removed gas was injected into an anerobic cuvette, filled with an assay mixture containing 0.9 mg/ml hemoglobin, 100 mM NaCl, 8 mM sodium dithionite, and 25 mM CAPSO buffer (pH 8.0) under an argon atmosphere (17). The assay mixture was equilibrated with the gas phase for 30 min at room temperature, by repeatedly turning the cuvette upside down on a rotating tilted plate. Then the absorption spectrum was recorded from 400 to 460 nm on a Hewlett-Packard 8452A Diode Array Spectrophotometer, using a cuvette without added CO as background. Binding of CO to reduced hemoglobin leads to a blue shift of the Soret band (increase in the extinction coefficient for Hb at 420 nm from 109.5 to 192 nm⁻¹cm⁻¹) and decrease of the extinction coefficient for Hb at 430 nm from 140 to 60 nm⁻¹cm⁻¹ (17), and hence a ΔΔε(420–430 nm) = 162.5 nm⁻¹cm⁻¹ was used for quantification of the formed Hb-CO species. The recovery of the overall procedure tested with samples of CO-saturated water was 91%.

**Determination of Protein-bound CN⁻**

A common approach to determine cyanide in aqueous solutions is to acidify the sample with sulfuric acid, followed by heating of the mixture. The evolved HCN gas (boiling point, 299 K) is transported by means of a carrier gas and led through an alkaline solution, which absorbs the HCN (18). The collected cyanide can be determined by spectrophotometric methods based on a modified König’s reaction (19), which starts with the production of cyanogen chloride. This is followed by a reaction of the cyanogen chloride with a pyridine derivative resulting in ring opening, thereby producing a 2-pentendial derivative. Subsequently, a condensation reaction between the 2-pentendial derivative and an active methyleneacarbonyl compound is performed, e.g. with barbituric acid, with pyrazolone or with 2-thiobarbituric acid (19). In the present work the isonicotinic acid/barbituric acid method was applied (Fig. 1), modified from Nagashima (19), using 1,3-dimethylbarbituric acid as described (20), under less extreme conditions (18). The blue product of the latter reaction is polymethine, having an absorption maximum at 600 nm. Color development using 1,3-dimethylbarbituric acid is faster and more intense compared with that of barbituric acid (20).

**The determination of cyanide in the hydrogenase of *C. vinosum* was performed by mild oxidative treatment with potassium permanganate in sulfuric acid (18), followed by detection of cyanide as described above. The methods have been optimized for the use with iron-sulfur proteins as described below.**

**Release of Cyanide from the Enzyme by Treatment in a Mildly Acidic and Oxidizing Environment—An appropriate setup was built using a 10-ml double-necked round-bottom flask, filled with 4.3 ml of distilled water, 0.3 ml of 3% (w/v) sodium anti-foaming agent (BDH, Poole, UK), and 250 ml of 3 M H₂SO₄. A small magnetic stirring bar was used for mixing. The flask contained a gas inlet, consisting of a thin glass tube reaching to the bottom. During the reaction, a flow of argon gas was bubbled through the solution (20–30 ml/min).**

As acid-labile sulfur is released upon destruction of iron-sulfur clusters in the protein by acid, it is likely, and was actually observed, that any S²⁻ formed reacts with cyanide to form thiocyanate (21). This would reduce the yield of the cyanide from the enzyme. We therefore added up to 200 μl of 5 mM KMnO₄ prior to heating in order to re-oxidize possible SCN⁻ to CN⁻ in the sulfuric acid environment (18). The reaction mixture was heated in a water bath to 95 °C, after which 50–250 μl of hydrogenate (about 10 nmol) was added by injection through a septum. Heating under a steady flow of argon was continued for 30 min. The gas mixture was then loaded into 1 ml of 100 mM NaOH to collect the HCN. The tube was weighed before and after the experiment to correct for evaporation.

**Oxidation of Cyanide with Chloramine T to Form Cyanogen Chloride—In a reaction tube, 0.5 ml of the NaOH solution containing the collected HCN, 1.5 ml of distilled water, 0.5 ml of sucinate solution (2 M), and 0.05 ml of 1% chloramine T were mixed and kept for about 1 min**
at room temperature at pH 5.6. Because of the volatility of cyanogen chloride, it was desirable to add the color reagent as soon as its formation had reached a maximum, which was after about 1 min, as also found previously (22). The succinate solution (2 M succinic acid, 2.6 M NaOH) was prepared by slow addition of NaOH to a cold suspension of succinic acid. Afterward the solution was filtered.

Reaction of Cyanogen Chloride with Isonicotinic Acid and 1,3-Dimethylbarbituric Acid to Form Polymethine—A color reagent solution was prepared by adding 2.8 g of 1,3-dimethylbarbituric acid and 2.3 g of isonicotinic acid to 150 ml of distilled water. Solid NaOH (1.4 g), required to dissolve the acids (23) and to adjust the pH to 5.6, was then slowly added, thereafter the solution was diluted to 250 ml with distilled water. To the mixture as obtained under “Oxidation of Cyanide with Chloramine T to Form Cyanogen Chloride,” 0.45 ml of color reagent was added and left for 30 min at room temperature. The reaction mixture was then transferred to a glass cuvette, and the polymethine concentration was determined by measuring its absorbance at 600 nm using a Zeiss M4 QII spectrophotometer. A sample of 0.5 ml of pure 100 mM NaOH, treated in the same way, was used as a blank. A series of KSCN samples (0–20 nmol), prepared from a 0.0998 M solution in water (volumetric KSCN standard, Aldrich), was used as a standard curve. This curve was linear in the used range and gave precisely the same color as a series of KCN solutions but is more convenient for routine analyses. Reaction of Cyanogen Chloride with Isonicotinic Acid and 1,3-Dimethylbarbituric Acid—A color reagent solution was prepared by adding 2.8 g of 1,3-dimethylbarbituric acid and 2.3 g of isonicotinic acid to 150 ml of distilled water. Solid NaOH (1.4 g), required to dissolve the acids (23) and to adjust the pH to 5.6, was then slowly added, thereafter the solution was diluted to 250 ml with distilled water. To the mixture as obtained under “Oxidation of Cyanide with Chloramine T to Form Cyanogen Chloride,” 0.45 ml of color reagent was added and left for 30 min at room temperature. The reaction mixture was then transferred to a glass cuvette, and the polymethine concentration was determined by measuring its absorbance at 600 nm using a Zeiss M4 QII spectrophotometer. A sample of 0.5 ml of pure 100 mM NaOH, treated in the same way, was used as a blank. A series of KSCN samples (0–20 nmol), prepared from a 0.0998 M solution in water (volumetric KSCN standard, Aldrich), was used as a standard curve. This curve was linear in the used range and gave precisely the same color as a series of KCN solutions but is more convenient for routine analyses.

Exchange Experiments with $^{13}$CN

In order to test whether the IR-detectable groups could be exchanged with added cyanide, hydrogenase of C. vinosum was treated with K$^{13}$CN (Aldrich). One sample was incubated with 5 mM K$^{13}$CN for 24 h at 30 °C in air at pH 8.0, and directly loaded to the IR transmittance cell. A similar incubation was carried out in the presence of 8 M urea, in order to loosen the protein structure to a certain extent (27). To verify this possibility in the C. vinosum enzyme, we have also prepared enzyme enriched in 15N—The results in Fig. 3 indicate that the two small bands are presumably due to CN$^{-}$ groups. The two bands may reflect the symmetrical and antisymmetrical vibrations of two vibrationally coupled CN$^{-}$ groups (25, 26) or they might be due to two different conformers of the enzyme, each with only one CN$^{-}$ ligand in the active site. Recently, a high resolution crystal structure (1.8 Å) has been published of the [NiFe]-hydrogenase from Desulfovibrio vulgaris, Miyazaki (27). Although the overall structure of the enzyme and its active site highly resembled the structure of the D. gigas enzyme (3), it was concluded that the diatomic ligands at the iron atom were one CO, one CN$^{-}$, and one SO group. FTIR spectra of this enzyme were very similar to those observed with the C. vinosum and D. gigas enzymes, and this was explained by assuming that the two bands in the 2100–2050 cm$^{-1}$ region were due to two different conformers of the enzyme (27).

RESULTS

FTIR Spectra of Hydrogenase Enriched in $^{15}$N or $^{13}$C—In Fig. 3, trace A, the FTIR spectrum of C. vinosum [NiFe]-hydrogenase in the ready form is shown. Two small bands (2090 and 2079 cm$^{-1}$) and one large band (1944 cm$^{-1}$) can be seen. Enzyme enriched in $^{13}$C showed the spectrum in trace B. The large band was shifted to 1899 cm$^{-1}$, whereas a small band (1943 cm$^{-1}$) was still detectable at the original position. The two bands at 2090 and 2079 cm$^{-1}$ shifted to 2046 and 2035 cm$^{-1}$. It is concluded that all groups responsible for the IR bands in trace A contain carbon.

Enrichment of the enzyme with $^{15}$N resulted in a shift of the two small bands (Fig. 3, trace C) to 2060 and 2049 cm$^{-1}$. The position of the large band was not affected. Hence, the groups responsible for the two small IR absorption bands contain nitrogen, whereas the group evoking the large absorption band does not. All band positions are summarized in Table I.

Exchange of Hydrogenase Enriched in 15N or 13C—In order to test whether the IR-detectable groups could be exchanged with added cyanide, hydrogenase of C. vinosum was treated with K$^{13}$CN (Aldrich). One sample was incubated with 5 mM K$^{13}$CN for 24 h at 30 °C in air at pH 8.0, and directly loaded to the IR transmittance cell. A similar incubation was carried out in the presence of 8 M urea, in order to loosen the protein structure to a certain extent (27). To verify this possibility in the C. vinosum enzyme, we have also prepared enzyme enriched in 15N. The result is shown in Fig. 4, trace B. For a proper understanding, the spectra of unenriched enzyme (trace A) and fully $^{15}$N-enriched enzyme (trace C) are shown in the same
figure as well. In case of two conformers of enzyme with one CN ligand only, a mixture of the spectra in A and C would be expected. This is clearly not the case. Instead, trace B shows two sets of three overlapping bands in the 2100 to 2040 cm\(^{-1}\) region. Two of the three bands in each set are at positions equivalent to those seen in traces A or C. The central band in each set can be visualized by appropriate subtractions (Fig. 4, trace D). The two bands, at 2085 and 2053 cm\(^{-1}\), are somewhat broader than those of the individual bands of the unenriched or fully enriched enzyme.

EPR—Possible effects of isotope enrichments on the EPR spectra of the nickel center and the [3Fe-4S]\(^{1}\) cluster in the ready or unready form of the C. vinosum enzyme as isolated have been studied as well. The preparations enriched for more than 98% in \(^{13}\)Co or \(^{15}\)N did not show any detectable broadening as compared with unenriched enzyme, however.

Chemical Analysis for CO and CN—To corroborate the implications of FTIR experiments, analytical procedures for the determination of intrinsic CO and CN in proteins and in particular hydrogenase have been designed. We encountered several complications. First, release of CO from the C. vinosum [NiFe]-hydrogenase was not observed using a variety of denaturing, aerobic, acid, alkaline, and temperature conditions other than the current anaerobic, neutral pH, SDS (100 °C) procedure. Although the method itself has a high recovery (91%), only up to 0.7 mol of carbon monoxide per mol of nickel was detected (Table II). When Hb-CO samples were used as a control, lower amounts of CO were also found (0.4–0.8 mol/mol Hb). No carbon monoxide evolution was seen in control experiments with other proteins. Second, the release of CN from hydrogenase was initially corrupted by reaction of acid-released cyanide with sulfur to form non-distillable thiocyanate. The subsequent use of KMnO\(_4\) to oxidize SCN\(^-\) back to CN\(^-\) led to side reactions with amino acids like tryptophan, tyrosine, cystine, and cysteine (18). The release of cyanide as a function of the quantities of permanganate, hydrogenase, and the non-cyanide-containing model protein BSA was therefore investigated (Fig. 2). By the use of 0.5–1.0 mg of protein amounts and low (0.05–0.2 mM) KMnO\(_4\) concentration-sensitive and specific detection of the cyanide contained in the hydrogenase active site could be achieved. The characteristics of the aspecific generation of cyanide from BSA and hydrogenase above 0.5 mM KMnO\(_4\) are so similar that it can be assumed that the cyanide released in the 0–0.2 mM KMnO\(_4\) range corresponds to cyanide not generated from amino acid breakdown but from SCN\(^-\) derived from reaction of CN\(^-\) with sulfur compounds. The above is exemplified by the very reproducible nature (six preparations) and limiting stoichiometry of 2 mol of cyanide per mol of nickel (Table II), mutually consistent with the FTIR results and x-ray crystallography.

Non-exchangeability of CO and CN—No changes in the IR spectra of C. vinosum hydrogenase were observed upon incubation with 5 mM K\(^{13}\)CN under the conditions tested (i.e. incubation of oxidized hydrogenase at 30 °C with 5 mM K\(^{13}\)CN in air for 24 h, in the presence or absence of 8 M urea, or incubation of H\(_2\)-activated enzyme with K\(^{13}\)CN). In our previous studies (4) it was already found that incubation of inactive, oxidized enzyme or active, reduced enzyme with \(^{13}\)CO did not shift or replace any of the FTIR bands either. This means that the CN\(^-\) groups and the CO molecule are not exchangeable under the examined conditions.

DISCUSSION

FTIR Spectra—The ready and unready forms of C. vinosum hydrogenase both have FTIR spectra with three bands in the 2150 to 1900 cm\(^{-1}\) region, be it with slightly different positions
Figs. 3. Effects of $^{13}$C and $^{15}$N isotopes on the infrared spectrum of C. vinson hydrogenase. A, enzyme isolated from cells grown in the presence of natural abundance carbonate and ammonium chloride; B, enzyme isolated from cells grown on $[^{13}$C]sodium carbonate (enrichment 99%); C, enzyme isolated from cells grown on $[^{15}$N]ammonium chloride (enrichment 98%). Enzyme samples were converted to the ready form and so the current data have an increased accuracy for the isotope shifts.

Effects of $^{13}$C and $^{15}$N isotopes on the infrared spectrum of C. vinson hydrogenase. Only the bands of the $\nu$(CN) stretching frequencies are shown. A, natural abundance enzyme (i.e., 0.9% $^{15}$N); B, enzyme 49% enriched in $^{15}$N; C, enzyme 98% enriched in $^{15}$N; D, trace B after suitable subtraction of the spectra from enzyme containing two $^{13}$N or two $^{15}$N molecules. Pretreatment of enzyme and spectrometer conditions were as in Fig. 3.

Infrared absorption bands in the 2150 to 1850 cm$^{-1}$ spectral region for unlabeled, $^{13}$C-enriched, and $^{15}$N-enriched C. vinson hydrogenase in the ready form.

| Enzyme                      | Band 1 (cm$^{-1}$) | Band 2 (cm$^{-1}$) | Band 3 (cm$^{-1}$) |
|-----------------------------|--------------------|--------------------|--------------------|
| Unlabeled                   | 2090.1$^a$         | 2079.1$^b$         | 1943.6             |
| $^{13}$C-Enriched (99%)     | 2046.2             | 2034.8             | 1988.3 (1943.0)    |
| Calculated                  | 2045.7             | 2034.8             | 1988.3 (1943.0)    |
| Observed frequency shift    | $\Delta v$         | $\Delta v$         | $\Delta v$         |
| $^{15}$N-Enriched (98%)     | 2057.8             | 2047.0             | 1943.0             |
| Calculated                  | 2060.4             | 2048.9             | 1943.0             |
| Observed frequency shift    | $\Delta v$         | $\Delta v$         | $\Delta v$         |
| $^{15}$N-Enriched (49%)     | 2085.4$^c$         | 2053.4$^d$         | 1943.1             |

$^a$ Symmetrical vibration of two vibrationally coupled cyanide groups.
$^b$ Antisymmetrical vibration of two vibrationally coupled cyanide groups.
$^c$ $\nu$(CN) stretch frequencies of $^{13}$C-ligands.
$^d$ $\nu$(CN) stretch frequencies of $^{15}$N-ligands.

TABLE II

Determination of nickel and intrinsically bound cyanide and carbon monoxide in C. vinson hydrogenase preparations

| Preparation                  | CN$^-$/nickel mol/mol | CO/nickel mol/mol |
|------------------------------|------------------------|-------------------|
| 1                            | 2.00 (n = 1)           | ND$^a$            |
| 2                            | 1.79 (n = 1)           | ND$^a$            |
| 3                            | 2.10 (n = 5)           | 0.60 (n = 3)      |
| 4                            | 2.02 (n = 3)           | 0.53 (n = 3)      |
| 5                            | 1.97 (n = 7)           | 0.66 (n = 5)      |
| 6                            | 2.07 (n = 5)           | 0.66 (n = 5)      |

$^a$ ND, not determined.

where $1/\mu = 1/m_1 + 1/m_2$; the expected band shifts upon isotope enrichment have been calculated (Table I). The values differ by less than 3 wave numbers from the experimental values. The calculated values for the $\nu$(CN) vibrations in enzyme molecules with a $^{14}$N/$^{15}$N couple differ only 0.2 cm$^{-1}$. Hence, both the frequency of the high frequency bands in the unlabeled enzyme and the pattern of shifts upon isotopic labeling are entirely consistent with the assignment of the two high frequency bands to CN$^-$. Assuming that the band at 1943 cm$^{-1}$ is due to CO, the calculated shift for $^{13}$CO differed 1.5 cm$^{-1}$ from the observed value. This indicates that our assumption is correct. The possibility of acetylene being a ligand can be ruled out, since a much larger shift in $^{13}$C-enriched samples would appear in that case. Carbon monoxide usually gives rise to strong bands in the region 2100 to 2000 cm$^{-1}$ in metal carboxyl complexes and in the region 1900 to 1800 cm$^{-1}$ if the CO bridges between two metals (26). From the position of the $\nu$(CO) stretch vibrations in normal and $^{15}$N-enriched samples, it is concluded that there is very little vibrational coupling between CO and the cyanide groups.

As previously stated, partial labeling of the center can be used to distinguish whether the two CN$^-$ bands detected arise from two CN$^-$ ligands on a single metal center or whether they arise from two different conformers of a single CN$^-$ ligand. The rationale is as follows. In the case of two CN$^-$ ligands located...
arising only from two C14N and two C15N. Hence, the resultant will consist of a total of 4 infrared bands, having frequencies the pattern of bands detected in a partial labeling experiment comparison, if the two CN
remaining two bands will arise from enzyme containing one

tical to the bands detected in the fully labeled enzyme. The
two sets in the 2100 to 2040 cm
bands. The relative intensity of the three bands in each of the
spectra for partially labeled enzyme consists of a total of 6
one cyanide but that they arise from two vibrationally coupled
with 49%15N (Fig. 4) clearly shows that the two small bands
are not due to two different conformers of the enzyme with only
one cyanide but that they arise from two vibrationally coupled
cyanide groups bound to the same metal ion. The resultant
spectra for partially labeled enzyme consists of a total of 6
bands. The relative intensity of the three bands in each of the
two sets in the 2100 to 2040 cm
region is as expected from a

ligands, Fe-C=N

lowest occupied sigma orbital is localized on the carbon atom,
iron very rarely complexes CN

the position where serine is found in the

coordinate as cyanides, Fe-C-N

CN makes contact with hydrophobic residues (3, 28), and this is
why CN
makes the M-C configuration the most stable one (31). In
iron-containing organometallic complexes CN
is able to bind as isocyanide and almost exclusively if the cyanide bridges
between two metals, using both its carbon and nitrogen for
binding. The crystal structures of the D. gigas (3) and D. vulgaris (27) hydrogenases rule out such a possibility.

**Non-exchangeability of CO and CN**—The crystallographic studies (3, 28) revealed that the diatomic ligands are tightly
buried in narrow protein cavities, the size of which was esti-
mated to be insufficient to hold triatomic molecules. This is probably the reason why we could not detect any exchange of the diatomic molecules with either $^{13}$CO or $^{13}$CN$^-$ and why the groups are so tightly associated with the enzyme.

**Localization of the Unpaired Spin in the Active Site**—From the observation that no detectable broadening of the nickel EPR signal could be observed in either $^{13}$C- or $^{15}$N-enriched enzyme, it is concluded that the unpaired spin in the active site has no appreciable spin density on carbon or nitrogen atoms from amino acid residues or the CN$^-$/CO ligands. As also no broadening is observed in samples enriched in $^{57}$Fe, this underlines the conclusion (1) that the unpaired spin is localized on the nickel ion and its immediate ligands in the active site.

**Model Compounds**—Recently two interesting iron model complexes with CN$^-$ and CO ligands have been described as a reaction in our preliminary report (13). One is a low spin Fe(II) thiolate complex with one CN$^-$ and one CO as ligands (32), being the first example of this kind. The CN$^-$ and CO stretch frequencies were found at 2079 cm$^{-1}$ and 1904 cm$^{-1}$, respectively. A second, even more interesting model is an iron compound in which iron is sandwiched within a cyclopentadiene ring and two cyanides and one carbon monoxide (33). The potassium salt of this compound matches the structural and infrared characteristics of the Fe(NO)$_2$CO site in [NiFe]-hydrogenases (33), having bands at 2094, 2088, and 1949 cm$^{-1}$ in aprotic media. As in the enzyme, very little vibrational coupling was observed between the ν(CN) and ν(CO) modes in this complex. The bands in the model compound are clearly broader than those observed in the enzymes.

**Biosynthesis**—Enrichment to more than 98% in either $^{13}$C or $^{15}$N resulted in a complete shift of the ν(CN) bands (Fig. 3). In the $^{13}$C-enriched enzyme, however, about one-fifth (22%) of the the culture from other possible sources, e.g. E. coli. The incorporation of one or more of these gene products is involved in the maturation of this class of enzymes. In view of the extraordinary nature of the active site structure, it has been proposed that one nickel and iron, synthesis (CO and CN$^-$), and incorporation of the several constituents of the active site. Indeed, Rey et al. (36) reported that one of the accessory genes (hypX) in *Rhizobium leguminosarum* codes for a protein resembling NADH-formyltetrahydrololate-dependent enzymes involved in C1-metabolism. This indicates that the diatomic ligands may be formed by the products of these accessory genes.

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