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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 lone ferrous iron ion 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[1] 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^5,N^{10}-methylenetetrahydromethanopterine 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 $^{15}NH_4Cl$ as nitrogen source or 58 mM $NaH^{13}CO_3$ as carbon source. Isotopes were purchased from Cambridge

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[1] The abbreviations used are: FTIR, Fourier transform infrared spectroscopy; EPR, electron paramagnetic resonance; BSA, bovine serum albumin; CAPSO, 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid.

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Isotope Laboratories (Cambridge, UK). Three different cultures were prepared with final, calculated enrichments of 98% ^{15}N, 49% ^{15}N, or 99% ^{13}C. 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 samples, it was decided to omit the TSK-DEAE column step in the purification procedure. The Ultragel ACA-44 column was replaced by a Hiload 16/60 Superdex 200 one. The purity of these samples, as determined by SDS-polyacrylamide gel electrophoresis (12%) (15), was therefore only 40–60%. Enzyme was dissolved in 50 mM Tris·HCl (pH 8.0) and stored in liquid nitrogen. Protein concentrations were determined according to Ref. 16, using bovine serum albumin (BSA) as standard.

EPR Measurements

X-band EPR measurements (9 GHz) were obtained with a Bruker ECS 106 EPR spectrometer, equipped with an Oxford Instruments ESR-900 helium-flow cryostat with an ITC-4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The frequency was measured with an HP 5350B Microwave Frequency Counter.

FTIR Measurements

FTIR measurements were performed on 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 CaF_2 windows kept at 50 μm distance with a Teflon spacer. A cell containing 50 mM Tris·HCl (pH 8.0) was used as reference. The small, variable contributions of water vapor was removed by subtraction of an appropriate water-vapor spectrum. The multi-point method of the Bio-Rad spectrometer was used for correction of the base line.

Measurement of Hydrogenase Activity

Hydrogenase activities were determined as previously (14). The *C. vinosum* enzyme samples used in this study displayed specific H_2 uptake activities (after activation under H_2 for 30 min at 45 °C) with benzyl viologen as acceptor in the range of 100–200 μmol of H_2/min/mg (30 °C, pH 8.0).

Metal Content Determination

Iron and nickel were determined with a Hitachi 180–80 polarized Zeeman Atomic Absorption Spectrophotometer using a standard series of the particular metal. Adventitious metal ions were eliminated from enzyme and buffer by passage through a Chelex 100 column (Bio-Rad). Relative metal contents were found to be 10–13 iron per nickel, in line with the presence of one iron atom in the active site and two [4Fe-4S] and one [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 EPR spectra and slightly different FTIR spectra. 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 H_2 at 50 °C for at least 30 min, cooled to 2 °C, evacuated, and flushed twice with argon, after which argon was substituted by 1.2 bar of O_2. After stirring for 10 min at 2 °C and 60 min at room temperature, the sample was concentrated by means of a Microcon PM10 to about 300 μl and inspected by EPR to verify the conversion into the ready form. The sample was recovered from the EPR tube and concentrated to 10 μl. As some spin coupling of nickel with the $\{X^{ox} = [3Fe\text{-}4S]^+\}$ moiety (X is an unknown, $n = 1$ redox component which, when oxidized, is strongly spin coupled to the oxidized $[3Fe\text{-}4S]^+$ cluster (7)) was detected, it was treated with freshly prepared phenazine methosulfate (5 μM) and neutralized ascorbic acid (25 mM), which removes this coupling by reduction of the unknown group X. The sample was loaded into the IR transmittance cell and incubated for 30 min at room temperature before recording the spectrum. No reduction of the active site itself was noticed in the FTIR spectra (5).

Before use, H_2 was passed over a Palladium catalyst (Degussa, Hanau, Germany; type E236P), and argon was passed through an Oxisorb cartridge (Messer-Griesheim, Düsseldorf, Germany) to remove residual O_2.

Determination of Protein-bound CO

The direct demonstration of $CO_{(g)}$, released from hydrogenase upon denaturation was performed by binding to ferrous hemoglobin, essentially as described earlier (17). Typically, hydrogenase of *C. vinosum* (2.5–5 nmol) 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 for 60 min at room temperature under continuous stirring. After a short 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 anaerobic 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 9.5) 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 $mM^{-1}\cdot cm^{-1}$ and decrease of the extinction coefficient for Hb at 430 nm from 140 to 60 $mM^{-1}\cdot cm^{-1}$ (17), and hence a $\Delta E(420-430\ nm) = 162.5\ mM^{-1}\cdot cm^{-1}$ 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 procedures 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-pentenedial derivative. Subsequently, a condensation reaction between the 2-pentenedial derivative and an active methylenecarbonyl 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, 20 μl of 3% (w/w) silicone anti-foaming agent (BDH, Poole, UK), and 250 μl of 3 M H_2SO_4. 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 S^0 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 $KMnO_4$ 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 hydrogenase (about 10 nmol) was added by injection through a septum. Heating under a steady flow of argon was continued for 30 min. The carrier gas was led through 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 succinate solution (2 M), and 0.05 ml of 1% chloramine T were mixed and kept for about 1 min

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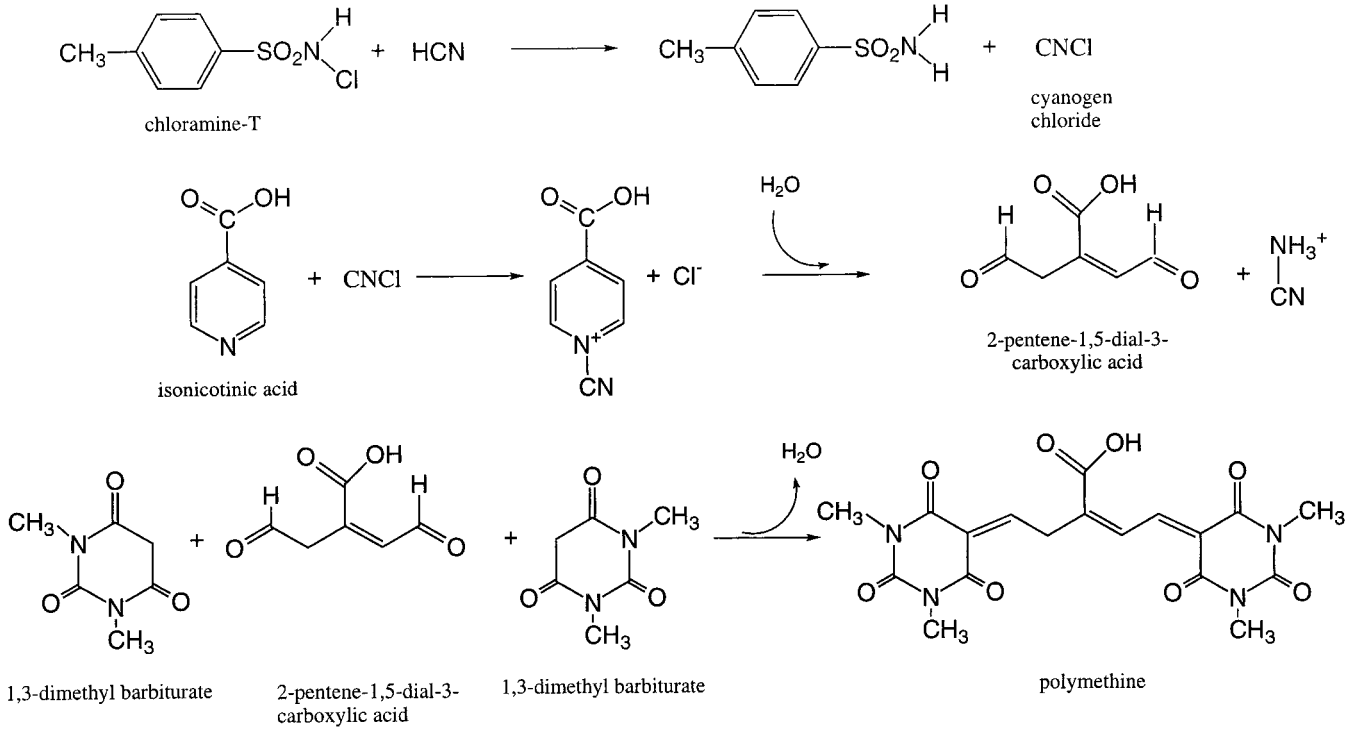

FIG. 1. **Summary of the reactions involved in the spectrophotometric assay for the determination of cyanide in hydrogenase.**

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 (~1.3 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 QIII 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.

It was observed that beyond a certain concentration of $KMnO_4$, the amount of HCN released from hydrogenase increased steadily (Fig. 2). By using BSA, it was found that HCN could develop from pure protein as well, if the concentration of $KMnO_4$ used per mg of protein exceeded a certain value (about 0.5 mM per mg; Fig. 2). Therefore, routinely cyanide determinations were carried out using 0.05–0.2 mM $KMnO_4$ and 0.5–1 mg of protein. With these amounts virtually no HCN was released from protein (Fig. 2). The recovery of KCN or KSCN added to the distillation setup as described above was determined to be at least 90%; therefore, no correction for loss during distillation of the cyanide was made.

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 (*C. vinosum* hydrogenase is stable in 8 M urea (24)) and to possibly make the active site more accessible. Prior to the measurements urea was removed from the sample by dialysis. In another experiment an incubation with 2 mM $K^{13}CN$ was carried out with enzyme activated under H_2 for 30 min at 50 °C.

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.

FTIR Spectra of Hydrogenase Enriched 49% in ^{15}N—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). To verify this possibility in the *C. vinosum* enzyme, we have also prepared enzyme enriched 49% in ^{15}N. 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

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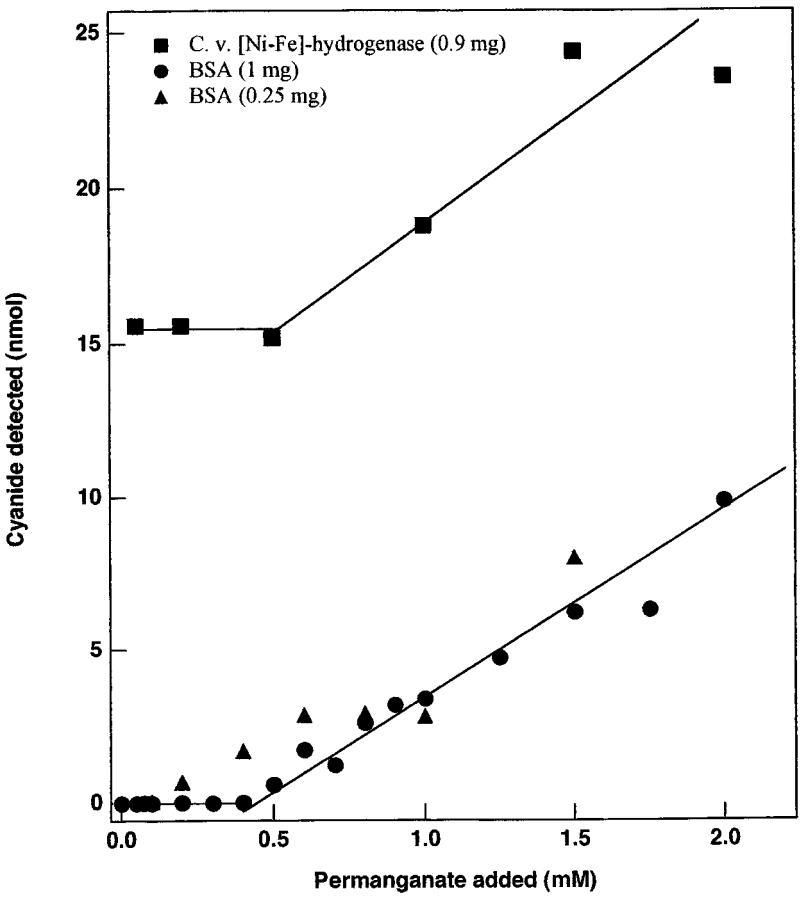

FIG. 2. **Release of cyanide from hydrogenase and BSA as function of the $KMnO_4$ concentration in the destruction mixture.** The indicated amounts of protein and $KMnO_4$ were incubated for 30 min at 95 °C in a home-built miniature distillation setup. Cyanide released from intrinsic ligands and/or protein was transferred by a nitrogen stream to an alkaline trap, of which the cyanide content was determined (details regarding the distillation and determination can be found under "Experimental Procedures"). *Squares,* 0.9 mg of *C. vinosum* hydrogenase; *circles,* 1 mg of BSA; *triangles,* 0.25 mg of BSA.

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 $[3Fe\text{-}4S]^+$ 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}C 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

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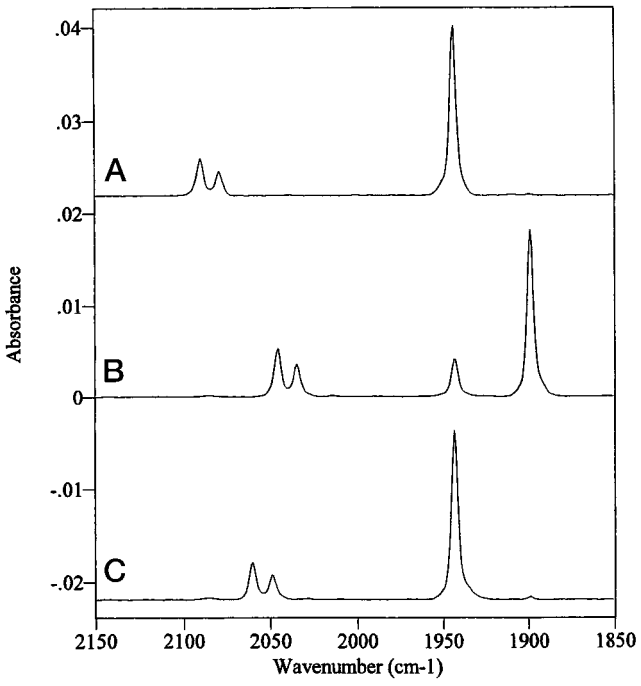

FIG. 3. **Effects of ^{13}C and ^{15}N isotopes on the infrared spectrum of *C. vinosum* hydrogenase.** *A,* enzyme isolated from cells grown in the presence of natural abundance carbonate and ammonium chloride; *B,* enzyme isolated from cells grown on [^{13}C]sodium carbonate (enrichment 99%); *C,* enzyme isolated from cells grown on [^{15}N]ammonium chloride (enrichment 98%). Enzyme samples were converted to the ready state as described under "Experimental Procedures." Samples (0.4–1.0 mM hydrogenase) were incubated at room temperature in 50 mM Tris·Cl, 25 mM sodium ascorbate, 5 μM phenazine methosulfate (pH 8.0), for 30 min to remove magnetic coupling (7). Spectra (780–1520 scans) were recorded at room temperature with a resolution of 2 cm^{-1}.

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

Enzyme	Band 1	Band 2	Band 3
	cm^{-1}	*cm^{-1}*	*cm^{-1}*
Unlabeled	2090.1[a]	2079.1[b]	1943.6
^{13}C-Enriched (99%)			
Calculated	2046.2	2035.4	1900.3
Observed	2045.7	2034.8	1898.8 (1943.0)
Observed frequency shift	−44.4	−44.3	−44.8
^{15}N-Enriched (98%)			
Calculated	2057.8	2047.0	
Observed	2060.4	2048.9	1943.0
Observed frequency shift	−29.7	−30.2	−0.6
^{15}N-Enriched (49%)			
Observed	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] ν(CN) stretch frequencies of C^{14}N in enzyme molecules with a combination of C^{14}N and C^{15}N ligands.
[d] ν(CN) stretch frequencies of C^{15}N in enzyme molecules with a combination of C^{15}N ligands.

(2090, 2079, and 1944 cm^{-1} for the ready enzyme and 2093, 2083, and 1945 cm^{-1} for the unready form). The previous preliminary results (13) were performed with mixtures of ready and unready enzyme. For a proper comparison of the effects of isotopes, we have therefore converted the enzyme samples into the ready form and so the current data have an increased accuracy for the isotope shifts.

By using the equation for a classical harmonic oscillator,

$$\nu = 1/2\pi \cdot \sqrt{(k/\mu)} \qquad \text{(Eq. 1)}$$

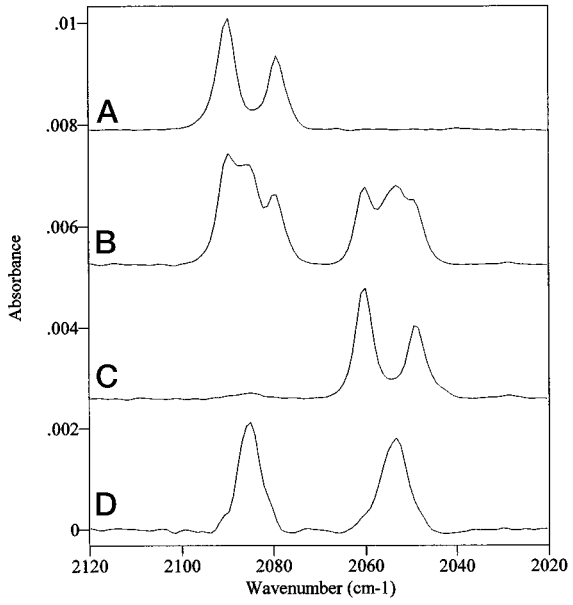

FIG. 4. **Evidence for the coupling between the vibrations of the two cyanides in the active site of the [NiFe]-hydrogenase from *C. vinosum* by partial ^{15}N enrichment.** Only the bands of the ν(CN) stretching frequencies are shown. *A,* natural abundance enzyme (*i.e.* 0.9% ^{15}N); *B,* enzyme 49% enriched in ^{15}N; *C,* enzyme 98% enriched in ^{15}N; *D, trace B* after suitable subtraction of the spectra from enzyme containing two C^{14}N or two C^{15}N molecules. Pretreatment of enzyme and spectrometer conditions were as in Fig. 3.

TABLE II
Determination of nickel and intrinsically bound cyanide and carbon monoxide in C. vinosum hydrogenase preparations

Preparation	CN$^-$/nickel	CO/nickel
	mol/mol	*mol/mol*
1	2.00 (n = 1)	ND[a]
2	1.79 (n = 1)	ND
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 ν(CN) vibrations in enzyme molecules with a C^{14}N/C^{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 carbonyl complexes and in the region 1900 to 1800 cm^{-1} if the CO bridges between two metals (26). From the position of the ν(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

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on the same active site iron, vibrational coupling of the CN^- vibrations is expected. Partial labeling of the CN^- ligands using ^{15}N (giving rise to a population of molecules containing one $C^{14}N$ ligand and one $C^{15}N$ ligand) will result in the partial loss of this coupling. This is in turn expected to give rise to detection of additional bands in the partially labeled spectra at frequencies intermediate to the bands arising from the fully unlabeled ($C^{14}N/C^{14}N$) and fully labeled ($C^{15}N/C^{15}N$) cases. Hence for a spectrum of a center with a partial $C^{15}N$ label, assuming that the CN^- ligands are in identical environments, a total of 6 infrared bands attributable to CN^- should be detected. Two of these bands are attributable to the case where both CN^- ligands contain ^{14}N ($C^{14}N/C^{14}N$) and will result in frequencies identical to those detected in the unenriched enzyme. Two of these bands will be due to centers in which both CNs contain ^{15}N ($C^{15}N/C^{15}N$) and will have frequencies identical to the bands detected in the fully labeled enzyme. The remaining two bands will arise from enzyme containing one CN^- labeled with ^{14}N and one CN^- labeled with ^{15}N ($C^{14}N/C^{15}N$). These bands will be located at frequencies slightly higher than the average of the frequencies for the bands arising from the $C^{14}N/C^{14}N$ case and slightly lower than the average frequency of the bands arising from the $C^{15}N/C^{15}N$ case. In comparison, if the two CN^- bands detected arise from two conformers of a single CN^- ligand on the active site iron, then the pattern of bands detected in a partial labeling experiment will consist of a total of 4 infrared bands, having frequencies arising only from two $C^{14}N$ and two $C^{15}N$. Hence, the resultant spectra should look like a sum of the infrared spectra for the unlabeled and fully labeled enzyme. Enrichment of the enzyme with 49% ^{15}N (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^{-1} region is as expected from a mixture of enzyme molecules with 26% $C^{14}N/C^{14}N$, 50% $C^{14}N/C^{15}N$, and 24% $C^{15}N/C^{15}N$ (49% ^{15}N enrichment). The bands of enzyme molecules with a $C^{14}N/C^{15}N$ couple are at 2085 cm^{-1} ($C^{14}N$) and 2053 cm^{-1} ($C^{15}N$) (Fig. 4, *traces B* and *D*). The frequencies are in good agreement with what is expected for partially labeling a center containing two coupled CN^- ligands, a small residual coupling being noticed even after this labeling. The resultant bands are, however, somewhat broader than those in the *traces A* and *C*. This is presumably caused by slight differences in the protein environment of the two CN^- ligands, as apparent from the x-ray structure, *i.e.* a CN hydrogen-bonded to a Ser residue and a CN hydrogen-bonded to an Arg residue (3, 28).

From the data in Figs. 3 and 4 we therefore conclude that the two small bands arise from two coupled cyanides and the large one from CO.

Chemical Analysis of CO and CN^-—The procedure to detect enzyme-bound CO is relatively straightforward. About 0.7 mol of CO/mol of nickel was found in *C. vinosum* [NiFe]-hydrogenase. The recovery of the method is quite good (91%), so it is unclear why less than 1 CO per nickel was found. It cannot be excluded that part of the CO is oxidized to CO_2 during denaturation of the enzyme, when the prosthetic groups degrade to a uncontrolled mixture of iron, nickel, sulfur, and CN^-. Lower amounts were also obtained with Hb-CO as a control. The chemical analysis quite clearly showed the presence of 2.0 mol of CN^-/mol of nickel.

Structure of the Active Site—From the combined results of present and previous (4–6) infrared spectroscopic and chemical analyses, it is concluded that the active site of *C. vinosum* [NiFe]-hydrogenase contains two cyanides and one carbon monoxide bound to the same metal ion. As most other [NiFe]-hydrogenases have basically the same infrared spectrum (6, 29), this conclusion can be extended to those enzymes as well. The x-ray structure of the *D. gigas* enzyme (2, 3) shows that these diatomic ligands are bound to iron in the bimetallic NiFe site. Our data do not support the suggestion of Higuchi *et al.* (27), based on studies of the *D. vulgaris* enzyme, that [NiFe]-hydrogenases have only one CN^- as a ligand to iron, in addition to SO and CO.

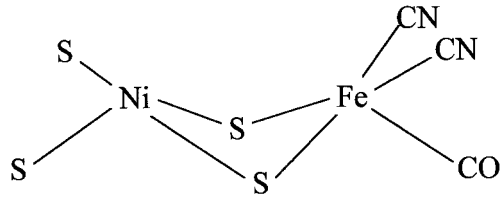

FIG. 5. **Model of the active site of [NiFe]-hydrogenases.** The model is based on the crystal structure of the *D. gigas* enzyme, determined by Volbeda and co-workers (3, 28) and the isotope studies reported here.

The results of our study enable a refinement of the model of the active site of [NiFe]-hydrogenases provided by the crystallographic studies (2, 3). This is shown in Fig. 5 . The strong ligand field due to the ligands around the active-site iron is expected to render it low spin, and this explains and confirms the observation of a lone low spin ferrous iron by Mössbauer spectroscopy (7) in the *C. vinosum* enzyme. This is unlike the remaining iron atoms in the enzyme, which are all contained in Fe-S clusters, and hence are high spin.

In view of the presence of similar infrared bands in [Fe]-hydrogenases (6), CO and cyanide are expected to be part of the active site in that class of hydrogenases as well. Hence, the activation of molecular hydrogen by all presently known, metal-containing hydrogenases obviously requires cyanide and carbon monoxide as ligands to iron in the active sites. The precise nature of these ligands in [Fe]-hydrogenases, as well as their stoichiometry, are currently under investigation in our laboratory.

Metal Cyanide Versus Metal Isocyanide—By examining the crystal structure, Volbeda and co-workers (3, 28) noted that ligand L1 could accept H bonds from the OH group of Ser-486 and its peptide NH and that L2 might form H bridges to the guanidine moiety of Arg-463, as well as to its peptide NH. Arg-463 is strictly conserved in [NiFe]-hydrogenases (30). At the position where serine is found in the *D. gigas* enzyme, either serine or threonine, which contains an hydroxyl group as well, is found in other [NiFe]-hydrogenases. Ligand L3 only makes contact with hydrophobic residues (3, 28), and this is why CN^- is preferred at the positions of L1 and L2. If the cyanide groups are indeed H-bridged, then these ligands will coordinate as cyanides, Fe-C-N···H, and not as isocyanides.

In inorganic compounds, iron very rarely complexes CN^- as isocyanide, due to the instability of the formed complex. The highest occupied sigma orbital is localized on the carbon atom, making this atom more basic than the nitrogen atom. This 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-

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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), this 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 between 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 $Fe(CN)_2CO$ 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 υ(CN) and υ(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 υ(CN) bands (Fig. 3). In the ^{13}C-enriched enzyme, however, about one-fifth (22%) of the υ(CO) band was still detectable at the original position. In principle, this could form an indication that all carbon for CN^- formation might come from bicarbonate, but this is not so for formation of CO. After considering the amounts of ^{12}C entering the culture from other possible sources, *e.g.* EDTA, cells, and products from the inoculum, we estimated these contaminations to be a few percent at most. We therefore have no explanation for the observed effect.

The biosynthesis of [NiFe]-hydrogenases is a highly complicated process (34, 35). A set of accessory genes in the hydrogenase operon is known to encode for proteins 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 or more of these gene products are involved in capture (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 N^{10}-formyltetrahydrofolate-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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ENZYMOLOGY:

Carbon Monoxide and Cyanide as Intrinsic Ligands to Iron in the Active Site of [NiFe]-Hydrogenases: NiFe(CN)2CO, BIOLOGY'S WAY TO ACTIVATE H2

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