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Similarities in the architecture of the active sites of Ni-hydrogenases and Fe-hydrogenases detected by means of infrared spectroscopy

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Three groups that absorb in the 2100–1800-cm^{-1} infrared spectral region have recently been detected in Ni-hydrogenase from *Chromatium vinosum* [Bagley, K. A., Duin, E. C., Roseboom, W., Albracht, S. P. J. & Woodruff, W. H. (1995) *Biochemistry 34*, 5527–5535]. To assess the significance and generality of this observation, we have carried out an infrared-spectroscopic study of eight hydrogenases of three different types (nickel, iron and metal-free) and of 11 other iron-sulfur and/or nickel proteins. Infrared bands in the 2100–1800-cm^{-1} spectral region were found in spectra of all Ni-hydrogenases and Fe-hydrogenases and were absent from spectra of any of the other proteins, including a metal-free hydrogenase. The positions of these bands are dependent on the redox state of the hydrogenase. The three groups in Ni-hydrogenases that are detected by infrared spectroscopy are assigned to the three unidentified small non-protein ligands that coordinate iron in the dinuclear Ni/Fe active site as observed in the X-ray structure of the enzyme from *Desulfovibrio gigas* [Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E. C., Frey, M. & Fontecilla-Camps, J. C. (1995) *Nature 373*, 580–587]. It is concluded that these groups occur exclusively in metal-containing H_2-activating enzymes. It is proposed that the active sites of Ni-hydrogenases and of Fe-hydrogenases have a similar architecture, that is required for the activation of molecular hydrogen.

Keywords: hydrogenase; Fourier-transform infrared spectroscopy; hydrogen activation; nickel; iron-sulfur protein.

Activation of H_2 in a wide variety of microorganisms is performed by enzymes called hydrogenases. Two types of enzymes are known that can activate H_2 without the need for added cofactors. Ni-hydrogenases (also called Ni-Fe hydrogenases or [NiFe] hydrogenases) contain one Ni atom and at least one [4Fe-4S] cluster; more [4Fe-4S] clusters and a [3Fe-4S] cluster are often detectable. The amino acid sequences of 30 such hydrogenases are known, only a few of which have been studied in detail [reviewed by Albracht (1994)]; Fe-hydrogenases [reviewed by Adams (1990)] contain Fe as their only metal constituent and are therefore also referred to as Fe-only hydrogenases. The amino acid sequences of four Fe-hydrogenases are known [reviewed by Albracht (1994)]. Except for the amino acid sequences that encode the binding sites of at least two [4Fe-4S] clusters, there is no obvious sequence similarity to the Ni-hydrogenases. The simplest Fe-hydrogenase, in terms of prosthetic groups, is that from *Desulfovibrio vulgaris* (Van der Westen et al., 1978). It contains two classical [4Fe-4S] clusters and an Fe-S cluster, termed the H-cluster, which has been proposed (Hagen et al., 1986) to contain approximately six Fe ions and to be directly involved in the activation of H_2. Fe-hydrogenases are usually about two orders of magnitude more active than Ni-hydrogenases but have a 100-fold-higher K_m value for H_2. In addition to the metal-containing hydrogenases, an enzyme from *Methanobacterium thermoautotrophicum* has been characterized which does not contain any transition metals and yet can activate H_2 while reducing methylenetetrahydromethanopterin (Zirngibl et al., 1992). However, this enzyme cannot activate H_2 in the absence of methylenetetrahydromethanopterin.

There is significant evidence that Ni is involved in the H_2-activating site of Ni-hydrogenases [reviewed by Albracht (1994)]. Mössbauer studies of the reduced *Chromatium vinosum* hydrogenase (Surerus et al., 1994) suggested the presence of a lone low-spin Fe ion, a [3Fe-4S] cluster and two [4Fe-4S] clusters. The redox equilibrium between this enzyme and H_2 (at pH 8.0 in the absence of mediating dyes) involves an $n = 2$ redox centre at the active site, in which an $S = 0.5$ Ni species with bound H_2 participates as one of the redox partners (Coremans et al., 1992a). The Fe-S clusters are not involved in this equilibrium (Ravi, N., Roseboom, W., Duin, E. C., Albracht, S. P. J. and Münck, E., unpublished results). This $n = 2$ centre has the same midpoint potential (E'_0) and pH dependence as the hydrogen electrode. It has therefore been hypothesized (Albracht, 1994) that the active site in Ni-hydrogenases involves a Ni ion and a lone Fe ion. The crystal structure of Ni-hydrogenase from *Desulfovibrio gigas* (Volbeda et al., 1995) indicates the possible presence of a lone Fe atom in close proximity to the Ni atom.

In Fe-hydrogenase from *D. vulgaris* in the presence of mediating dyes, the EPR signal proposed to represent the H-cluster

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Abbreviation. FTIR, Fourier-transform infrared.

behaves as an $S = 0.5$ Fe species with an E_0' of -307 mV ($n = 1-2$; pH 7.0; Pierik et al., 1992). In *Clostridium pasteurianum* Fe-hydrogenase this EPR signal disappears within 6 ms of mixing with H_2 (Erbes et al., 1975). The active sites in Ni-hydrogenases and Fe-hydrogenases have therefore been assumed to be very different.

Three absorption bands in the 2100–1850-cm^{-1} spectral region were described in infrared spectra of Ni-hydrogenase from *C. vinosum* (Bagley et al., 1994, 1995). The frequencies and intensity of these bands were novel for a protein. The bands were found to have a unique position in seven forms of the enzyme, which differed either in the redox state or in the state of coordination of the Ni centre. It was concluded that the bands represent groups that contain polar triple bonds and/or two adjacent double bonds very close to the Ni centre, and probably arise from ligands attached to this centre.

Recently, another enzyme has been reported to show bands at approximately 1850 cm^{-1}. In nitrile hydratase from *Rhodococcus* two bands at 1847 cm^{-1} and 1855 cm^{-1} have been ascribed to Fe-bound NO (Noguchi et al., 1995).

To examine how general the infrared-spectroscopy features of the *C. vinosum* enzyme are in Ni-hydrogenases, we have extended our infrared-spectroscopy investigations. Examination of the infrared spectra of four additional Ni-hydrogenases from widely different organisms establishes that the groups responsible for these bands are due to components of Ni-hydrogenases. For comparison, a variety of Fe-S proteins and some Ni-containing non-hydrogenase enzymes were also studied. None of the spectra from these proteins showed any bands in the 2100–1800-cm^{-1} spectral region. We investigated whether other H_2-activating enzymes showed such bands. Similar bands were also observed in two Fe-hydrogenases, but not in the H_2-forming methylenetetrahydromethanopterin dehydrogenase.

MATERIALS AND METHODS

A number of enzymes were purified by published procedures: Ni-hydrogenase from *Alcaligenes eutrophus* (soluble NAD$^+$-reducing enzyme; cells were obtained from G. Haverkamp and C. G. Friedrich, Dortmund, Germany; Schneider et al., 1979; Friedrich et al., 1982); Ni-hydrogenase from *C. vinosum* (strain DSM 185) (Coremans et al., 1992b); Fe-hydrogenase (Van der Westen et al., 1978) and prismane protein (Stokkermans et al., 1992) from *D. vulgaris* (Hildenborough); dissimilatory sulfite reductase from *Desulfosarcina variabilis* (Arendsen et al., 1993); ferredoxin from *Megasphaera elsdenii* (Gillard et al., 1965); and rubredoxin from *Pyrococcus furiosus* (Blake et al., 1991).

Other proteins were kind gifts from several laboratories: *Wolinella succinogenes* hydrogenase (Albracht et al., 1986) from A. Kröger (Frankfurt, Germany); *Methanococcus voltae* hydrogenase (Sorgenfrei et al., 1993) from O. Sorgenfrei and A. Klein (Marburg, Germany); *M. thermoautotrophicum* hydrogenases (Zirngibl et al., 1992) from G. Hartmann, R. Hedderich and R. K. Thauer (Marburg, Germany); Fe-hydrogenase from *M. elsdenii* (Van Dijk et al., 1980, Filipiak et al., 1989) from M. Filipiak (Wageningen, The Netherlands); the water-soluble Fe-S fragment of the Rieske protein of bovine heart bc_1 complex (Link et al., 1992) from T. A. Link (Frankfurt, Germany); *Rhodopseudomonas gelatinosa* high-potential iron protein (Bartsch, 1978) from T. E. Meyer (Tucson, AZ, USA); MoFe-protein (component I) of *Azotobacter chroococcum* nitrogenase (Yates and Planque, 1975) from R. R. Eady (Brighton, UK); *Methanothrix soehngenii* CO dehydrogenase (Jetten et al., 1991) and methyl-CoM reductase (Jetten et al., 1990) from M. S. M. Jetten (Wageningen, The Netherlands); and bovine heart Complex I (Finel et al., 1992) from R. van Belzen (Amsterdam, The Netherlands). *Spirulina platensis* [2Fe-2S] ferredoxin was from Sigma.

For the infrared-spectroscopy comparisons of the various hydrogenases and non-hydrogenase proteins, the proteins were dissolved in either 50 mM Tris/HCl, pH 8.0, or 20–120 mM potassium phosphate, pH 7–8, and concentrated to 0.12–2 mM by means of Centricon PM30 or PM10 filters. Examination of the infrared spectra of the buffers revealed no specific infrared absorption bands in the 2100–1800-cm^{-1} region. O_2-sensitive proteins were kept under Ar in the presence of 2–50 mM sodium dithionite. Samples were treated as indicated in Results and loaded into a gas-tight infrared-transmittance cell (Bagley et al., 1994) with CaF_2 windows and Teflon spacers of 50–60 µm (total sample approximately 10 µl). Aerobic samples were directly loaded into the cell and the infrared spectrometer was purged with dry air. O_2-sensitive samples were placed in a glove box, purged with Ar, and loaded into the anaerobic infrared-transmittance cell. Prior to loading the samples, the cell was filled with a mixture of glucose (80 mM) and glucose oxidase (0.4 mg/ml) to remove O_2. After 15 min the cell was extensively rinsed with Ar-saturated buffer. After loading the sample, the cell was transported to the Fourier-tansform infrared (FTIR) spectrometer in an anaerobic container and the FTIR spectrometer was purged continuously with N_2. Infrared spectra were collected at room temperature on a BioRad FTS-60A FTIR spectrometer with an MCT detector. Spectra were corrected for the water background by subtraction of the corresponding buffer spectrum. The multiple-point method of the programme WIN-IR (BioRad) was used for further correction of the base line. Spectral resolution was 2 cm^{-1}.

Studies of the pH dependence of the bands were performed with oxidized *C. vinosum* enzyme dissolved in 50 mM of each of the following buffers: Mes, pH 6.0; Mops, pH 6.75; Tris/HCl, pH 8.0; Taps, pH 9.0; glycine/NaOH, pH 9.75. Spectra were collected by means of a Perkin Elmer 1600 FTIR instrument equipped with a DTGS detector. Spectra were collected at 4-cm^{-1} resolution. For comparison of spectra measured at different pH values, the intensities of the bands were scaled with the absorbance at 280 nm for the enzyme loaded in the infrared-transmittance cell.

RESULTS

In previous infrared-spectroscopy studies, at cryogenic temperatures, of the Ni-hydrogenase from *C. vinosum* (Bagley et al., 1994, 1995) it was reported that this enzyme showed three bands in the 2100–1800-cm^{-1} spectral region. The groups responsible for these bands responded to all changes in the status of the Ni centre. We have now extended these studies by examination of the infrared absorption spectra at room temperature of several other Ni-hydrogenases to determine whether the presence of these bands is a general property of this class of enzymes.

C. vinosum hydrogenase. As only limited quantities of the other enzymes were available, we first tested the minimal concentration required for reliable detection of the major infrared absorption band of the *C. vinosum* enzyme. Infrared spectra of the enzyme at different concentrations are shown in Fig. 1. A direct linear relationship was observed between the amplitude of the 1945-cm^{-1} band and the enzyme concentration. The band could still be observed at 50 µM enzyme. All subsequent samples were tested at 0.12–2 mM protein.

The bands at 2092, 2082 and 1945 cm^{-1} are typical of enzymes with trivalent Ni (Bagley et al., 1995). The bands at 2066,

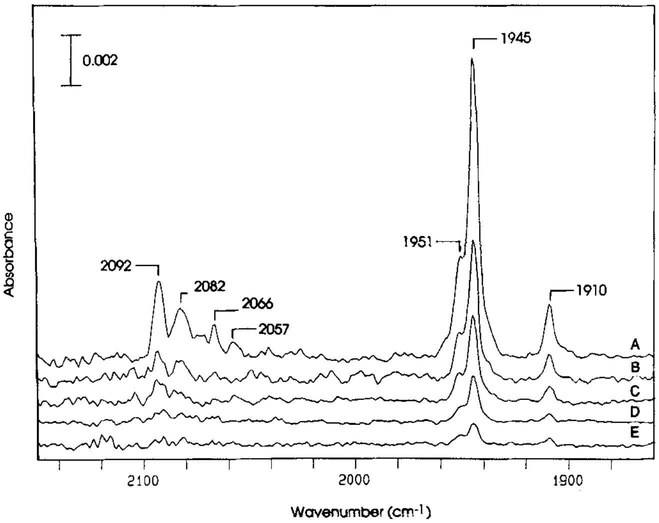

Fig. 1. Infrared spectra at room temperature of *C. vinosum* Ni-hydrogenase at several concentrations. The spectra were averages of scans at 2-cm^{-1} resolution. (A), 640 μM, 2048 scans; (B), 320 μM, 5000 scans; (C), 213 μM, 5000 scans; (D), 107 μM, 5000 scans; (E), 53 μM, 5000 scans.

2057 and $1910\ \text{cm}^{-1}$ (Fig. 1) are here ascribed to an enzyme with divalent Ni. Although in an earlier study (Bagley et al., 1995) divalent Ni was ascribed to have bands at 2067, 2051 and $1910\ \text{cm}^{-1}$ in low-temperature infrared spectra, we consistently found the second band at $2057\ \text{cm}^{-1}$ in room-temperature spectra.

The shoulder at $1951\ \text{cm}^{-1}$ detected in these room-temperature studies was not previously reported in spectra of *C. vinosum* at cryogenic temperatures (Bagley et al., 1994, 1995). Examination of the infrared spectra of unready enzyme (i.e., inactive enzyme which is unready to react with H_2; Fernandez et al., 1985) in its fully oxidized state suggested that this band displays significant temperature sensitivity. The band was quite large and well resolved in spectra taken at room temperature (1-cm^{-1} resolution) but decreased significantly in intensity (to nearly background level) when the sample was cooled to cryogenic temperatures (data not shown). In addition, it appeared that the 1951-cm^{-1} band was maximal in preparations where the Ni^{3+} ion was spin coupled to a system that consisted of an unknown redox group, X, and a $[3Fe\text{-}4S]^+$ cluster $\{X^{ox} = [3Fe\text{-}4S]^+\}$ (Surerus et al., 1994; Albracht, 1994), and minimal in the oxidized enzyme where no such spin coupling could be detected (Ni^{3+} X^{red} $[3Fe\text{-}4S]^+$).

A study of the bands detected by infrared spectroscopy as a function of pH in the range 6.0−9.75 suggested that there was no significant change in frequency or intensity for any of the bands in the $2100-1800\text{-cm}^{-1}$ spectral region of oxidized *C. vinosum* enzyme over this pH range.

Other Ni-hydrogenases. To determine whether the bands detectable by infrared spectroscopy of the *C. vinosum* enzyme are a general property of Ni-hydrogenases, we studied several enzymes from other sources. As an example, the spectrum of an aerobic sample of the soluble hydrogenase from *A. eutrophus* is shown in Fig. 2. The major infrared band is at $1956\ \text{cm}^{-1}$, whereas three minor bands are present at 2087, 2081 and $2071\ \text{cm}^{-1}$.

Examination of the infrared spectra of three other Ni-hydrogenases in their oxidized forms shows the presence of at least one intense infrared band between $1960\ \text{cm}^{-1}$ and $1850\ \text{cm}^{-1}$ (Table 1). The spectrum of F_{420}-non-reducing enzyme from *M. thermoautotrophicum* showed a band at $1955\ \text{cm}^{-1}$, that of ^{77}Se-containing F_{420}-reducing enzyme from *M. voltae* showed bands at $1930\ \text{cm}^{-1}$ and $1921\ \text{cm}^{-1}$, and that of the Ni-hydrogenase from *W. succinogenes* showed a band at $1942\ \text{cm}^{-1}$ with a shoulder at $1953\ \text{cm}^{-1}$. In addition, as previously reported (Fernandez, V. M. and Hatchikian, E. C., personal communication), the enzyme from *D. gigas* shows intense bands in this region. From the observation of these bands in six Ni-hydrogenases, we conclude that the groups responsible for these bands are probably present in all Ni-hydrogenases and that they arise from very similar chemical structures.

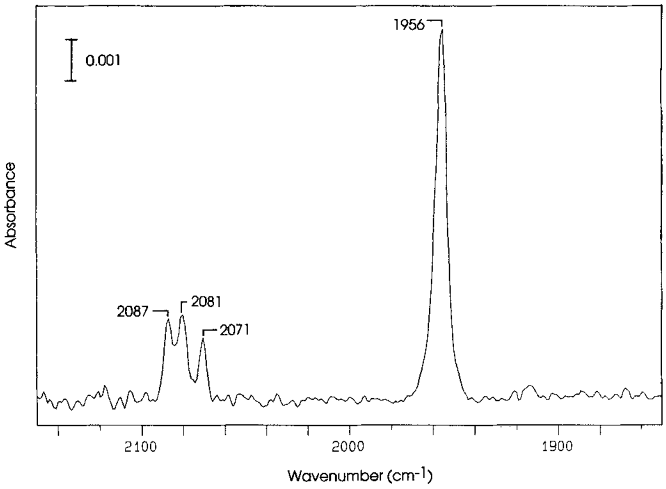

Fig. 2. Infrared spectrum at room temperature of the soluble Ni-hydrogenase from *A. eutrophus*. The spectrum was an average of 5000 scans at 2-cm^{-1} resolution.

Fe-S proteins and Ni-enzymes (non-hydrogenases). Ni-hydrogenases contain Ni and Fe-S clusters as prosthetic groups. To confirm that the presence of these types of prosthetic groups in other proteins do not give rise to the infrared absorbances described above, we examined the infrared spectra of a number of non-hydrogenase proteins that contain either Fe-S clusters, Ni ions or both. We could not detect infrared bands in the $2100-1800\text{-cm}^{-1}$ spectral region in any of the following proteins (Table 1): rubredoxin from *P. furiosus*; ferredoxins from *M. elsdenii* and *S. platensis*; the Rieske Fe-S protein from bovine heart; high-potential iron protein from *R. gelatinosa*; the prismane protein from *D. vulgaris* (Hildenborough); the MoFe-protein of nitrogenase from *A. chroococcum*; dissimilatory sulfite reductase from *D. variabilis*; and methyl-CoM reductase and CO-dehydrogenase from *M. soehngenii*. The published infrared spectra of CO dehydrogenase from *Clostridium thermoaceticum* (Kumar and Ragsdale, 1992) also do not display bands in this region.

The sequence similarity (Albracht, 1993) of the 49-kDa subunit and the PSST subunit, named after the first four amino-acid residues of this subunit (Arizmendi et al., 1992), of mitochondrial NADH:ubiquinone oxidoreductase (Complex I) with the large and the small subunits of Ni-hydrogenases, respectively, prompted us to study Complex I from bovine heart. No bands in the $2100-1800\text{-cm}^{-1}$ region could be detected in this enzyme.

Non-Ni-hydrogenases. We investigated whether other H_2-activating enzymes showed these bands. We could not find any such bands in the purified H_2-forming methylenetetrahydromethanopterin dehydrogenase. We have not tested whether such bands were induced by addition of the substrate methylenetetrahydromethanopterin. This substrate alone has no bands in

Table 1. Proteins studied by means of infrared spectroscopy. The presence (+) or absence (−) of absorption bands in the 2100–1800-cm^{-1} spectral region was investigated.

Protein	Prosthetic groups	Source	Infrared bands
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Chromatium vinosum*	+
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Alcaligenes eutrophus*	+
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Wolinella succinogenes*	+
Ni-hydrogenase	Ni, Fe, [4Fe-4S]	*Methanococcus voltae*	+
Ni-hydrogenase	Ni, Fe, [4Fe-4S]	*Methanobacterium thermoautotrophicum*	+
Metal-free hydrogenase	none	*Methanobacterium thermoautotrophicum*	−
Fe-hydrogenase	H-cluster, [4Fe-4S]	*Desulfovibrio vulgaris*	+
Fe-hydrogenase	H-cluster, [4Fe-4S]	*Megasphera elsdenii*	+
Rubredoxin	$Fe(Cys)_4$	*Pyrococcus furiosus*	−
Ferredoxin	[2Fe-2S]	*Spirulina platensis*	−
Rieske Fe-S protein	[2Fe-2S]	Bovine heart	−
High-potential iron protein	[4Fe-4S]	*Rhodopseudomonas gelatinosa*	−
Ferredoxin	[4Fe-4S]	*Megasphaera elsdenii*	−
Complex I	FMN, [2Fe-2S], [4Fe-4S]	Bovine heart	−
MoFe-protein	P-cluster, FeMo cofactor	*Azotobacter choococcum*	−
Prismane protein	[6Fe-6S]	*Desulfovibrio vulgaris*	−
Dissimilatory sulfite reductase	siroheme, [*x*Fe-4S]	*Desulfosarcina variabilis*	−
CO dehydrogenase	Ni, [*x*Fe-*y*S]	*Methanothrix soehngenii*	−
Methyl-CoM reductase	Ni (F_{430})	*Methanothrix soehngenii*	−

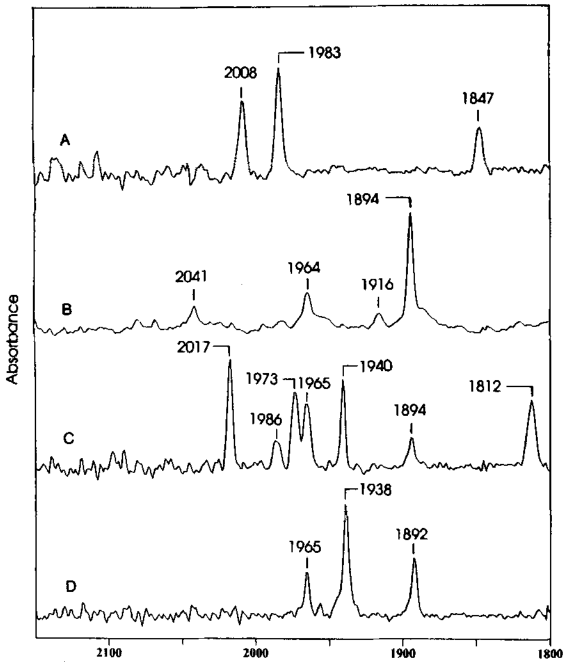

Fig. 3. Infrared spectra of Fe-hydrogenases from *D. vulgaris* and *M. elsdenii*. The spectra were averages of 5000 scans at 2-cm^{-1} resolution. (A), enzyme from *D. vulgaris* isolated in air; (B), *D. vulgaris* enzyme reduced with 50 mM dithionite for 2 h at room temperature; (C), *D. vulgaris* enzyme treated with H_2 for 15 min at room temperature then with Ar for 2 h at room temperature; (D), *M. elsdenii* enzyme prepared in the presence of 10 mM dithionite.

the 2100–1800-cm^{-1} region (G. Hartmann and A. Klein, personal communication).

The Fe-hydrogenases from *D. vulgaris* and *M. elsdenii*, however, showed bands in the 2100–1800 cm^{-1}-region (Fig. 3). The *D. vulgaris* enzyme, isolated in air (Fig. 3A), showed bands at 2008, 1983 and 1847 cm^{-1}. Upon reduction with dithionite a major band at 1894 cm^{-1} and minor bands at 2041, 1964 and 1916 cm^{-1} were observed (Fig. 3B). When the enzyme was reduced with H_2 and reoxidized with protons (i.e. by replacement of H_2 with Ar for several vacuum/Ar cycles), a number of bands appeared (2017, 1986, 1973, 1965, 1940, 1894 and 1812 cm^{-1}; Fig. 3C). The dithionite-reduced Fe-hydrogenase from *M. elsdenii* (Fig. 3D) only showed bands at 1965, 1938 and 1892 cm^{-1}. With this enzyme we found that the bands at 1965 cm^{-1} and 1938 cm^{-1} bands were maximal, and the 1892-cm^{-1} band was minimal, in the absence of dithionite, whereas the opposite occurred in the presence of excess dithionite. The major band detected by infrared spectroscopy of fully reduced Fe-hydrogenases from both bacteria was at 1892–1894 cm^{-1}. The enzyme in less-reduced states showed a variety of bands at different positions, presumably due to the presence of enzyme molecules in various redox states. These observations suggest that the Fe-hydrogenases also display the infrared features detected in the Ni-hydrogenases and that, like the Ni-hydrogenases, the bands detected by infrared spectroscopy in the Fe-hydrogenases are sensitive to the oxidation state of the active site.

DISCUSSION

***C. vinosum* hydrogenase.** The band at 1951 cm^{-1} has only been detected reproducibly in preparations which showed a large amount of spin coupling between Ni^{3+} and the $\{X^{ox} - [3Fe\text{-}4S]^+\}$ species. This coupling is not well understood. X behaves like an $n = 1$ Nernst redox component with an E_0' of +150 mV (Coremans et al., 1992b). There are indications (Surerus et al., 1994) that X might be an Fe ion or a radical species close to the [3Fe-4S] cluster. The observation of the spin-spin interaction in EPR experiments suggests that the distance between the Ni^{3+} and the $\{X^{ox} = [3Fe\text{-}4S]^+\}$ system is much shorter than that between the Ni atom and the [3Fe-4S] cluster (approximately 2.1 nm) in the X-ray structure of the *D. gigas* enzyme (Volbeda et al., 1993, 1995). After reduction of X, the Ni^{3+} spin has no interaction with the $S = 0.5$ of the oxidized [3Fe-4S] cluster (or the $S = 2$ of the reduced cluster), which shows that they are far apart under these conditions (Albracht et al., 1984; Asso et al., 1992). It is possible that a structural change takes place when X changes oxidation state. Given the apparent correlation between the detection of the 1951-cm^{-1} band and the spin-coupled state, we suspect that the band at 1951 cm^{-1} might represent X in its oxidized state. This possibility is currently under investigation.

Groups in metal-containing hydrogenases detectable by infrared spectroscopy. The results presented here indicate that the bands in the 2100–1800-cm^{-1} spectral region are exclusively present in metal-containing H_2-activating enzymes. They are not simply due to the presence of Fe-S clusters or Ni. This finding and the response to specific changes of the active sites in these enzymes strongly indicate that the groups involved are an integral part of the H_2-activating site. The major bands in the Ni-hydrogenases and Fe-hydrogenases are in the 2020–1810-cm^{-1} region.

As previously discussed (Bagley et al., 1994, 1995), it is unlikely that the bands in the spectrum of the *C. vinosum* enzyme are due to exchangeable CO molecules, bonds that involve an exchangeable proton, or a metal hydride. The frequency and intensity of the bands in the 2100–1800-cm^{-1} spectral region are most consistent with chemical groups that contain polar triple bonds (e.g., cyanide, CO, metal-bound N_2) or a system of adjacent double bonds (e.g., azide, thiocyanate, isothiocyanate) (Nakamoto, 1978; Colthup et al., 1990). Metal-coordinated NO has recently been proposed to be present in the Fe-containing enzyme nitrile hydratase (Noguchi et al., 1995) based on detection of two ^{15}N-sensitive bands at 1855 cm^{-1} and 1847 cm^{-1} in the infrared spectrum of this enzyme. The frequency detected in the nitrile hydratase enzyme is most consistent with a neutral or negatively charged ligand since neutral NO and negatively charged NO have $v(NO)$ stretching frequencies below 1880 cm^{-1}. Of the metal-containing hydrogenases studied only one of the Fe-hydrogenases showed bands which were so low in frequency (Fig. 3), which suggests that most, if not all, of the bands detected are not due to a neutral or negatively charged nitrosyl ligand. However, positively charged NO ligands (nitrosium) coordinated to metals have significantly higher frequencies and may be consistent with a number of the infrared frequencies detected in the 2100–1800-cm^{-1} spectral region of the metal-containing hydrogenases.

The X-ray structure of Ni-hydrogenase from *D. gigas* indicates that there are three non-protein groups coordinated to the Fe at the binuclear Ni/Fe active site (Volbeda et al., 1995). The three groups detectable by infrared spectroscopy in Ni-hydrogenases described here are probably the same three non-protein groups detected in the X-ray structure. Since the position of the bands detected by infrared spectroscopy is highly sensitive to the status of the Ni-centre as monitored by EPR, it was concluded (Bagley et al., 1995) that the groups were ligands to Ni. However, the X-ray data indicate that the three non-protein groups are ligands to the Fe atom. None of the EPR signals ascribed to Ni in the *C. vinosum* enzyme showed any broadening when inspected in preparations more than 90% enriched in ^{57}Fe, but all of them showed broadening or splitting in ^{61}Ni-enriched preparations (Duin, E. C. and Albracht, S. P. J., unpublished results). This finding suggests that unpaired spins in the active site are localized on Ni, rather than on Fe. If the groups detectable by infrared spectroscopy are ligands to the Fe ion, then electronic changes on the Ni ion, as monitored in EPR, are effectively transferred to the Fe ion, resulting in changes of the infrared frequency of the three unknown groups.

The finding of three minor bands in the infrared spectra of the soluble *A. eutrophus* enzyme (Fig. 2), instead of two bands in the *C. vinosum* enzyme, might be related to the finding that in the *Alcaligenes* enzyme no significant EPR signals of Ni can be evoked under any redox conditions (Cammack et al., 1986), unless artificial redox dyes are added (Happe, R., Massanz, C., Friedrich, B. and Albracht, S. P. J., unpublished results). This enzyme is not inhibited by O_2 or CO (Schneider et al., 1983). We speculate that the active site in this enzyme contains an extra group detectable by infrared spectroscopy that fixes the Ni in the divalent state and blocks the site where CO and O_2 can attack other Ni-hydrogenases.

The presence of these groups detectable by infrared spectroscopy in the active sites of metal-containing hydrogenases suggests some similarities in the structure of the H_2-activating centres of Ni-hydrogenases and Fe-hydrogenases. As one of the possibilities we speculate that the H_2-activating centre in both enzymes consists of a bi-metallic centre (Ni/Fe in Ni-hydrogenases or Fe/Fe in Fe-hydrogenases) involving a low-spin Fe ion, the groups detectable by infrared spectroscopy and thiols from Cys residues.

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