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Paramagnetic centers in the nickel-containing, deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*

(methanogenesis/archaebacteria/factor 420)

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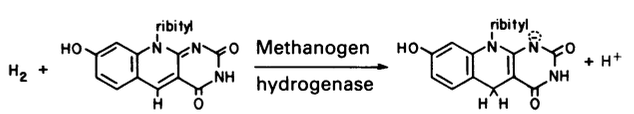
ABSTRACT **Two hydrogenases from the methanogenic bacterium *Methanobacterium thermoautotrophicum* strain ΔH have been purified and contain tightly bound nickel as well as the anticipated iron/sulfur atoms with a fixed ratio of 15–20 iron atoms per nickel. One hydrogenase reduces the 8-hydroxy-5-deazaflavin coenzyme factor 420 (F_{420}), whereas the other has been purified as a methyl viologen-reducing hydrogenase. Both enzymes possess an EPR signal attributed to paramagnetic nickel as demonstrated by hyperfine coupling in ^{61}Ni-containing hydrogenases. Comparison to model compounds suggests a nickel(III) oxidation state in the inactive forms of these aerobically purified enzymes. Loss of the nickel(III) signal accompanies reductive activation but is not kinetically correlated with regain of high specific activity. On replacement of H_2 by argon in the gas phase over reduced, active, F_{420}-reducing enzyme, several EPR signals appear, including a signal at $g = 2.004$ that is probably enzyme-bound FADH semiquinone, two signals at $g = 2.140$ and 2.196 that reflect a new form of paramagnetic nickel(III), and also a signal at $g = 2.036$ that may be an iron signal. The F_{420}-reducing hydrogenase in the second paramagnetic nickel form is either itself active or in facile equilibrium with active enzyme. The size of the signal at $g = 2.036$ may correlate with the degree of activation of the enzyme. In contrast to the hydrogenase of *Clostridium pasteurianum* [Erbes, D. L., Burris, R. H. & Orme-Johnson, W. H. (1975) *Proc. Natl. Acad. Sci. USA* 72, 4795–4799], which appears to use only iron/sulfur prosthetic groups and which reacts with one-electron-transfer agents, this methanogen hydrogenase seems to utilize iron, nickel, and flavin redox sites and to reduce obligate one-electron (viologen) and two-electron (deazaflavin) oxidants.**

Methanogenic bacteria reduce CO_2 to CH_4 in an overall eight-electron reduction process that involves the cooxidation of four molecules of hydrogen gas, H_2.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

The H_2 oxidation steps, discrete from the carbon reduction steps, are catalyzed by one or more hydrogenases which reduce some cosubstrate X to its dihydro form XH_2.



Scheme 1

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A variety of studies (1, 2), mostly from Wolfe and his colleagues, have suggested that the naturally occurring 8-hydroxy-5-deazaflavin coenzyme factor 420 (F_{420}) might be the acceptor of H_2-derived electrons because the H_2-mediated reduction of F_{420} to dihydro F_{420}, $F_{420}H_2$, could be monitored in crude extracts. We have reported (3) the purification from *Methanobacterium thermoautotrophicum* strain ΔH of a three-subunit hydrogenase that does, in fact, use F_{420} as cosubstrate. This hydrogenase comprises about 2% of the cell protein and was separated from a second hydrogenase, present in about an equal amount, which could not reduce the deazaflavin cofactor but was assayable by methyl viologen reduction.

The growth of methanobacteria is stimulated by nickel ions, and much of the added nickel is incorporated specifically into a prosthetic group, F_{430} (4, 5). This cofactor is bound to the enzyme involved in the last reductive step in methane biosynthesis, methyl-coenzyme M (CoM) methylreductase (6). Thauer, Eschenmoser, and colleagues have just determined that factor 430 is a novel nickel-liganded tetrapyrrole (7). In addition, radioactive ^{63}Ni is incorporated stoichiometrically into the methyl viologen-reducing hydrogenase of *M. thermoautotrophicum*, Marburg strain (8), whereas we have reported tightly bound nickel in the F_{420}-reducing hydrogenase from the ΔH strain by atomic absorption analysis (3). Recently, stoichiometric amounts of nickel have been reported in a pure hydrogenase from *Desulfovibrio gigas* (9, 10) and *Alcaligenes eutrophus* (11).

In this paper, we report studies on the oxidation state of the nickel in the two hydrogenases from *M. thermoautotrophicum* strain ΔH and on the redox modulation of the nickel ion and the bound FAD and iron/sulfur atoms associated with the F_{420}-reducing hydrogenase.

EXPERIMENTAL

Hydrogenases were prepared as described from *M. thermoautotrophicum* strain ΔH (3). Enzymes isotopically labeled with ^{61}Ni were prepared from cells grown on 10 μM $Ni(NO_3)_2$, 88.84% ^{61}Ni enriched from Oak Ridge National Laboratory. Cells were grown in a 14-liter New Brunswick glass fermentor from an inoculum of nickel-starved cells and harvested with a Millipore Pellicon Membrane filtration system. Ni-containing hydrogenase from normal cells is referred to as ^{59}Ni-hydrogenase or enzyme, and isotopically enriched enzyme, as ^{61}Ni-hydrogenase or enzyme.

EPR spectra were recorded on a Varian E-9 spectrometer operating in the X band. Except where otherwise stated, the

Abbreviations: F_{420}, naturally occurring 8-hydroxy-5-deazaflavin coenzyme factor 420; CoM, coenzyme M.

enzyme (≈50% pure) was used at ≈2.5 mg/ml (total protein, 5 mg/ml) in 50 mM Tris·HCl buffer (pH 7.5 at room temperature). Intensities of EPR lines were calculated by double integration compared to a Cu-EDTA standard.

RESULTS

Composition of F_{420}-Reducing and Methyl Viologen-Reducing Hydrogenases. We have found (3) by $NaDodSO_4$ assay three subunits of M_rs 40,000, 31,000, and 26,000 in the *M. thermoautotrophicum* ΔH F_{420}-reducing hydrogenase. Purification of the methyl viologen-reducing hydrogenase activity from this same organism led to enrichment of a two-subunit protein with subunit M_rs of 52,000 and 40,000 as noted in Table 1. In both hydrogenases, an iron/nickel ratio of 15–20:1 was observed from cells grown on either $^{59}Ni(NO_3)_2$ or $^{61}Ni(NO_3)_2$. No value for iron/sulfur content was reported by Thauer and colleagues for the partially purified hydrogenase from the Marburg strain (8, 12). Whether the M_r 40,000 subunit is common to the two hydrogenases listed in Table 1 (and, for example, contains the iron/sulfur clusters and the nickel) is not known at this time.

EPR Analysis of F_{420}-Reducing and Methyl Viologen-Reducing Hydrogenases. We had noted briefly (3) the existence of unusual EPR signals in the inactive, isolated F_{420}-reducing hydrogenase and, therefore, tested the proposition that they may signal a paramagnetic nickel species in the enzyme. To determine whether the EPR signals observed at 150 K at g values of 2.309, 2.237, and 2.017 in the ^{59}Ni-enzyme represented a nickel paramagnet, we prepared enzyme from cells grown on 10 μM $^{61}Ni(NO_3)_2$ at 89% isotopic enrichment. The EPR spectra of the F_{420}-reducing ^{61}Ni- and ^{59}Ni-hydrogenases are compared in Fig. 1*A*; the spectrum of the ^{61}Ni-enzyme shows broadening of the signals at g = 2.309 and 2.237, while the signal at g = 2.017 is resolved into the four-line hyperfine coupling pattern anticipated for ^{61}Ni, which has a nuclear spin $I = 3/2$. The ^{59}Ni isotope has $I = 0$. This is unambiguous evidence for paramagnetic nickel in the F_{420}-reducing hydrogenase as isolated.

The corresponding EPR spectra for methyl viologen-reducing ^{59}Ni- and ^{61}Ni-hydrogenase from this ΔH strain (Fig. 1*B*) showed essentially identical patterns to the F_{420}-reducing enzyme. These data suggest similar ligand environments, coordination numbers, and geometries for the nickel paramagnets in both hydrogenases from this organism. The g values (Table 2) approximate those reported by Margerum and colleagues (also in Table 2) for nickel(III)–tetraglycine complexes made by iridate oxidation of nickel(II) species and quick freezing for EPR analysis (13). Margerum suggested tetragonally distorted octahedral coordination geometry for those complexes. We ascertained that there is no factor 430 or other porphyrin-like material bound to the purified F_{420}-reducing hydrogenase, so the ligands to the nickel(III) ion are likely to be amino acid side chains from the enzyme.

An essentially identical set of ^{59}Ni and ^{61}Ni EPR lines to those described above have just been described by Thauer and colleagues (12) for the partially purified hydrogenase from *M. thermoautotrophicum*, Marburg strain, an organism that, despite the same species name as the ΔH strain, has only about 40% DNA homology and so is very different (14). In studies on crude membrane particles from *Methanobacterium bryantii*, Lancaster also has noted resolution of an EPR signal into four lines on ^{61}Ni substitution (15), and we now tentatively assign this to a hydrogenase in those membranes. Finally, two preliminary reports also have appeared documenting an almost identical ^{59}Ni EPR signal (but no ^{61}Ni data) in a pure hydrogenase, assayed with methyl viologen, from *D. gigas*, a nonmethanogenic, sulfate-reducing bacterium (9, 10). We note that, in all these cases, the initial nickel(III) signal is associated with inactive forms of the hydrogenases, all of which require reductive activation to regain catalytic activity. By integration of the nickel(III) spins in the F_{420}-reducing hydrogenase, we found that 50% of the total nickel is detectable as this paramagnetic oxidation state. For comparison, about 46% of the *D. gigas* hydrogenase nickel is detectable paramagnetically (10).

Because we found large amounts of iron and sulfur associated with each of the two methanogen hydrogenases, and each enzyme has the brown color and broad peak around 400 nm associated with iron/sulfur clusters, we examined the enzymes at lower temperatures in the EPR spectrometer. At 11 K, EPR signals from iron paramagnets were detectable only in reduced enzyme, as shown in Fig. 2 for both the F_{420}- and the methyl viologen-reducing hydrogenases; the complex set of broad lines in the area typical of iron/sulfur clusters may represent a set of interacting iron species yet to be deconvoluted. The integrated spin is about 0.5 electrons, but this may be an underestimate if there are several interacting paramagnets; proper analysis must await the quantitation of the types and amounts of iron/sulfur clusters present (16).

EPR Studies on F_{420}-Reducing Hydrogenase on H_2 Reduction. We monitored the EPR spectrum of the F_{420}-reducing hydrogenase during the period of reductive activation to analyze whether there was detectable change in the EPR-active nickel signal. Fig. 3 shows several changes during conversion to catalytically competent enzyme on exposure to H_2 under anaerobiosis. Spectrum B indicates that after 60 min the original nickel(III) species disappeared presumably by reduction, probably to nickel(II), and a new signal appeared at g = 2.036. During reduction by H_2, there was transient formation of an EPR signal at g = 2.004 with a linewidth of 20 gauss. This signal is typical of a flavin semiquinone and, given the presence of tightly bound FAD in the enzyme, we assign this provisionally as the

Table 1. Components of the F_{420}-reducing and methyl viologen-reducing hydrogenases from *M. thermoautotrophicum*

Hydrogenase	Subunits M_r	Subunits Ratio	Cofactor content per M_r 170,000	Iron/nickel ratio
F_{420}-reducing (M_r, 170,000)	40,000	2	FAD, 2.3	^{59}Ni-enzyme, 17:1
	31,000	2	Iron, 33–43 atoms	
	26,000	1	Sulfide, 24–30 atoms	^{61}Ni-enzyme, 15:1
			Nickel, 2.5–3.1 atoms	
Methyl viologen-reducing	52,000	—	Iron	^{59}Ni-enzyme, 21:1
	40,000	—	Nickel	^{61}Ni-enzyme, 14–18:1

FAD, inorganic sulfide, and subunit molecular weights were determined as described (3). Iron and nickel were quantitated by using a Perkin-Elmer 2380 atomic absorption spectrophotometer fitted with an HGA-400 graphite furnace assembly.

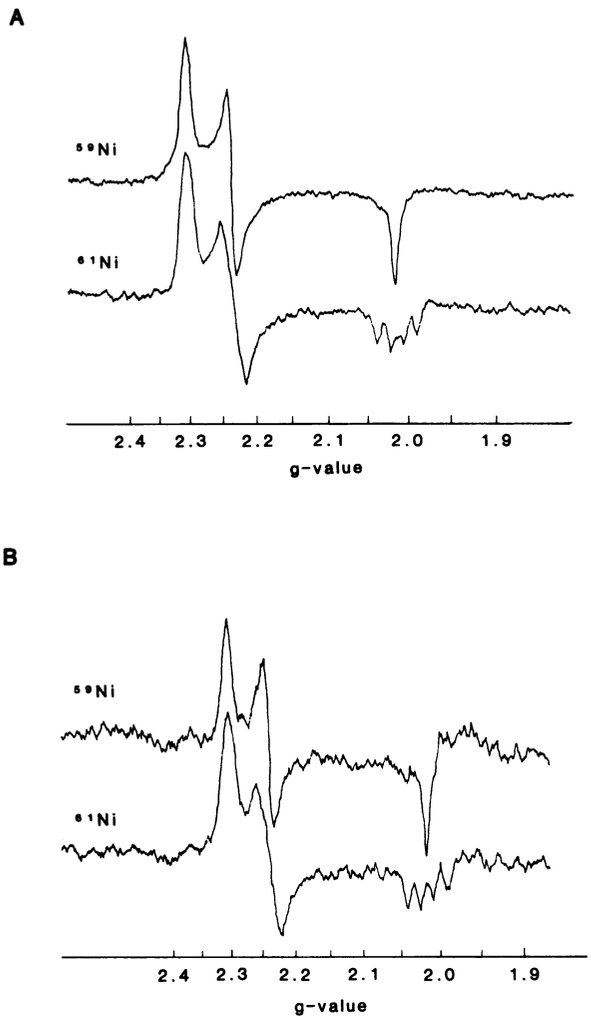


FIG. 1. EPR spectra of hydrogenases as isolated from *M. thermoautotrophicum*. (*A*) F_{420}-reducing hydrogenase (5 mg/ml). (*B*) Methyl viologen-reducing hydrogenase (^{59}Ni-hydrogenase, 10 mg/ml; ^{61}Ni-hydrogenase, 20 mg/ml). Upper spectra in *A* and *B* are of ^{59}Ni-hydrogenase, and lower spectra are of ^{61}Ni-hydrogenase. EPR spectra were recorded under the following conditions: microwave frequency, 9.17 GHz; modulation frequency, 100 kHz; modulation amplitude, 10 G; temperature, 150 K; microwave power, 10 mW; time constant, 1 sec; and scanning rate, 250 G/min. Field positions are shown on the frequency-independent *g* scale.

FADH semiquinone arising on one-electron reduction from oxidized FAD. The nickel EPR signal disappeared before the flavin semiquinone appeared (not shown). Continued exposure

Table 2. Nickel(III) EPR parameters for the F_{420}-reducing hydrogenase, the hydrogen-reduced enzyme after replacement of hydrogen by argon, and nickel(III)–tetraglycine (13)

EPR parameters	F_{420}-reducing hydrogenase		Nickel(III)–tetraglycine
	As isolated	H_2/Ar-treated	
g values			
g_{xx}	2.309	2.196	2.24–2.32
g_{yy}	2.237	2.140	2.28–2.29
g_{zz}	2.017	—	2.00–2.01
Hyperfine coupling of ^{61}Ni			
A_{xx}	7	≈1.5	
A_{yy}	14	≈2	
A_{zz}	26	—	

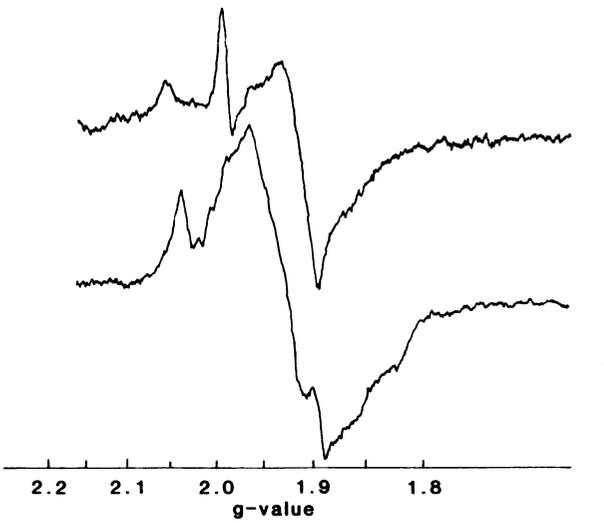


FIG. 2. EPR spectra of reduced methyl viologen-reducing hydrogenase (*Upper*) and F_{420}-reducing hydrogenase (*Lower*). Hydrogenases were reduced under H_2 in 1 M KCl/40 μM F_0 (riboflavin level of F_{420})/10 mM 2-mercaptoethanol/50 mM Tris·HCl buffer (pH 7.5 at room temperature) and incubated at 45°C for 1 hr. EPR conditions were the same as in Fig. 1 except microwave power (0.2 mW) and temperature (11 K).

to H_2 led to dissipation of the flavin semiquinone, presumably reflecting further reduction to the $FADH_2$ diamagnetic state.

Because H_2 is the reducing substrate in the catalytic reaction and the reductant in this enzyme activation sequence, we wanted to examine the EPR spectrum of the activated enzyme in the absence of H_2. To that end we removed H_2 on the vacuum line and replaced it with an argon atmosphere. Enzyme treated

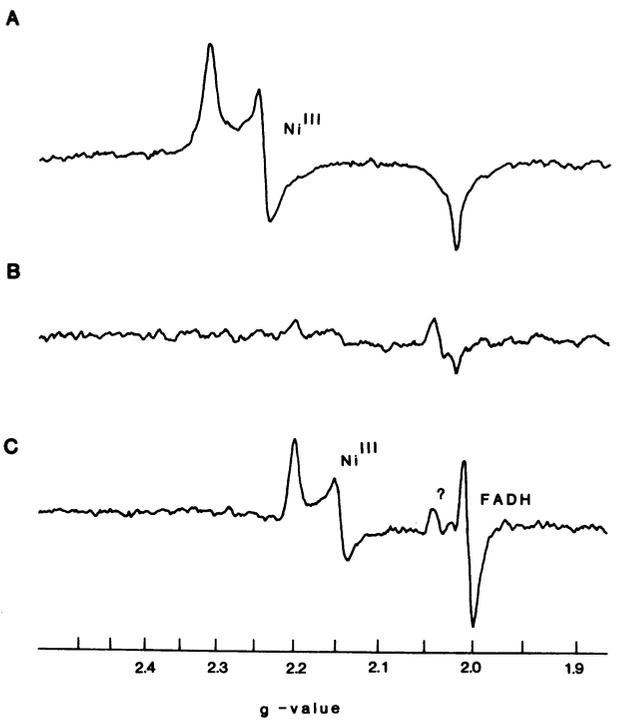


FIG. 3. EPR spectra of F_{420}-reducing ^{59}Ni-hydrogenase as isolated (spectrum A), reduced with H_2 (spectrum B), and after argon replacement of H_2 (spectrum C). The enzyme was reduced under H_2 in 1 M KCl/50 mM Tris·HCl buffer, pH 7.5, at room temperature and incubated at 45°C for 1 hr. EPR conditions were as in Fig. 1.

in this fashion showed catalytic activity comparable to enzyme maintained under H_2. Argon replacement for hydrogen had a dramatic effect on the EPR spectrum of the enzyme with appearance of a g signal at 2.004, retention of the signal at 2.036, and development of two signals at $g = 2.140$ and 2.196 (Fig. 3, spectrum C). The removal of H_2 from the incubation constitutes a shifting of the equilibrium of the hydrogenase reaction, and the fully reduced enzyme is likely to evolve H_2 from protons and electrons stored in the enzyme and become more oxidized. This interpretation is consistent with formation of the FADH semiquinone from the fully reduced enzyme-bound $FADH_2$.

To determine if the appearance of the two signals at $g = 2.140$ and 2.196 might reflect similarly a reoxidation of the enzyme-bound ^{59}Ni, we repeated these experiments with ^{61}Ni-enzyme to see if the signals became broader. There were small but detectable hyperfine differences between the ^{59}Ni- and ^{61}Ni-enzymes (Table 2), consistent with assignment of these two EPR lines to paramagnetic nickel. The simplest interpretation of these new nickel EPR signals is an oxidative conversion of nickel(II) in the hydrogen-reduced enzyme back to nickel(III) on hydrogen removal and replacement by argon. The new nickel(III)-containing species with different g and A values from the original inactive nickel(III)-enzyme could be in a different coordination geometry or could have a different coordination number. This new nickel(III)-enzyme form is either itself active or in facile, rapid equilibrium with active enzyme because, on readmission of H_2 (needed to determine catalytic activity), the enzyme was active without the prolonged preincubation period required for the initial nickel(III) form of the enzyme. These data do not *per se* suggest that the bound nickel cycles between nickel(II) and nickel(III) in turnover, but only that there can be ready and reversible interconversion between nickel(II) and this second paramagnetic nickel species. Finally, we have not yet ruled out the possibility that the g signals of 2.140 and 2.196 reflect not a nickel(III) but a nickel(I) species. It seems unlikely that H_2 removal from reduced enzyme would lead to nickel(I) not nickel(III), but until we determine the distribution of the electrons in each of the redox components of this multicenter enzyme, this is an unresolved issue.

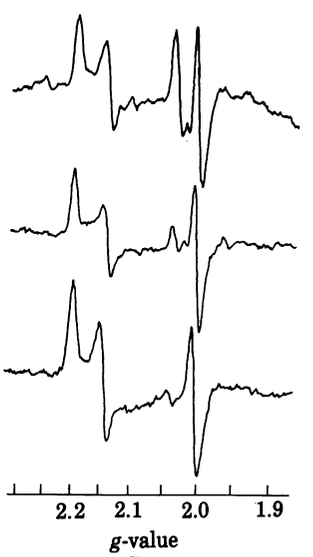
We do not yet know the nature of the paramagnet with the g value of 2.036. We have no evidence that this species undergoes redox cycling during catalytic turnover, but there is a hint that its appearance may mirror the catalytic competence of the F_{420}-reducing hydrogenase. The optimal reductive activation of the enzyme requires high salt, 1 M KCl in these experiments, and also 2-mercaptoethanol for full regain of high specific activity (Table 3). In particular, reduction of the aerobically isolated, inactive enzyme with H_2 alone in low salt led to disappearance of the initial nickel(III) signal, appearance of flavin semiquinone, and its eventual reduction as in Fig. 3, but the resulting reduced enzyme had only 5% of the specific activity obtained from a high-salt, thiol-containing reductive activation sequence. The difference between lines 1 and 3 of Table 3 could be seen on the subsequent replacement of hydrogen by argon and EPR analysis. The g signal of 2.036 was prominent in the high-activity form of the enzyme but almost absent in the low activity form; it was present at an intermediate level in enzyme with 75% of the specific activity. Whether this paramagnet may reflect a regulatory iron/sulfur cluster evincing a high-activity form of enzyme akin to those in aconitase (17) and amidophosphoribosyltransferase (18) remains to be investigated.

DISCUSSION

F_{420} is postulated to function as a low-potential redox shuttle in methanogenic bacteria accepting electrons from H_2 or $HCOO^-$ and subsequently transferring reducing equivalents to such molecules as NADP or to CO_2 in the reductive carboxylation of acetyl-CoA to pyruvate (19, 20) and possibly to provide some (or all) of the eight electrons required in the reduction of CO_2 to CH_4 (1, 2). We have recently demonstrated that F_{420} is a direct cosubstrate with H_2 for a F_{420}-reducing hydrogenase by purification of such an enzyme from *M. thermoautotrophicum* strain ΔH (3). The most novel feature of that hydrogenase

Table 3. Effect of the incubation conditions on enzyme activity and on the EPR signal of H_2-reduced enzyme after replacement of hydrogen by argon

Exp.	Conditions of incubation with H_2*	Specific activity, μmol/min·mg	% activity	$g = 2.036$	EPR spectra (150 K, 10 mW)
A	50 mM Tris·HCl/1 M KCl with 40 μM F_0 and 10 mM 2-mercaptoethanol	39–45	100	++	
B	50 mM Tris·HCl/1 M KCl	31	69–79	+	
C	50 mM Tris·HCl alone	1.8	4.6	−	



* Incubation conditions were the same (under H_2 at 45°C for 1 hr) except for the contents of the 50 mM Tris·HCl buffer, which was pH 7.5 at room temperature in all cases. F_0, F_{420} at the riboflavin level.

is the presence of tightly associated nickel among the redox inventory of bound FAD and bound iron/sulfur atoms.

The presence of tightly-bound stoichiometric nickel is rare in known enzymes, the first example being that of urease, for which the stoichiometric nickel requirement for catalysis was established only in 1975 (21). There is no obvious role for a redox function of the nickel(II) in that enzyme, and its function as a superacid catalyst has been proposed (22). Thauer and associates (23) in 1979 proposed that nickel was involved in the conversion of carbon monoxide to carbon dioxide by a clostridial CO dehydrogenase, and this has been supported by the detection of stoichiometric nickel after purification of the enzyme from two strains of *Clostridia* (24). The third case of enzyme-bound nickel is in the methanogenic enzyme methyl-CoM methylreductase, which catalyzes the last step in methane biogenesis from the methyl thioether, methyl-S-CoM. The nickel is part of a cofactor F_{430} (4, 5). The structure of a methanolysis product from F_{430} has been determined to be a nickel tetrapyrrole derivative by the groups of Thauer and Eschenmoser (7). The methanogen hydrogenases described here and hydrogenases from the related *Desulfovibrio* sulfate-reducing class of bacteria (9, 10), comprise the fourth example of nickel-containing enzymes. Unlike the methyl-CoM methyl-reductase, the other three enzymes probably use amino acid side chains as ligands.

The nickel(III) oxidation state has been relatively rare in the solution chemistry of nickel, but the recent model studies of Margerum's group are relevant here (13). Their characterization of the EPR signal of nickel(III)–tetraglycine complexes prepared by the iridate oxidation of the nickel(II) peptides allowed the unambiguous assignment of the three-line EPR signal to nickel(III) and presaged the report of the signals described in this paper and by Albracht *et al.* (12), Lancaster (15), LeGall *et al.* (9), and Cammack *et al.* (10) on related hydrogenases. This precedent and the four-line hyperfine splitting for the ^{61}Ni-hydrogenases leave no doubt that enzyme-associated paramagnetic nickel is being observed. About 50% of the nickel in the F_{420}-reducing hydrogenase from *M. thermoautotrophicum* and a similar amount in the *D. gigas* methyl viologen-reducing hydrogenase is detectable as nickel(III) in the isolated enzymes (10). Because the methanogen enzymes as isolated are inactive after aerobic purification, we feel that the initial nickel(III) signal is an artifact of the isolation procedure. Indeed, Lappin *et al.* (13) noted that oxygen converted nickel(II) to nickel(III), which was kinetically and thermodynamically stabilized in the tetraglycine complexes. Nonetheless, the nickel(III) signal will be a useful probe of the coordination number and geometry in the hydrogenases in some oxidation states.

Given the presence of nickel in these hydrogenases, does it function in catalysis, either in a redox or a nonredox role? Reduction of the initial nickel(III) signal to an EPR-inactive one [presumably nickel(II), as shown by the titrimetric studies on the *D. gigas* hydrogenase (10)] accompanies reductive activation, but the loss of nickel(III) is not correlated kinetically with rate of regain of enzyme activity and so is not causally related; that is, it is not a sufficient event for generation of catalytic competence. H_2 removal from active enzyme and replacement with argon leads to a regain of a paramagnetic nickel species with different *g* values and hyperfine coupling on ^{61}Ni substitution, which is still active or in facile equilibrium with active enzyme. This suggests that the nickel may be able to take up or give out electrons either to H_2 or to the other redox centers (FAD, iron/sulfur) in the hydrogenase. Also, if the new nickel paramagnet is indeed nickel(III), then in the active enzyme it may have a different coordination number or geometry, or both, than in the aerobic, inactive nickel(III) form of the enzyme. Because the new paramagnetic nickel species is produced by removal of the reducing substrate, we believe it more likely to be the nickel(III) oxidation state rather than nickel(I). The preliminary results of LeGall and colleagues on the *D. gigas* hydrogenase suggest a similar, if not identical, second paramagnetic form of nickel after H_2 reduction and argon replacement in that enzyme as well (9). Such a similar pattern, plus the presence of nickel in other hydrogenases (11), makes it unlikely that nickel is just an inert bystander in catalysis.

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