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The Role of Nickel in Hydrogenases: Implications for a Heterodinuclear Active Site

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Hydrogenases (H_2ases) are enzymes that catalyze the reversible two-electron redox chemistry of H_2. Most (but not all) H_2ases are now known to contain Ni, which is intimately associated with unusual epr signals that are characteristic of the enzymes. A recent crystal structure of the H_2ase from *Desulfovibrio gigas* reveals that the Ni in these enzymes is not an isolated metal center, but part of a Ni,Fe dinuclear cluster. The structure of the biological Ni,Fe cluster is compared with those of model complexes, and the redox chemistry of the model systems is examined for mechanistic insights into the role played by the active site cluster. Coupled with biophysical studies designed to probe the role of the Ni center, these studies fail to provide support for schemes that involve Ni-centered redox chemistry or utilize Ni as a binding site for the substrate (H_2) or for inhibitors like CO. Instead, they point to the involvement of the cysteinate ligands and/or the Fe center in the catalysis of H_2 redox chemistry.

Key Words: *hydrogenase structure and function, thiolate redox chemistry, biophysical and model studies*

I. BACKGROUND

Hydrogenases (H_2ases) are redox enzymes found in both prokaryotes and eukaryotes that catalyze the reversible two-electron oxidation

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of molecular hydrogen.[1] Thus they play a central role in the anaerobic metabolism of many microorganisms and are of additional interest as models for the photoproduction of H_2 and its utilization as an energy source. Several recent reviews focusing on the molecular biology and biochemistry of Ni-containing H_2ases have appeared.[2–5] Likewise, the chemistry of complexes that model aspects of biological Ni sites have been reviewed.[6–8] This commentary will focus on the structure and function of the Ni center and specifically explore its role as a potential redox center and substrate binding site in the enzyme in light of the recently published crystal structure of *Desulfovibrio gigas* hydrogenase.[9]

With the exception of a recently discovered hydrogenase from *Methanobacterium* that contains an organic redox cofactor, methylenetetrahydromethanopterin, that serves as a source of hydride ion,[10] all hydrogenases are classified as Fe,S proteins. The majority of hydrogenases also contain one Ni atom, in addition to various Fe,S clusters. Although a nutritional requirement for Ni has long been recognized, it is only since 1975 that specific biological roles for Ni have come to light. Four Ni-dependent enzymes have been characterized, including urease, methylcoenzyme M reductase, some carbon monoxide dehydrogenases, and most hydrogenases.[3,4,11,12] Curiously, with the exception of urease, all of these enzymes are redox enzymes. The choice of Ni for redox enzymes is provocative, given the normally inaccessible potentials of the Ni(III/II) and Ni(II/I) redox couples[13–15] and the bioavailability of metals with facile redox chemistry, such as Fe and Cu.

In H_2ases, the Ni-containing center has been associated, via the observation of ^{61}Ni hyperfine coupling,[16,17] with a series of rhombic epr signals (g = 2.4–2.0) that indicate the redox state of the enzyme and are sensitive to the presence of the substrate (H_2) and inhibitors (e.g., CO).[18,19] Thus, the Ni atom appears to be part of the active site of the enzyme. The appearance and disappearance of the epr signals associated with the Ni center as a function of the redox poise of the enzyme has led to a number of mechanistic proposals based on Ni redox chemistry and involving formal oxidation states IV–0.[2,17,20,21] These epr signals are unusual from the standpoint that Ni has only one common oxidation state in coordination compounds (II), and it features an even number of electrons and thus cannot give rise to the S = 1/2 epr signals associated with the enzyme.

Further, these signals are observed at liquid N_2 temperatures (77 K and above) and cannot be assigned to conventional Fe,S clusters whose epr spectra are observed only at temperatures below ~30 K. If the epr signals are assigned to Ni, they must be due to formal oxidation states of III or I. Alternatively, it is possible that the signals arise from other redox-active groups that are associated with the Ni center in the enzyme.

The typical Ni,Fe H_2ase is a heterodimer composed of subunits with molecular weights of ca. 30 and 60 kDa. The amino acid sequences of a number of Ni,Fe hydrogenases are known from the structural genes that code for these two subunits,[22,23] although it is now recognized that the amino acid sequence of the mature large subunit reflects a post-translational modification of the protein.[24,25] This modification is Ni dependent and involves the cleavage of several (e.g., 15 in *D. gigas* H_2ase) amino acids from the C-terminal region. The amino acid sequences of several large subunits reveal several conserved regions, including two Cys–X–X–Cys sequences, one near the N-terminus and one near the C-terminus, that have been associated with the Ni binding site. In some enzymes, notably *Desulfovibrio baculatus* and *Methanococcus voltae,* the first cysteine in the C-terminal Cys–X–X–Cys sequence is substituted by selenocysteine, which has been shown to be a Ni ligand by EXAFS analysis[26] and by the observation of ^{77}Se hyperfine coupling in the spectra of enzymes enriched in this isotope.[27] These results strongly indicate a role for the C-terminal Cys–X–X–Cys sequence in binding Ni in the enzyme.

II. STRUCTURAL MODELS

A recent breakthrough in the understanding of the structure of Ni,Fe hydrogenases occurred with the publication of the 2.85 Å resolution crystal structure of *Desulfovibrio gigas* H_2ase.[9] This structure reveals that the two subunits in the heterodimeric enzyme are intimately associated to form a roughly spherical molecule. The Ni center is found in the large subunit of the enzyme, and all the Fe,S clusters are bound to the small subunit. The Fe,S cluster composition of Ni,Fe H_2ases is variable, but in *D. gigas* consists of 2 Fe_4S_4 clusters and one Fe_3S_4 cluster. The crystal structure reveals that the Fe,S

clusters are arranged in a linear fashion, with the Fe_3S_4 cluster in the middle. The roughly 10 Å M–M spacing of the redox centers in the enzyme suggests an electron transfer pathway with the Ni site on one end and the distal Fe_3S_4 cluster on the other end. The distal Fe_4S_4 cluster is unique in that one of the Fe ligands is a histidine imidazole, rather than the usual cysteinate ligand. This His residue is solvent accessible, and thus provides a possible path for transferring electrons to or from another redox protein.

The crystal structure also reveals that the Ni site is actually a heterodinuclear cluster composed of a Ni atom and another metal center, presumably Fe.[9] The assignment of Fe as the second metal in the cluster is based on metals analysis, the strong anomalous scattering of Cu–K^{α} radiation and the electron density associated with the metal. Interestingly, the site contains only four endogenous ligands—the four conserved Cys residues found in the large subunit (Fig. 1). Two of the cysteinate ligands are bound as terminal thiolates to the Ni center, and the remaining two conserved Cys residues form bridges to the Fe center. There are no other protein ligands to the Fe, which resides in a five-coordinate site with the remaining three ligands modeled as H_2O molecules. Thus this biological Ni site is a dithiolato bridged cluster, with a Ni–Fe separation of ~2.7 Å and a fold angle (defined as the dihedral angle between the two MS_2 planes) of ~96°.

The coordination environment of the Ni center can be described as roughly trigonal pyramidal. Three of the cystenate ligands have average Ni–S bond lengths of 2.25 Å. The remaining Ni–S distance (cys 533) is 2.6 Å and constitutes the apical donor. Although at 2.85 Å resolution these differences in Ni–S distances are not very significant, this arrangement is nonetheless consistent with the analysis of X-ray absorption spectroscopic data, indicating that one likely geometry for the Ni site is trigonal bipyramidal. This assumes that the other axial ligand position on Ni, which would place the fifth ligand into another Ni,Fe bridging position, could be occupied by another exogenous ligand, such as H_2O, O_2, CO, H_2 (or H^-) in various states of the enzyme. The thiolate ligation observed in the protein is also consistent with the analysis of EXAFS data, which in general detects 2–4 S-donor ligands at 2.23 (3) Å.[26,28–34] In some cases, an additional long Ni–S interaction is also indicated.[31,34,35] In some H_2ases, the best fits of the Ni EXAFS data also include some

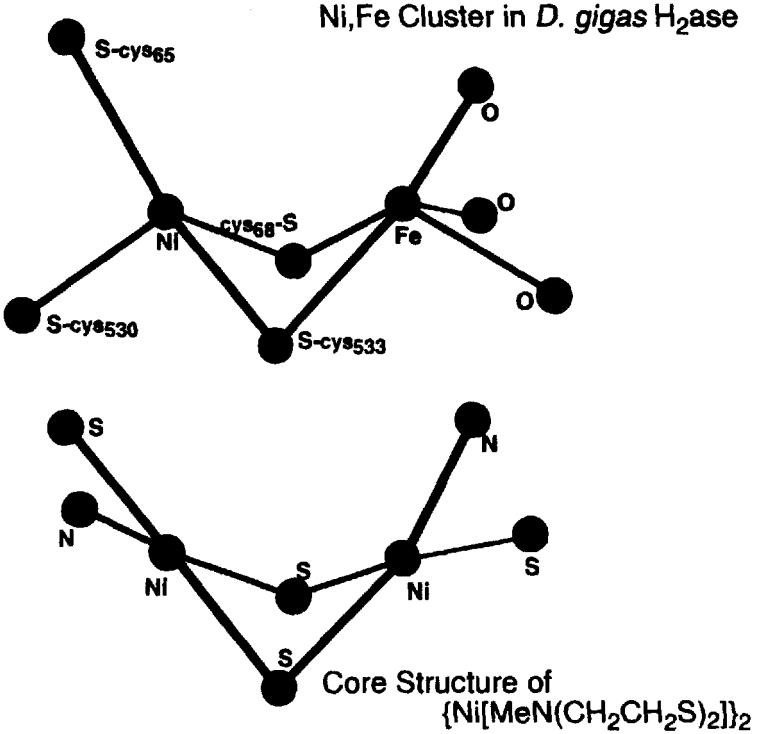

FIGURE 1 A comparison of the core structures of the Ni,Fe cluster in *D. gigas* H_2ase and the dinickel model compound, $\{Ni[MeN(CH_2CH_2S)_2]\}_2$. For the Ni,Fe cluster: ave. Ni–S ($cys_{65,68,530}$) = 2.25 Å, Ni–S (cys_{533}) = 2.6 Å; Fe–S (cys_{68}, cys_{533}) = 2.35, 2.15 Å; Ni–Fe = 2.7 Å; fold angle = 96° (Ref. 9). For $\{Ni[MeN(CH_2CH_2S)_2]\}_2$: Ni–$S_{br}$ = 2.221(4), 2.210(4), 2.180(4), 2.179(4) Å; Ni–S_t = 2.172 (4), 2.136(4) Å; Ni–Ni = 2.679(2) Å; fold angle = 108.4 (1)° (Ref. 42).

O- or N-scattering atoms.[26,31,33] The presence of these ligands in the best EXAFS fits may reflect missing water molecules in the crystallographic model of the oxidized enzyme (for example, the other axial ligand), species variations, the difficulty of accurately detecting O- or N-scattering atoms in coordination sphere dominated by S-donor ligands, or some combination of these factors.

The characterization of the Ni site as a Ni,Fe cluster is something of a surprise, since the presence of an Fe atom at ~ 2.7 Å was not indicated from the analysis of EXAFS data.[31,33] Although it has long

been suspected that the Ni site might be associated with an Fe atom,[2,33] there is no distinctive feature in the EXAFS spectrum that indicates that the Ni is associated with another metal at ~2.7 Å. Analyses carried out subsequent to the publication of the crystal structure indicate that an Fe atom at a distance of 2.4–2.8 Å can be accommodated by the data.[36] However, it is not possible to distinguish it clearly from a long Ni–S interaction. Thus, either or both possibilities can be accommodated by the EXAFS data.

The structure that emerges for the Ni site is consistent with the known chemistry of Ni thiolates.[37–41] Many four-coordinate Ni thiolate complexes are known in both planar and tetrahedral geometries. Many of these complexes, particularly those with alkyl thiolate ligands, exhibit a marked tendency to polymerize by forming thiolate bridges. This is exemplified by a series of model dinickel compounds with μ-alkylthiolato ligands, such as the one shown in Fig. 1.[38,42] The structures of the cores of these models are quite similar to the structure of the biological Ni,Fe site and to the core structure found in Fe,S clusters. The Ni_2S_2 rhombs found in S-bridged model compounds typically have M–M distances of about 2.6–2.9 Å and are folded along the shared S–S edge, forming angles of 90–120° between the two MS_2 planes. These angles result from the use of p-orbitals on S in the formation of the Ni-thiolate bonds, and are also reflected in M–S–M angles of 73–83°.

The use of bridging thiolates to form heteronuclear clusters is well known, and has recently been demonstrated for Ni and Fe.[43,44] A model cluster, **2**, shown in Fig. 2 contains a central Fe (II) atom in an S_5 coordination environment composed entirely of thiolate ligands that bridge to three Ni centers.[43] These bridges involve two of the μ-dithiolato variety and one single thiolate bridge. It appears that nature has taken advantage of this chemistry to create the heterodinuclear active site found in Ni,Fe H_2ases.

The crystallographic model for the putative Fe center in the structure of the Ni,Fe cluster is very unusual.[9] First, the second metal must be magnetically silent, or its presence would be detected by epr and other techniques. Epr spectra obtained on samples of H_2ase labeled with ^{57}Fe do not reveal resolved hyperfine or obvious line broadening.[45] This means that if the second metal is Fe, it must be low-spin Fe(II) in all the epr-active redox states accessible at potentials of 0 V or lower (Forms A, B and C). However, this is not likely

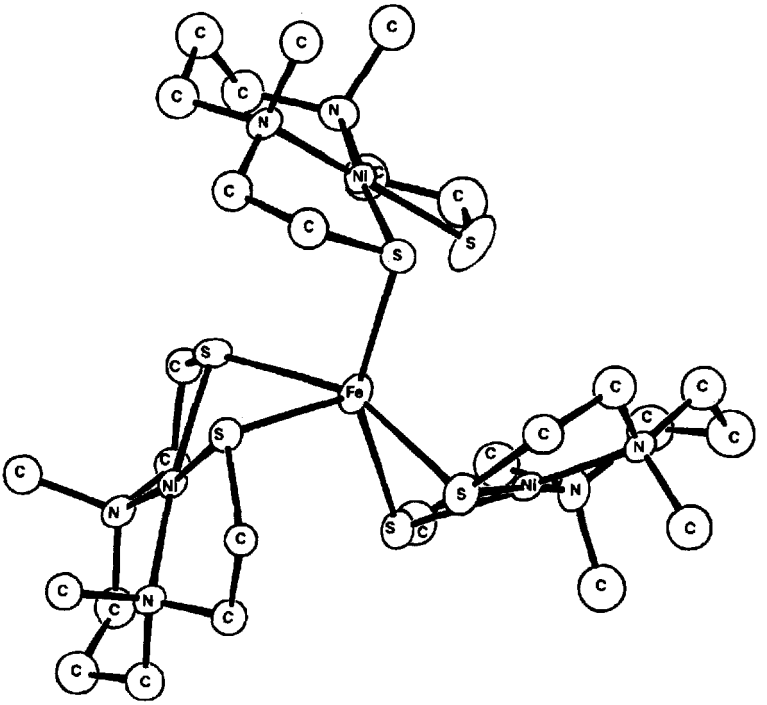

FIGURE 2 The structure of a tetranuclear cluster containing two μ-dithiolato Ni,Fe units and a single μ-thiolato Ni,Fe unit. For the μ-dithiolato units: Ni–S = 2.16–2.17 Å; Fe–S = 2.46–2.62 Å; Ni–Fe = 2.98, 3.12 Å; fold angles = 106°, 115°. For the μ-thiolato unit: Ni–S_{br} = 2.16 Å; Ni–S_t = 2.16 Å; Fe–S = 2.48 Å (Ref. 43).

for an Fe(II) center in a ligand environment composed of two μ-thiolato and three aquo (or hydroxo) ligands. Even the S_5 environment found in **2** gives rise to a *high-spin* Fe(II) center.[43] Thus, it would appear that either the second metal is not Fe, that its coordination sphere is incomplete, or that the exogenous ligands are not derived from water.

III. NI AS A REDOX CENTER

When isolated in air, Ni,Fe H_2ases typically exist as a combination of catalytically inactive forms displaying epr signals associated with

the Ni center (Form A, g = 2.31, 2.23, 2.02) and (Form B, g = 2.33, 2.16, 2.01) at 77 K, and a signal arising from an oxidized Fe_3S_4 cluster at lower temperatures (Table I).[2,17,21] Both of the oxidized forms may be activated by reduction with H_2 or other reducing agents, and are initially reduced to a redox level that is epr-silent at 77 K, SI. However, the kinetics of the reduction differs for the two oxidized forms. Form B is instantaneously reduced upon exposure to H_2 (it is the "ready" form), whereas Form A requires a period of incubation under H_2 (it is the "unready" form).[21]

Recently, Albracht and co-workers studied in detail another redox active center in the H_2ase from *Chromatium vinosum*.[2,46] This center becomes epr-active when the enzyme is oxidized at potentials higher than those associated with Forms A and B (+105–+160 mV), and is coupled to epr signals arising from the Ni center and the Fe_3S_4 cluster that is present in that enzyme. An analysis of Mössbauer spectra obtained on samples of *C. vinosum* H_2ase indicates that one possibility for the new redox center is a single Fe atom with an unusually small isomer shift (δ = 0.05–0.15 mm/s). Given the weakness of the coupling involved (6 mT) and the distance between the Ni,Fe cluster and the Fe_3S_4 cluster in the crystal structure of the *D. gigas* enzyme, it seems unlikely that this new epr-active species is associated with the recently discovered Fe atom in the Ni,Fe cluster (*vide infra*), although this is the only possibility suggested by the 2.85 Å resolution crystal structure.

TABLE I

A summary of redox chemistry associated with Ni in hydrogenases

Sample	77 K epr Signal	Approx. E_m (mV vs. NHE, pH 7)	Scheme A	Scheme B	Scheme C
Form A (Ni–A, unready)	g = 2.31, **2.23**, 2.02	A → SI −150	Ni(III)	Ni(III)	Ni(I)
Form B (Ni–B, ready)	g = 2.33, **2.16**, 2.01		Ni(III)	Ni(III)	Ni(I)
SI	none		Ni(II)	Ni(II)	Ni(II)
Form C	g = **2.19**, 2.14, 2.02	SI → C −270	Ni(I)	Ni(III)	Ni(I)
R	none	C → R −390	Ni(0)	Ni(II)	Ni(II)

Reduction of the enzyme under more reducing conditions produces another epr-active redox state of the enzyme, Form C (g = 2.19, 2.14, 2.02). This form of the enzyme has long been associated with an intermediate in the catalysis of H_2 redox chemistry, since the signal reaches a maximum intensity at the same potential associated with the onset of catalytic activity.[21] However, recent work has shown that Form C is stable indefinitely in the absence of H_2[47,48] an observation that is inconsistent with it being a catalytic intermediate. If it were an intermediate representing an H_2-bound complex, it should lose H_2 upon standing. It is possible that Form C represents an intermediate that lies off the catalytic cycle, because it is a one-electron redox product in a two-electron redox cycle.

Form C also displays coupled and uncoupled forms at low temperature. The weakly coupled signal presumably arises from the interaction of the Ni-containing center with one Fe_4S_4 cluster, which are reduced in the potential range where the Form C 77 K epr signal is observed.

Form C may be reduced further to another redox level that is epr-silent at 77 K and contains only fully reduced redox cofactors (R). Redox titrations have been performed in tandem with epr spectroscopy to determine the potentials and the number of electrons involved with the changes in the 77 K epr spectra associated with Ni.[2,17,21,47,49] These titrations indicate that all the redox chemistry associated with the Ni site occurs between ca. −100–−400 mV (Table I). At least in the presence of dyes, the reductions all appear to be one-electron processes. However, for *C. vinosum,* redox titrations conducted using H_2 partial pressure to determine the redox potential indicate that the Form C/R couple is a two-electron process.[48] It is not clear how this is achieved, since the addition of two electrons to a half-integer spin system should give another half-integer spin system, and R is epr-silent at 77 K.

The association of the epr signals with Ni in an oxidized enzyme, coupled with their general similarity to epr spectra from Ni(III) complexes,[17] led to the assignment of these signals to a *formally* Ni(III) center in the oxidized enzyme. The sequential disappearance (in SI), reappearance (in Form C) and disappearance (in R) of the epr signals associated with Ni led to a number of mechanistic proposals based on Ni-centered redox chemistry. These proposals range from invoking oxidation states III–0 (Ni as a three-electron redox

center)[2] to proposals utilizing only one-electron redox chemistry for Ni (either the III/II couple or the II/I couple) with the odd oxidation states assigned to epr-active species (Table I).[6,17,21] The scheme involving both oxidation states III and I requires that the potentials associated with the III/II and II/I redox couples be compressed into a 300 mV potential range between −100 and −400 mV. This is unprecedented redox chemistry for a single Ni complex. Although it has been demonstrated that thiolate ligation will lower the potential of the III/II couple into this range,[50,51] it invariably also lowers the potential of the II/I couple. In fact, only one series of complexes that is stable in both the III and I formal oxidation states is known, and the generation of these species involves oxidants and reductants (for example, $Fe(CN_6)^{3-}$ and NaS_2O_4) with redox potentials outside of the relevant range.[52] Presumably, if metal-centered redox chemistry involving both Ni(III) and Ni(I) were to occur in the enzyme, a major structural change in the Ni site would be required. Similarly, the schemes based on a one-electron redox couple would also require a change in structure at the Ni site. For example, the III → II → III → II scheme requires that the Ni(II) complex derived from reduction of the oxidized enzyme (Forms A or B → SI) be reoxidized (SI → Form C) at a *lower* potential.

The redox activity of the Ni center has been examined by using X-ray absorption spectroscopy.[31] Examination of the Ni K-edge energy provides a method for detecting changes in the charge density residing on the Ni center.[53] This technique has been applied to many redox enzymes, and has proved useful in detecting redox reactions that are ligand-centered rather than metal-centered,[54,55] provided the ligand environment is relatively constant.[56] If the redox reactions observed for H_2ase were Ni-centered, one would expect a ca. 2 eV shift in the edge energy for each oxidation state change. At least in *Thiocapsa roseopersicina,* the only enzyme to be examined in each of the five forms defined by the 77 K epr spectrum, no shift of this magnitude is observed.[31] Within the precision of the measurement, the maximum shift observed is ~1 eV. This result, coupled with the EXAFS analysis of each form that fails to reveal any significant structural change in the Ni site, effectively rules out mechanisms involving both Ni(III) and Ni(I) as realistically describing the changes in electron density that occur during redox catalysis.

The lack of a structural change in the Ni site also argues against mechanisms involving Ni-centered one-electron redox chemistry, and can be contrasted with the results of a model study that serves to define the structural changes one might expect for a Ni-centered redox couple.[57] The structures of $[Ni(pdmt)]^{2-}$ and $[Ni(pdmt)]^{-}$ have been reported and indicate that a 0.14 Å shortening of the Ni–S bonds occurs upon oxidation of the Ni(II) complex to Ni(III). In contrast, the average Ni–S bond lengths found in the *T. roseopersicina* H_2ase samples vary only 0.03 Å in fully oxidized and fully reduced enzymes.[31] Further, the average Ni–S distance found (2.23 Å) is most consistent with a low-spin Ni(II) center,[58] an electronic configuration that is consistent with magnetization[59] and MCD spectroscopic studies[2] carried out on the SI redox level of the enzyme.

Clearly, the redox chemistry associated with the Ni site must involve changes in electron density that are either very delocalized or localized on atoms other than Ni. One possibility is that the Fe center is directly involved. However, the number of electrons associated with the redox titrations of the Ni site (3–4) are greater than could be accommodated by a single Fe center. Further, since the Fe is magnetically silent in Forms A, B and C, one cannot invoke Fe(III) or Fe(I) to account for the one-electron redox chemistry.

One clue that helps explain at least the oxidized states of the enzyme comes from studies of various oxidations of model Ni(II) alkylthiolate complexes, which yield Ni(II) products with oxidized thiolate ligands.[41,56,60–62] This chemistry is summarized in Fig. 3 for one model system. Reaction of $Ni(OAc)_2$ with a series of tridentate ligands $[RN(CH_2CH_2SH)_2]$ leads to a series of dimeric complexes, like **1** (Fig. 1) or like **A** in Fig. 3. These dimers contain distorted planar Ni(II) centers coordinated by three thiolate donors and a tertiary amine. Like the Ni,Fe site in the enzyme, the two metal centers are bridged by two thiolate ligands and the Ni centers are also ligated by terminal thiolates. The two planes containing Ni meet at an angle (fold angle) at the shared edge composed of the bridging thiolate S-donor atoms. One-electron oxidation of the dimers gives rise to the only oxidation products in this study that have not been crystallographically characterized (**B**). The one-electron oxidation products are thermally unstable $S = 1/2$ species with epr spectra that integrate to ca. one spin/dimer.[61,63] The assignment of the one-electron oxidation product to a *formally* Ni(II),(III) dimer is based

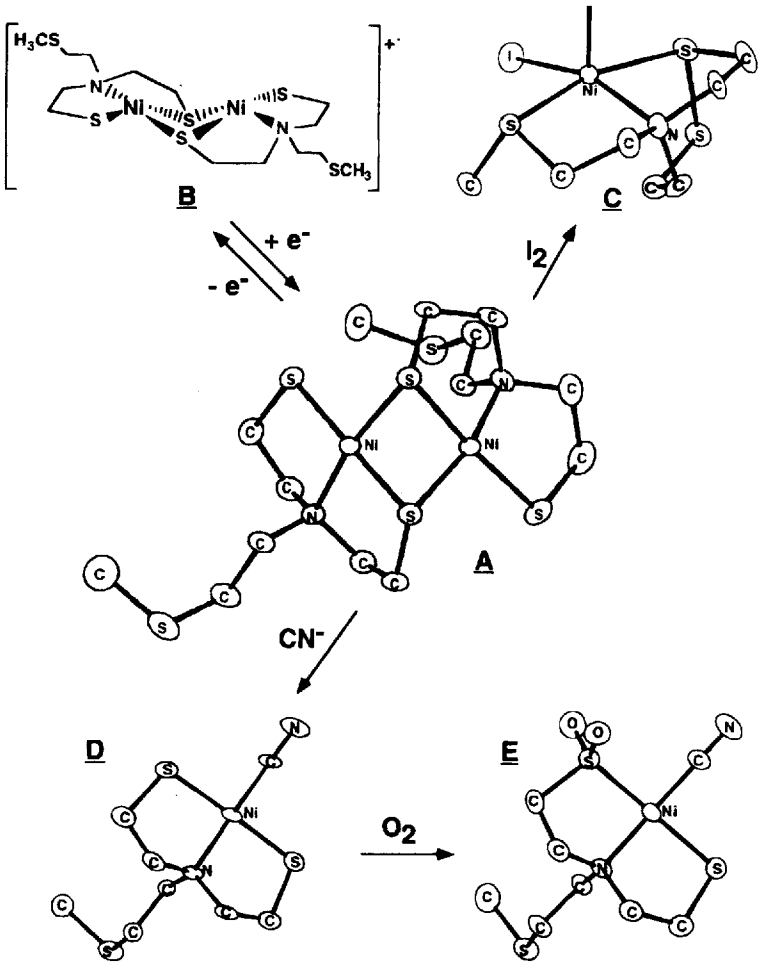

FIGURE 3 Reactions leading to S-oxidation products in Ni(II) thiolate complexes.

on the spin quantitation, the quasi-reversible electrochemistry exhibited by some of the dimers, epr spectroscopy of ^{61}Ni labeled analogs where the observed hyperfine is best fit assuming two Ni centers, and on the structural analogy with $\{Ni[P(o\text{-}C_6H_4S)_3]\}_2^-$.[64] This Ni(II,III) dimer is composed of two five-coordinate pyramidal Ni centers with PS_4 ligation containing the same $Ni_2(SR)_2$ core formed by bridging thiolates that is found in the Ni(II) dimers formed by the $[RN(CH_2CH_2SH)_2]$ ligands.

The electronic structures of these $S = 1/2$ dimers have been examined by epr, ^{1}H-ENDOR, electronic absorption spectroscopies, and *ab initio* theoretical methods.[56,63,65] The (g-values obtained from the epr spectra (g = 2.20, 2.14, 2.02) match those obtained from Form C H_2ase (Fig. 4). Since it is possible to match the g-values in formally Ni(I), Ni(III) and both mononuclear and dinuclear complexes,[52] this is an observation that is significant only because of the structural similarity between the metal sites. The epr spectra also reveal very small ^{61}Ni hyperfine coupling constants that average, for the two Ni centers, to $|A_1|$, $|A_2|$, and $|A_3| = 10.5$, 0.0 and 4.1 G (for R = CH_2Ph).[56,65] These values are about half of the magnitude of

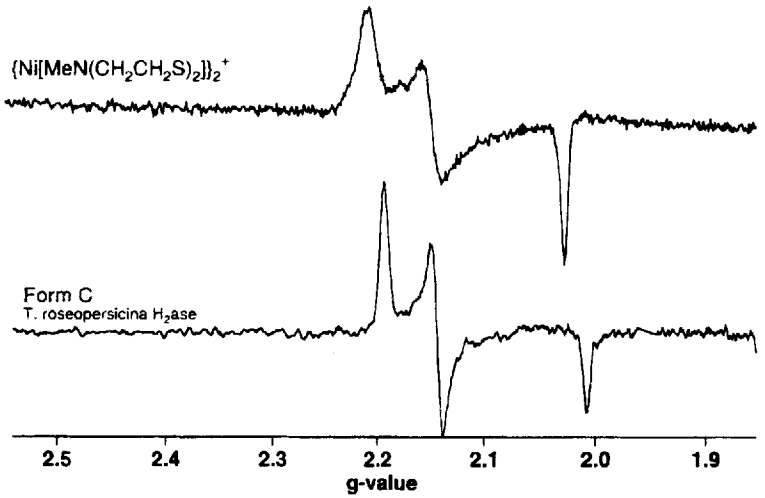

FIGURE 4 A comparison of the X-band epr spectra obtained from Form C hydrogenases and the dinickel model compound, $\{Ni[MeN(CH_2CH_2S)_2]\}_2^+$.

the hyperfine couplings observed in H_2ase,[66] which are in turn about half of what are observed in Ni(III) complexes with O- or N-donor ligands.[14,15] The ^{1}H-ENDOR spectrum reveals couplings to S–CH_2 protons with a coupling of 12–14 MHz.[56,65] In contrast to {Ni[P(o-$C_6H_4S)_3$]{ }$_2^-$, no intervalence transfer transition could be observed in the NIR spectrum of these dimers. All of these observations are consistent with a large amount of spin density residing on one or more of the thiolate ligands. Direct evidence of spin density on S in H_2ases has been obtained via the observation of hyperfine coupling to ^{33}S[67] and to cysteine β-CH_2 protons.[34,68]

At first glance, the observation of g-values > 2.0 suggests that a Ni(III) center has been produced and that spin-orbit coupling from the metal gives rise to the observed g-values. However, electrons localized on S also experience spin-orbit coupling of sufficient magnitude to lead to the observed g-values. Epr spectra of thiyl radicals (for example, cysteinyl radical) taken in MeOH reveal axial spectra with $g_\parallel = 2.3$ and $g_\perp = 2.0$ ($g_{ave} = 2.1$).[69] The spectra of the $S = 1/2$ dimers also have $g_{ave} = g_{iso} = 2.1$. The spectra of the thiyl radicals are typically axial due to the degeneracy of the p-orbitals that are not involved in the S–C σ-bond. This degeneracy could easily be lifted in a metal complex, giving rise to a rhombic spectrum. Thus, the g-values cannot be used to distinguish largely M- vs. largely S-centered oxidation.

Although the analysis of hyperfine couplings is complicated by the combination of contact vs. dipolar couplings and the angular dependence of the latter, the small ^{61}Ni hyperfine couplings observed and the substantial coupling to ligand protons are both consistent with considerable spin density on one or more S-donor ligands. This situation is reminiscent of Cu hyperfine couplings and cysteinate β-CH_2 couplings that are observed for blue Cu centers.[70]

The theoretical model that emerges from the calculation of the oxidized dimers indicates that the spin density resides in a molecular orbital that has mainly S p-orbital character from one of the terminal thiolate ligands (89%).[65] Thus, all the available evidence points to an electronic description that is closer to Ni(II)–$^{\cdot}$SR than to Ni(III)–SR for the one-electron oxidation products of Ni alkylthiolate complexes. This theoretical result is in line with expectations based on blue Cu, where the unpaired spin density is distributed nearly equally between the Cu(II) center and a thiolate S-donor atom.[71]

Given a similar ligand environment, a qualitative argument (which is easier to oxidize, Cu(I) or Ni(II) ?) leads to the correct prediction that the Ni–alkylthiolate bond should have more S character. The importance of the thiolates in the oxidative chemistry of the enzyme is also supported by the redox potentials (−100–−400 mV), which lie in an unusual range for Ni(II) complexes, but are typical of cysteine oxidations.[72]

The intimate involvement of the S-donor atoms in the oxidative chemistry of Ni alkylthiolate complexes is also supported by the two- and four-electron oxidative chemistry of model complexes.[61] When R = CH_2CH_2SMe and I_2 is used as a two-electron oxidant, a reaction occurs, leading to the formation of a five–coordinate pyramidal high-spin (S = 1) Ni(II) complex with a disulfide ligand (Fig. 3, **C**). The stoichiometry of the reaction is 1 I_2:1 Ni, and leads to the complete oxidation of all the thiolates in the dimer to disulfides. If substoichiometric I_2 is used, one obtains a mixture of the oxidation product and unreacted dimer.

Nickel thiolates have also been shown to be sensitive to oxidation by O_2.[60,62,73,74] Reaction of various thiolates with O_2 yields a series of Ni(II) sulfinate complexes, reflecting the four-electron oxidation of the thiolate (Figs. 3 **E** and 5). The second thiolate in dithiolato starting materials is not converted to a sulfinate, an observation that has been traced to the electronic structure of the complexes. *Ab initio* theoretical calculations of planar trans- and cis-Ni(II) dithiolates reveal that the occupied frontier molecular orbitals of the thiolates are dominated by S p-orbitals, whereas the occupied frontier molecular orbitals of the monosulfinates are dominated by the sulfinate group.[75]

Kinetic investigations of this oxidation reveal that the reactions are first order in [Ni] and in [O_2] and have half-lives that range from hours to days.[62] Other mechanistic studies demonstrate that there are no stable intermediates, no radicals involved, and no free singlet O_2. Using a mixture of $^{18}O_2$ and $^{16}O_2$, it has been shown that the reaction follows a dioxygenase-like mechanism, with the incorporation of both atoms of O from a single O_2 molecule.[62,74] A mechanism drawn in analogy with the $^1O_2$ oxidation of organic thioethers[76–78] has been proposed (Fig. 6). This mechanism, which proceeds through a persulfoxide/thiadioxirane intermediate, is consistent with the known features of the reaction and predicts that the reaction rate will increase

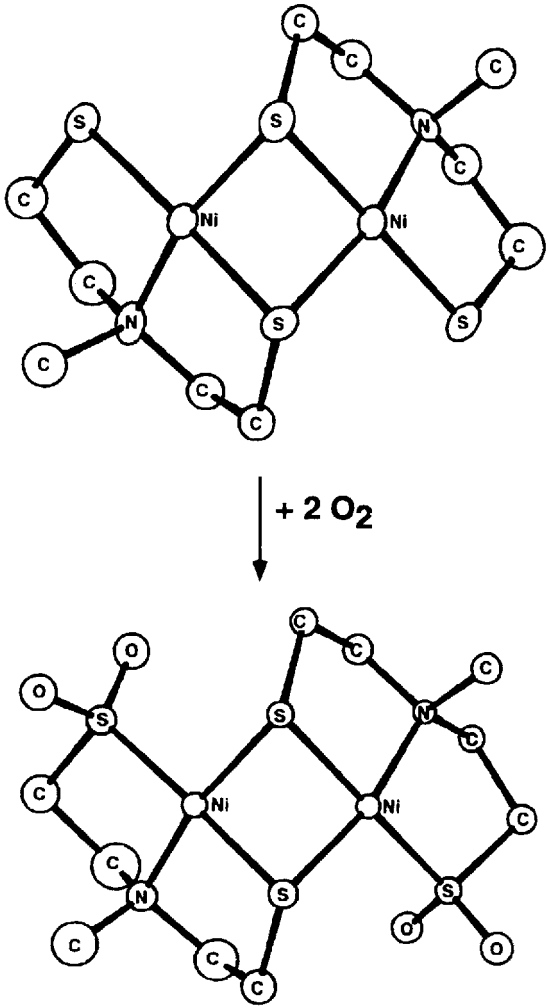

FIGURE 5 The oxidation of a dinickel model complex with O_2 leads to the production of a dinickel complex where the terminal thiolate ligands have been oxidized to sulfinate ligands.

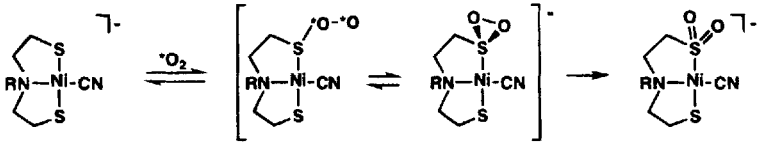

FIGURE 6 A hypothetical mechanism for the oxidation of Ni thiolate complexes by O_2 (Ref. 62).

with increasing nuclophilicity of the thiolate ligands. This is confirmed by the theoretical models.[75]

In the case of dimeric complexes, oxidation converts the two terminal thiolate ligands to sulfinates (one per Ni) without oxidizing the bridging S atoms (Fig. 5).[74,79] Thus, one would expect that the Ni thiolate complex in H_2ases would be susceptible to this oxidation unless the enzyme has taken precautions to prevent it from happening. From the model oxidation of the dimer, one way to accomplish this would be to constrain the O_2 to bind between the metals over the unreactive bridging thiolates, forming a third bridge between the metals. Given that oxidations of the enzyme with $^{17}O_2$ (I = 5/2) lead to the observation of only a small amount of line broadening, this model would not be applicable in a Ni-centered redox mechanism.[18] On the other hand, if the spin density resides mostly on S, then binding O_2 in a bridging position will not necessarily give rise to resolved hyperfine couplings.

The effect of O_2 on the enzyme is dependent on the redox state of the enzyme. The fully reduced enzyme is rapidly and irreversibly oxidized by O_2. In contrast, intermediate redox levels are oxidized to Forms A and B, which may be reductively reactivated in the absence of O_2. The reversible oxidative deactivation of the enzyme is a rather slow process, proceeding with a half-life that is similar to the thiolate oxidations with O_2.[21] One exception to the oxidative deactivation of the enzymes appears to be the H_2ase from *D. baculatus*, which is isolated aerobically in an epr-silent state and requires no reductive activation.[80] This enzyme contains a conservative replacement of one cysteine residue by a selenocysteine residue, suggesting another way that nature might control the oxidation of the Ni ligands.

Studies comparing the chemistry of Ni(II) thiolate complexes with the analogous selenolate complexes have been carried out (Fig. 7).[42]

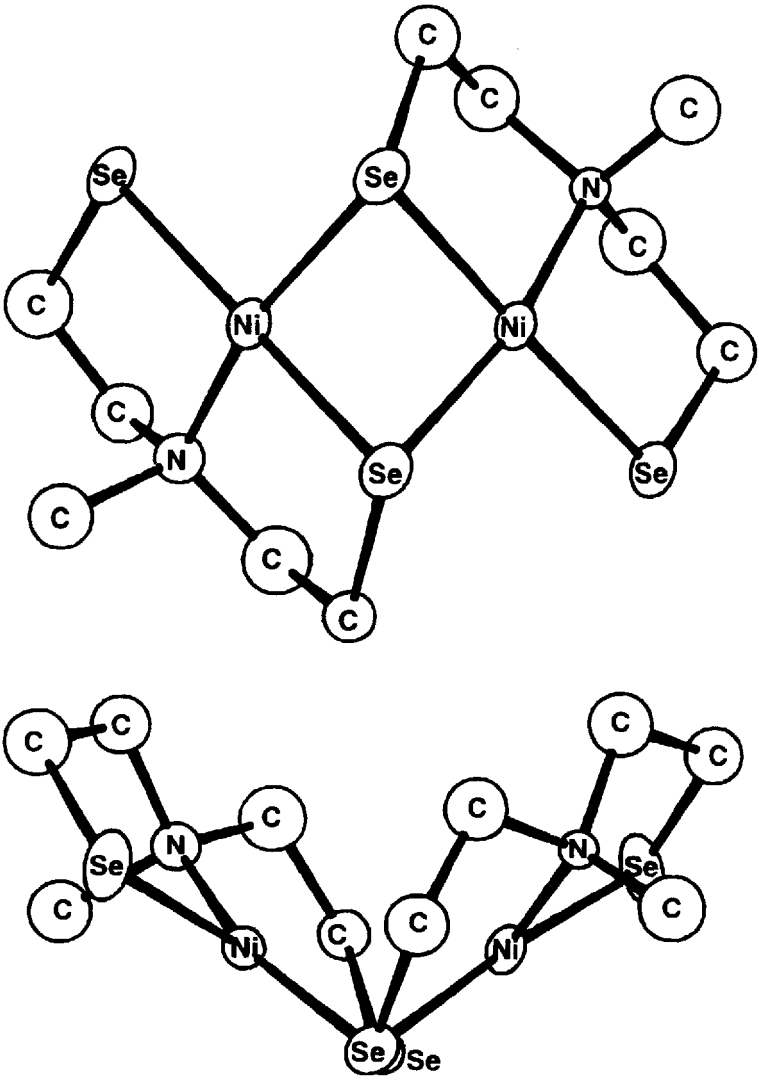

FIGURE 7 The structure of $\{Ni[MeN(CH_2CH_2Se)_2]\}_2$, the selenolate analog of $\{Ni[MeN(CH_2CH_2S)_2]\}_2$ (Ref. 42).

These studies reveal chemistry that is analogous in every way except for one: the oxidation of the selenolate complexes by O_2 does not proceed at a measurable rate and does not lead to the formation of selenoxy species. This observation is consistent with the descriptive chemistry of group 16, and suggests that the selenocysteine serves as an antioxidant in the enzyme. This substitution is indicated by a TGA codon in the structural gene for the enzyme and corresponds to cys-530 in the *D. gigas* sequence. It is probably not purely coincidental that cys-530 is a terminal thiolate. Thus, the thiolate involved in the seleno cysteine substitution would be expected to be one of the more nucleophilic centers and reactive with respect to oxidation by O_2. However, there is no evidence to support O-incorporation of cys-530 in the crystal structure.

Since several H_2ases that do not contain Ni have been characterized,[1] Ni is not an absolute requirement for the construction of a H_2ase active site. The active site in Fe-only enzymes is a spectroscopically distinct Fe,S cluster called the H-cluster. In fact, the Fe-only enzymes are better H_2 redox catalysts than are the Ni-containing enzymes. The activities of the Ni,Fe enzymes are only 1–10% as large as those of many Fe-only enzymes. The special property that is most closely associated with the incorporation of Ni in the enzyme is the *reversibility* of the oxidative deactivation of the enzyme by air. Many of the Fe-only enzymes (e.g., *Clostridium* H_2ases[1]) are rapidly and irreversibly inactivated by exposure to air. One view of the redox role of Ni that emerges from the biophysical and model studies presented here is that it is involved in modifying the reactivity of chemistry that involves active site Fe-thiolate bonds to make it more O_2 tolerant. This property is even further developed in enzymes incorporating selenocysteine.

IV. NI AS THE SUBSTRATE/INHIBITOR BINDING SITE

Another potential role that has been suggested for the Ni site in H_2ases is as a binding site for the substrate (H_2) or inhibitors, such as CO. Most of the discussion has focused on the reduced epr-active redox state designated Form C. The epr signal associated with Form C has been attributed to Ni(III) or Ni(I) complexes of H^- or H_2, based on epr and ENDOR spectroscopic studies of Form C and the changes observed upon exposure to CO.[2,18,19,68,81] ^{1}H-ENDOR spectroscopy on *D. gigas* H_2ase reveals three sets of signals: one

set corresponding to solvent exchangeable protons with a coupling constant of 17 MHz, one solvent nonexchangeable set with a coupling constant of 12 MHz, and another solvent exchangeable set of protons that are weakly coupled (4 MHz). The nonexchangeable set, attributed to cysteine β-CH_2 protons, is detected in both oxidized (Forms A and B) and reduced (Form C), and is direct spectroscopic evidence of spin density on cysteinate ligands in the enzyme. Further, since the coupling constant is not very sensitive to redox state, it suggests that the electronic structures of the Ni centers in the oxidized and reduced enzyme are similar. The weakly coupled ^{1}H-ENDOR resonance was assigned to a protonated Ni ligand, such as a water molecule. Interestingly, this solvent exchangeable proton is not solvent accessible in Form A, suggesting a conformational change that blocks access to the active site. The remaining solvent exchangeable ^{1}H-ENDOR resonance (17 MHz) is the best candidate for a H^- or H_2 ligand. Although this is a relatively large ^{1}H-ENDOR coupling constant, it does not resemble Ni–H coupling constants in paramagnetic hydrides, which are in the 300–500 MHz range and are clearly detected in epr spectra.[82,83] To account for the small coupling constant, it was suggested that this proton could be a hydride ligand bound to a Ni(III) center through a d-orbital in the *x,y* plane that does not contain much spin density.[68,84] This possibility is supported by epr spectra obtained on $[Ni(CN_4)(H_2O)_2]^-$, where no hyperfine splitting due to equatorial $^{13}CN^-$ ligands is observed.[85]

Form C is light sensitive and is converted to an epr-active photoproduct (Ni–C*, g = 2.29, 2.13, 2.05) by exposure to visible light at low temperature.[2,19,34,86] The photochemistry is reversible upon annealing the sample at ca. 200 K, and thus has all the characteristics of a ligand photodissociation and recombination reaction. It was suggested that the ligand involved was the putative hydride. This possibility was explored using a combination of ^{1}H-ENDOR and XAS measurements.[34] The ^{1}H-ENDOR studies reveal that the photochemistry does indeed lead to the loss of the strongly coupled ^{1}H-ENDOR resonance. However, there is no corresponding change in the Ni K-edge energy or in the remaining ligand distances, suggesting that the proton is not bound to Ni. Two explanations that are consistent with all the data currently available are possible. First, the heterodinuclear structure of the Ni site suggests that the Fe center could be the substrate/inhibitor binding site. Second, it is possible that Form

C corresponds to an enzyme containing a Ni site that features a thiol ligand. The latter model is attractive from a photochemical perspective, since it is likely that the transition(s) that would be excited by visible light correspond to S → M LMCT transitions. This excitation could lead to the dissociation of the thiol ligand and removal of the S–H proton from the spin system. This model is also attractive since it provides a possible explanation for the recent observation that Form C is stable in the absence of H_2. This might be due to the fact that it represents a form of the enzyme that has retained the H^+ produced from heterolytic cleavage of H_2, but lacks the H^- produced. The protonated enzyme might well be stable in the absence of redox agents, and also may account for the pH dependence of the redox potential and epr signal intensity (for example, spin quantitation) associated with Form C.[87,48]

The potential involvement of the thiolate ligands as bases is also consistent with model chemistry. The reaction of $Ni(cyclam)^{2+}$ with KSH in THF/MeOH solution results in a compound containing the complex cation $[Ni_2(cyclam)_2(\mu\text{-}SH)_2]^{2+}$.[88] The structure of this dimer (Fig. 8) reveals a typical Ni_2S_2 core structure, except that the orientation of one SH^- ligand is in a side-on fashion that suggests a Ni–H interaction with the SH^- proton. When this compound is heated in acetonitrile under N_2, it is converted into a complex cation containing a η^2, η^2-μ-S_2^{2-} ligand, $[Ni_2(cyclam)_2(\mu\text{-}S_2)]^{2+}$ (Fig. 8). This reaction couples the oxidation $2HS^- \rightarrow S_2^{2-}$ with the production of the equivalent of H_2, which apparently reduces the solvent. This suggests that the bridging thiolates in the core of the Ni,Fe active site could be responsible for H_2 activation without requiring metals to act as binding sites.

Work involving the stoichiometric protonation of various metal thiolate complexes has led to the characterization of complexes with coordinated thiol ligands. These systems have been shown to catalyze H/D exchange or to stoichiometrically produce H_2 from the reduction of H^+.[89,90] The Fe(II) containing system that produces H_2 proceeds through a series of intermediates that feature thiol complexes, ultimately coupling the oxidation of a μ-dithiolato diferrous complex to a μ-dithiolato diferric complex with the production of H_2 (Fig. 9).

A weakness of the XAS experiments on Forms C and C* is that the presence or absence of a H^-/H_2 ligand cannot be directly detected. Recent experiments have examined the Ni ligand environment in

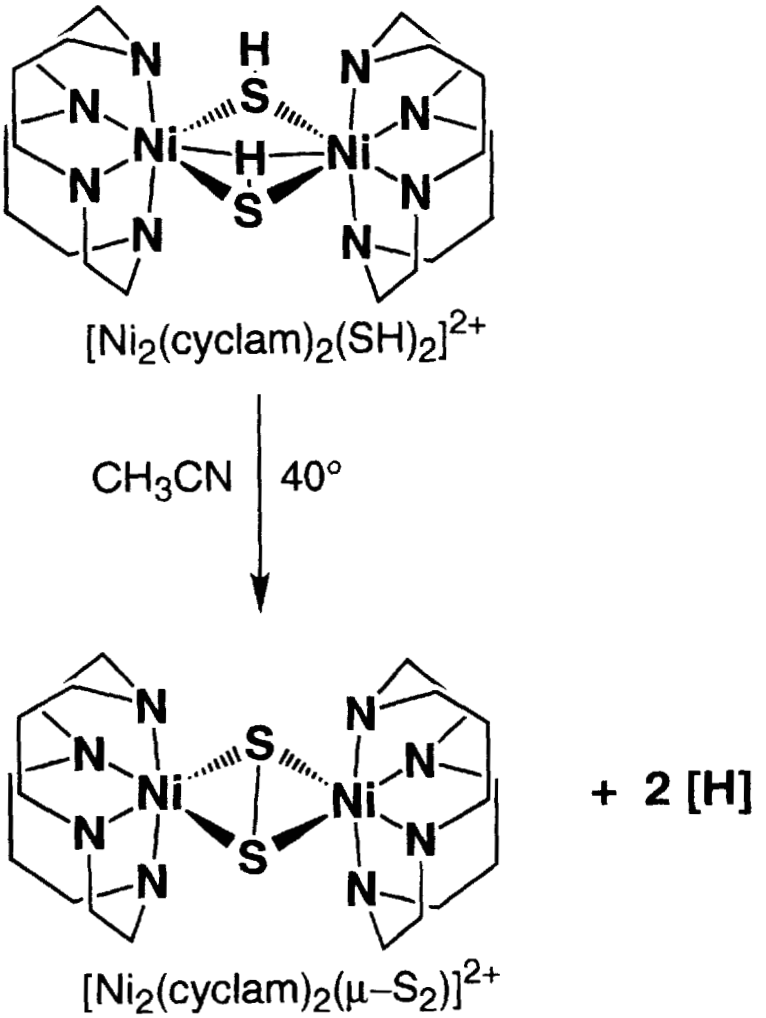

FIGURE 8 The oxidation of μ-SH^- ligands to a disulfide ligand in a dinuclear Ni (II) complex.

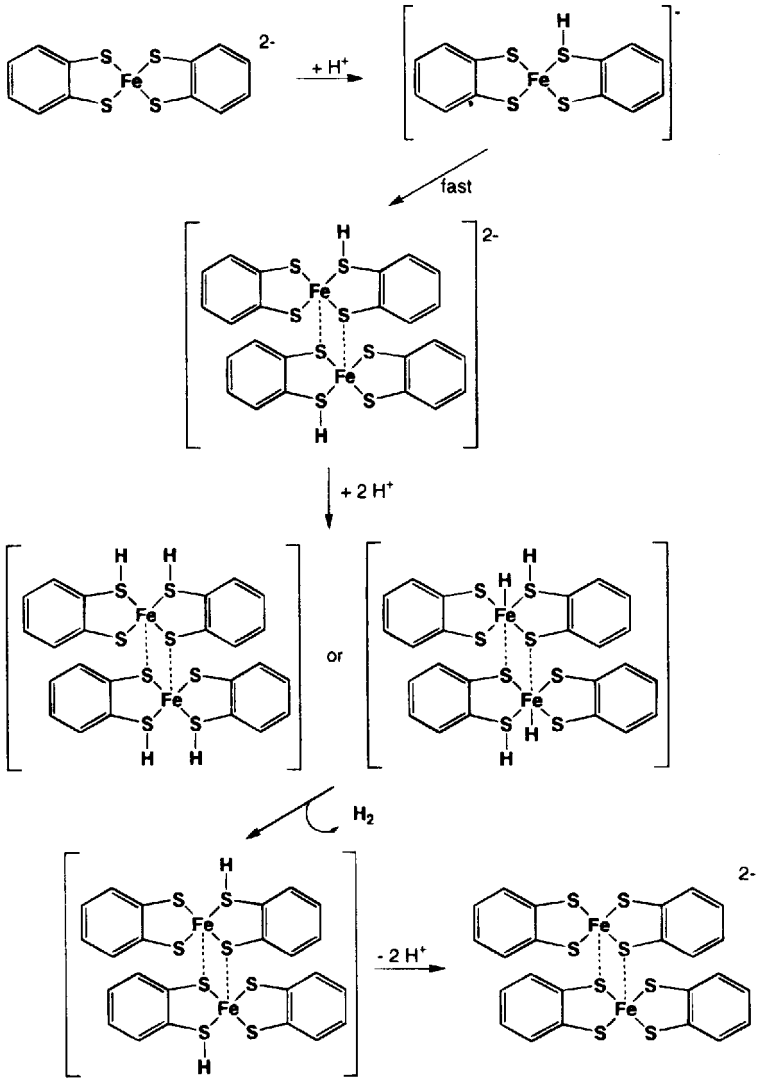

FIGURE 9 The stoichiometric production of H_2 from H^+ in a dinuclear Fe system (adapted from Ref. 89).

another photoactive complex. Carbon monoxide is a competitive inhibitor of H_2ase.[91] One likely mechanism would involve a competition between CO and H_2 for the same binding site. Albracht and coworkers demonstrated that when CO binds to Form C, a new epr signal is generated that shows coupling to both ^{61}Ni and to ^{13}CO, thus implicating Ni as the binding site of CO.[18] Exposure to light led to the observation of the same epr signal observed in Form C*, suggesting that the photoproduct produced from the dissociation of the CO ligand is the same as that produced from Form C. Given the dinuclear structure of the active site, it is possible that ligands bound to either Ni or Fe could lead to hyperfine couplings in the epr spectrum.

Upon extensive exposure to CO, an epr-silent CO complex is produced. It is not clear what is oxidized to produce this epr-silent intermediate, but this CO complex has been extensively examined using IR spectroscopy.[81] The CO complex has a $\nu_{CO} = 2060\ cm^{-1}$ and is also light sensitive. Exposure to visible light leads to the reversible photodissociation of the CO ligand. The Ni K-edge XAS studies have probed the role of the Ni in this photochemistry. No evidence for a Ni–CO ligand is found in the EXAFS analysis and photodissociation of the CO leads to very small changes in the edge energy. Given that it is unlikely that the thiolates bind CO and that the IR spectrum clearly indicates a terminally bound CO ligand, these results support the model wherein the Fe serves as the binding site for this competitive inhibitor. Thus, Fe remains a potential H^-/H_2 binding site, although it is not required, since binding of CO to Fe could alter the electronic structure of the cluster in a way that inhibits H_2 activation at another binding site (for example the bridging cysteinate ligands).

V. CONCLUSIONS

Detailed spectroscopic studies of redox-poised H_2ases fail to provide definitive evidence that Ni participates directly in redox chemistry or in the binding of substrates/inhibitors. The lack of a significant shift in the Ni K-edge energy or in the Ni–ligand bond lengths in H_2ase are inconsistent with a redox role for the Ni center. The bond lengths obtained from EXAFS analysis and the $S = 0$ determination for the Ni center in the SI redox level are both consistent with an oxidation state of II for the Ni. Combined with the edge energy data

and the lack of bond length changes in EXAFS spectra from various redox states, the data indicate that the oxidation state of Ni is closer to II in all redox states of the enzyme.

Although this conclusion is appealing in view of the redox chemistry of classical Ni(II) complexes, it does not explain the epr spectra associated with the Ni site or provide any insight into the nature of the redox active species. Further, the absence of spectroscopic evidence indicating that the Fe center is directly involved in redox chemistry, plus the number of electrons that are involved, indicate that the redox chemistry cannot be attributed to the Fe center in the dinuclear active site. It is clear from the observation of ^{61}Ni hyperfine coupling in the epr spectra that the radical giving rise to the epr signals observed at 77 K intimately involves the Ni center. However, we have argued that the epr data is not unequivocal evidence for Ni-centered redox chemistry or the presence of either Ni(III) or Ni(I) in the enzyme. It is possible that the primary redox-active species in the active site, at least with respect to the oxidized enzyme, are the conserved cysteine residues that constitute the endogenous ligands for the Ni, Fe cluster. This proposal is supported by the products of the oxidations of Ni alkylthiolate complexes that invariably reflect S-oxidation.

The possibility that Ni serves as the substrate (or inhibitor) binding site in the enzyme is also not supported by the data. Although the examination of the photochemistry that is associated with an enzyme/substrate complex (Form C) and the complex formed with the competitive inhibitor CO clearly indicate that ligand dissociation and recombination are involved, there is no evidence to associate these processes clearly with the Ni center. Model studies implicate the thiolates as potential binding sites for H^+ and possibly for the H^- produced by heterolytic cleavage of H_2.

The most striking result that arises from the biophysical studies of the role of the Ni site in H_2ase is the insensitivity of the Ni center to redox chemistry and the binding of substrates and inhibitors. Thus, it appears that the role of Ni is to modify the active site, rather than to serve as the active site. This conclusion is supported by enzymology of H_2ase, since the biochemical characteristics that are most clearly associated with the presence of Ni in the enzyme (and the modification of the Ni site by selenocysteine) are a much lower turnover rate and O_2 tolerance. Thus, the role of Ni is more closely

associated with producing an O_2 stable enzyme at the expense of optimal catalysis of H_2 redox chemistry.

Acknowledgments

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