Stability of the Ni-C State and Oxidative Titrations of *Desulfovibrio gigas* Hydrogenase Monitored by EPR and Electronic Absorption Spectroscopies

David P. Barondeau, Lee Melvin Roberts, and Paul A. Lindahl*

Contribution from the Department of Chemistry, Texas A&M University, College Station, Texas 77843

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Abstract: The Ni-C state of the NiFe hydrogenase from *Desulfovibrio gigas* was found to be stable for over 40 h at pH 8.0 in the strict absence of H_2. This demonstrates that Ni-C is unable to spontaneously reduce protons to H_2 under these conditions, a result that is contrary to earlier reports. The form of the nickel that spontaneously reduces protons is probably Ni-R, the EPR-silent state that appears to be one electron more reduced than Ni-C. The stability of Ni-C permitted, for the first time, stoichiometric oxidative titrations of the H_2-free, reduced enzyme. Four such titrations were performed, using the oxidant thionin and monitoring the progress of the titrations by electronic absorption (at 410 nm) and EPR spectroscopies. Redox changes in the enzyme's two Fe_4S_4 clusters were readily followed by changes at 410 nm, while those of the Ni were observed by EPR. Redox changes of the Fe_3S_4 cluster were monitored by both spectroscopic methods. At the start of the titrations, the Ni center was in the Ni-C state and the Fe–S clusters were partially oxidized. Adding thionin caused disappearance of Ni-C, development of the EPR-silent intermediate state, and eventually the appearance of Ni-B. The Fe–S clusters oxidized gradually throughout every stage of the titrations. An average of 4.2 oxidizing equiv/mol of thionin was consumed overall. After the number of equiv/mol consumed by the Fe–S clusters and the EPR-active Ni ions were substracted, an average of 1.2 oxidizing equiv/mol remained unassigned. The activity of the enzyme appears to be correlated to the ability of the Ni site to undergo redox chemistry.

Introduction

Nickel–iron hydrogenases catalyze the reversible oxidation of H_2 to $2H^+$. The enzyme from *Desulfovibrio gigas* (Hase[1]) has an $\alpha\beta$ quaternary structure (M_r = 89 500 Da) containing one $[Fe_3S_4]^{+/0}$ cluster, two $[Fe_4S_4]^{2+/+}$ clusters, and one mononuclear nickel complex.[2-5] In the presence of air, the enzyme is essentially inactive.[6] The Ni in this state has a 3+ valance and yields an EPR signal known as ***Ni-A*** (g_1 = 2.31, g_2 = 2.23, g_3 = 2.02). A second EPR signal known as ***Ni-B*** (g_1 = 2.33, g_2 = 2.16, g_3 = 2.02) is often present as well.[2] The proportion of analytically-detected Ni in the Ni–B form can be increased at the expense of Ni–A by reducing and then anaerobically reoxidizing the enzyme. The Fe_3S_4 cluster in the oxidized enzyme is in the 1+ core oxidation state and yields an EPR signal at ***g* = 2.02**.

The enzyme is activated by prolonged exposure to H_2. During activation, the Ni displays an unusual sequence of redox and magnetic changes. Ni-A disappears in accordance with $E^{\circ\prime}$ = −220 mV[7] (at pH 7.8), resulting in an EPR-silent intermediate state[8] to be called ***Ni-SI***. This disappearance is generally thought to correspond to the reduction of Ni^{3+} to Ni^{2+}. The enzyme can be reduced further, to a state yielding another Ni EPR signal known as ***Ni-C*** (g_1 = 2.19, g_2 = 2.16, g_3 = 2.02),[2,3,9] and even further to yet another EPR-silent reduced state to be called ***Ni-R***. At temperatures below *ca.* 15 K, a complex signal known as the g = 2.21 signal develops, apparently arising from magnetic coupling of the Ni center to one of the reduced $[Fe_4S_4]^+$ clusters.[2] When the enzyme is fully reduced, both Fe_4S_4 clusters are in their reduced 1+ core oxidation states and exhibit extremely broad EPR signals.[5,10]

The Ni center is thought to be the active site of the enzyme, largely because of the unusual properties of Ni-C. Van der Zwaan et al. discovered that exposing samples exhibiting Ni-C to visible light at low temperatures alters the signal.[11] They also found that this photoconversion occurs nearly six times slower when the enzyme is in D_2O rather than H_2O. They suggested that the Ni center in the Ni-C state might be a photolabile Ni hydride. Fan et al. identified two types of exchangeable protons associated with Ni-C,[12] and Whitehead et al. found that one such proton dissociates upon illumination.[13] Hase catalyzes H/D exchange and cleaves H_2 heterolytically,[14] which suggests that a hydride forms somewhere on the enzyme. The results demonstrating the close association of an exchangeable hydrogenic species with Ni-C implicate the Ni center as the active site of the enzyme, and Ni-C as a catalytically important hydride-bound state.

* Author to whom correspondence should be addressed.

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(1) Abbreviations: Hase, hydrogenase; EPR, electron paramagnetic resonance; A_{410} and A_{600}, absorbance at 410 and 600 nm, respectively: A_{max}, maximum absorbance at 410 nm; A_{min}, minimum absorbance at 410 nm; $\Delta\epsilon$, extinction coefficient of oxidized Hase at 410 nm minus that of reduced Hase at 410 nm; mN, 10^{-3} equiv/L.

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Other studies reveal a correlation between Ni-C and enzyme activity. Teixeira et al. found that Ni-C develops synchronously with enzyme activity as solution potentials are lowered.[4] Fernandez et al.[10] and others[8,15-17] report that activation of the enzyme correlated with the development of the Ni-C signal. The fact that Ni-C is a *transient* or *intermediate* in the activation of the enzyme, developing and then decaying with time, has also been used as evidence that Ni-C is an important intermediate in catalysis.[2,4] Other unusual aspects of the signal, such as its low spin intensity[12,18] and variable spin intensity during a potentiometric titration,[4] have also been attributed to its transient nature. Van der Zwaan et al. found that Ni-C is unstable in the absence of H_2 and that it oxidizes to Ni-SI in 20 h at 8 °C.[11] Moreover, solution potentials of Hase prepared in the Ni-C state drift to higher values. They suggested that this occurs because the H_2 production activity of the enzyme in the Ni-C form consumes reducing equivalents; i.e., that Ni-C is in redox equilibrium with H_2.[11]

In summary, the generally accepted view is that Ni-C represents the active state of the enzyme: an unstable transient intermediate in the catalytic cycle, with substrate (hydride) bound, capable of spontaneously reducing protons to H_2 and becoming oxidized in the process.

This view has been modified somewhat by the recent results of Coremans et al.[16] They report that Ni-C is stable in the absence of H_2 as long as redox mediators are not present in solution. We estimate from their data (Figures 1, 2, and 4 of ref 16) that Ni-C decayed at rates ranging from only 7–40% per h over the course of their experiments (1.7 h). They suggested that mediators accelerated the oxidation of Ni-C to the Ni-SI state by providing a kinetic pathway for this one-electron oxidation. Without mediators, only the further-reduced Ni-R state appeared to be in redox equilibrium with H_2.

We have obtained evidence that confirms and extends the recent work of Coremans et al.[16] We have found that Ni-C is stable in the absence of H_2 for at least 40 h and is probably stable indefinitely as long as oxygen is completely excluded. The stability did not depend on the presence or absence of mediators, and it allowed us to perform the first stoichiometric oxidative titrations of a NiFe hydrogenase. The significance of the titrations and the Ni-C stability are discussed.

Experimental Procedures

Purification and Characterization of Hase. Three batches of *Desulfovibrio gigas* (ATTC 19364) (designated A, B, and C) were grown as described.[19] Hase was purified from each batch as described,[20] except for the following details: (i) the purifications were performed anaerobically at room temperature in an argon-atmosphere glovebox (Vacuum/Atmospheres, Model HE-453) containing 0.5–1 ppm O_2 (monitored by a Teledyne Model 310 analyzer); (ii) sodium dithionite (1 mM) was added to all degassed buffer solutions; (iii) cells were ruptured by sonicating 5 min (Heat Systems model W-380); and (iv) an ultracentrifugation step (100 000 g, 1 h) replaced the low-speed spin.

Metal contents were determined by inductively-coupled plasma emission or atomic absorption spectrophotometry. Protein concentrations were determined by the biuret[21] or Bradford[22] method using bovine serum albumin as a standard. The enzyme from each batch appeared *ca.* 90% homogeneous by visual inspection of SDS–polyacrylamide electrophoretic gels.

Specific activities for H_2 oxidation were determined as described[23] using the Bradford protein method. Test tubes containing 3–8 mL of assay mix (100 mM Tris pH 8 + 0.5 mM benzyl viologen) were sealed with a septum, purged 2 min with H_2, and heated to 30 °C. Samples were injected into the tubes and the absorbance at 555 nm, due to reduced viologen (ϵ = 10 400 M^{-1} cm^{-1}), was monitored with time. The specific activities obtained here should be compared with caution to those obtained from other laboratories, as subtle differences in the method used might substantially affect the values obtained. Crude extracts or fractions from the first few purification steps did not require H_2 activation. Highly purified fractions or samples that had been frozen and rethawed were found to require a 2-h incubation under H_2 for maximal activity.

Ni-C Stability Studies. All procedures involving the enzyme were performed in the glovebox, on a Schlenk line, or in a quartz optical cuvette modified with a double-septum seal.[24] A sample of Hase (batch A) was reduced by incubation for 2 h under H_2 in buffer containing the redox mediator triquat ($E^{\circ\prime}$ = −540 mV). The sample (specific activity 970 units/mg) was subjected to Sephadex G-25 (Pharmacia) chromatography (1 X 15 cm), to separate it from excess, unreacted H_2. The absorbance at 410 nm (A_{410}) of the H_2-free sample was determined (Perkin-Elmer Model λ3B), and an aliquot was loaded into an EPR tube and then frozen. The remaining sample was incubated in an optical cuvette for 18 h at 27 °C. A_{410} was measured thereafter, and another aliquot was frozen in an EPR tube for analysis. Solution potentials were measured at room temperature using a potentiostat (Princeton Applied Research Model 273), a Au working electrode, and a Ag/AgCl reference. The reference was calibrated from the midpoint potential of methyl viologen (assumed to be −440 mV), obtained by cyclic voltammetry. EPR spectra were obtained and quantified as described.[25,26]

The H_2 concentration in the glovebox was determined by an enzymatic method to be 10 ppm on one occasion and 19 ppm on another. (The enzymatic method involved adding a trace of Hase to a vial containing 0.5 mL of activity-assay mix and a known volume of box atmosphere and calculating the amount of H_2 from the total amount of benzyl viologen reduced.) A second experiment was designed to eliminate any trace amounts of H_2 from the enzyme solution. A sample of Hase (batch B) that had been exposed to 1 atm of H_2 overnight at 27 °C (specific activity 1200 units/mg) was subjected to gel filtration chromatography (Sephadex G-25, in 100 mM potassium phosphate pH 8.0 and 1 mM triquat) to separate it from excess H_2. A small portion of the eluate was oxidized by a slight excess of thionin (3,7-diaminophenothiazin-5-ium chloride, Aldrich). The maximum absorbance at 410 nm obtained upon oxidation was designated A_{max}, and the enzyme concentration was calculated therefrom (18 μM).[27] The major portion of the eluate was used to completely fill a 13-mL double-septum-sealed optical cuvette (i.e. with no gas bubble), and A_{410} was determined. Three gas-tight syringes were inserted into the cuvette, including a 100-μL syringe containing thionin, a 500-μL syringe filled with the remaining eluate, and another 500-μL syringe that was empty. All syringes were prepared so they contained no gas bubbles. Volumes of a thionin solution, totaling 1.4 equiv/mol, were incrementally added to the sample. After each addition, the solution in the optical cuvette was mixed by slowly injecting the solution of the filled 500-μL syringe into the cuvette and simultaneously withdrawing that same volume into what had been the empty syringe. After 12 such cycles, the enzyme in the cuvette and in the filled syringe was of homogeneous concentration and oxidation state (as determined by the cessation of changes at A_{410} upon further cycling). Two modified EPR tubes (4 mm i.d. X 4 cm) were fitted with rubber septa that had been degassing in the box for at least one month and intentionally punctured as described.[28] The material in the filled syringe was injected

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Table 1. Determination of Enzyme Concentration (values are in μM)

property measured	sample 1	sample 2	sample 3	sample 4
[Ni]	24	20	14, 15	22
[Fe]/11	22	n.d.	22	22
A_{max} at 410 nm/ϵ_{410}	23	21	23	25
$[g = 2.02]_{max}$ intensity	15	13	15	23
[protein] (biuret method)	17	n.d.[a]	30	n.d.[a]
$[Hase]_{avg}$	20	18	20	23
σ_{n-1}	4.0	4.4	6.3	1.4

[a] Not determined.

rapidly into one of the EPR tubes so as to fill it completely and leave no gas bubble. The EPR sample was frozen by rapid immersion into cold isopentane within 30 s of filling the tube. The enzyme in the filled optical cuvette was incubated for 41 h at 27 °C inside the glovebox and A_{410} was obtained thereafter. An aliquot was removed from the cuvette, transferred to the other EPR tube, and frozen immediately. The remaining enzyme was reduced with 1 atm of H_2, and A_{min} was obtained.

Titration Procedure. Four titrations were performed. For **titration 1**, a sample of Hase (batch A) was exposed to 1 atm of H_2 for 2 h. Excess H_2 was then separated from the protein using a Sephadex G-25 column (1 × 15 cm) equilibrated in 100 mM potassium phosphate pH 8.0. The enzyme was buffered with phosphate to minimize temperature-dependent changes in pH and potential ($\Delta pK_a/\Delta T = -0.0028$ C^{-1}).[29] The eluted sample was collected in the optical cuvette and diluted to 4.78 mL with buffer. A_{410} was measured and then 350 μL was transferred to an EPR tube and frozen for later analysis. Aliquots of a standardized 1.89 mN thionin solution were added slowly with gentle mixing. Equilibrium (taken as the condition when A_{410} stopped changing) was established within ~2 min of mixing. Equilibrium A_{410} and A_{600} values were recorded for each thionin aliquot added. At various points during the titration, aliquots were removed for EPR. A_{410} values measured both before and after removing aliquots were consistently within 1% of each other, indicating that essentially no redox changes took place during the 20 min required for the sample-removal process. The maximum A_{410} obtained during the titration was designated A_{max}. At the end of the titration, the remaining solution was exposed to an atmosphere of H_2 for 1 h to obtain A_{min}. Data plotted as $(A_{410} - A_{min})/(A_{max} - A_{min})$ *vs* the number of equivalents/mole of thionin added will be called the ***A_{410} titration curve***. $(A_{410} - A_{min})/(A_{max} - A_{min})$ multiplied by 100 will be referred to as ***percent oxidized at 410 nm***. The $g = 2.02$, Ni-B and Ni-C signals were plotted as spin/mol *vs* the number of equivalents/mole of thionin added, yielding the ***$g = 2.02$, Ni-B*** and ***Ni-C titration curves***, respectively. The ***A_{600} titration curve***, reflecting the amount of residual thionin present past the titration end point, was obtained from the A_{600} absorbance values divided by the extinction coefficient of oxidized thionin (2.80×10^4 N^{-1} cm^{-1}). The other titrations were performed similarly, except that the buffer was 50 mM phosphate pH 7.0 for titration 2 (batch A), 50 mM Tris pH 8.0 + 100 mM NaCl for titration 3 (batch A), and 50 mM Tris pH 8.0 for titration 4 (batch C).

We estimated the Hase concentrations of the samples used in the titrations to be the average of values obtained from several different types of determinations including the following (i) the biuret protein concentration; (ii) the nickel concentration; (iii) the iron concentration divided by 11 Fe/mol; (iv) A_{max} divided by the published ϵ;[27] and (v) the maximum spin concentration of the $g = 2.02$ signal (which tends to be the signal with the greatest intensity). The standard deviations of these values were used as realistic estimates of the relative uncertainties in the enzyme concentration. The concentrations obtained are given in Table 1.

Results

Absorption Spectra of Hase. The spectrum of thionin-oxidized enzyme (Figure 1, solid line) exhibits a shoulder in the 400-nm region that declined substantially after introducing an atmosphere of H_2 (dashed line). The oxidized-minus-reduced difference spectrum (inset) reveals that the maximum absorbance difference occurs at 410 nm. Spectra of the four samples used in the titrations were similar. The molar extinction coefficients (ϵ) of oxidized and reduced Hase at 410 nm are given in Table 2. The average values are 51 000 and 33 000 M^{-1} cm^{-1} for oxidized and reduced enzyme, respectively, yielding an overall molar extinction coefficient difference ($\Delta\epsilon$) of 18 000 M^{-1} cm^{-1}. An ϵ of 46 500 M^{-1} cm^{-1} at 400 nm for oxidized Hase has been reported previously.[27]

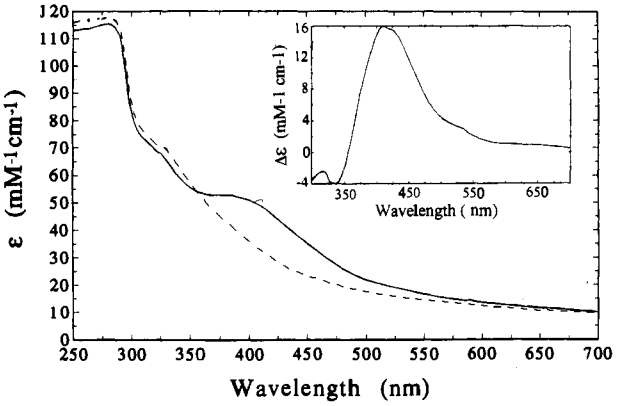

Figure 1. Electronic absorption spectra of Hase: (—), Hase sample 1 oxidized by thionin; (– –) same sample after H_2 reduction. Inset shows oxidized-minus-reduced difference spectrum.

We wanted to estimate the proportion of $\Delta\epsilon$ due to each redox site in Hase. Oxidized Fe-S clusters exhibit S-to-Fe charge-transfer transitions in the 400-nm region. When such clusters are reduced, the transition intensities decline by *ca.* 50%. The average $\Delta\epsilon$ at 390 nm for the Fe_4S_4 clusters in three well-characterized ferredoxins is 6600 M^{-1} cm^{-1}.[25,30] $\Delta\epsilon$ for Fe_3S_4 clusters appears to be somewhat more variable. $\Delta\epsilon$ for the $[Fe_3S_4]^{+/0}$ cluster in *Desulfovibrio gigas* Ferredoxin II is 6100 M^{-1} cm^{-1},[31] similar to that for $[Fe_4S_4]^{2+/+}$ clusters. However, the $\Delta\epsilon$ at 410 nm for the Fe_3S_4 cluster of inactive aconitase is only ~45% of that for the corresponding Fe_4S_4 cluster in the active enzyme.[32] Given the uncertainties involved, the overall $\Delta\epsilon$ expected for 1 Fe_3S_4 and 2 Fe_4S_4 clusters (~4500 + 2 × 6600 = ~17 700 M^{-1} cm^{-1}) is quite near to that observed for Hase (18 000 M^{-1} cm^{-1}). This indicates that the Fe-S clusters in Hase are responsible for most, if not all, of the observed spectral changes at A_{410}.

The spectral features of known Ni complexes with S and N ligands vary widely in intensity and energy. In the region between 300 and 600 nm, molar extinction coefficients for such complexes commonly range from *ca.* 0 to 6000 M^{-1} cm^{-1}.[33-35] Since there were no obvious features due to the Ni in any part of the spectra, and all A_{410} changes could be accounted for by the Fe–S clusters, we assumed that the Ni in Hase did not contribute significantly to A_{410}.

Stability of Ni-C. A sample of Hase containing the mediator triquat was reduced with H_2 and then separated from any unreacted H_2 as described in Experimental Procedures. The H_2-free sample was 35% oxidized at 410 nm and yielded the Ni-C signal (0.25 spins/mol) presented in Figure 2A. Eighteen hours later, the sample was 41% oxidized and yielded the Ni-C signal with an unchanged intensity (Figure 2B, 0.25 spin/mol). The solution potential after the 18-h period was –314 mV.[36] The essentially invariant A_{410} and Ni-C signal intensities demonstrate

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(36) The experiment was repeated using the mediator methyl viologen rather than triquat. In this case, the solution potential stabilized at –220 mV, the sample was 77% oxidized at 410 nm, and it exhibited Ni-B with an intensity of 0.44 spins/mol.

Table 2. Results of Oxidative Titrations

	titration 1	titration 2	titration 3	titration 4	avg
specific activity (units/mg)	1000[a]	1000[b]	1000[a]	300[a]	820
ϵ_{max} at 410 nm (M^{-1} cm^{-1})	49,000	55,000	50,000	50,000	51,000
ϵ_{min} at 410 nm (M^{-1} cm^{-1})	33,000	35,000	32,000	32,000	33,000
$\Delta\epsilon$ at 410 nm (M^{-1} cm^{-1})	16,000	20,000	19,000	19,000	18,000
$[Ni\text{-}B]_{max}$ (spins/mol)	0.66	0.34	0.64	0.20	0.46
$[Ni\text{-}C]_{max}$ (spins/mol)	0.32	0.28	0.36	0.12	0.27
$[g = 2.02]_{max}$ (spins/mol)	0.74	0.71	0.68	0.86	0.75
A_{600} slope past end point	0.80	1.1	1.3	0.9	1.0
% oxidized at 410 nm at start point	25	38	19	16	24
% oxidized at 410 nm at null point	69	67	65	69	68
% oxidized at 410 nm at end point	98	95	97	98	97
fully reduced point (equiv/mol)	0.0	0.0	0.0	0.0	0.0
start point (equiv/mol)	2.0	2.8	1.4	0.6	1.7
null point (equiv/mol)	5.0	4.8	4.4	2.4	4.2
end point (equiv/mol)	6.6	6.3	6.8	3.9	5.9

[a] Measured just prior to the titration. [b] Measured immediately after purification.

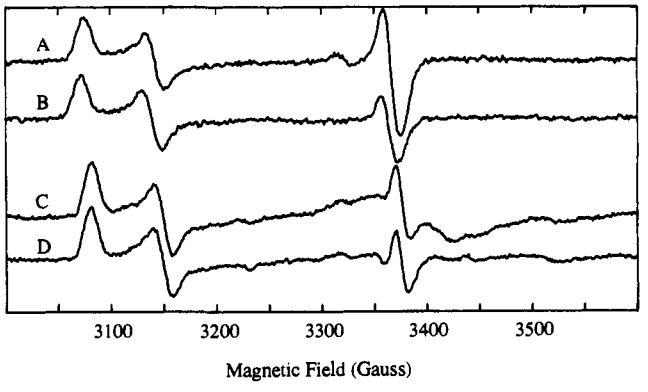

Figure 2. Stability of Ni-C in the Absence of H_2: (A) Hase reduced with H_2 and separated from excess H_2 (the first such experiment described in Experimental Procedures); (B) sample A after 18 h; (C) Hase reduced with H_2, separated from excess H_2, and partially oxidized with thionin (the second such experiment described in Experimental Procedures); (D) sample C after 41 h. EPR conditions for A and B: temperature, 10 K; microwave power, 0.02 mW; microwave frequency, 9.428 GHz. EPR conditions for C and D: temperature, 100 K; microwave power, 2 mW; microwave frequency, 9.46 GHz. The isotropic signals in A–D at g = 2.0 have not been assigned but they do not contribute substantially to the overall spectral intensities.

that the redox states of both the Fe-S clusters and the Ni center are stable for at least 18 h in the absence of H_2 and the presence of a redox mediator. Moreover, the potential of the solution in which Ni-C was stable was more positive than that required to spontaneously reduce protons to H_2.

Apart from concluding that Ni-C is incapable of reducing $2H^+$ to H_2 and is stable in the absence of H_2, how might these results be explained? If the chromatographic separation of H_2 from Hase had been incomplete, and a small amount of H_2 remained in solution, this small amount might have been sufficient to stabilize Ni-C. In addition, the atmosphere in which the experiment was performed, our glovebox, contained 10–20 ppm H_2. Trace amounts of H_2 from either of these sources might have been present in this experiment, and they could have stabilized Ni-C.

To eliminate this possibility, a second experiment (described in Experimental Procedures) was performed that essentially guaranteed the complete absence of H_2 from the enzyme solution. This condition was achieved by intentionally oxidizing an H_2-free sample using a slight amount of thionin. The sample was 29% oxidized at 410 nm prior to the addition and 46% oxidized thereafter. Even in this partially-oxidized condition, Ni-C was stable for 41 h; the Ni-C signal intensity corresponded to 0.15 spins/mol before (Figure 2C) and 0.13 spin/mol after (Figure 2D) the 41-h incubation. The small change observed in spin intensity is within the uncertainty of spin quantitation measurements.

The A_{410} increase that occurred after thionin was added confirms that the Fe–S clusters had been oxidized by the thionin. Had a substantial amount of H_2 been present initially, it would have been preferentially oxidized, and no increase in A_{410} would have been observed. The increase in A_{410} thus indicates that any trace of H_2 possibly present before adding thionin had been eliminated and that the sample was incubated for 41 h in the strict absence of H_2.

Titration 1. Ideally, Hase samples would be fully reduced and reductant free at the beginning of a titration. Unfortunately, this condition could not be obtained. We incubated samples in 1 atm of H_2 for 2 h and then separated excess H_2 by gel filtration chromatography. Inevitably, the samples were partially oxidized at 410 nm by this treatment.

The partially-oxidized, reductant-free samples were oxidatively titrated with thionin and monitored by both optical (at 410 and 600 nm) and EPR spectroscopies. The titration curves obtained for Titration 1 are shown in Figure 3, and the associated EPR spectra are shown in Figure 4. The parametrized results of this and three other titrations are listed in Table 2.

A_{600} increased dramatically during the latter part of the titration (Figure 3) due to the presence of unreacted, excess, oxidized thionin.[37] The A_{600} data points in this region were least-squares fitted to a first-order polynomial line, the x-axis intercept of which defined the ***end point*** of the titration. Since all thionin added beyond the end point should have been detected at A_{600}, the slope of the best-fit line (with units of moles of *detected thionin* per mole of *added thionin*) should equal 1. The actual slope was 0.8 (Table 2).

The A_{410} titration curve (Figure 3A, circles) was assumed to arise entirely from changes in the redox states of the Fe–S clusters. The point at which the titration began will be called the ***start point***. The sample was 25% oxidized at 410 nm at the start point. The A_{410} titration curve increased monotonically throughout the

(37) We do not understand the origin of the broad "hump" in the A_{600} titration curve occurring before the endpoint. It is probably not due to unreacted, excess oxidized thionin; if it were, the dramatic increase indicating the titration end point would have occurred in place of the hump. Nor does the hump appear to arise because the A_{600} values were recorded before the reactions with thionin were complete; if this were the case, the A_{600} values would have declined slowly with time and would have been in decline when the A_{600} values were recorded. To the contrary, A_{600} values declined rapidly and were generally stable within 2 min of each thionin addition. Moreover, if this interpretation was correct, the A_{600} changes would not have yielded a hump, but a gradual increase. The A_{600} titration curves from the other three titrations also exhibited a hump region, and it is interesting to speculate that some redox center in Hase, with a state that develops and decays during the region of the titrations that yielded the hump (such as the Ni-SI state of the Ni center), absorbs at 600 nm. Further studies are required to evaluate this possibility.

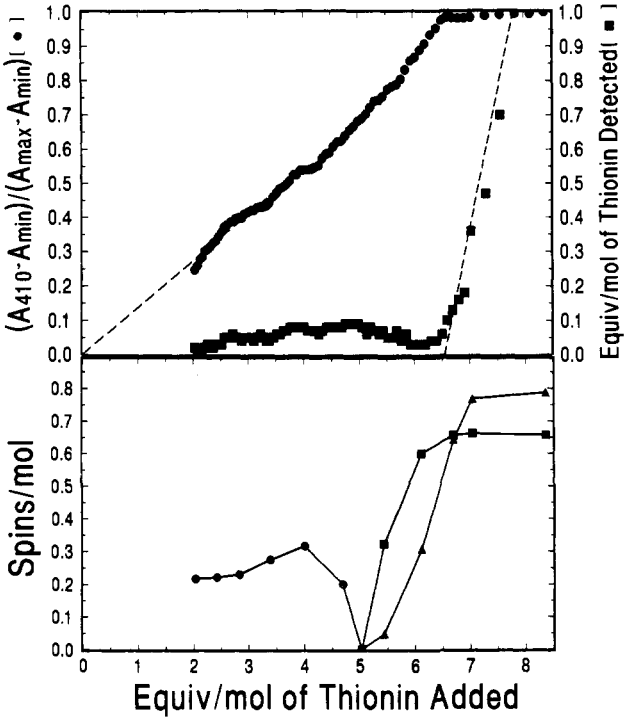

Figure 3. Thionin oxidative titration 1 of Hase: (A) circles—A_{410} titration curve, squares—A_{600} titration curve (dashed lines are least-squares fits, as described in the text); (B) circles—Ni-C titration curve, squares—Ni-B titration curve, triangles—g = 2.02 titration curve.

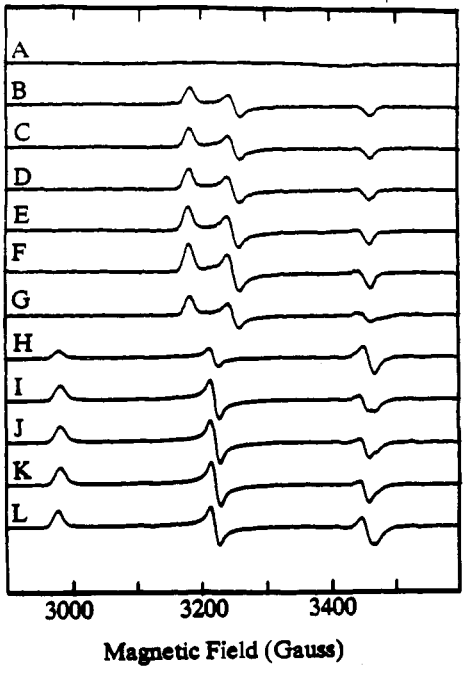

Figure 4. EPR spectra from titration 1: (B, C, D, E, F, G, H, I, J, K, and L) aliquots frozen after adding 0.0, 0.4, 0.8, 1.4, 2.0, 2.7, 3.4, 4.1, 4.7, 5.0, and 6.3 equiv/mol of thionin, respectively. (A) Sample 1 after the titration and exposure to H_2 for 1 h. EPR conditions: temperature, 10 K for A–G, 100 K for H–L; microwave power, 0.02 mW for A–G, 1 mW for H–L; microwave frequency, 9.457 GHz for A–G, 9.427 GHz for H–L; modulation amplitude, 11.5 G.

titration, indicating that Fe–S clusters underwent oxidation with every addition of thionin. The lack of a "plateau" region indicates that "A_{410}-insensitive" redox-active species (such as the Ni center) titrated along with one or more of the Fe–S clusters. The A_{410} titration curve maximized at the end point, indicating that the last site or sites in the enzyme to be oxidized were or included an Fe–S cluster. This was almost certainly the Fe_3S_4 cluster, since it has the most positive redox potential of all sites in the enzyme and because the g = 2.02 signal, originating from this cluster, developed in this region (see below).

We wanted to estimate the number of oxidizing equivalents that would have been used in the titration had the sample been fully reduced initially. To do this, the data constituting the first 1.5 equiv/mol of the A_{410} titration curve were least-squares fitted to a first-order polynomial line. The origin of the abscissa was redefined to be the point where the least-squares-fitted line crossed the abscissa. This point will be called the ***fully-reduced point***. According to this procedure, the sample was 2.0 equiv/mol oxidized at the start point, and a fully-reduced sample titrated to the end point would have consumed 6.6 oxidizing equiv/mol.

The partially-oxidized sample at the beginning of the titration exhibited only Ni-C (Figure 4). The Ni-C intensity increased slightly with added thionin and then declined and disappeared as more thionin was added. The Ni-B signal developed after more thionin was added. The g = 2.02 signal, arising from the $[Fe_3S_4]^+$ cluster, developed last. None of the samples exhibited Ni-C and Ni-B simultaneously, and none exhibited Ni-A. Ni-A was probably absent because the sample had been purified anaerobically and had never been exposed to oxygen.

That the Ni-C and Ni-B signals were never observed simultaneously during the titration is of some significance. If Ni-C and Ni-B arose from the same Ni ions, in isoelectronic states, and were distinguished only because they arose from two different protein conformations (for example, protein conformations that would be determined by the redox status of a species besides the Ni center), the decline of Ni-C would occur simultaneously with the development of Ni-B. The absence of Ni-C/Ni-B overlap suggests that the Ni-C state is more reduced than Ni-B and that the two states are separated by at least one EPR silent state (Ni-SI).

We define the ***null point*** of the titration to be the point where both Ni-C and Ni-B are absent and Ni-SI is present. The sample was 68% oxidized at 410 nm at the null point. Assuming that the Fe–S clusters have equal $\Delta\epsilon$ values at 410 nm and that the Fe_3S_4 cluster oxidized last in the titration, this result suggests that both Fe_4S_4 clusters were in their oxidized states and the Fe_3S_4 cluster was in its reduced state at the null point. This suggestion is supported by the Mössbauer and EPR results of Teixeira et al.[5] They found that samples prepared in the vicinity of the null point (poised at *ca.* –200 mV, EPR silent in the g = 2 region) have both Fe_4S_4 clusters oxidized and the Fe_3S_4 cluster reduced. This result also supports our assumption of equal $\Delta\epsilon$ values at 410 nm for each Fe–S cluster.

The quantified spin intensities of Ni-C, Ni-B, and the g = 2.02 signal (Table 2) were all substantially below 1 spin/mol. Although the reasons for such low values are not understood, similarly low values have been reported previously. Quantified intensities of 0.1, 0.25, 0.4, and 0.4–0.6 spin/mol for Ni-C have been reported, as have values of 0.3, 0.46, 0.5–1.0, 0.43, and 0.52 spin/mol for Ni-A and 0.59 and 0.89 spin/mol for the g = 2.02 signal.[4,8,9,12,18,19,38] No quantified Ni-B spin intensities have been reported. These low values indicate that only a fraction of the Ni (and Fe_3S_4) centers were EPR active in the samples titrated. Nothing is known regarding the fate of the EPR-silent Ni ions, including whether they are redox active.

Other Titrations. Three other samples were titrated. The shapes of the resulting titration curves were qualitatively very similar to those of titration 1, though some quantitative aspects (percent oxidized at the start points, maximum EPR signal intensities, and number of equivalents/mole consumed) varied significantly. Parametrized results are summarized in Table 2.

Relative to the other samples, sample 4 consumed substantially fewer equivalents/mole of thionin (Figure 5). Fully-reduced

(38) Cammack, R.; Patil, D.; Aguirre, R.; Hatchikian, E. C. *FEBS Lett.* **1982**, *142*, 289.

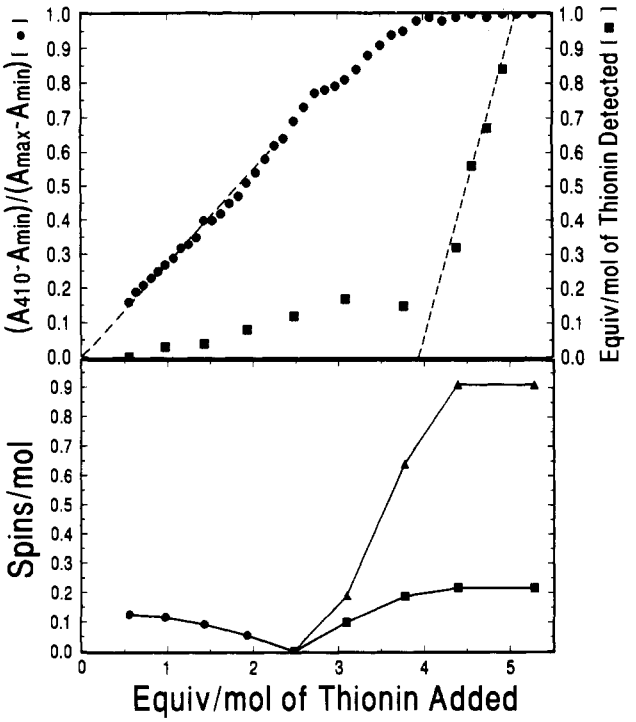

Figure 5. Thionin oxidative titration 4. Symbols are defined in Figure 3.

sample 4 would have consumed ca. 3.9 equiv/mol, rather than the 6.6 equiv/mol estimated as the average of the other three samples. This difference may have arisen from an overestimate of the Hase concentration in sample 4 (Table 1), but this is incongruent with the small relative uncertainty in the concentration of that sample. Fewer equivalents may have been consumed because a smaller proportion of Fe–S clusters were oxidized. However, the ϵ_{410} values obtained for sample 4 were near those of the other samples (Table 2), suggesting that the same number of Fe–S clusters underwent the same $n = 1$ redox changes in all four samples. Thus, it appears that A_{410}-insensitive redox centers (presumably the Ni center) consumed fewer oxidizing equivalents per mole in sample 4. Indeed the Ni-C and Ni-B signal intensities for titration 4 were the lowest of any titration. However, the situation may be more complex, since the Ni signals of titration 2 were also very low, even though sample 2 consumed substantially more oxidizing equivalents than sample 4. The only obvious characteristic that distinguished sample 4 is specific activity; it was *ca.* $^1/_3$ as active as the others. This suggests that enzyme activity may correlate with the number of oxidizing equivalents that the Ni site can consume.

Discussion

The Nature of Ni–C. As mentioned in the Introduction, Ni-C is generally thought to represent the *active state* of the enzyme,[10] serving as a *key intermediate*[12] in the catalytic cycle. Ni-C has been reported to be an unstable *transient*,[2,4,5,9,18] in redox equilibrium with H_2.[11] Our results demonstrate that Ni-C is stable in the absence of H_2 for at least 40 h and that it is probably stable indefinitely. Thus, Ni-C does not appear to be an unstable transient, nor does it appear to spontaneously reduce protons to H_2 or to be in redox equilibrium with H_2.

The function to which Ni-C had been assigned also appears incompatible with the slow rate at which Ni-C has been reported to decay. If Ni-C spontaneously reduced protons to H_2 during catalysis, it would decay at a rate equal to or faster than the turnover number of the enzyme. Since the turnover number is *ca.* 650 s^{-1} at 30 °C, Ni-C should be oxidized within milliseconds of removing H_2. This is too fast for Ni-C to be observed in H_2-free samples using conventional freezing methods. But such samples have been observed to be stable for minutes to hours.[4,11,39,40] We suggest that the slow decay rate may have been due, instead, to oxidation of Ni-C by trace amounts of O_2. Initially, such oxidations occurred with some of our samples, causing us to employ numerous procedures to exclude O_2 in the experiments presented here.[41]

Our conclusion that Ni-C is not a transient in the absence of H_2 is actually completely compatible with earlier reports indicating that Ni-C is a transient intermediate during potentiometric redox titrations[4] and reductive activation.[10] The transient nature observed for redox titrations refers only to the fact that Ni-C develops and declines as a function of solution potential, not that it changes as a function of time. Activation is a complex process and we have no doubt that Ni-C behaves transiently with time, as reported. But the properties of the Ni center during activation need not be those of the activated enzyme. We have attempted to avoid the complexities of activation by purifying and handling our samples anaerobically.

Ni-C has been reported to represent the active state of the enzyme (enzyme reductively activated by incubation in H_2).[10] But how can it represent the active state and also be an intermediate in the catalytic mechanism? If Ni-C is a catalytic intermediate, the Ni center must cycle between Ni-C and other states during catalysis. These other Ni states thus "represent" active enzyme as much as Ni-C does. In our view, active enzyme *can exhibit* Ni-C as well as other Ni states, depending on the potential at which such samples are poised, but no single Ni state represents active enzyme. We are uncertain which other Ni states active enzyme can exhibit, but Ni-R is certainly among them. Ni-R is more reduced than Ni-C and is readily obtained by exposing samples to H_2 (Figure 4A), the condition used to activate the enzyme.

The Active-Reductant State of Hase. The term "active state" can be used ambiguously. It can refer not only to the activated state of the enzyme (the way we have used it), but also to states capable of spontaneously reducing protons or oxidizing H_2. We will call the state capable of spontaneously reducing protons the ***active-reductant*** state and that capable of oxidizing H_2 the ***active-oxidant*** state. Accordingly, Ni-C had been thought to be the active-reductant state of Hase, though our results suggest this is not the case.

If Ni-C is not the active-reductant state, what is? This state must be more reduced than Ni-C and impossible to isolate in the absence of H_2, using conventional freezing methods. The four samples prepared for oxidative titration and the two prepared for the Ni-C stability studies were all in the Ni-C state. Since Ni-R is the only known Ni state more reduced than Ni-C, we propose that *it* is the active-reductant state and that Ni-C is the oxidation product of the reaction of Ni-R with a proton.

Since Ni-R is EPR silent and Ni-C is an odd-electron state, Ni-R is probably one electron more reduced than Ni-C. But $2H^+ \rightarrow H_2$ is a two-electron reduction. Thus, Ni-C would not appear to be the *direct* oxidation product of the reaction of Ni-R with protons. Assuming that (i) Ni-R is one electron more reduced than Ni-C, (ii) Ni-SI is one-electron more oxidized than Ni-C,

(39) Cammack, R.; Patil, D. S.; Hatchikian, E. C.; Fernandez, V. M. *Biochim. Biophys. Acta* **1987**, *912*, 98.

(40) Kojima, N.; Fox, J. A.; Hausinger, R. P.; Daniels, L.; Orme-Johnson, W. H.; Walsh, C. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 378.

(41) The experiments were performed anaerobically in a glovebox containing *ca.* 0.5 ppm O_2. The rubber septa employed were stored in the box at least one month prior to use. Samples were not exposed to any plexiglass, as this material slowly releases O_2. Van der Zwaan reported that Ni-C was unstable, but their samples were prepared in a plexiglass electrochemical cell.[11] The more recent results of Coremans et al., in which Ni-C was more stable, also used a plexiglass cell but considerable precautions were employed to remove impregnated O_2 from this material before enzyme samples were introduced.[16] These precautions may have been responsible for the increased stability of Ni-C, rather than the presence or absence of redox mediators.

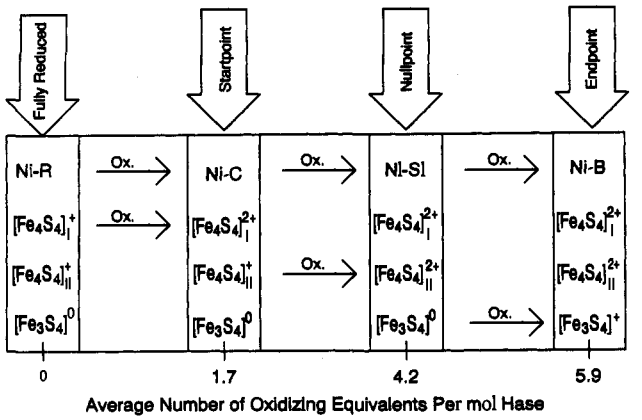

Figure 6. Analysis of oxidative titrations. The four designated titration points (fully reduced, start point, null point, and end point) are indicated by the vertical arrows along the titration axis. The redox states of the four centers in Hase (Ni center, two Fe_4S_4 clusters, one Fe_3S_4 cluster) are designated at each titration point. Horizontal arrows between titration points indicate the redox centers that oxidize predominantly within those regions.

and (iii) Ni-C and Ni-R are hydrides, we conjecture that Ni-R reacts with a proton as described in reaction 1.

$$H^+ + \text{Ni-R} \rightleftharpoons H_2 + \text{Ni-SI} \quad (1)$$

$$H^+ + \text{Ni-SI} + [Fe_4S_4]^{1+} \rightleftharpoons \text{Ni-C} + [Fe_4S_4]^{2+} \quad (2)$$

$$\text{net: } 2H^+ + \{\text{Ni-R}[Fe_4S_4]^{1+}\} \rightleftharpoons H_2 + \{\text{Ni-C}[Fe_4S_4]^{2+}\} \quad (3)$$

Besides exhibiting Ni-C, the samples we prepared in the absence of H_2 were also partially oxidized at 410 nm (16–38%). Assuming equal $\Delta\epsilon$ values for each Fe–S cluster, the extent of oxidation observed suggests that one of the Fe_4S_4 clusters (probably that designated ***Fe–S center II*** by Teixeira et al.[4]) was partially oxidized. We suggest that after removing H_2, the $[Fe_4S_4]^+$ state of this cluster reduces Ni-SI to Ni-C as shown in reaction 2. The sum of reactions 1 and 2, namely reaction 3, can be used to explain why removing H_2 from our fully-reduced Hase samples resulted in the Ni-C state and partially oxidized Fe–S clusters. It also specifies the states of the metal centers in Hase that appear to be in redox equilibrium with H_2 and protons.

Redox Status of Metal Sites at Different Titration Points. The redox status of the metal sites in Hase at designated points of the titrations are summarized in Figure 6. We propose that the fully-reduced state of Hase consists of Ni-R and the reduced forms of the three Fe–S clusters. In the absence of H_2, our samples spontaneously oxidized, yielding Ni-C, partially oxidized Fe_4S_4 clusters, and the fully-reduced Fe_3S_4 cluster. This was the state of the enzyme at the start point of the titrations, occuring *ca.* 1.7 equiv/mol more oxidized than the fully-reduced state. Between the start point and the null point, the Ni center oxidized from the Ni-C state to Ni-SI, and both Fe_4S_4 clusters oxidized as well. The enzyme consumed an average of 2.5 oxidizing equiv/mol during this step. Between the null point and the end point, Ni-SI oxidized to Ni-B and the Fe_3S_4 cluster oxidized, consuming an average of 1.7 oxidizing equiv/mol.

Table 3. Assignment of Oxidizing Equivalents in Hase (values are in equiv/mol)

	titration 1	titration 2	titration 3	titration 4	avg
total (start point to end point)	4.6	3.5	5.4	3.3	4.2
assigned to Fe–S clusters	2.2	1.9	2.4	2.5	2.3
assigned to EPR-active Ni	1.0	0.6	1.0	0.3	0.7
unassigned	1.4	1.0	2.0	0.5	1.2

The order of the redox changes that occurred during the titrations agrees well with that implied from the reported reduction potentials of the various redox couples in the enzyme. The reported couples and redox potentials, adjusted to pH 8.0, are as follows: $[Fe_3S_4]^{+/0}$ (–70 mV[8]); Ni-A/Ni-SI (*ca.* –200 mV[8,38]); $[Fe_4S_4]^{2+/+}$ center I (–350 mV[5,39]); Ni-SI/Ni-C (–390 mV[39]); $[Fe_4S_4]^{2+/+}$ center II (–400 mV[5,39]); and Ni-C/Ni-R (–450 mV[39]). According to these potentials, the redox centers in fully-reduced enzyme should be oxidized at pH 8.0 in the following order (ignoring overlap between reactions with similar potentials): (1) Ni-R → Ni-C; (2) $[Fe_4S_4]^+ \rightarrow [Fe_4S_4]^{2+}$ (center II); (3) Ni-C → Ni-SI; (4) $[Fe_4S_4]^+ \rightarrow [Fe_4S_4]^{2+}$ (center I); (5) Ni-SI → Ni-B; and (6) $[Fe_3S_4]^0 \rightarrow [Fe_3S_4]^+$. This appears to be approximately the order indicated by our titrations.

These titrations were performed to determine the number of oxidizing equivalents consumed by each redox center in the enzyme, and our analysis of this is summarized in Table 3. From start points to end points, totals of 3.3–5.4 oxidizing equiv/mol were consumed by the samples. Between 1.9 and 2.5 equiv/mol were consumed by the Fe–S clusters, as determined using eq 4.

$$\left[\frac{\%\text{ oxidized at end point} - \%\text{ oxidized at start point}}{100}\right] \times [3\text{ equiv/mol}] \quad (4)$$

The number of oxidizing equivalents consumed by the Ni center was more difficult to assess, because the spin intensities of Ni-B and Ni-C were signficantly less than unity. The minimum number attributed to the Ni was assumed to equal the sum of the maximum Ni-B and Ni-C signal intensities, or 0.3–1.0 equiv/mol for the four titrations. This leaves 0.5–2.0 oxidizing equiv/mol unaccounted for. These unassigned oxidizing equivalents may be artifacts of systematically underestimating the true enzyme concentrations by an average of 30%, or they may be evidence of other redox-active species in the samples. We are presently analyzing these titration curves in more detail by simulating them using different models of the redox properties of the enzyme. This analysis may help to establish the electronic state of Ni-C, an issue of current debate.

Acknowledgment. This work was supported by the Robert A. Welch Foundation (A-1170) and the National Institutes of Health (GM46441).