Heterogeneous Nickel Environments in Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*

Woonsup Shin,† Mark E. Anderson, and Paul A. Lindahl*

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

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Abstract: Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* has an $(\alpha\beta)_3$ quaternary protein structure and contains a novel Ni-and-Fe-containing complex (the NiFe complex) that exhibits an EPR signal (the NiFeC signal) of unusually low spin intensity. The Ni in the NiFe complex can be removed by reaction with 1,10-phenanthroline, yielding enzyme devoid of CO/acetyl-CoA exchange activity and unable to exhibit the NiFeC signal. On average, each CODH $\alpha\beta$ dimer was found to react rapidly and stoichiometrically with as few as 1.0 ± 0.2 phenanthrolines. Metal analyses of the enzyme before and after phenanthroline treatment, and of the phenanthroline-containing products of the reaction, revealed that only $\sim$0.3 Ni per $\alpha\beta$ were removed. Incubation of phenanthroline-treated enzyme with radioactive $^{63}Ni^{2+}$ followed by chromatographic separation of the ^{63}Ni-containing enzyme from unreacted $^{63}Ni^{2+}$ demonstrated that only 0.3 Ni per $\alpha\beta$ could be reinserted into the empty labile sites. These results indicate that the enzyme is heterogeneous; 30% of the $\alpha\beta$ protein subunits contain a labile Ni ion while the remaining 70% do not. Only those $\alpha\beta$ subunits with labile Ni ions can exhibit the NiFeC EPR signal and contain the NiFe complex in the form commonly recognized as such. Enzyme solutions lacking labile Ni are completely devoid of CO/acetyl-CoA exchange activity, suggesting that only $\alpha\beta$ subunits with labile Ni ions are capable of catalyzing CO/acetyl-CoA exchange. However, this activity may only be afforded to $(\alpha\beta)_3$ molecular assemblies that include both types of subunits, thereby precluding assignment of activity to a particular type of subunit. This analysis explains the low-spin intensity of the NiFeC signal and suggests that the NiFe complex contains significantly more irons than previously thought. The unusually mild conditions required for removal of the labile Ni suggests that this Ni may be coordinatively unsaturated.

Introduction

Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* (CODH[1]) catalyzes the reversible oxidation of CO to CO_2, the synthesis of acetyl-coenzyme A from CO, a methyl group and coenzyme A, and various exchange reactions including that of the carbonyl group of acetyl-coenzyme A with free CO (CO/acetyl-CoA exchange).[2] The synthase activity can be estimated from the CO/acetyl-CoA exchange activity since they share a similar mechanism.[3] The enzyme is rapidly inactivated by exposure to trace amounts of oxygen.

CODH has an $(\alpha\beta)_3$ hexameric protein structure.[4] The α and β subunits have molecular masses of 72 928 and 81 730 Da, respectively.[5] The three $\alpha\beta$ dimers that constitute one CODH molecule are commonly assumed to be identical in metal content and all other respects. From the metal contents of purified enzyme solutions, each $\alpha\beta$ dimer appears to contain 2 Ni, 11-13 Fe, and $\sim$14 sulfides,[4] organized into at least four complexes and clusters[6,7] including the *NiFe complex*.

The defining feature of the NiFe complex is an $S = 1/2$ EPR signal with principal g-values 2.08, 2.075, and 2.028. The so-called *NiFeC* signal arises when the cluster is in its one-electron reduced state and bound with CO.[8-13] The signal exhibits hyperfine broadening when ^{61}Ni or ^{57}Fe are substituted into the enzyme, and when ^{13}CO is bound.[8-11] EPR and ENDOR studies of the signal are most consistent with there being 1 Ni and 3-4 Fe in each NiFe complex.[11] The irons of the complex exhibit Mössbauer parameters typical of $[Fe_4S_4]^{2+}$ clusters.[7] The Ni does not appear to be part of a cubane-type Fe-S cluster; rather it is thought to be chemically linked to an Fe_4S_4 cluster.[7,11,14]

Besides the NiFe complex, each $\alpha\beta$ subunit is thought to contain an $[Fe_4S_4]^{2+/1+}$ cluster yielding two $g_{av} = 1.94$ signals, another cluster that yields the $g_{av} = 1.82$ and $g_{av} = 1.86$ signals, and an iron-containing species evident only as a Mössbauer quadrupole doublet known as *ferrous component II*.[7] Little is known about the structure and properties of the second Ni ion in the enzyme, but some circumstantial evidence suggests that it might be part of the $g_{av} = 1.82$ cluster or ferrous component II.[7] EXAFS studies indicate that the Ni ions in CODH are predominantly coordinated by S ligands.[14,15]

* To whom correspondence should be addressed.
† Present address: Department of Chemistry, Stanford University, Stanford, CA 94305.

(1) Abbreviations: CODH, carbon monoxide dehydrogenase; phen, 1,10-phenanthroline; $\alpha\beta_{LAB}$, CODH $\alpha\beta$ dimer containing a labile Ni ion; $\alpha\beta_{NONLAB}$, CODH $\alpha\beta$ dimer without a labile Ni ion; CoA, coenzyme A; DTT, dithiothreitol; EXAFS, extended X-ray absorption fine structure; ENDOR, electron nuclear double magnetic resonance.

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The spin intensities of the EPR signals from CODH are unusually low. The intensity of the NiFeC signal, for example, corresponds to 0.04–0.35 spin/$\alpha\beta$ rather than the expected value of 1 spin/$\alpha\beta$.[6,7,12,13,16] The reason for these low values has not been determined, but the suggestion has been made that some CODH molecules lack a full complement of metal complexes and clusters.[7]

Recently we found that Ni in the NiFe complex can be removed by adding 1,10-phenanthroline (phen) to CODH.[13,16] Removal causes loss of CO/acetyl-CoA exchange activity and renders the NiFe complex unable to yield the NiFeC signal. The CO oxidation activity and the enzyme's other EPR signals are completely unaffected by adding phen. Incubating phen-treated CODH with Ni^{2+} restores the CO/acetyl-CoA exchange activity and the NiFeC signal near to their original values.[16] These results indicate that the NiFe complex contains a labile Ni ion required exclusively for CO/acetyl-CoA exchange activity and for development of the NiFeC signal.

We have characterized the reactions of CODH with phen and of phen-treated CODH with Ni^{2+} in more detail. In this paper we report that only ~30% of CODH $\alpha\beta$ protein subunits contain labile Ni ions and that only these subunits can yield the NiFeC signal. The remaining subunits do not have these properties and do not appear to contain the NiFe complex. The Ni ion environments in CODH are thus shown to be heterogeneous. Possible reasons for the heterogeneity and its implications are discussed.

Experimental Procedures

CODH Purification. CODH was purified and characterized as described previously.[3,17,18] Preparations were homogeneous according to SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by the Biuret method.[19] A molecular mass of 154 700 Da for each CODH $\alpha\beta$ dimer was used in calculations. Ni and Fe contents were determined by inductively-coupled plasma emission spectrophotometry. The EPR and electronic absorption spectrometers used have been described.[20] Spin concentrations were determined by a published method.[21]

Preparation of Phen-Treated CODH. All procedures involving purified and active CODH were performed anaerobically at 25–28 °C in an argon atmosphere glovebox (Vacuum/Atmospheres HE-453) containing ~0.5 ppm oxygen (monitored by a Teledyne model 310 analyzer). Dithionite and DTT were removed from CODH samples (2.0 mL of 10.6 mg/mL) by gel filtration chromatography using a Sephadex G-25 (Pharmacia) column (1.6 cm × 15 cm) equilibrated in 50 mM Tris·Cl pH 8.0. To the eluted CODH samples (3.7 mg/mL final concentration) were added between 0 and 2 equiv/$\alpha\beta$ of phen (from a 0.714 mM stock in 50 mM Tris·Cl pH 8.0). After 15 h, samples were loaded into EPR tubes, exposed to CO for 1 min, and frozen. For the metal analyses, the phen-containing products ($[Ni(phen)_3]^{2+}$ and $[Fe(phen)_3]^{2+}$) were chromatographically separated from phen-treated CODH as above and concentrated by evaporation under reduced pressure prior to analysis.

^{63}Ni Titration. Various amounts of $^{63}NiCl_2$ (New England Nuclear) doped with isotopically unenriched $NiCl_2$ (to a final specific activity of 0.26 mCi/mg Ni, 3650 cpm/nmol Ni) were added to samples of phen-treated CODH (400 μL of 5.8 mg/mL Tris pH 8.0) in EPR tubes. After 3 days, samples were exposed to CO and frozen for EPR analysis. After obtaining EPR spectra, samples were thawed, and the ^{63}Ni-containing enzyme was isolated from any unincorporated $^{63}Ni^{2+}$ by Sephadex G-25 chromatography. The radioactive content of the fractions were determined by scintillation counting (Beckman LS 6000SE).

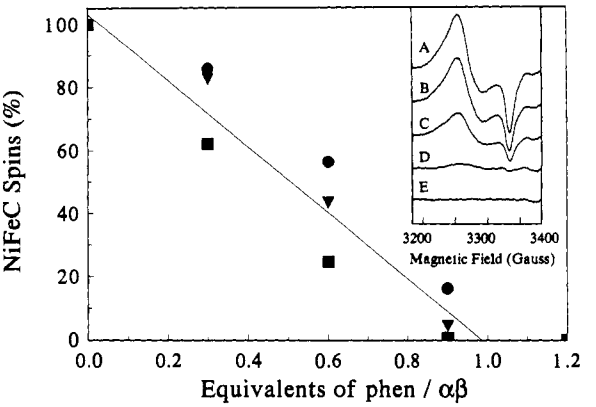

Figure 1. Titrations of CODH with phen. Samples from three independent preparations (batches no. 1, no. 3, and no. 4, designated by circles, triangles, and squares, respectively) were prepared as described in Experimental Procedures. Inset shows the EPR spectra obtained in one experiment (triangles) where 0 (A), 0.3 (B), 0.6 (C), 0.9 (D), and 1.2 (E) equiv/$\alpha\beta$ of phen were added. The solid line is the least-squares best fit to the data. The samples used for one experiment (circles) contained a significant amount (0.3 Fe/$\alpha\beta$) of adventitious Fe^{2+}. For this presentation, the amount of phen used to complex this Fe was subtracted from the total amount of phen used.

Enzyme Activities. CO/acetyl-CoA exchange and CO oxidation activity measurements were performed as described.[4,22] The five batches of CODH used, labeled no. 1–5, had CO oxidation activities of 250, 270, 260, 270, and 240 units/mg, CO/acetyl-CoA exchange activities of 0.31, 0.11, 0.12, 0.14 and 0.11 units/mg, and NiFeC spin intensities of 0.21, 0.14, 0.07, 0.16, and 0.12 spin/$\alpha\beta$, respectively. After phen-treatment, batch no. 1 had a CO oxidation activity of 240 units/mg and no CO/acetyl-CoA exchange activity. The activities of the other batches after phen treatment were not determined. The NiFeC signal could not be generated from any preparation of phen-treated CODH. After incorporating Ni, batch no. 1 had CO oxidation and exchange activities of 240 and 0.29 units/mg, respectively, and a NiFeC signal corresponding to 0.19 spin/$\alpha\beta$; batch no. 2 had an exchange activity of 0.10 units/mg and a NiFeC signal corresponding to 0.13 spin/$\alpha\beta$; and batch no. 5 had a NiFeC signal corresponding to 0.10 spin/$\alpha\beta$. The activities and NiFeC spin intensities of the other batches after phen treatment and Ni incorporation were not determined. The activity patterns observed here are in complete accord with earlier reports.[13,16]

Results

Quantifying the Reaction of CODH with Phen. We wanted to determine the amount of Ni removed from CODH by phen, the amount of phen required for the removal, and the amount of Ni that could be reinserted into phen-treated CODH. First, the amount of phen used in its reaction with CODH was quantified. Various amounts of phen were added to samples of CODH. After 15 h, samples were reduced with CO and frozen for EPR analysis. The NiFeC signal intensities from these samples and from two other similar experiments declined monotonically with increasing amounts of phen (Figure 1), and disappeared after 1.0 ± 0.2 equiv/$\alpha\beta$ of phen had been added.

To determine whether the reaction of CODH with phen had reached equilibrium after 15 h, the NiFeC signal intensity was followed with time after adding 1.6 equiv/$\alpha\beta$ phen. As shown in Figure 2, the NiFeC signal disappeared in under 2 h. Similar results were obtained when less phen was used. Thus, 15 h was long enough for equilibrium to be established, and we can conclude that 1.0 ± 0.2 equiv/$\alpha\beta$ of phen are required to remove the labile Ni from CODH.

Next, we measured the metal content of CODH before and after phen treatment and the metal content of the phen-containing products of the reaction. Phen (2.2 equiv/$\alpha\beta$) was added to a sample of CODH containing 1.85 Ni/$\alpha\beta$ and 10.6 Fe/$\alpha\beta$. After

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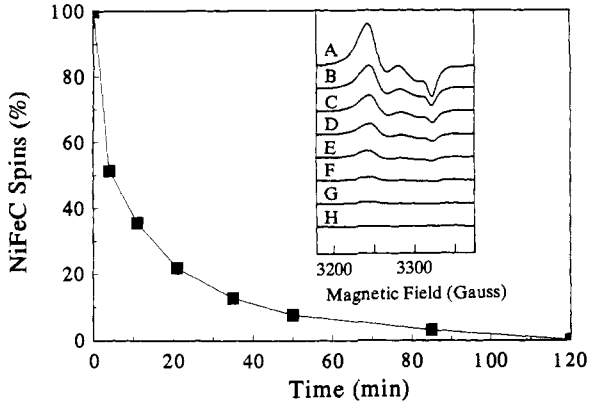

Figure 2. Disappearance of the NiFeC signal with time after adding phen. Phen was added to a sample of CODH (batch no. 5) as described in Experimental Procedures. At different reaction times aliquots were reduced with CO and frozen for EPR analysis. Inset shows the EPR spectra obtained. Reaction times were (A) before addition, (B) 5 min, (C) 10 min, (D) 20 min, (E) 37 min, (F) 50 min, (G) 83 min, and (H) 120 min after adding phen.

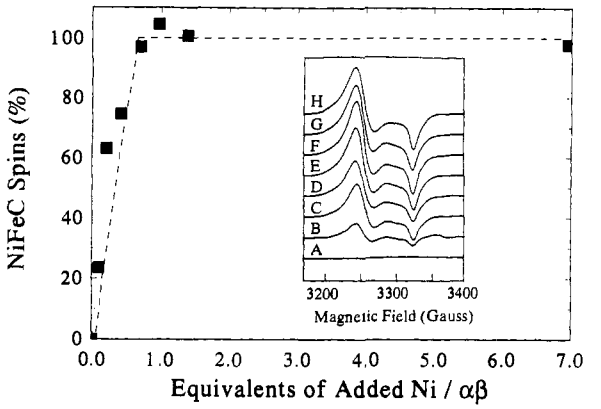

Figure 3. Titration of phen-treated CODH with $^{63}NiCl_2$. Various amounts of radioactive $^{63}Ni^{2+}$ were added to aliquots of phen-treated CODH (batch no. 2) as described in Experimental Procedures. Inset shows the EPR spectra obtained. The amounts of $^{63}Ni^{2+}$ added, in equiv/$\alpha\beta$ were (A) 0, (B) 0.2, (C) 0.4, (D) 0.6, (E) 0.8, (F) 1.0, (G) 1.4, and (H) 7.0.

6 h, the reaction products were separated by gel filtration chromatography. The phen-treated CODH sample contained 1.65 Ni/$\alpha\beta$ and 10.7 Fe/$\alpha\beta$. The Ni content of the low molecular weight, nonproteinaceous products of the reaction was 0.29 Ni/$\alpha\beta$. In two other experiments, 0.30 and 0.32 Ni/$\alpha\beta$ were detected in similar fractions. The amounts of Ni detected in the nonproteinaceous products (0.29–0.32 Ni/$\alpha\beta$) are probably more accurate than that determined from the difference in the Ni contents of CODH before and after phen treatment (i.e., 0.2 Ni/$\alpha\beta$), because the 0.2 Ni/$\alpha\beta$ value was obtained from the difference of two large and similar values (1.85–1.65 Ni/$\alpha\beta$).

That only 1.0 ± 0.2 phenanthrolines per $\alpha\beta$ were required to remove 0.29–0.32 Ni/$\alpha\beta$ suggests that the very stable complex[23] $[Ni(phen)_3]^{2+}$ was formed as a product of the reaction. Thus, we formulate the reaction of CODH $\alpha\beta$ dimers with phen as

$$\left\{\begin{matrix}0.3\alpha\beta_{LAB}[Ni^{2+}]\\0.7\alpha\beta_{NONLAB}[Ni^{2+}]\end{matrix}\right\} + 0.9\text{phen} \longrightarrow \left\{\begin{matrix}0.3\alpha\beta_{LAB}[\ ]\\0.7\alpha\beta_{NONLAB}[Ni^{2+}]\end{matrix}\right\} + 0.3[Ni(phen)_3]^{2+}$$

where $\alpha\beta_{LAB}$ and $\alpha\beta_{NONLAB}$ represent CODH $\alpha\beta$ dimers with and without labile Ni ions. In this formulation we have ignored the presence of the second Ni ion in each $\alpha\beta$ dimer since it does not appear to be involved in the reaction of CODH with phen.

Release of Fe During Reaction of CODH with Phen. The low molecular weight, nonproteinaceous products of the reaction of CODH with phen also contained Fe in an amount corresponding to 0.46 Fe/$\alpha\beta$. In two other experiments, 0.07 and 0.24 Fe/$\alpha\beta$ were detected in similar fractions. Thus, a small amount of Fe was removed during the reaction of phen with CODH. We were better able to quantify this amount using electronic absorption spectroscopy. CODH solutions turned slightly red after phen was added, with spectral features at 508 nm. $[Fe(phen)_3]^{2+}$ exhibits such spectra, and we assumed that the red color originated from this complex.[24] Using the molar absorptivity for $[Fe(phen)_3]^{2+}$ at this wavelength (ϵ = 12 500 M^{-1} cm^{-1}, obtained from a standard curve), between 0.05 and 0.45 equiv/$\alpha\beta$ of Fe were calculated to have been complexed in five different reactions of phen and CODH. We were unable to establish conditions that consistently precluded Fe complexation, probably because our samples contained small, variable amounts of adventitiously-bound Fe. The Fe released here does not appear to be functionally associated with CODH.[25] More $[Fe(phen)_3]^{2+}$ slowly formed when enzyme was exposed to greater concentrations of phen for longer periods of time. The additional Fe complexed under these more extreme conditions probably arose from degradation of functional iron-containing clusters in CODH.

Quantifying the Reaction of Phen-Treated CODH with Ni^{2+}. We determined the maximum amount of Ni^{2+} that could be incorporated into phen-treated CODH, by a titration with radioactive $^{63}NiCl_2$. Prior to phen treatment, the sample used exhibited a NiFeC signal representing 0.14 spin/$\alpha\beta$. Increasing amounts of $^{63}Ni^{2+}$ were added to aliquots of the phen-treated sample, and after 3 days the aliquots were reduced with CO and analyzed by EPR. NiFeC signal intensities developed in proportion to the amount of $^{63}Ni^{2+}$ added, reaching a maximum of 0.13 spin/$\alpha\beta$ after ~0.7 $^{63}Ni/\alpha\beta$ were added (Figure 3). Past this point, the intensities remained constant regardless of the amount of $^{63}NiCl_2$ added. By monitoring the NiFeC signal as a function of time after adding Ni, 3 days were found to be more than sufficient to establish equilibrium. The sharp bend in the titration curve indicates that Ni^{2+} bound tightly to the phen-treated enzyme.

After EPR spectra were obtained, samples were thawed, and the enzyme incorporated with ^{63}Ni was separated from any unincorporated $^{63}Ni^{2+}$ using gel filtration chromatography. The elution profile of a typical sample, shown in Figure 4 demonstrates that complete separation was achieved. No more than 0.3 equiv/$\alpha\beta$ of ^{63}Ni was incorporated into the phen-treated enzyme regardless of how much ^{63}Ni had been added. As shown in Figure 5, the NiFeC signal intensity was directly proportional to the amount of $^{63}Ni^{2+}$ *incorporated* into phen-treated CODH.

(23) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1974–1989; Vol. 1–6. The logarithms of the overall stability constants (log β) for $[Ni(phen)_3]^{2+}$, $[Ni(phen)_2]^{2+}$, and $[Ni(phen)_1]^{2+}$ are 24.3, 16.1, and 8.6, respectively (at 25 °C, 0.1 M ionic strength).

(24) McWhinnie, W. R.; Miller, J. D. *Adv. Inorg. Chem. Radiochem.* **1969**, *12*, 135–215. The red color does not appear to have originated from $[Ni(phen)_3]^{2+}$, since ϵ = ~20 M^{-1} cm^{-1} for this complex at 508 nm in 50 mM Tris pH 8.0.

(25) The only reagent required to reactivate phen-treated CODH was Ni^{2+}. We considered that the Fe removed by phen *did* originate from a functional site in CODH and that Fe was not required for reactivation because of few slowly inactivating CODH molecules released Fe into solution and these ions bound to vacated functional Fe sites as Ni^{2+} bound to its site. A related situation occurs during the autoactivation of aconitase.[26] However, this possibility does not appear to occur here. Adding Fe^{2+} to phen-treated CODH rapidly results in a new EPR signal due to the binding of Fe into the empty Ni site (unpublished), but this signal was not observed days after CODH had been treated by phen. If molecules of CODH were slowly inactivating and releasing Fe into solution, this new EPR signal would have appeared. By contrast, the NiFeC signal developed within an hour of adding Ni^{2+} to phen-treated CODH.

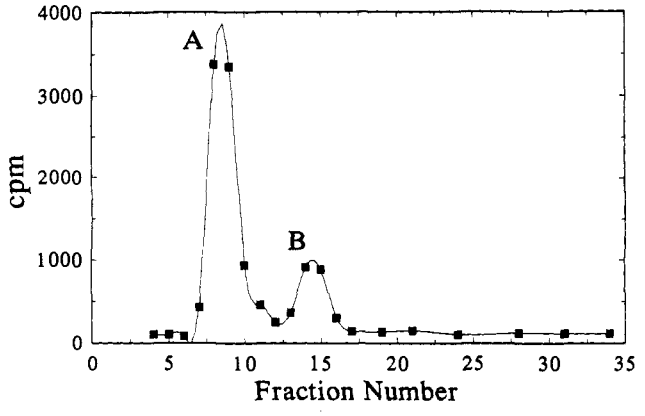

Figure 4. Separation of CODH incorporated with ^{63}Ni from excess ^{63}Ni by gel filtration. Phen-treated CODH sample (batch no. 2) to which 1.0 equiv/$\alpha\beta$ of $^{63}NiCl_2$ had been added was fractionated by Sephadex G-25 chromatography. Fractions were monitored for radioactivity by scintillation counting. Peak A contained CODH that had been incorporated with ^{63}Ni. Peak B and later fractions contained unincorporated $^{63}Ni^{2+}$. The total recovery of radioactivity was 70%.

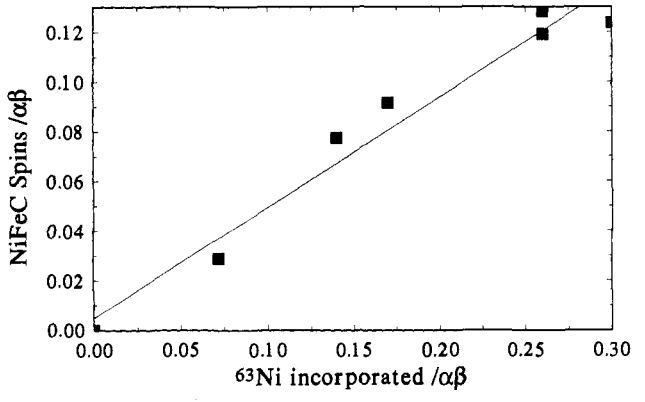

Figure 5. Linear relationship between NiFeC signal intensity and amount of ^{63}Ni incorporated into phen-treated CODH. The EPR signal intensities that redeveloped after Ni incorporation and the amounts of ^{63}Ni incorporated into phen-treated CODH (batch no. 2) were determined as described in Experimental Procedures. The solid line, with a slope of 0.44 spins/Ni, is the least-squares best fit to the data.

The NiFeC spin intensities obtained after reincorporation were routinely ~90% of the original (prior to phen-treatment) intensities, *independent* of how intense those original signals were. For the experiment just described, the NiFeC intensity was 0.14 spin/$\alpha\beta$ before and 0.13 spin/$\alpha\beta$ after phen-treatment and Ni reincorporation. In another, the NiFeC signal intensity was 0.21 spin/$\alpha\beta$ before and 0.19 spin/$\alpha\beta$ after treatment. In a third experiment, the intensity was 0.07 spin/$\alpha\beta$ before and 0.06 spin/$\alpha\beta$ after treatment. In no experiment was the NiFeC intensity of the original sample less than the intensity after Ni reincorporation. These results indicate that the nickel removal and replacement reactions are highly reversible and that a factor intrinsic to the particular sample used limits the intensity of the NiFeC signal.

Discussion

We recently found that phen reacts with CODH to remove Ni^{2+} from the NiFe complex.[13,16] Phen-treated CODH is devoid of CO/acetyl-CoA exchange activity and is unable to exhibit the NiFeC signal. CO/acetyl-CoA exchange activity and NiFeC signal intensity are restored after incubating phen-treated CODH with Ni^{2+} ions.

In the present study, we have quantified the amount of Ni removed from CODH and the amount reinserted into phen-treated CODH. A maximum of only ~0.3 Ni/$\alpha\beta$ could be removed and reinserted. Given that each NiFe complex contains 1 Ni (see Introduction), these results indicate that only ~30% of $\alpha\beta$ dimers (those called $\alpha\beta_{LAB}$) contain NiFe complexes and that ~70% (those called $\alpha\beta_{NONLAB}$) do not contain NiFe complexes, at least not in the form having labile Ni ions and able to yield NiFeC signals. This conclusion is surprising because the presence of one NiFe complex per $\alpha\beta$ dimer had been commonly assumed.

The NiFeC signal intensity has been reported to quantify to only 0.04–0.35 spin/$\alpha\beta$ rather than the expected value of 1 spin/$\alpha\beta$. Comparably low NiFeC spin intensities (0.1–0.2 spin/$\alpha\beta$) were obtained in the present study. The similarity between the amount of Ni removed by phen and the amount of spin lost as a consequence of that removal suggests that the two events are related quantitatively; that is, each NiFe complex appears to give rise to a NiFeC signal with an intensity corresponding to 1 spin/NiFe complex. This suggests that the NiFeC signal quantifies to such low values because only ~30% of the $\alpha\beta$ subunits contain NiFe complexes.

Actually, with our samples, the molar amount of Ni removed and reincorporated was similar but not *exactly* equal to the molar amount of NiFeC intensity lost and regained; slightly more Ni was labile than could be accounted for by the NiFeC spin intensity. Moreover, there was more variability in the NiFeC intensities than in the amounts of Ni removed. For example, the amounts of phen required for labile Ni removal from three different CODH samples (Figure 1) were fairly constant, 1.0 ± 0.2 phen/$\alpha\beta$, and the amounts of Ni lost by phen-treatment and reincorporated into phen-treated CODH were routinely 0.3 Ni/$\alpha\beta$. Thus 0.3 Ni/$\alpha\beta$ appear to have been labile in all of our samples. By contrast, the original NiFeC spin intensities of the samples just referred to range from 0.07 to 0.21 spin/$\alpha\beta$. Given that the total range of NiFeC intensities previously reported is 0.04–0.35 spin/$\alpha\beta$, the range observed for these samples is relatively quite large. Thus, the NiFeC spin intensities exhibited greater variation than expected, given the variation in the amounts of Ni labilized. These considerations suggest that 30% of CODH $\alpha\beta$ units are *consistently* $\alpha\beta_{LAB}$, but that only a (highly variable) fraction of $\alpha\beta_{LAB}$ can exhibit the NiFeC signal.

What factors determine the fraction of $\alpha\beta_{LAB}$ able to exhibit the NiFeC signal? Our results show that the cycle of Ni removal and replacement does not significantly alter the fraction of $\alpha\beta_{LAB}$ exhibiting the NiFeC signal. One factor known to affect the NiFeC intensities is oxygen damage. Trace amounts of oxygen rapidly and irreversibly alter the NiFe complex, rendering it nonfunctional and unable to exhibit the NiFeC signal.[18] Thus, the sizable variation of NiFeC spin intensities probably reflects the variable amounts of oxygen to which the samples were historically exposed. However, since approximately the same molar amount of Ni was removed from each sample, oxygen damage appears to alter the proportion of $\alpha\beta_{LAB}$ able to yield the NiFeC signal but not the proportion of $\alpha\beta$ units that are $\alpha\beta_{LAB}$.

Since CODH molecules contain three $\alpha\beta$ subunits in an $(\alpha\beta)_3$ hexameric structure, solutions of CODH must now be considered to consist of molecules with the arrangements $(\alpha\beta_{LAB})_3$, $(\alpha\beta_{LAB})_2(\alpha\beta_{NONLAB})_1$, $(\alpha\beta_{LAB})_1(\alpha\beta_{NONLAB})_2$, and $(\alpha\beta_{NONLAB})_3$, present in proportions that yield the observed 7:3 ratio of $\alpha\beta_{NONLAB}/\alpha\beta_{LAB}$. One possibility (referred to as the *nonrandom* arrangement) is that all of the molecules are $(\alpha\beta_{LAB})_1(\alpha\beta_{NONLAB})_2$. Another possibility (the *random* arrangement) is that the two types of subunits are distributed randomly, resulting in a 1:7:16:13 population ration for the molecular forms listed above.[27] We favor the nonrandom arrangement because it provides a simple explanation as to why the observed $\alpha\beta_{NONLAB}/\alpha\beta_{LAB}$ ratio is near 2:1, and it does not require that hexameric molecules of CODH

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(27) The relative population distribution for $(\alpha\beta_{LAB})_3$, $(\alpha\beta_{LAB})_2(\alpha\beta_{NONLAB})_1$, $(\alpha\beta_{LAB})_1(\alpha\beta_{NONLAB})_2$, and $(\alpha\beta_{NONLAB})_3$, would be $1(0.3)^3$, $3(0.3)^2(0.7)^1$, $3(0.3)^1(0.7)^2$, and $1(0.7)^3$, respectively.

be heterogeneous. No chromatographic evidence for heterogeneous populations of CODH $(\alpha\beta)_3$ molecules has been reported.

CODH solutions from which 0.3 Ni/$\alpha\beta$ had been removed were completely devoid of CO/acetyl-CoA exchange activity. Again this result suggests that only $\alpha\beta_{LAB}$ are capable of catalyzing CO/acetyl-CoA exchange. However, whether $\alpha\beta_{LAB}$ can catalyze CO/acetyl-CoA exchange independently of the two other subunits to which it is associated in $(\alpha\beta)_3$ molecules is not known. Thus, activity may be afforded only when one $\alpha\beta_{LAB}$ is associated with two $\alpha\beta_{NONLAB}$. In this case concluding that only $\alpha\beta_{LAB}$ have CO/acetyl-CoA exchange activity would be incorrect because activity would require the presence of both types of subunits.

We do not know the origin of the heterogeneity. The analysis given above suggests that oxygen damage is not the fundamental cause. The heterogeneity also does not appear to originate from damage incurred during protein purification. We recently purified a batch of CODH without damaging a significant portion of enzyme molecules.[18] That this condition had been achieved was concluded because 98 ± 10% of the CO oxidation activity originally in the crude extract were recovered in the purified enzyme and side fractions, and because the (oxygen-sensitive) NiFeC signal intensities were virtually unchanged in fractions obtained after each step of the purification. Importantly, the NiFeC signal intensities in these fractions, including the supernatant of the bacterial crude extract, corresponded to 0.2–0.3 spin/$\alpha\beta$. Since we now know that such low intensities are due to heterogeneous Ni ion environments in CODH, it appears that this heterogeneity originates prior to the purification process.

The heterogeneity demonstrated here dramatically alters our understanding of CODH. The enzyme's metal content (2 Ni/$\alpha\beta$ and 11–13 Fe/$\alpha\beta$) must now be regarded as the *weighted average* of the metal contents of 30% $\alpha\beta_{LAB}$ and 70% $\alpha\beta_{NONLAB}$. The four known metal complexes/clusters in CODH may not be evenly distributed between $\alpha\beta_{LAB}$ and $\alpha\beta_{NONLAB}$. For example, the cluster yielding the g_{av} = 1.82 and g_{av} = 1.86 signals may be present only in ~30% of the $\alpha\beta$ units, given the low spin intensities of these signals (0.1–0.3 spin/$\alpha\beta$).[6,7] The $[Fe_4S_4]^{1+}$ cluster yielding the g_{av} = 1.94 signals may be present in only two out of three $\alpha\beta$ dimers, given that these signals together quantify to only ~0.6 spin/$\alpha\beta$.[6,7] Much work remains to examine these possibilities.

On the basis of their analysis of CODH Mössbauer spectra, Lindahl et al. suggested that CODH was heterogeneous.[7] The irons associated with the NiFe complex (yielding in this sample a NiFeC signal corresponding to 0.35 spin/$\alpha\beta$) represented 18% of total Mössbauer absorption, with spectroscopic parameters typical of $[Fe_4S_4]^{2+}$ clusters. Since there are 11–13 Fe/$\alpha\beta$, 18% of the iron corresponds to approximately 2 Fe/$\alpha\beta$. To explain how an Fe_4S_4 cluster could correspond to only 2 Fe/$\alpha\beta$, Lindahl et al. proposed that only a fraction (~50%) of CODH molecules contained NiFe complexes.[7] With half the NiFe sites empty, the NiFe complex would only contribute Mössbauer spectral intensity corresponding to 2 irons and NiFeC signal intensity corresponding to 0.5 spin/$\alpha\beta$, near to that observed. The results presented here suggest that only ~30% of the $\alpha\beta$ units of that Mössbauer sample probably contained NiFe complexes and that only those NiFe complexes contributed to the 18% spectral intensity. Since 2 Fe/$\alpha\beta$ divided by 0.3 NiFe complexes/$\alpha\beta$ equals ~7 Fe/NiFe complex, the NiFe complex appears to contain significantly more than the 3–4 irons previously proposed.

Phen is commonly used to remove metal ions from many metalloenzymes. The conditions typically include a lengthy dialysis (1 day-1 week) against buffer at slightly active pH and containing a high concentration (1 mM) of phen.[28] By comparison, the conditions required to remove and reinsert the labile Ni of CODH (pH 8, 0.05 mM phen for 2 h) are unusually mild. Such mild conditions suggest that the labile Ni site is significantly less stable than $[Ni(phen)_3]^{2+}$ and that it has a lower kinetic barrier to substitution by phen than do most other metal ions in enzymes. One possibility supported by the unusually mild reaction conditions and by studies of well-characterized Ni complexes[29–32] is that the labile Ni in CODH is coordinated to fewer than six tight-binding ligands. Further studies are required to examine this possibility.

Lu and Ragsdale reported that the Scatchard plot of the binding of coenzyme A to CODH fit best if two inequivalent binding-sites were assumed.[33] They suggested that CODH exists in two forms, a "highly active" form that binds coenzyme A tightly (K_d = 52 μM), and another that binds the coenzyme weakly (K_d = 2600 μM). The results presented here are congruent with this possibility, in that coenzyme A might bind $\alpha\beta_{LAB}$ tightly and $\alpha\beta_{NONLAB}$ weakly.

In summary, our results suggest that Ni-ion lability, NiFeC EPR signal-intensity, and possibly CO/acetyl-CoA exchange activity are properties of only ~30% of CODH $\alpha\beta$ dimers. Exposure to oxygen lowers the activity and signal intensity still further. The remaining $\alpha\beta$ dimers do not contain labile Ni ions and cannot exhibit the NiFe EPR signal; they do not appear to contain NiFe complexes, at least not in the form recognized as such. The reason for the heterogeneity is unknown, but it originates prior to protein purification. The NiFe complex appears to be a large cluster containing significantly more than 3–4 irons, and the labile Ni ion in this complex may be coordinatively unsaturated.

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