Substitution of Valine for Histidine 265 in Carbon Monoxide Dehydrogenase from \textit{Rhodospirillum rubrum} Affects Activity and Spectroscopic States*

(Nathan J. Spangler†, Monica R. Meyers‡, Karin L. Gierke‡, Robert L. Kerby§, Gary P. Roberts§, and Paul W. Ludden‡)†‡

From the Departments of †Biochemistry and §Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

In carbon monoxide dehydrogenase (CODH) from \textit{Rhodospirillum rubrum}, histidine 265 was replaced with valine by site-directed mutagenesis of the \textit{cooS} gene. The altered form of CODH (H265V) had a low nickel content and a dramatically reduced level of catalytic activity. Although treatment with NiCl$_2$ and CoCl$_2$ increased the activity of H265V CODH by severalfold, activity levels remained more than 1000-fold lower than that of wild-type CODH. Histidine 265 was not essential for the formation and stability of the Fe$_5$S$_4$ clusters. The $K_a$ and $K_D$ for CO as well as the $K_D$ for cyanide were relatively unchanged as a result of the amino acid substitution in CODH. The time-dependent reduction of the [Fe$_5$S$_4$]$^{2+}$ clusters by CO occurred on a time scale of hours, suggesting that, as a consequence of the mutation, a rate-limiting step had been introduced prior to the transfer of electrons from CO to the cubanes in centers B and C. EPR spectra of H265V CODH lacked the $g_{av}$ = 1.86 and $g_{av}$ = 1.87 signals characteristic of reduced forms of the active site (center C) of wild-type CODH. This indicates that the electronic properties of center C have been modified possibly by the disruption or alteration of the ligand-mediated interaction between the nickel site and Fe$_5$S$_4$ chromophore.

The photosynthetic bacterium \textit{Rhodospirillum rubrum} is able to utilize CO as its sole energy source when grown anaerobically in the dark (1). The key enzyme in this metabolism is the CO-induced, carbon monoxide dehydrogenase (CODH), which is the product of the \textit{cooS} gene (2, 3). CODH catalyzes the reversible oxidation of CO to CO$_2$ and the reduction of a special ferredoxin that is the \textit{cooF} gene product (3, 4). CODH has only two metal clusters, which have been designated as centers B and C (5). The latter contains a nickel atom bridged by an unknown ligand to an iron atom in one of the two [Fe$_5$S$_4$]$^{2+/-}$ clusters found in CODH (5, 6). The second cubane, which is not linked to nickel, is the sole component of center B. CO oxidation occurs at center C, and center B is proposed to mediate the transfer of electrons to CO$_2$ or artificial electron acceptors such as methyl viologen (4, 7, 8). When purified from \textit{R. rubrum} grown on nickel-depleted medium, CODH lacks both nickel and the ability to oxidize CO, yet retains both [Fe$_5$S$_4$]$^{2+/-}$ clusters (5, 9). Nickel-deficient CODH can be fully activated by treatment with Ni$^{2+}$.

CODH from \textit{R. rubrum} is closely related to another class of anaerobic CO-oxidizing enzymes that, unlike CODH, also catalyze the synthesis of acetyl coenzyme A from CO, coenzyme A, and a methyl donor. The CO-oxidizing acetyl-CoA synthase (CODH/ACS), found in acetogenic and methanogenic bacteria, possesses center B and center C analogs as well as a third and unidentified redox center, which has been invoked in recently proposed mechanisms for the enzymatic oxidation of CO (25, 28). CODH/ACS also contains a second NiFeS cluster (center A) that serves as the site of acetyl-CoA synthesis (for a recent review of CODH/ACS, see Ragsdale and Kumar (10)). Unlike the monomeric CODH from \textit{R. rubrum}, the CODH/ACS enzymes are composed of multiple subunits among which the CODH and ACS activities are divided. In the \textit{Clostridium thermoacetieum} and \textit{Methanothermobacter\textit{ soehngenii}} enzymes, the CODH activity appears to be localized in the β (CmbB) and α (CdHA) subunits, respectively (3, 11). This is consistent with the significant sequence similarity of cmbB and cdHA genes to the \textit{cooS} gene, which encodes the single subunit of the \textit{R. rubrum} CODH (3).

Although the structures of neither \textit{R. rubrum} CODH nor the CODH/ACS enzymes are known, analysis of the deduced amino acid sequences of \textit{cooS}, \textit{cmbB} (24), and \textit{cdHA} (21) as well as two related genes (22, 23) from genome sequences allows the identification of conserved motifs and amino acid residues. In the absence of structural information, site-specific substitution of conserved amino acid residues is most likely to provide insight into the immediate environment of the metal clusters of CODH. Of the conserved residues, histidine 265 of the \textit{R. rubrum} CODH was chosen for substitution as it is the only histidine conserved in all known CooS-like gene products. The selection of histidine was also guided by previous extended x-ray absorption fine structure studies that suggest one or more nitrogen-donor ligands to the nickel center of \textit{R. rubrum} CODH (6).

In this study, the effect of substitution of valine for histidine 265 resulted in an intact CODH enzyme (designated as H265V) which partially lacked nickel and had a dramatically lower activity. The ability of nickel-deficient H265V CODH to incorporate nickel in vitro is shown here, and the catalytic activity and binding of CO and cyanide to nickel-treated H265V CODH has been investigated. The nickel-containing form of the enzyme does not exhibit the unique C$_{red}$ and C$_{red^2}$ EPR signals attributed to reduced forms of center C that were characterized in previous spectroscopic studies of CODH and CODH/ACS.

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enzymes (see Hu et al. (5) and references therein). From these studies, we have developed the hypothesis that the ligand-mediated, electronic coupling between nickel and iron in center C has been disrupted or altered by the amino acid substitution. This does not adversely affect substrate or inhibitor binding; however, the modification greatly diminishes the ability of the enzyme to oxidize bound substrate.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of cooS—The conversion of CODH His-265 to Val was achieved by incorporating the restriction site elimination plasmid modification (13). Standard media, antibiotic usage, and mating protocols were employed (14). The pUC19-derived template plasmid (pCO11, in Escherichia coli strain UQ1155) bears a 2.2-kb Sall fragment that encompasses all of cooS, the gene encoding CODH. This template was mutagenized in vitro using a selection primer that converted an ApIII site to a BglII site in the vector, with a mutagenic primer that converted the His-265 codon (CAT) to a Val (GTT), and simultaneously created a HpaI restriction cleavage site (GTTAAC). The desired Km ’Gn’N’x’ recombinant was isolated. The merodiploid recombinant was resolved by growth for several generations in SMN liquid culture supplemented with Nt, followed by isolation of a Km ’Gn’ derivative, indicative of homologous recombination. The construct was verified by Southern analysis of chromosome DNA, and the sequence of one strand of the Nco1-HindIII fragment cloned into pCO12 was verified (data not shown).

Cell Growth and Enzyme Purification—Nickel-supplemented cell cultures of R. rubrum, strain UR499, were grown in medium (15) supplemented with 0.05 mM NiCl2 and 3 g/liter each of yeast extract and casamino acids. Nickel-depleted cultures were grown according to previously published methods (9). Enzyme purification was performed according to the method of Bonam and Ludden (15). All buffers used in the purification of nickel-deficient H265V from nickel-depleted cultures were passed over a metal-chelating column of Bio-Rad Chelex-100 cation exchange resin, and all buffer solutions contained 1 mM EDTA.

Assays—Protein concentrations were determined by the bicinchoninic acid colorimetric method using bovine serum albumin (grade A, Sigma) as a standard (16). The CO oxidation activity of H265V was measured by the CO-dependent, methyl viologen reduction assay (7, 9). One unit of activity equals 1 μmol of CO oxidized per min. The catalytic activity of the reverse reaction, CO2 reduction, was measured according to the method described by Ensign (17). For the reverse reaction, 1 unit of activity equals 1 μmol of CO produced per min. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to published methods (18).

Sample Preparations—All samples were prepared in an anaerobic glove box (Vacuum/Atmospheres Dri-Lab glove box model HE-493) with an N2 atmosphere containing less than 1 ppm O2. The buffer used in each experiment was 100 mM MOPS that was adjusted to pH 7.3 with NaOH unless otherwise stated. Trace metal contaminants in buffers were removed by passing buffer solutions through a column of Bio-Rad Chelex-100.

Assay of Purified H265V in the Presence of Divalent Metals—The ability of divalent metal cations to activate nickel-deficient H265V was tested by adding solutions of CoCl2, ZnCl2, MnCl2, NiCl2, or Fe(II) to solutions of nickel-deficient enzyme containing equimolar amounts of methyl viologen and sodium dithionite or titanium(III) citrate (7, 19). Activity was measured versus time following the addition of metal salt. The Km for nickel activation was determined according to published methods (19).

Preparation of Nickel-treated H265V—Nickel-treated samples of H265V, freed from exogenous nickel, were prepared according to published methods (7). Metal Content of H265V CODH—The metal content of H265V CODH isolated from UR499 cultures grown in nickel-supplemented or nickel-depleted media were compared with the metal content of CODH from wild-type cultures grown similarly (Table I). Also included in the comparison is H265V CODH from nickel-supplemented cells that was treated with Ni2+ following purification. All forms of H265V contained 8 iron atoms, consistent with the presence of an intact center B and Fe3+ component of center C. Thus, the substitution of valine for histidine 265 has no effect on the ability of the enzyme to ligand a full compliment of iron; UV-visible spectra of H265V CODH confirms this conclusion (see below).

The H265V CODH isolated from UR499 cells grown with normal levels (0.05 mM) of Ni2+ in the medium consistently...
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Metal content and activity of H265V and wild-type CODH

| Metal content* | Ni^{2+} | Fe^{2+} | CO oxidation | CO_{2} reduction |
|----------------|---------|---------|--------------|------------------|
| Ni-deficient H265V | 0.014 ± 0.006 | 8.1 ± 1.2 | <0.01 | not measured |
| As-purified H265V | 0.24 ± 0.02 | 7.9 ± 0.6 | 0.67 | not measured |
| Ni-treated H265V | 2.4 ± 0.1 | 8.1 ± 0.4 | 2.4 | 0.003 |
| Wild-type | 0.97 ± 0.09 | 8.3 ± 0.9 | 4000 | 4.3 |

* Manganese and cobalt levels were less than 0.01 mol of metal/mol of protein, and zinc was less than 0.13 mol of metal/mol of protein in all samples.

When purified from cells grown on nickel-depleted medium, wild-type CODH has 0.01 to 0.02 mol of nickel and 7–8 mol of iron/mol of enzyme purified from cells grown on nickel-supplemented medium. Because the activity and the nickel content of H265V CODH did not decrease following gel filtration, metal-chelating chromatography, and treatment with 1.3 mM EDTA for over 1 h (data not shown), the low nickel content of H265V CODH samples does not appear to result from nickel loss during purification and handling. It seems likely, therefore, that nickel is poorly incorporated into H265V CODH in vivo as a consequence of the amino acid substitution.

When H265V CODH purified from nickel-supplemented cultures was treated with Ni^{2+}, the enzyme’s nickel content increased by 10-fold to 2.4 nickel atoms per monomer, a level above that of the wild-type enzyme. It is not known if the greater than 1:1 molar ratio of nickel versus H265V CODH is significant, or if it reflects nonspecifically bound nickel in addition to that which is presumed to be incorporated into center C. The K_{m} for the in vitro incorporation of nickel into nickel-deficient H265V CODH was determined by measuring the initial rate of activity increase versus nickel concentration. Compared with the nickel-deficient wild-type enzyme, the K_{m} for nickel incorporation into H265V CODH is about 4-fold higher (Table II), which may in part explain the low efficiency of nickel incorporation in vivo.

Manganese and cobalt levels in H265V CODH were negligible, and the level of zinc was only 0.13 atom per monomer (Table I). Thus, the center C nickel site of H265V CODH, as purified, did not appear to be occupied by a competing metal ion. Moreover, the ability of both the nickel-deficient and as-purified H265V CODH to incorporate nickel, as judged by the nickel-dependent increase in activity, suggests a vacancy in the nickel site, given that nickel-deficient wild-type CODH can neither bind nor be activated by Ni^{2+} if first treated with Co^{2+}, Zn^{2+}, or Fe^{2+} (7).

Treatments of nickel-deficient H265V CODH with divalent metal cations—As mentioned above, the activity of as-purified H265V CODH increased from 0.7 to 2.4 units/mg following treatment with Ni^{2+}. Likewise, the activity of nickel-deficient H265V CODH increased from 0.01 to 2.2 units/mg when treated with Ni^{2+} over a 3-h period (Fig. 1). Nickel-deficient H265V CODH was also treated with Co^{2+}, Zn^{2+}, Fe^{2+}, or Mn^{2+}. Of these, only Co^{2+} gave a form of the enzyme having measurable activity, which was approximately 20% of that observed with Ni^{2+} (Fig. 1; data for Zn^{2+}, Fe^{2+}, and Mn^{2+} not shown). This is in contrast to the 1000-fold greater increase in activity produced by Ni^{2+} versus Co^{2+} treatments of the nickel-deficient wild-type enzyme (7). The activity of the cobalt-containing wild-type CODH, judged by the rate at which the Fe_{4}S_{4} are reduced by CO (7), is similar to the activity levels measured

![Fig. 1. Time-dependent increase in activity following treatment of nickel-deficient H265V CODH (0.013 nickel atom per monomer) with Ni^{2+} or Co^{2+}. At t = 0, a solution of CoCl_{2} (●), NiCl_{2} (▲), or buffer (▲, control) was added to buffer solutions containing enzyme, methyl viologen, sodium dithionite and titanium(III) citrate (7). At the indicated time points, 15-μl aliquots were removed and assayed for activity by the CO-dependent, methyl viologen reduction assay. Mn^{2+}, Zn^{2+}, and Fe^{2+} were also tested; however, treatments of nickel-deficient H265V CODH with these metal cations did not produce measurable levels of activity (data not shown). Concentrations were as follows: CODH, 3.1 μM; metal salts, 5 mM; methyl viologen, 0.4 mM; sodium dithionite, 0.1 mM; and titanium(III) citrate, 0.3 mM.](Image)
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**Fig. 2.** Time-dependent reduction of Fe₄S₄ by CO. The reduction of H265V CODH in a solution of CO-saturated buffer was determined by measuring the magnitude of absorbance loss at 420 nm. Fully oxidized, 3-ml samples of A, nickel-deficient H265V CODH (0.01 nickel atom per monomer), and B, nickel-treated H265V CODH (2.4 nickel atoms per monomer) were placed under 100% N₂ in double-stoppered cuvettes. After measuring the initial absorbance at 420 nm (100% oxidation), the cuvettes were evacuated and flushed with 100% CO and mixed for three minutes before resuming the time-course readings at 420 nm. The absorbance of fully-reduced (i.e., 0% oxidation) samples was measured following the addition of sodium dithionite. CODH concentrations were A, 3.9 μM, and B, 3.6 μM.

In this work for the nickel and cobalt-treated forms of H265V CODH. It seems interesting, therefore, that the amino acid substitution appears to greatly impair the activity of the nickel but not the cobalt-containing form of CODH. Because the Km for CO for nickel-treated H265V CODH is similar to that of the wild-type enzyme (Table II; discussed below), low activity does not reflect an inability to bind CO; rather, it suggests an inability to catalyze the oxidation of CO that is bound to center C of H265V CODH.

**UV-visible Spectra of H265V CODH—UV-visible absorption spectra of oxidized and reduced H265V CODH were found to be identical to those of wild-type CODH (data not shown), demonstrating that histidine 265 is not required for assembly of the Fe₄S₄ clusters. The molar extinction coefficients at 420 nm (ε₄20) for oxidized and reduced H265V CODH were 36.2 and 20.1 mmol⁻¹ cm⁻¹, respectively. These values are essentially identical to those of the wild-type enzyme (7). Nickel does not contribute to the UV-visible spectrum of the enzyme (7).

It was previously shown by Ensign et al. (7) that the Fe₄S₄ centers of nickel-deficient CODH are neither reduced by CO directly nor indirectly through protein to protein electron transfer from the trace of active (i.e., nickel-containing) CODH background. In the present study, the catalytic competence of H265V CODH samples was assessed by monitoring at 420 nm the rate and magnitude of reduction of Fe₄S₄ clusters in the presence of CO. Shown in Fig. 2, nickel-treated H265V CODH (containing 2.4 nickel atoms per monomer) became 80% reduced by 3 h (Fig. 2B); full reduction was achieved at this point by adding dithionite (data not shown). This suggests that the majority of nickel-treated H265V CODH (at least 80%) binds to and is reduced by CO. Therefore, the low activity cannot be attributed to a very small quantity of active enzyme in a population consisting of mostly inactive enzyme. If this were true, one would expect that only a small fraction of the Fe₄S₄ centers would become reduced following exposure to CO, as is seen with the nickel-deficient forms of H265V (Fig. 2A) and wild-type CODH (7).

**Activity of H265V CODH—**As noted above, nickel-containing H265V CODH has at least 1000-fold lower catalytic activity compared with the wild-type enzyme. Both the forward (CO oxidation) and reverse (CO₂ reduction) activities were decreased by similar factors (Table I) although the Km values for CO and methyl viologen for H265V CODH were not significantly different from those of the wild-type enzyme (Table II). With the exception of V₅₀, the kinetic parameters of H265V CODH were very similar to those of the wild-type enzyme. Therefore, we conclude that H265V CODH binds substrates with normal affinities but somehow lacks the ability to catalyze the oxidation of CO. The defect in activity does not appear to be in the ability to transfer electrons from center C to center B (or the reverse). If this were the case, one would expect that, upon treatment of the oxidized enzyme with CO, the Fe₄S₄ component of center C would become rapidly reduced followed by a very slow transfer of electrons to center B. This would be observed as an initially rapid decrease in A₄20 due to reduction of one Fe₄S₄ center (center C), followed by a very slow decrease in A₆30, reflecting the reduction of the second Fe₄S₄ center (center B). Based upon the data shown in Fig. 2, it appears that the defect occurs prior to or at the point of electron transfer to the Fe₄S₄ clusters of H265V CODH.

**pH Dependence of CO Oxidation Activity by H265V CODH—**The mechanism of CO oxidation may involve the attack of a hydroxyl on bound CO, and it was suggested to us by Dr. S. Ensign that the pH profiles of the wild-type CODH and H265V CODH might, therefore, differ dramatically. The pH profile of H265V CODH activity showed a nearly linear increase from 1.3 units/mg at pH 6.5 to 4.2 units/mg at pH 9.0, and the activity level reached a plateau of approximately 4.6 units/mg from pH 9.5 to 11 (data not shown). This is nearly identical to the effect of pH on the wild-type CODH, and the two pH profiles are nearly superimposable (data not shown). The defect in CODH activity would not appear to be an inability to generate a hydroxyl group for attack on metal-bound CO, otherwise, a much greater increase in activity at higher pH would be expected for the H265V CODH.

**Inhibition of H265V CODH by Cyanide—**Cyanide is a slow, tight-binding, competitive inhibitor of wild-type CODH (8). Analysis of the inhibition of nickel-treated H265V CODH by cyanide in the absence or presence of CO yielded results similar to those previously reported for the wild-type enzyme (Table II). Both wild-type and H265V forms of the enzyme bind substrate and inhibitor with similar affinity; therefore, H265V CODH does not appear to suffer from any loss of binding affinity for substrate or substrate analog.

**EPR Spectroscopy of H265V CODH—**The EPR spectrum of dithionite-reduced H265V CODH exhibits several overlapping S = ½ signals (Fig. 3). The most prominent has g values of 2.04, 1.92, and 1.88 (gav = 1.94) and is characteristic of the [Fe₄S₄]⁺ form of center B (B₁(B₂)) of wild-type CODH. Another signal having g values of 2.07, 1.92 and 1.88 (gav = 1.96) is evident (Fig. 3A), and this signal appeared to lose intensity with increasing nickel content (Fig. 3, B and C) and, therefore, was presumed to be due to a reduced version of center C in H265V CODH that lacked nickel. This signal did not shift significantly following the addition of cyanide (data not shown), which is what one would anticipate considering that cyanide is not expected to bind to the active site lacking nickel (8). Note that cyanide strongly perturbs the Cred₁ (gav = 1.86) EPR signal (20, 26).

Following treatment with CO, nickel-treated H265V CODH has an EPR spectrum exhibiting only one S = ½ signal with g values of 2.04, 1.92, and 1.88 (gav = 1.94) which is characteristic of the Bred state (spectrum of nickel-treated H265V CODH shown in Fig. 4A). Notably absent in Fig. 4A is the gav = 1.87 signal characteristic of the three-electron-reduced form (25) of center C, designated Cred₂, which develops following reduction of wild-type CODH with CO. The gav = 1.94 signal shown in
2 h (data not shown). This is consistent with the data in Fig. 2, which demonstrate that the Fe₄S₄ clusters of nickel-deficient H265V CODH do not undergo reduction in the presence of CO.

All attempts also failed to produce the \( g_{av} = 1.86 \) (Cred₁) signal, which is characteristic of the 1-electron-reduced form of center C (designated Cred₁) of wild-type CODH. Neither oxidation with the indigo carmine nor oxidative titration with thionine resulted in the formation of the Cred₁ state in samples of H265V CODH that were initially reduced with sodium dithionite. During the thionine titration, the \( g_{av} = 1.94 \) (Bred) and \( g_{av} = 1.96 \) intensities diminished proportionally with increasing amounts of thionine added, and new signal formation was not observed at any point during the titration.

The Cred₁ and Cred₂ spectroscopic states of center C of the wild-type CODH were previously explained by an electronic model describing an \( S = \frac{1}{2} [Fe₄S₄]^{2+} \) cluster and an \( S = 1 Ni^{2+} \) site weakly coupled via the bridging ligand X (5). The Cred₁ and Cred₂ spectroscopic states correlate with the presence of a unique iron subsite, designated ferrous component II (FCII) that is coordinated by five or six ligands in center C (5). The FCII subsite, however, is not observed in nickel-deficient CODH. In the absence of nickel, all iron atoms appear to be tetracoordinated and neither Cred₁ nor Cred₂ spectroscopic state is observed. In this study, the failure to observe the \( g_{av} = 1.86 \) (Cred₁) and \( g_{av} = 1.87 \) (Cred₂) EPR signals is consistent with our conclusion that the involvement of the nickel site with the chromophore electronic system of center C has been modified in H265V CODH. Our working hypothesis is that this modification affects and possibly prevents the formation of the penta or hexacoordinated FCII subsite when nickel is incorporated into center C. This is supported by the observation of unidentified, CO-dependent and cyanide-sensitive signals in spectrum of CO-reduced H265V CODH (Fig. 4). Assuming that these signals are due to a paramagnetic oxidation state of center C, the low intensity of the \( g_{av} = 1.94 \) signal combined with the appearance of signals in the \( g = 4–6 \) region (Fig. 4A) suggests a spin-state equilibrium of \( S = \frac{1}{2} \) and \( S > \frac{1}{2} \) (3/2, 5/2, and so forth) states. Such a spin-state equilibrium has been invoked in previous studies of center C of wild-type CODH (5, 20, 27) to rationalize the low spin intensities of the \( g_{av} = 1.86 \) (Cred₁) and \( g_{av} = 1.87 \) (Cred₂) EPR signals.

Summary—When purified from nickel-grown cultures, H265V CODH has a low nickel content that appears to result from a diminished capacity to incorporate nickel in vitro. When treated with either Co²⁺ or Ni²⁺ in vitro, the activity level of nickel-deficient H265V CODH increases, yet remains more than 1000-fold less active than does wild-type CODH. The kinetic data and slow rate of CO-dependent reduction of Fe₄S₄ clusters implicate a rate-limiting step that occurs prior to or at the point of electron transfer to the cubanes. The affinity of H265V CODH for substrates, however, is not diminished as a result of the substitution. The complete absence of \( g_{av} = 1.86 \) (Cred₁) and \( g_{av} = 1.87 \) (Cred₂) EPR signals suggest that the unique FCII subsite is not formed in the presence of nickel, perhaps as a consequence of the alteration of the ligand-mediated, electronic interaction between nickel and iron components of the center C of H265V CODH.

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