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ORIGINAL ARTICLE

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Carbon monoxide dehydrogenase from *Rhodospirillum rubrum* produces formate

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Abstract Carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum* reversibly catalyzes the oxidation of CO to CO_2 at the active site C-cluster. In this article, the reduction of CO_2 to formate is reported as a slow side reaction catalyzed by both Ni-containing CODH and Ni-deficient CODH. Recently, the structures of *R. rubrum* CODH and its active site NiFeS cluster (the C-cluster) have been solved. The data in this manuscript describe the formate-producing capability of CODH with or without Ni in the active site.

Keywords Carbon monoxide dehydrogenase · Nickel-iron-sulfur enzyme · *Rhodospirillum rubrum* · Formate production

Introduction

Carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum* is a nickel-iron-sulfur enzyme that carries out the reversible oxidation of CO to CO_2 [1, 2, 3]. Studies with Ni-deficient enzyme have demonstrated that Ni is required for CODH activity [4, 5, 6]. Spectroscopic and crystallographic studies have indicated that the dimeric CODH contains five metal clusters: a pair of all-cysteinyl liganded $[Fe_4S_4]$ clusters referred to as "B-clusters," an all-cysteinyl-liganded $[Fe_4S_4]$ cluster that bridges the two subunits, and a Ni- and FeS-containing C-cluster [7, 8]. Recent chemical and kinetic analyses have revealed that a C-cluster CO ligand (CO_L) is required for CODH activity [9]. The slow displacement of this CO_L by cyanide (CN^-) results in the complete loss of CODH activity [9]. This displacement occurs in both Ni-deficient and Ni-containing CODH [9]. Based upon the lack of a requirement for the presence of Ni for the observation of CO_L, and taking into consideration the spectroscopic properties of CODH, it was proposed that CO_L is liganded to a unique Fe site of the C-cluster. These spectroscopic and kinetic studies indicate that at least two sites (the Ni site and the CO_L-ligated Fe site) of the C-cluster are essential for the catalytic interconversion of CO and CO_2.

During NMR analysis of CODH, a proton peak was observed at approximately 8.4 ppm and was originally assigned as arising from a proton of histidine. Quantitative analysis, however, showed that this peak increased in intensity with time and that it represented a proton present at significantly higher concentration than the sum of the histidine residues of CODH. NMR and chromatographic analyses show that several CODH samples (including Ni-containing and Ni-deficient wild-type and variant CODH) are able to reduce CO_2 to formate. The production of formate by CODH is inhibited by CN^-, suggesting that the presence of CO_L is necessary for the reaction to occur. This also indicates that the production of formate is more specific than simply being a function of the presence of metal entities.

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Materials and methods

All preparations and treatments of samples were performed in an anaerobic glove box (Vacuum Atmospheres Dri-Lab glovebox, model HE-493; $O_2 < 2$ ppm) unless otherwise indicated.

Preparation of chemicals

Na_2CO_3 and $NaHCO_3$ stocks were prepared in 1.0 M phosphate buffer (Sigma, pH 7.5) to avoid pH changes as a result of the bicarbonate addition. Potassium cyanide (KCN) stock was freshly prepared in 0.01 M NaOH solution. $NaHCO_3$-free sodium dithionite (DTH) was provided as a gift from Dr. Robert Burris and was originally purchased from Fluka Chemicals.

Cell growth and purification of CODH

Wild-type and H265V strains of *Rhodospirillum rubrum* were cultured [10, 11, 12] and CODH was purified as described previously [9, 12].

Protein assays

CODH samples were determined to be >95% pure by SDS-PAGE analysis. CODH concentrations were determined colorimetrically using carbonic anhydrase (Sigma) as a standard [13].

Oxidation of CODH samples

As-isolated CODH was thionin-oxidized as described previously [9, 14] except that phosphate buffer (50 mM, pH 7.5) was used instead of MOPS buffer.

Cyanide treatment of CODH

CODH was incubated with CN^- (100 μM final concentration) and DTH (1 mM final concentration) for 30 min. Unbound CN^- was removed by applying the sample mixture onto a Sephadex G-25 column (0.5 cm×12 cm, Pharmacia). The Sephadex G-25 column was equilibrated with 50 mM phosphate buffer (pH 7.5) prior to use.

NMR spectroscopy

One-dimensional ^1H NMR spectra were collected on a Bruker DMX 750 spectrometer (for Fig. 1) and Bruker DMX 500 (for Fig. 2) with a 5 mm ^1H probe at 300 K.

Results

Unusual NMR spectra of CODH

Figure 1A shows the ^1H NMR spectra of wild-type CODH in the presence of Na_2CO_3 (as a source of CO_2) and a reductant (DTH). The predominant sharp resonance at 8.37 ppm is indicated by the arrow, and is present in the general region where protons of histidine and aromatic residues (at ~8 ppm) normally occur. However, the integrated peak area was much greater than the concentration of CODH, and increased over time. Therefore, this resonance could not be attributed to an amino acid side-chain of the protein. Additionally, the variable intensity of the resonance indicated that the resonance was not protein-based.

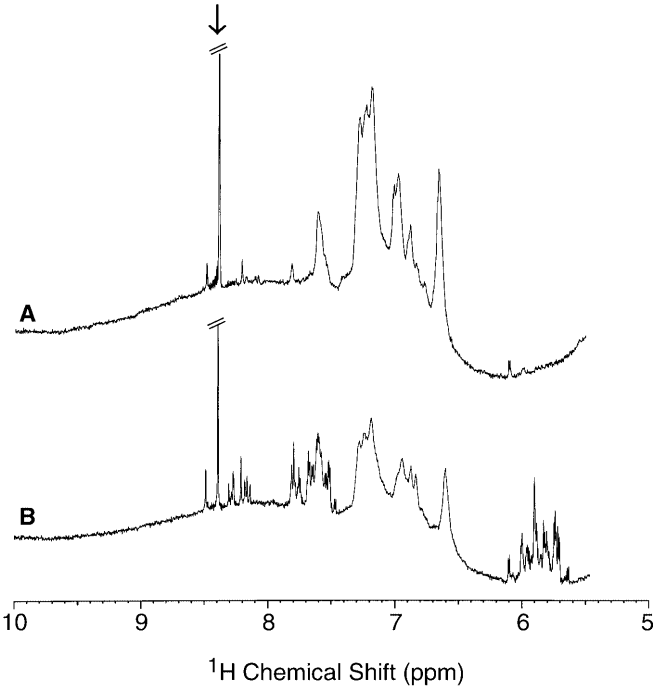

Fig. 1A, B. ^1H NMR signals from wild-type and Ni-deficient H265V CODH samples in ^1H_2O (90%) + ^2H_2O (10%). Sample **A** (thionin-oxidized, wild-type Ni-containing CODH) and sample **B** (thionin-oxidized, Ni-deficient H265V CODH) were transferred anaerobically in 50 mM phosphate buffer (pH 7.5) in the presence of DTH (2 mM final concentration) and Na_2CO_3 (10 mM final concentration) to the NMR tubes. The final concentrations of wild-type Ni-containing CODH and Ni-deficient H265V CODH were 2.0 and 1.5 mM, respectively. Conditions for NMR spectroscopy are described in the Materials and methods section. Data collection occurred over a period of 10 min following sample preparation. ^1H NMR peaks at 8.37 ppm (as indicated by the *arrow*) accumulated in both samples **A** and **B** as a function of time

Determination of the optimal conditions to produce the 8.37 ppm signal

Once it was ascertained that the 8.37 ppm signal arose from a non-protein entity in the reaction mix, various conditions were used to maximize the appearance of this signal. As shown in Table 1, the presence of Na_2CO_3 as a source of CO_2 provides the maximum activity. Note that for assays performed in the presence of Na_2CO_3 the 8.37 ppm signal is observed after 5 min, while longer treatments are necessary to observe the signal in reaction mixtures in the absence of added Na_2CO_3 (compare lines 1 and 5). The source of CO_2 in the initial experiment in which the 8.37 ppm signal was first observed was likely the reductant, sodium dithionite. Commercial preparations of sodium dithionite contain 5–10% Na_2CO_3 to maintain the pH of dithionite solutions. A purified batch of carbonate-free sodium dithionite was obtained from Professor R.H. Burris at the University of Wisconsin and used in the experiments presented here. Scarcely any 8.37 ppm signal is observed when wild-type CODH is incubated with carbonate-free sodium dithionite (line 7, Table 1).

CN^- is a potent inhibitor of both the CO oxidation and CO_2 reduction activities of CODH and, as shown in the second line of Table 1, CN-treated enzyme loses its ability to generate the 8.37 ppm signal in the presence of Na_2CO_3. Note that excess CN^- is not present in the reaction mixture; as described in the Materials and methods section, CODH was pre-treated with CN^- and then excess CN^- was removed by gel filtration.

The form of CODH with valine substituted for the C-cluster ligand H265 (H265V) was tested for the ability to generate the 8.37 ppm signal. H265V CODH for this experiment was prepared in the Ni-deficient form by growing the cells in the absence of Ni^{2+}. Neither H265V CODH nor the Ni-deficient H265V CODH exhibit CO oxidation activity. Surprisingly, the Ni-deficient H265V CODH showed nearly the same level of generation of the 8.37 ppm signal as the wild-type CODH. This suggests that the generation of the 8.37 ppm signal proceeds by a reaction that differs from the mechanism of CO oxidation.

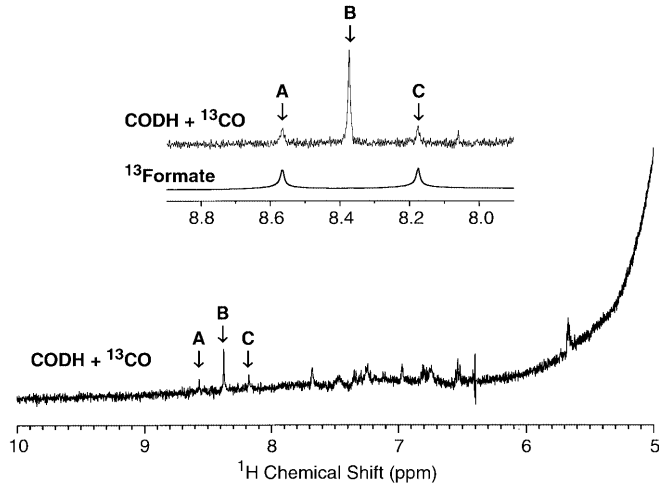

Fig. 2. Assignment of ^{1}H NMR signals. Thionin-oxidized, wild-type CODH (0.1 mM) in ^1H_2O (90%) + ^2H_2O (10%) in 50 mM phosphate buffer (pH 7.5) was incubated with saturated ^{13}CO (0.88 mM) for 14 h prior to performing ^{1}H NMR. NMR conditions are described under the Materials and methods section. Three peaks, *A*, *B*, and *C* (as indicated by *arrows*), were observed upon incubation of CODH with ^{13}CO. The ^{1}H NMR spectrum of ^{13}C-labeled formate was obtained under identical solution conditions to that of the CODH sample. The *inset* shows a comparison of resonances observed when CODH was incubated with ^{13}CO and those observed when ^{13}C-labeled formate was added to the reaction mixture. Peaks labeled *A* and *C* correspond to the ^{1}H resonances split by the nearby ^{13}C nuclear spin ($I = 1/2$) of labeled formate

Both CO_2 and CO are substrates for CODH, so the generation of the 8.37 ppm signal by CODH in the presence of CO was tested. As shown in lines 5 and 6 of Table 1, much less 8.37 ppm signal was observed under these conditions, even when the incubation was continued for 1 h. The small amount of activity was inhibited by CN^- as well. We propose that the small amount of activity here results from CO_2 generated by CO oxidation by CODH. Note that not much CO oxidation is expected, as no external electron acceptor is supplied.

Analyses of the chemical species that produces the NMR resonance at 8.37 ppm

In order to identify the species that produces the 8.37 ppm resonance and to prove that this entity was a reaction product of CODH, ^{13}CO was used as a substrate, and production of the resonance was monitored under the conditions for line 5 in Table 1; the results are shown in Fig. 2. The ^{1}H resonance at 8.37 ppm was indeed split into a doublet by the ^{13}C atom ($I = 1/2$), with resonances at 8.17 and 8.57 ppm (Fig. 2; peaks marked A and C). These are at the expected positions for a hydrogen ($I = 1/2$) atom interacting with an adjacent ($I = 1/2$) atom, confirming that the hydrogen atom is directly bound to the ^{13}C atom. Furthermore, the split resonances occur at the same positions as those of a control NMR spectrum of [^{13}C]formate, indicating that the resonance at 8.37 ppm is due to the production of formate by CODH (Fig. 2, inset). Figure 2 also reveals the presence of a fraction of unsplit signal at 8.37 ppm (Fig. 2, peak B) in addition to the split signal. It is

Table 1. Intensity of the 8.37 ppm peak under various conditions[a]

Form of CODH (incubation time)	DTH	Na_2CO_3	CO	Intensity of 8.37 ppm peak
1. Wild-type (5 min)	+	+	–	100
2. CN^--treated wild-type (5 min)	+	+	–	1
3. Ni-deficient H265V (5 min)	+	+	–	96
4. Ni-deficient, CN^--treated H265V (5 min)	+	+	–	1
5. Wild-type (1 h)	–	–	+	7
6. CN^--treated wild-type (1 h)	–	–	+	1
7. Wild-type (1 h)	+	–	–	2

[a]The 8.37 ppm peak was detected by NMR as described in Materials and methods. Enzyme concentrations were as follows: wild-type enzyme, 1.5 mM; CN^--treated wild-type enzyme, 1.4 mM; Ni-deficient H265V CODH, 1.5 mM; CN^--treated, Ni-deficient H265V CODH, 1.1 mM. CN^- treatment was as described in the Materials and methods section and enzyme was re-isolated by gel filtration before NMR analysis. Sodium dithionite (DTH) was present at 2 mM where indicated. Na_2CO_3 was added to a final concentration of 10 mM where indicated. Where indicated, reaction mixtures were equilibrated with an atmosphere of 100% CO. The amount of the 8.37 ppm peak observed for line 1 was set as 100% and other values are relative to that value

possible that this fraction arises from formate derived from a portion of the ligand CO (CO_L) which dissociated and reassociated with $^{13}CO_L$-bound CODH as a ^{12}CO substrate. The enzyme used in this experiment had been stored in solution with carbonate-containing dithionite, and we suggest that the unsplit 8.37 ppm resonance arises from carbonate that remained in the enzyme solution.

Detection of formate by chromatography

High performance liquid chromatograph (HPLC) analysis also indicated formate production and confirmed the NMR results. Figure 3 shows the HPLC trace (amperometric column monitor) for a reaction mixture containing wild-type CODH and Na_2CO_3. The product eluted at the retention time for a formate standard. This evidence supports the NMR data indicating that CODH produces formate in the presence of CO_2 and reductant. The estimated rate of accumulation of formate is slow [0.04 ± 0.02 μmol formate formed min^{-1} (mg protein)$^{-1}$] compared to the specific activity of the enzyme for CO oxidation (> 7000 μmol CO oxidation min^{-1} (mg protein)$^{-1}$ [9, 15]). The rate is also at least 1000-fold slower than the rate of CO_2 reduction [2].

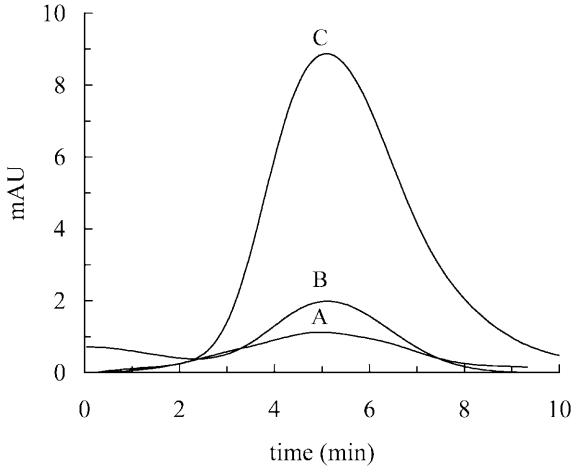

Fig. 3. Quantitative analyses of formate production by CODH. The production of formate by CODH was initiated by adding thionin-oxidized, wild-type CODH (50 μg) to a sealed, anaerobic reaction mixture (1 mL). The reaction mixture contained Na_2CO_3 (10 mM) and DTH (2 mM) in 10 mM MOPS buffer, pH 7.5. The reactions were terminated by the addition of concentrated HCl (10 μL) at time intervals of 1 and 2 min. The sample solutions were centrifuged (1500*g* for 1 min), and the supernatant was injected into an IonPac AS11 anion-exchange HPLC column anaerobically. The elution time of the species produced by CODH catalysis (*A*, 1 min reaction; and *B*, 2 min reaction) was identical to that of a formate standard (50 μmol of commercial formate, *C*). The elution of formate was monitored amperometrically. The amount of formate formed as a function of time was determined by injection of the samples into the column, and standardizing versus a formate sample of known concentration. The rate of formate production by CODH was $(4.3 \pm 2.0) \times 10^{-2}$ μmol formate formed min^{-1} (mg protein)$^{-1}$

Discussion

Highly purified CODH from *R. rubrum* catalyzes the reduction of CO_2 to formate at a rate of approximately 40 nmol formate produced per min per mg of CODH. While this activity is unlikely to be physiologically significant, it may provide insight into the catalytic capabilities of the enzyme. CODHs from *R. rubrum* and other organisms have been found to perform other redox reactions, including H_2 oxidation [16] and the reduction of 2,4,6-trinitrotoluene (TNT) [17]. The discovery of CO_2 reduction to formate adds another activity to the repertoire of CODHs.

Because this activity is novel and because *R. rubrum* is known to produce a formate dehydrogenase, it is important to establish that the activity observed here is not due to a trace contamination of formate dehydrogenase. The argument that the activity is due to CODH and not a contaminant rests on the following points: (1) formate dehydrogenases are readily reversible, whereas, to the limits of our detection, CO_2 is not produced from formate by CODH (we would easily have detected 100-fold less CO_2); (2) formate dehydrogenases contain Mo or W and to the limits of detection [by ICP-MS (ppb)], no Mo or W is present above background in the batches of enzyme used in these experiments; (3) the tight-binding inhibitor of CODH (CN^-) strongly inhibits the formate-producing activity; (4) the 8.37 ppm NMR signal that arises from formate is observed when either CO_2 or CO is provided to the enzyme; formate dehydrogenases are not known to interact with CO (however, CODH could convert CO to CO_2); (5) finally, the CODH used here was highly purified through a preparative gel electrophoresis step and is unlikely to be contaminated with FDH, which is a significantly larger enzyme. For these reasons, we conclude that the formate observed here is produced by CODH.

The structures of the *R. rubrum* CODH and the *Carboxydothermus hydrogenoformus* CODH have recently been solved and the active site clusters (the C-cluster) contain four Fe atoms, a Ni atom, and four or five non-protein bridging S atoms [7, 8]. The Ni atom sits at one corner of a cubane arrangement and one Fe atom is outside of the cube and linked to the cube by either a bridging S (the *C. hydrothermus* CODH structure) or Cys531 (the *R. rubrum* structure) to the Ni atom. His265 is a ligand to the Fe atom that sits outside the cube. A problem in interpreting the spectroscopic data from CODHs is explaining the two-electron oxidation of CO to CO_2 without invoking a change in the formal redox state of the Ni atom of the enzyme. No EPR-detectable change in the redox state of Ni has been observed. One possibility that has been raised is the presence of an Fe-bound hydride on the C-cluster [14]. The production of formate from CO_2 demands that a hydride equivalent (two electrons and a proton) be added to CO_2. An Fe-bound hydride would allow nucleophilic attack by the hydride on the electron-poor

carbon atom of a M-COO- entity. The fact that the Ni-deficient enzyme is also capable of formate production suggests that the Ni atom of the C-cluster is neither necessary nor involved in this process.

The formate-producing activity of CODH is not the primary chemistry of CODH. Iron-sulfide (pyrite) and iron-sulfide-nickel are known to catalyze the production of formate from CO and CO_2 in the absence of protein [18, 19], but in very low quantities at high temperature and pressure. It is well known that a process for the production of formate involves the insertion of CO_2 into a metal-hydride bond [20]. However, there have not been reports of an iron-sulfur cluster-containing protein producing formate even as a side reaction. The production of formate by CODH is of interest because it demonstrates a new catalytic capability of Fe-S proteins.

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