Unleashing Hydrogenase Activity in Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase and Pyruvate:Ferredoxin Oxidoreductase†

Saurabh Menon and Stephen W. Ragsdale*

Department of Biochemistry, Beadle Center, University of Nebraska, Lincoln, Nebraska 68588-0664

Received June 27, 1996; Revised Manuscript Received September 25, 1996⊗

ABSTRACT: These results demonstrate that two well-studied metalloenzymes, carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) and pyruvate:ferredoxin oxidoreductase (PFOR), can reduce protons to H_2 and, at much lower rates, oxidize H_2 to protons and electrons. To our knowledge, this if the first time that PFOR has been shown to have hydrogenase activity. CODH/ACS and PFOR evolved H_2 at maximum rates when CO and pyruvate were the electron donors, respectively, and when electron acceptors are absent; dithionite was a very poor substitute. PFOR, when purified to greater than 99% homogeneity, exhibited a specific activity for pyruvate-dependent H_2 production of 135 nmol min^{-1} mg^{-1}. The H_2 evolution activity divided by the H_2 uptake activity was 282:1; the highest ratio previously reported (22:1) was with the membrane-bound hydrogenase from *Rhodospirillum rubrum* [Fox, J. D., Kerby, R. L., Roberts, G. P., & Ludden, P. W. (1996) *J. Bacteriol. 178*, 1515−1524]. Highly purified samples of CODH/ACS (>99% homogeneity) exhibited a specific activity of CO-dependent H_2 evolution in the absence of electron carrier of 590 nmol min^{-1} mg^{-1}. Equivalent rates of CO oxidation and H_2 production were observed when determined in the absence of electron acceptor. This level of activity can account for the rate of H_2 production that has been observed by growing cultures of *Clostridium thermoaceticum* and could solve the paradox that the highly CO-sensitive hydrogenases from acetogenic bacteria evolve H_2 when grown on CO. The ratio of the rates of (H_2 evolution):(H_2 uptake) for purified CODH/ACS is between 20:1 and 30:1. H_2 evolution and uptake by CODH/ACS were strongly inhibited by cyanide (K_i = 1 μM), indicating that these reactions are catalyzed by cluster C, the site of CO oxidation. Our results extend earlier findings that the CODHs from *Methanosarcina barkeri* [Bhatnagar, L., Krzycki, J. A., & Zeikus, J. G. (1987) *FEMS Microbiol. Lett. 41*, 337−343] and *Oligotropha carboxydovorans* [Santiago, B., & Meyer, O. (1996) *FEMS Microbiol. Lett. 136*, 157−162] exhibit hydrogenase activity. Mechanistic implications of hydrogenase activity are discussed. Several physiological roles for proton reduction by CODH/ACS and PFOR are discussed, including the prevention of radical formation from reduced metal clusters when electron carriers (ferredoxin, flavodoxin, etc.) are limiting.

Hydrogenases play an important metabolic role. Some hydrogenases are geared toward H_2 uptake and allow organisms to couple the oxidation of the low-potential H_2 molecule to the reduction of various organic and inorganic compounds. For example, electrons derived from H_2 can be transferred to different electron carriers such as ferredoxin, NAD, flavin, or cytochrome, depending on the bacterium. These electrons ultimately reduce compounds like CO_2 to form reduced compounds like methane and acetic acid, sulfate to make sulfide, or nitrate or dinitrogen to generate ammonia. The protons derived from H_2 oxidation can generate a transmembrane proton gradient that can be coupled to ATP synthesis by a chemiosmotic mechanism. Another class of hydrogenases evolves H_2. This reaction aids in maintaining the redox balance in the cell; excess electrons combine with protons to generate H_2 which escapes from the cell. This paper focuses on conditions under which hydrogenase activity is unleashed in two proteins whose main physiological functions are to oxidize or reduce substrates other than H_2 or protons.

Hydrogenases can be classified by their metal content. The Fe-only hydrogenases lack other transition metals and have active sites that consistent solely of FeS clusters (Adams *et al*., 1981). The best-studied enzyme in this class is the *Clostridium pasteurianum* hydrogenase. The site of H_2 activation appears to be an FeS cluster of unknown structure called the H cluster. The Fe-only hydrogenases generally have a high specific activity (>1000 μmol min^{-1} mg^{-1}) and are very sensitive to oxygen and CO. NiFe hydrogenases contain four metal clusters: a center in which a Ni and an Fe ion are bridged by two thiols, two Fe_4S_4 clusters, and a Fe_3S_4 cluster (Albracht, 1994). The NiFeS cluster is viewed as the site of H_2 activation. Some NiFe hydrogenases also contain Se which is a component of the heterobimetallic site. A hydrogenase that lacks metals also exists (Berkessel & Thauer, 1995; Klein *et al*., 1995).

Another class of hydrogenases in which H_2 evolution or oxidation is exhibited in lieu of or in addition to another redox reaction exists. Nitrogenase, for example, catalyzes the formation of 1 mol of H_2 with each molecule of ammonia generated (Peters *et al*., 1995). It was shown earlier that the carbon monoxide dehydrogenases (CODHs[1]) from *Methanosarcina barkeri* (Bhatnagar *et al*., 1987) and *Oligotropha carboxydovorans* have hydrogenase activity (Santiago & Meyer, 1996). Here, we demonstrate that pyruvate:ferre-

† This work was supported by NIH Grant GM39451 (to S.W.R.).
* Corresponding author. Phone: 402-472-2943. Fax: 402-472-7842. E-mail: sragsdale@unlinfo.unl.edu.
⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

S0006-2960(96)01559-0 CCC: $12.00 © 1996 American Chemical Society

doxin oxidoreductase (PFOR) and carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) from *Clostridium thermoaceticum* have hydrogenase activity. When these enzymes are incubated with their electron donor (pyruvate or CO, respectively) in the absence of an electron acceptor, the low-potential electrons are shunted toward the generation of H_2. Electron carriers inhibit H_2 formation.

MATERIALS AND METHODS

Organism and Enzyme Purification. *C. thermoaceticum* strain ATCC 39073 was grown on glucose at 55 °C (Andreesen *et al*., 1973). PFOR was purified as described (Wahl & Orme-Johnson, 1987) except that ion exchange chromatography was performed on a DEAE-Sephacel column and the enzyme was eluted with a 2 L linear gradient from 0 to 0.4 M NaCl in 50 mM 2-(*N*-morpholino)-propanesulfonate buffer (pH 7.5) CODH/ACS (Ragsdale *et al*., 1983) and ferredoxin (Elliott & Ljungdahl, 1982) were purified as described under strictly anaerobic conditions at 17 °C in a Vacuum Atmospheres chamber maintained below 1 ppm oxygen.

PFOR activity during the purification procedure was monitored by a spectroscopic assay that was modified from Zeikus *et al*. (1977). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM thiamin pyrophosphate (TPP), 1 mM CoA, and 10 mM methyl viologen in a final volume of 1 mL. The assay was performed at 25 °C. PFOR concentrations are expressed as the dimer (240 kDa). The average specific activity of CODH was 300 u mg^{-1} (1 u = 1 μmol of CO oxidized min^{-1}) at 55 °C as measured by the standard methyl viologen-linked assay at pH 7.6 (Ragsdale *et al*., 1983). CODH/ACS concentrations were expressed in terms of the $\alpha_2\beta_2$ tetramer (310 kDa)[2] (Xia *et al*., 1996). Protein concentrations were determined by the Rose Bengal method (Elliott & Brewer, 1978). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed under denaturing conditions (Laemmli, 1971) as described.

Materials. N_2 (99.98%) and CO (99.99%) were obtained from Linweld (Lincoln, NE). N_2 was deoxygenated by passing through a heated column containing BASF catalyst. Reagents were of the highest purity available.

Enzyme Assays. Acetyl-CoA formation from carbons 2 and 3 of pyruvate was measured by a coupled malate dehydrogenase/citrate synthase assay. The reaction mixture contained 200 nM PFOR, 10 mM pyruvate, 1 mM CoA, 1 mM TPP, and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) in a total volume of 1 mL. At different times, 30 μL aliquots were removed and assayed as described (Pearson, 1965). The CO concentration in solution was derived by taking into account that the concentration of a saturated solution of CO at 55 °C is 670 μM.

Measurement of Hydrogenase Activity. H_2 evolution was quantified by gas chromatography on a Varian 3700 gas chromatograph using 10% AT-1000 on a Chromosorb 80/100 column (Alltech) and a TCD detector. Measurement of H_2 formation from pyruvate by PFOR was asayed at 25 °C in serum-stoppered cuvettes containing 10 mM pyruvic acid, 1 mM CoA, and 1 mM thiamin pyrophosphate in 50 mM MES buffer (pH 6.0). The reaction was initiated by injecting PFOR. The head space was analyzed for H_2. A similar reaction was used to measure the H_2 evolution activity of CODH/ACS. In this case, the 1.0 mL reaction mixture contained 50 mM MES buffer (pH 6.0) and 1.0 mM CO, equilibrated by bubbling the cuvette for 5 min with 1 mM CO prior to addition of CODH/ACS. In some cases, electron acceptors were added to the reaction mixture. H_2 evolution was also measured with 20 mM sodium dithionite in the presence and absence of 1 mM methyl viologen as the electron carrier in 50 mM MES buffer (pH 6.0).

Cyanide inhibition of H_2 evolution from CO by CODH/ACS was measured by adding varying concentrations of cyanide to the reaction mixture containing 50 mM MES (pH 6.0) and 1.0 mM CO incubated at 55 °C, and the formation of H_2 was monitored by gas chromatography. Cyanide inhibition of H_2 evolution from dithionite and reduced methyl viologen was performed similarly in reaction mixtures containing 1 mM reduced methyl viologen and sufficient sodium dithionite to reach an absorbance value at 604 nm of 2.0. The reaction was monitored by following the oxidation of methyl viologen at 604 nm. The reaction was initiated by adding CODH.

Attempts to measure H_2 evolution from ferredoxin were performed in an assay mixture containing 20 mM sodium dithionite in 50 mM MES buffer (pH 6.0) at 25 °C. The ferredoxin concentration was varied from 1 to 20 μM, and quantitation of H_2 was performed by gas chromatography.

H_2 uptake activities of CODH/ACS and PFOR were determined spectrophotometrically by measuring the reduction of methyl viologen at 604 nm. The reaction mixture contained 50 mM MES (pH 6.0) and 10 mM methyl viologen in a final volume of 1 mL. The reaction was performed in serum-stoppered cuvettes that had been bubbled with H_2 and incubated at 25 °C for PFOR and at 55 °C for CODH/ACS. The reaction was initiated by adding enzyme.

RESULTS

Hydrogenase Activity of PFOR

Hydrogen Evolution and Uptake Activities of PFOR. SDS−PAGE analysis of purified pyruvate:ferredoxin oxidoreductase revealed a single protein band corresponding to a molecular mass of 120 kDa, as had been determined earlier (Drake *et al*., 1981). On the basis of densitometric analysis, PFOR was 99% pure. The specific activity of the purified enzyme was 17.8 u/mg (k_{cat} = 2000 min^{-1}) at pH 7.6 and 25 °C in the presence of 10 mM methyl viologen, which compares favorably with a specific activity of 12 u/mg at pH 7.5 and 30 °C determined previously for the purified *C. thermoaceticum* PFOR (Wahl & Orme-Johnson, 1987).

Reaction of purified PFOR with pyruvate and CoA in the absence of electron acceptors[3] would be expected to result in a single turnover of pyruvate to produce 1 mol each of acetyl-CoA and CO_2 per mole of PFOR present. Unexpectedly, pyruvate underwent multiple turnovers; acetyl-CoA and CO_2 were produced, albeit more slowly, in amounts as high

[1] Abbreviation: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; PFOR, pyruvate:ferredoxin oxidoreductase; TPP, thiamin pyrophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Fd, ferredoxin; tmtss, tetramethylsalicyldehyde thiosemicarbozone.

[2] CODH/ACS recently was shown by sedimentation equilibrium (Xia *et al*., 1996) and electron micrographic and chemical cross-linking (Sundaresh *et al*., 1996) studies to be a tetramer. Previously, it was considered to be a hexamer (Ragsdale *et al*., 1983a).

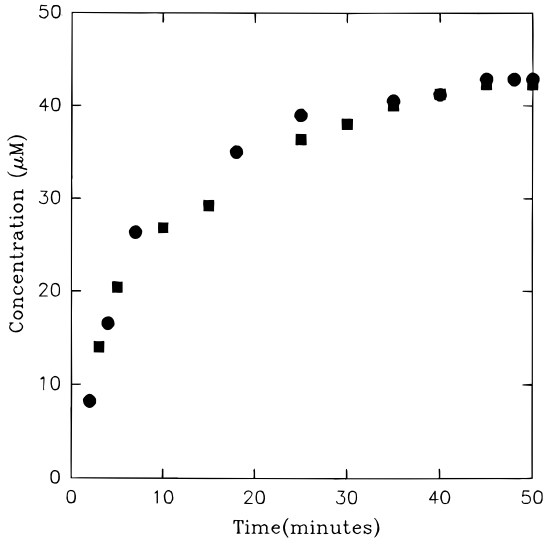

FIGURE 1: Production of H_2 and acetyl-CoA from pyruvate by PFOR. The assay for the synthesis of acetyl-CoA and H_2 from pyruvate was performed at 25 °C in MES (pH 6.0) and contained 200 nM PFOr, 10 mM pyruvic acid, 1 mM CoA, and 1 mM TPP. Acetyl-CoA (■) was quantitated by the malate dehydrogenase/citrate synthase assay, and H_2 evolution (●) was monitored by gas chromatography as described in Materials and Methods.

as those observed in the presence of electron acceptors (Figure 1). This indicated that the reduced protein had a mechanism for "self-oxidation" in the absence of electron acceptor. We found that this catalytic activity derived from the reduction of protons to H_2. The specific activity for H_2 evolution in the absence of electron carrier was 135.4 nmol min^{-1} mg^{-1} (0.3 s^{-1}), which equaled that measured for pyruvate decarboxylation. As far as we know, this activity has never been measured. The standard PFOR assays are performed in the presence of an electron acceptor where the rate of pyruvate decarboxylation is ~130-fold higher than that measured in its absence. We observed a constant 1:1 stoichiometry between the total amount of acetyl-CoA formed and the amount of H_2 produced. This activity was linear with PFOR concentration. Thus, the reaction is represented by eq 1.

$$\text{pyruvate} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + H_2 + CO_2 \quad (1)$$

There were two possible explanations for the H_2 evolution activity. It could result from an inherent hydrogenase activity of PFOR or from a contaminant hydrogenase in the sample, since *C. thermoaceticum* contains a hydrogenase with a high specific activity (Drake, 1982; Kellum & Drake, 1984; Pezacka & Wood, 1984). One way to distinguish between these possibilities was to compare the pyruvate-dependent H_2 evolution activity (in the absence of electron acceptor) with the standard pyruvate:ferredoxin oxidoreductase activity at each step in the purification of PFOR (Table 1). Both specific activities increase by nearly identical amounts with each purification step, and in the assays, the amount of H_2 produced always equaled the amount of pyruvate oxidized. Attempts to separate the PFOR activity from the electron carrier-independent hydrogenase activity were unsuccessful. If the hydrogenase activity detected in the PFOR preparation is due to a contaminant, it should share similar properties with other known hydrogenases. H_2 evolution is generally measured by incubating the enzyme with a solution of dithionite in the presence of reduced methyl viologen. Purified PFOR had a very low activity under these conditions. When PFOR was incubated with dithionite, the rate of H_2 production was only 2.6 nmol min^{-1} mg^{-1}, 50-fold lower than with pyruvate as the electron donor. Addition of 1.0 mM methyl viologen stimulated this activity to 10.8 nmol min^{-1} mg^{-1}. Therefore, the H_2 evolving activity was pyruvate-dependent.

The previously identified hydrogenase in *C. thermoacticum* is predominantly a H_2 uptake enzyme and requires an electron acceptor. Hydrogen uptake was measured by incubating samples with 1 atm of H_2 in the presence of 10 mM methyl viologen. Since this is the major hydrogenase present in *C. thermoaceticum* cells, the H_2 uptake activity in cell extracts predominantly reflects the activity of this enzyme. This has been shown to be 4 μmol min^{-1} mg^{-1} at pH 8.5−9 and 0.07 μmol min^{-1} mg^{-1} at pH 7.0 in *C. thermoaceticum* extracts assayed at 50 °C (Drake, 1982; Kellum & Drake, 1984). Table 1 shows that this activity decreases during the purification of PFOR. The H_2 uptake activity of purified PFOR was 0.48 nmol min^{-1} mg^{-1} and was linear with PFOR concentration. The H_2 evolution activity (in the presence of pyruvate and in the absence of a dye mediator) divided by the H_2 uptake activity (at 10 mM methyl viologen) was found to be 282:1. This is the highest ratio ever measured for a hydrogenase.

Most hydrogenases are highly sensitive to CO, which serves as a competitive inhibitor. At high concentrations, CO inhibited the hydrogenase activity of PFOR and exhibited an apparent K_i of 14 μM (Figure 2). This is quite different from the known acetogenic hydrogenases; they are strongly inhibited by CO. The hydrogenase from *Acetobacterium woodii*, for example, exhibits a K_i for CO of 14 nM. The combined results from the protein purification, pyruvate dependence, evolution:uptake ratio, and CO inhibition strongly suggest that the observed H_2 evolution activity results from PFOR, not from a contaminating hydrogenase.

Since the production of H_2 was pyruvate-dependent, it was important to determine whether CO inhibits the pyruvate oxidation step (and, therefore, only indirectly affects H_2 production) or if it directly interferes with H_2 evolution. This was tested by measuring the effect of CO on pyruvate oxidation in the presence of an electron acceptor. CO was unable to inhibit PFOR when pyruvate was saturating at 10 mM and an electron carrier was present (data not shown). When the concentration of pyruvate was decreased to 1 mM in the presence of electron carrier, weak inhibition of the enzyme could be observed; the apparent K_i under these conditions was 730 μM (Figure 2, inset). These experiments suggest that CO inhibits the proton reduction step(s).

Inherent Hydrogenase Activity of CODH/ACS

We have observed that the redox centers in CODH/ACS undergo slow oxidation after CO is removed from the enzyme under strictly anaerobic conditions, suggesting that a mech-

[3] Hydrogen evolution is generally measured in the presence of a redox mediator like methyl viologen, benzyl viologen, or ferredoxin. The maximum activity for PFOR and CODH/ACS described here occurs when only the electron donor (pyruvate or CO) and the catalyst (PFOR or CODH/ACS) are present; i.e., when redox mediators are absent. Although, strictly speaking, CODH/ACS and PFOR are electron acceptors, we have described the assays as lacking electron carriers, which emphasizes the omission of redox mediators like methyl viologen.

Table 1

	PFOR			H_2 evolution			H_2 uptake[a]	
purification step	specific activity[a] (u/mg)	fold purification	total units	specific activity[b] (mu/mg)	fold purification	total mu	specific activity (mu/mg)	total mu
DEAE-cellulose	0.1	1	4340	0.83	1.0	1600	0.003	8630
DEAE-Sephacel	1.1	11	2760	8.3	10	1080	0.03	1310
red agarose	12.0	120	2460	66.2	80	652	0.30	1250
gel filtration	13.4	134	1960	83.3	100	485	0.33	665
Q Sepharose	17.8	178	1610	135	160	445	0.48	578

[a] Measured by the standard assays in the presence of 10 mM methyl viologen. [b] Measured in the absence of electron acceptor. One milliunit is defined as 1 nmol of H_2 taken up per minute per milligram of protein.

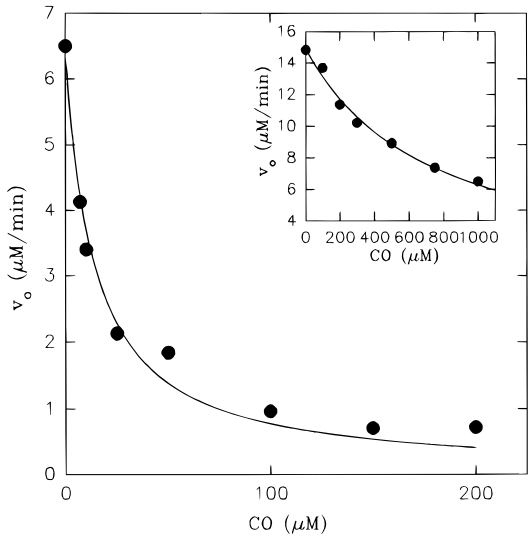

FIGURE 2: CO inhibition of pyruvate-dependent H_2 evolution by PFOR. For the inhibition reaction, the reaction mixture contained 50 mM MES (pH 6.0), 10 mM pyruvic acid, 1 mM CoA, and 1 mM TPP at varying concentrations of CO at 25 °C. The reaction was initiated by the addition of PFOR. The value of K_i obtained from the fit was 14 ± 2 μM. (Inset) CO inhibition of PFOR activity in the presence of electron carrier. The reaction mixture contained 50 mM MES (pH 6.0), 1 mM pyruvic acid, 1 mM CoA, and 1 mM TPP at varying concentrations of CO at 25 °C. The reaction was initiated by the addition of 200 nM PFOR. The value of K_i obtained from the fit was 730 ± 50 μM.

anism for releasing low-potential electrons from the enzyme exists. As with PFOR, the level of H_2 evolution from CO in the absence of electron acceptor was determined at each step in the purification and compared with the standard CO oxidation activity. Both activities copurified and increased by the same extent at each step in the purification procedure (Table 2). CODH/ACS was purified to at least 99% purity, on the basis of densitometric analysis of SDS−PAGE. H_2 evolution measured in the absence of electron carrier was retained throughout the purification procedure. The specific activity of CO-dependent H_2 evolution in the absence of electron carrier was 590 nmol min^{-1} mg^{-1} (3 s^{-1}) (Figure 3). This activity is ~1000-fold lower than that of CO oxidation measured at high concentrations of electron acceptor; however, it is even faster than the rate of CO_2 reduction. The rate of H_2 evolution was found to equal the rate of CO oxidation when determined in the absence of added electron acceptor. Therefore, under these conditions, CODH/ACS catalyzes the water−gas shift reaction, represented by eq 2.

$$CO + H_2O \leftrightarrow H_2 + CO_2 \qquad (2)$$

Hydrogen evolution from CODH/ACS required CO; replacing CO with dithionite yielded a rate of H_2 production of 6.4 nmol min^{-1} mg^{-1}, ~90-fold lower than with CO at saturating levels. This activity increased to 13 nmol min^{-1} mg^{-1} when 1 mM methyl viologen was present. The K_m for CO was 96 μM (Figure 3), which is similar to the K_m for CO in the CO oxidation reaction at pH 6.0 (125 μM) (Seravilli *et al*., 1995). CODH/ACS and PFOR are completely separated during the first step in the purification procedure; therefore, this activity is not due to contamination from PFOR. In addition, pyruvate had no effect on the rate of CO-dependent H_2 evolution and could not serve as an electron donor for H_2 evolution with the purified CODH/ACS sample.

CODH/ACS, like PFOR, catalyzes H_2 evolution at a much faster rate than it catalyzes H_2 uptake. The H_2 uptake activity of purified CODH/ACS was 19 nmol min^{-1} mg^{-1} with 10 mM methyl viologen and 30.5 nmol min^{-1} mg^{-1} using 50 μM methylene blue, and the rates were strictly linear with CODH/ACS concentration. Therefore, the ratio of the H_2 evolution and H_2 uptake activities was between ~20:1 and 30:1. Similarly, the *M. barkeri* CODH was reported to catalyze only H_2 evolution and not H_2 uptake (Bhatnagar *et al*., 1987).

We found that H_2 evolution from CO was strongly inhibited by cyanide (Figure 4); the apparent K_i value at saturating concentrations of CO was ~1 μM. In order to determine if cyanide inhibited the CO oxidation step or the proton reduction step, the effects of cyanide on H_2 evolution with dithionite as electron donor and on H_2 uptake were measured (Figure 4). Cyanide inhibited strongly suggests that proton reduction occurs at the site of CO oxidation (cluster C), since CO oxidation is strongly inhibited by cyanide (K_i <10 μM) (Morton, 1991), whereas, acetyl-CoA synthesis, which occurs at cluster A, requires a much higher level of cyanide for inhibition (Ragsdale *et al*., 1983; Ragsdale & Wood, 1985; Raybuck *et al*., 1988). The extreme cyanide sensitivity of the hydrogenase activity of CODH/ACS lends further weight to our proposal that this activity is a property of CODH/ACS, not a contaminating hydrogenase, since most hydrogenases are cyanide-insensitive. H_2 evolution by the *M. barkeri* CODH was also shown to be inhibited by cyanide (Bhatnagar *et al*., 1987).

Given that both PFOR and CODH/ACS catalyze substrate-dependent proton reduction, we wondered if ferredoxin might catalyze this reaction. However, the *C. thermoaceticum* FdII was unable to perform H_2 evolution. When the Fd concentration was increased to 20 μM, even a single turnover of proton reduction would have been measurable; however, H_2 was not detected.

Table 2

purification step	CO oxidation: specific activity[a] (u/mg)	CO oxidation: fold purification[a]	CO oxidation: total units	H_2 evolution: specific activity[b] (mu/mg)	H_2 evolution: fold purification[b]	H_2 evolution: total mu	H_2 uptake: specific activity (mu/mg)	H_2 uptake: total mu
cell extract	8.0	1.0	210 000	16.3	1.0	3920	51.2	70440
DEAE-cellulose	16.5	2.1	188 000	31.6	1.9	3690	0.62	17820
Q Sepharose	54.0	6.8	183 120	102	6.2	3210	3.9	3390
phenyl-Sepharose	170	22	86 625	320	19.4	2870	9.2	1040
gel filtration	320	40	57 500	590	36.1	1330	18.7	910

[a] Measured by the standard assays in the presence of 10 mM methyl viologen. [b] Measured in the absence of electron acceptor. One milliunit is defined as 1 nmol of H_2 evolved or taken up per minute per milligram of protein.

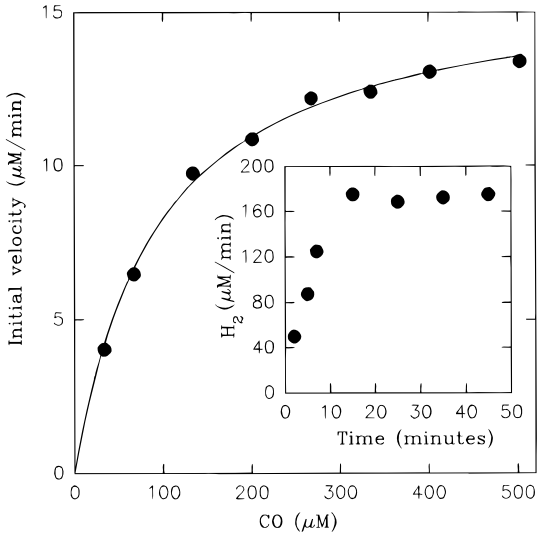

FIGURE 3: Determination of the K_m for CO of H_2 evolution activity by CODH/ACS. The K_m for CO was determined by following H_2 evolution at varying concentrations of CO at 55 °C as described in Materials and Methods. The value of K_m obtained from the initial velocity measurement was determined to be 96 ± 5 μM. (Inset) Representative data for CO-dependent H_2 production by CODH/ACS at 1 atm (670 μM) CO. The specific activity obtained from the initial velocity measurement was 590 nmol of H_2 produced min^{-1} mg^{-1}.

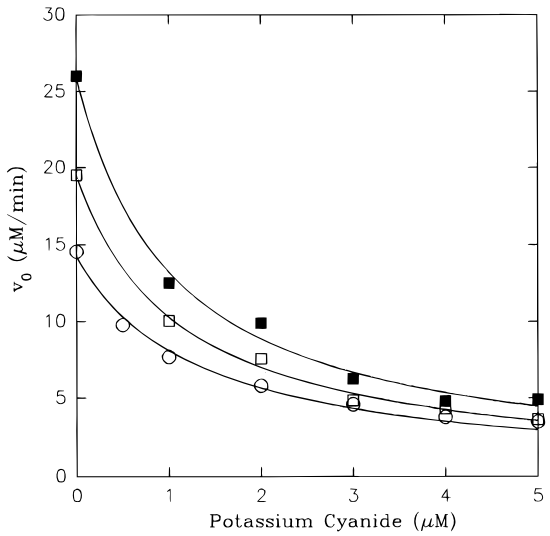

FIGURE 4: Cyanide inhibition of hydrogenase activity of CODH/ACS. Cyanide inhibition of H_2 evolution with CO (■) and with dithionite and 1 mM reduced methyl viologen (○) was monitored in a reaction mixture containing MES buffer (pH 6.0) at 55 °C as described in Materials and Methods. The reactions were initiated with 45 ng (■) or 1 μg (○, □) of CODH/ACS. The initial velocities for H_2 evolution and methyl viologen reduction were measured at varying concentrations of potassium cyanide. The K_i values for cyanide were 1.04 ± 0.08 and 1.32 ± 0.09 μM for the reactions containing CO and dithionite/viologen as electron donors, respectively. Cyanide inhibition of H_2 uptake (□) was determined by measuring the H_2-dependent reduction of methyl viologen at 604 nm. The apparent K_i was 1.12 ± 0.06 μM.

DISCUSSION

The results presented here demonstrate that two well-studied metalloenzymes, CODH/ACS and PFOR, can use CO or pyruvate to reduce protons to H_2 and, at a much lower rate, oxidize H_2 to protons and electrons. On the basis of several criteria, we have provided convincing evidence that the hydrogenase activities are inherent properties of CODH/ACS and PFOR and are not due to contamination from discrete hydrogenases. First, during the purification of both CODH/ACS and PFOR to greater than 99% homogeneity, the specific activities of substrate-dependent H_2 evolution tracked those of substrate-dependent reduction of electron acceptors. Therefore, CO- or pyruvate-dependent reduction of protons to H_2 could also be viewed as a specific assay for CODH/ACS or PFOR when the assays are performed in the absence of added electron acceptor. Second, CODH/ACS and PFOR exhibited the highest H_2 evolution activity when CO and pyruvate were the electron donors, respectively, and when electron acceptors were absent. Dithionite, for example, was a very poor substitute for the natural substrate. Third, the ratios of H_2 evolution to H_2 uptake were quite unlike those reported for other hydrogenases; they were between 20:1 and 30:1 for CODH/ACS and 280:1 for PFOR. The highest ratio previously reported (22:1) was with the membrane-bound hydrogenase from *Rhodospirillum rubrum* (Fox *et al*., 1996). Fourth, the H_2 evolution activities of CODH/ACS and PFOR are tolerant to CO, another unusual property shared with the *R. rubrum* hydrogenase (Fox *et al*., 1996). Fifth, the hydrogenase activity of CODH/ACS (including H_2 evolution from both CO and dithionite/reduced viologen and H_2 uptake) was strongly inhibited by cyanide; most hydrogenases are either completely insensitive or weakly inhibited by cyanide.

The CODH from *O. carboxydovorans* was recently shown to exhibit hydrogenase activity (Santiago & Meyer, 1996). This CODH activity resides in a multienzyme complex containing molybdopterin, flavin, and an FeS cluster, but no nickel (Meyer *et al*., 1993). In contrast to the two enzymes studied here, the *O. carboxydovorans* CODH oxidizes H_2 approximately 20-fold faster than it reduces protons (Santiago & Meyer, 1996). There are other enzymes that are hydrogenases in disguise. The best-studied example is nitrogenase, which evolves 1 mol of H_2 for every mole of N_2 reduced to ammonia (Simpon & Burris, 1984). Another example is a methanogenic enzyme that uses H_2 to reversibly reduce methenyltetrahydrometanopterin (methenyl-H_4MPT) to

methylene-H_4MPt (Berkessel & Thauer, 1995; Klein *et al*., 1995). Unlike the metal-containing hydrogenases, this enzyme is not inhibited by CO and does not reduce dyes like methyl viologen with H_2 or reduce protons to H_2.

Both CODH/ACS and PFOR contain several prosthetic groups; which one(s) are responsible for H_2 evolution? Is this activity physiologically relevant? Hydrogenase activity by CODH/ACS and PFOR can be viewed as a leakage of electrons from the reduced state of low-potential redox centers by capturing solvent-derived protons. This activity is possibly an inherent response of FeS clusters to the simultaneous exposure to water and low-potential electrons. However, simple model Fe_4S_4 clusters (Adams *et al*., 1980) and the *C. thermoaceticum* ferredoxin that contains two Fe_4S_4 clusters do not evolve H_2. This is possibly because H_2 is cleaved heterolytically, generating a hydride ion and a proton as intermediates (Krasna & Rittenberg, 1954). Thus, it is expected that the site of H_2 production/evolution must accommodate the influx/efflux of two electrons and two protons. This presumably would preclude simple FeS clusters from participating in hydrogenase reactions since they function almost exclusively as one-electron carriers, although, at very low potentials, apparently Fe_2S_2 and Fe_4S_4 clusters can undergo two-electron reduction (Watt & Reddy, 1994; Im *et al*., 1995). The metal center itself may not need to undergo two-electron redox chemistry. For example, in one mechanism for H_2 evolution, the metal center is proposed to first undergo one-electron reduction; two thiol ligands then accept two protons followed by a concerted one-electron/one-proton transfer step to generate a metal hydride which captures a proton to generate H_2 (Roberts & Lindahl, 1994). Ni-substituted rubredoxin from *Desulfovibrio gigas* has been reported to slowly catalyze proton reduction and proton/deuterium exchange (Saint-Martin *et al*., 1988). Several Ni complexes also have been shown to reduce protons to H_2 [discussed in Halcrow and Christou (1994)]. Although it is clear that nitrogenase catalyzes proton reduction to H_2, it is not known whether this reaction occurs at the eight-Fe-containing P cluster or at the MoFeS cofactor. It also is not known which metal cluster in the Mo-CODH catalyzes proton reduction.

The *C. thermoaceticum* PFOR apparently contains only three sites at which proton reduction could occur: TPP, where pyruvate binds and forms the hydroxyethyl-TPP radical, and two Fe_4S_4 clusters. One possibility is that H_2 is produced during the oxidation of an intermediate bound to TPP; however, the observation that CO inhibits this reaction suggests that one of the FeS clusters serves as the hydrogenase active site. However, both of the clusters in PFOR are considered to be standard ferredoxin-like Fe_4S_4 clusters (Wahl & Orme-Johnson, 1987), and ferredoxins are unable to catalyze H_2 evolution. Further studies will be required to determine the site of H_2 production by PFOR.

One way to determine which active site on CODH/ACS catalyzes the H_2 evolution activity is to evaluate how strongly the activity is inhibited by cyanide. CODH/ACS is a bifunctional enzyme that oxidizes CO to CO_2 and assembles acetyl-CoA. CO oxidation is catalyzed by a metal cluster called cluster C (Anderson *et al*., 1993; Kumar *et al*., 1993), and electrons are transferred to cluster B, which interacts with external electron acceptors. Since Fe_4S_4 clusters apparently are unable to catalyze H_2 production (above), it is highly unlikely that cluster B can catalyze proton reduction. CO oxidation is strongly inhibited by cyanide (Morton, 1991). Acetyl-CoA synthesis is catalyzed by cluster A (Gorst & Ragsdale, 1991; Shin & Lindahl, 1992; Kumar *et al*., 1993) and is cyanide-insensitive. On the basis of the observed strong inhibition of both H_2 production and uptake by cyanide, we propose that the site of H_2 production on CODH/ACS is the mixed-metal cluster known as cluster C. This is a Fe_4S_4 cluster bridged to a Ni ion (Qiu *et al*., 1995, 1996; Xia & Lindahl, 1996). Cluster C serves as the site of a similar reaction, the two-electron reduction of CO_2 to CO. Therefore, the reaction could be viewed as a competition between CO_2 and solvent-derived protons for the CODH active site.

The scenario of proton/CO_2 competition at the active sites of CODH/ACS and PFOR is interesting from both mechanistic and physiological points of view. A proposed role for proton reduction by nitrogenase is keeping the site of N_2 reduction free of H_2, which is a strong inhibitor of N_2 reduction (Mortensen, 1978). Likewise, we have observed here that protons can be reduced even faster than CO_2 by CODH/ACS. However, when CODH/ACS and PFOR were incubated with pyruvate and CoA at pH 6.0, CO was produced as fast as pyruvate underwent decarboxylation. This is important because it is CO produced at cluster C that is incorporated into the carbonyl group at cluster A. Further studies of the levels of CO *versus* H_2 production at varied concentrations of pyruvate, protons, and CO_2 are required to fully understand the balance between electron transfer and proton reduction at the active site of CO oxidation.

That CODH can catalyze CO-dependent hydrogen production may have important mechanistic implications. This reaction is equivalent to a well-known industrial process called the water−gas shift reaction (WGSR) (Parshall, 1980). This reaction utilizes the reducing power of CO to produce H_2 from water under mild conditions. The hydrogen is used as the reductant for processes such as the Haber−Weiss process, which produces ammonia from N_2. The WGSR can be performed by both heterogeneous and homogeneous metal-based catalysts (Laine & Crawford, 1988). The reaction appears to involve the following steps: (i) coordination of CO to a metal center, (ii) nucleophilic attack of hydroxide ion on the metal carbonyl to generate a metal−carboxyl species, (iii) a β hydride shift to eliminate CO_2 and generate a metal−hydride complex, and (iv) attack of water by the metal−hydride to regenerate the hydroxide nucleophile, H_2, and the active catalyst. It appears likely that the enzymatic mechanism of CO-dependent H_2 production includes a metal−hydride intermediate and involves a β hydride shift. If so, does the metal−hydride only occur under conditions that favor H_2 production and not electron acceptor reduction? Or could it be a component of both reactions? Detection of such an intermediate would connect the CODH mechanism with that of hydrogenase which appears to utilize a metal−hydride intermediate (above). Dinuclear nickel(II) complexes containing iminothiolate ligands were shown to catalyze CO oxidation to CO_2 and protons in aqueous solution with methyl viologen as the electron acceptor (Lu *et al*., 1993; Lu & Crabtree, 1995). It was suggested that this complex reduces methyl viologen instead of releasing H_2 because it precludes formation of a nickel−hydride intermediate.

A role for substrate-dependent proton reduction by PFOR and CODH/ACS that can be considered is aiding in the release of excess reducing equivalents from the cell when electron carriers are limiting or when CO is present at levels high enough to inhibit the conventional hydrogenases. The specific activity of H_2 production can be calculated to be ~2 nmol min^{-1} (mg of cell extract protein)$^{-1}$ for *C. thermoaceticum* cells growing on glucose and 14 nmol min^{-1} mg^{-1} on glucose plus CO.[4] On the basis of the observation that H_2 production and hydrogenase activity were highest in cells cultured in the presence of CO, it was concluded that the *C. thermoaceticum* hydrogenase functions in both the production and consumption of H_2 (Kellum & Drake, 1984). However, since CODH/ACS alone can evolve H_2 at a rate of 18 nmol min^{-1} (mg of cell extract protein)$^{-1}$, another possibility is that H_2 evolution could have come from the combined activities of CODH/ACS and PFOR and not the intrinsic hydrogenase, which is highly sensitive to CO (Drake, 1982). Therefore, perhaps catalysis of proton reduction by CODH/ACS can solve the puzzle that cells grown under CO produce H_2 even though the acetogenic hydrogenases are highly sensitive to CO. This activity is likely to be augmented by traditional hydrogenases that are CO-insensitive in some organisms and possibly even in some acetogens. For example, an electron transfer chain from CODH through a ferredoxin-like protein to a membrane hydrogenase has been described for methanogenic bacteria (Terlesky & Ferry, 1988) and photosynthetic bacteria (Ensign & Ludden, 1991; Kerby *et al*., 1995).

Another possible role for proton reduction by CODH was proposed by Meyer, e.g., serving as an electron detoxification reaction to prevent cell damage (Santiago & Meyer, 1996). When oxidized substrates (protons or CO_2) are absent, the reduced states of metal centers such as center C could serve as powerful one-electron reductants of a number of biomolecules. Such chemistry could be detrimental to the enzyme because it could initiate radical chemistry. For example, oxygen activation is initiated by one-electron reduction. Proton reduction, therefore, would serve as a safety valve to protect the protein and the cell from radical-induced damage. Maximum rates of H_2 evolution would occur when the cell is in a highly reducing state and electron acceptors (ferredoxin, flavodoxin, etc.) are limiting, since, when these electron transfer proteins are available, CODH/ACS or PFOR is oxidized by the electron acceptor which then transfers its electrons to other enzymes, including traditional hydrogenases or dehydrogenases.

[4] Assuming that CODH/ACS is present at a concentration of 3% of the cell protein (Roberts *et al*., 1989), the rate of H_2 production at saturating levels of CO from CODH/ACS alone would be 18 nmol min^{-1} mg^{-1} (i.e., 590 × 0.03). Proton reduction from PFOR would slightly increase this value. This is considered the maximum rate since substrates are likely to be below their K_m value, and electron acceptors (ferredoxin, flavodoxin, etc.) are probably not limiting. *C. thermoaceticum* was shown to produce H_2 during growth on glucose and CO (Martin *et al*., 1983). The rates of H_2 evolution can be calculated from the data presented in Table 1 of Martin *et al.* to be 0.04 mM h^{-1} when the cells are grown on dextrose and 0.22 mM h^{-1} on dextrose plus CO (Martin *et al*., 1983). The approximate concentration of cells during maximal H_2 production was 4.5 g wet weight L^{-1}. We have found that 1 g wet weight of cells yields ~60 mg of cell extract protein. Therefore, one can roughly calculate the rate of H_2 production to be 2.5 nmol min^{-1} mg^{-1} with cells grown on dextrose and 14 nmol min^{-1} mg^{-1} with cells grown on dextrose plus CO. A similar number (1.7 nmol min^{-1} mg^{-1}) can be calculated by dividing the rate of acetate production by cells of *C. thermoaceticum* growing on glucose [calculated to be 115 nmol min^{-1} mg^{-1} by Fuchs (1986)] by the ratio of (acetate production):(H_2 production), measured to be 67:1 (Martin *et al*., 1985).

REFERENCES

Adams, M. W. W., Rao, K. K., Hall, D. O., Christou, G., & Garner, C. D. (1980) *Biochim. Biophys. Acta 589*, 1−9.

Adams, M. W. W., Mortenson, L. E., & Chen, J.-S. (1981) *Biochim. Biophys. Acta 594*, 105−176.

Albracht, S. P. J. (1994) *Biochim. Biophys. Acta 1188*, 167−204.

Anderson, M. E., DeRose, V. J., Hoffman, B. M., & Lindahl, P. A. (1993) *J. Inorg. Biochem. 51*, Abstract B149.

Andreesen, J. R., Schaupp, A., Neurater, C., Brown, A., & Ljundahl, L. G. (1973) *J. Bacteriol. 114*, 743−751.

Berkessel, A., & Thauer, R. K. (1995) *Angew. Chem. Int. Ed. Engl. 34*, 2247−2250.

Bhatnagar, L., Krzycki, J. A., & Zeikus, J. G. (1987) *FEMS Microbiol. Lett. 41*, 337−343.

Drake, H. L. (1982) *J. Bacteriol. 150*, 702−709.

Drake, H. L., Hu, S.-I., & Wood, H. G. (1981) *J. Biol. Chem. 256*, 11137−11144.

Elliott, J. I., & Brewer, J. M. (1978) *Arch. Biochem. Biophys. 190*, 351−357.

Elliott, J. I., & Ljungdahl, L. G. (1982) *J. Bacteriol. 151*, 328−333.

Ensign, S. A., & Ludden, P. W. (1991) *J. Biol. Chem. 266*, 18395−18403.

Fox, J. D., Kerby, R. L., Roberts, G. P., & Ludden, P. W. (1996) *J. Bacteriol. 178*, 1515−1524.

Fuchs, G. (1986) *FEMS Microbiol. Rev. 39*, 181−213.

Gorst, C. M., & Ragsdale, S. W. (1991) *J. Biol. Chem. 266*, 20687−20693.

Halcrow, M. A., & Christou, G. (1994) *Chem. Rev. 94*, 2421−2476.

Im, S. C., Lam, K. Y., Lim, M. C., Ooi, B. L., & Sykes, A. G. (1995) *J. Am. Chem. Soc. 117*, 3635−3636.

Kellum, R., & Drake, H. L. (1984) *J. Bacteriol. 160*, 466−469.

Kerby, R. L., Ludden, P. W., & Roberts, G. P. (1995) *J. Bacteriol. 177*, 2241−2244.

Klein, A. R., Hartmann, G. C., & Thauer, R. K. (1995) *Eur. J. Biochem. 233*, 372−376.

Krasna, A. I., & Rittenberg, D. (1954) *J. Am. Chem. Soc. 76*, 3015.

Kumar, M., Lu, W.-P., Liu, L., & Ragsdale, S. W. (1993) *J. Am. Chem. Soc. 115*, 11646−11647.

Laemmli, U. K. (1971) *Nature (London) 227*, 680−685.

Laine, R. M., & Crawford, E. J. (1988) *J. Mol. Catal. 44*, 357.

Lu, Z., & Crabtree, R. H. (1995) *J. Am. Chem. Soc. 117*, 3994−3998.

Lu, Z., White, C., Rheingold, A. L., & Crabtree, R. H. (1993) *Angew. Chem., Int. Ed. Engl. 32*, 92−94.

Martin, D. R., Lundie, L. L., Kellum, R., & Drake, H. L. (1983) *Curr. Microbiol. 8*, 337−340.

Martin, D. R., Misra, A., & Drake, H. L. (1985) *Appl. Environ. Microbiol. 49*, 1412−1417.

Meyer, O., Frunzke, K., & Mörsdorf, G. (1993) in *Microbiol. growth on C_1 compounds* (Murrell, J. C., & Kelly, D. P., Eds.) Intercept Ltd., Andover, U.K.

Mortensen, L. E. (1978) *Biochimie 60*, 219−223.

Morton, T. A. (1991) Sequencing and active site studies of carbon monoxide dehydrogenase/acetyl-CoA synthase from *Clostridium thermoaceticum*, Ph.D. Thesis, University of Georgia, Athens, GA.

Parshall, G. (1980) in *Homogeneous Catalysis*, Wiley-Interscience, New York.

Pearson, D. J. (1965) *Methods Enzymol. 13*, 545−548.

Peters, J. W., Fisher, K., & Dean, D. R. (1995) *Annu. Rev. Microbiol. 49*, 335−366.

Pezacka, E., & Wood, H. G. (1984) *Arch. Microbiol. 137*, 63−69.

Qiu, D., Kumar, M., Ragsdale, S. W., & Spiro, T. G. (1995) *J. Am. Chem. Soc. 117*, 2653−2654.

Qiu, D., Kumar, M., Ragsdale, S. W., & Spiro, T. G. (1996) *J. Am. Chem. Soc.* (in press).

Ragsdale, S. W., & Wood, H. G. (1985) *J. Biol. Chem. 260*, 3970−3977.

Ragsdale, S. W., Clark, J. E., Ljungdahl, L. G., Lundie, L. L., & Drake, H. L. (1983) *J. Biol. Chem. 258*, 2364−2369.
Raybuck, S. A., Bastian, N. R., Orne-Johnson, W. H., & Walsh, C. T. 1988) *Biochemistry 27*, 7698−7702.
Roberts, D. L., James-Hagstrom, J. E., Smith, D. K., Gorst, C. M., Runquist, J. A., Bauer, J. R., Haase, F. C., & Ragsdale, S. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 32−36.
Roberts, L. M., & Lindahl, P. A. (1994) *Biochemistry 33*, 14339−14350.
Saint-Martin, P., Lespiant, P. A., Fauque, G., Berliner, Y., LeGall, J., Moura, I., Teixeira, M., Xavier, A. V., & Moura, J. J. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9378−9380.
Santiago, B., & Meyer, O. (1996) *FEMS Microbiol. Lett. 136*, 157−162.
Seravalli, J., Kumar, M., Lu, W. P., & Ragsdale, S. W. (1995) *Biochemistry 34*, 7879−7888.
Shin, W., & Lindahl, P. A. (1992) *Biochemistry 31*, 12870−12875.
Simpson, F. B., & Burris, R. H. (1984) *Science 224*, 1095−1097.
Sundaresh, C. S., Beegen, H., Shenoy, B. C., Wall, J. S., & Kumar, G. K. (1996) *J. Biol. Chem*. (in press).
Terlesky, K. C., & Ferry, J. G. (1988) *J. Biol. Chem. 263*, 4075−4079.
Wahl, R. C., & Orme-Johnson, W. H. (1987) *J. Biol. Chem. 262*, 10489−10496.
Watt, G. D., & Reddy, K. R. N. (1994) *J. Inorg. Biochem. 53*, 281−294.
Xia, J. Q., & Lindahl, P. A. (1996) *J. Am. Chem. Soc. 118*, 483−484.
Xia, J. Q., Sinclair, J. F., Baldwin, T. O., & Lindahl, P. A. (1996) *Biochemistry 35*, 1965−1971.
Zeikus, J. G., Fuchs, G., Kenealy, W., & Thauer, R. K. (1977) *J. Bacteriol. 132*, 604−613.

BI9615598