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Inactivation of Formate Dehydrogenase from *Methanobacterium formicicum* by Cyanide†

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ABSTRACT: Formate dehydrogenase from *Methanobacterium formicicum* contains Mo, FAD, and Fe/S clusters as prosthetic groups. Partial reduction of the native enzyme with either formate or dithionite yielded a Mo(V) electron paramagnetic resonance (EPR) signal, $g_1 = 2.020$, $g_2 = 2.006$, and $g_3 = 1.997$, exhibiting nearly isotropic superhyperfine interaction to two, equivalent, strongly coupled protons ($A_1 = 0.45$, $A_2 = 0.55$, and $A_3 = 0.5$ mT), identified by ^2H substitution. Incubation of oxidized formate dehydrogenase with cyanide resulted in an irreversible loss of enzyme activity, which could not be restored by treatment with sulfide. Equimolar amounts of thiocyanate were released from cyanide-treated formate dehydrogenase, indicating the loss of one terminal sulfur ligand to molybdenum. Cyanide inactivation of the oxidized native enzyme resulted in a shift of the Mo(V) EPR signal to higher field ($g_1 = 2.005$, $g_2 = 1.998$, and $g_3 = 1.989$) and a reduction in the complexity of the superhyperfine splitting pattern due to loss of a strongly coupled, exchangeable proton, the remaining coupled proton yielding coupling constants of $A_1 = 1.05$, $A_2 = 0.85$, and $A_3 = 0.88$ mT, respectively. In addition, cyanide treatment altered the behavior of the Mo(V) EPR signal during potentiometric titration of the enzyme, yielding midpoint potentials for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) redox couples of −319 and −321 mV, respectively. Comparison of the spectroscopic and thermodynamic properties of the native and cyanide-treated forms of formate dehydrogenase indicates the mode of inactivation to be analogous to that established for the related enzyme, xanthine oxidase, resulting in the loss of a sulfur residue yielding the "desulfo" form of formate dehydrogenase.

Formate dehydrogenase (FDH)[1] (EC 1.2.1.2) catalyzes the oxidation of formate to CO_2 in aerobic, anaerobic, and facultatively anaerobic eubacteria. Among the archaebacteria, only methanogens have so far been shown to contain FDH, with approximately half of the strains examined capable of utilizing formate as an electron donor for methanogenesis (Balch et al., 1979). Formate dehydrogenase isolated from *Methanococcus vannielli* (Jones & Stadtman, 1981) has been shown to contain Se, Mo, and Fe/S centers.

The FDH purified from *Methanobacterium formicicum* is an extremely O_2-sensitive, soluble enzyme (M_r 177 000) that contains FAD, Fe/S, and Mo prosthetic groups (Barber et al., 1983; Schauer & Ferry, 1982, 1983). The latter has been identified as present in combination with a substituted or modified pterin which together comprise a form of the Mo cofactor (Johnson et al., 1984). The Mo cofactor, while possibly existing in a number of very similar forms, has been demonstrated to be present in a wide variety of Mo-containing enzymes isolated from diverse sources.

With the exception of nitrogenase (Shah et al., 1984), which contains Mo as part of a spin-coupled cluster including Fe and inorganic S, termed "Fe–Mo–Co", molybdenum-containing enzymes are known to be sensitive to inhibition by cyanide with inactivation occurring by either of two possible mechanisms. The oxidized forms of the xanthine-utilizing molybdenum hydroxylases, xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase, are irreversibly inactivated by cyanide (Wahl & Rajagopalan, 1982) which results in the loss of a terminal sulfur ligand of the Mo and its release as thiocyanate (Massey & Edmondson, 1970). The Mo enzymes that do not possess a terminal sulfur ligand, such as sulfite oxidase and nitrate reductase, resist inhibition by cyanide in their oxidized state. In contrast, in the reduced state all the molybdenum-containing enzymes are reversibly inhibited by exposure to cyanide. Formate dehydrogenase has previously been shown to be inhibited, noncompetitively, by cyanide with a K_i of 6 μM (Schauer & Ferry, 1982). However, the effects of this inhibitor on the properties of the enzyme's cofactors and site of action have not been studied. In this paper, we report the results of studies using EPR spectroscopy to define the mode of inhibition of FDH by cyanide and to facilitate comparison with other molybdenum hydroxylases.

Materials and Methods

Cell Material. *M. formicicum*, strain JF-1, was cultured with either formate or H_2/CO_2 as the sole energy source as previously described (Schauer & Ferry, 1980). In addition to the standard growth conditions, *M. formicicum* was also cultured on a medium enriched in $^{95}MoO_4^{2-}$ and a medium depleted of Mo and containing WO_4^{2-} under which conditions

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[1] Abbreviations: FDH, formate dehydrogenase; Fe/S, non-heme iron acid-labile sulfur cluster; EPR, electron paramagnetic resonance; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; MV, methyl viologen; XO, xanthine oxidase; BSA, bovine serum albumin; AFR, activity flavin ratio; EDTA, ethylenediaminetetraacetic acid; EXAFS, extended X-ray absorption fine structure.

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a H_2/CO_2 mixture was used as the source of carbon and reducing equivalents.

Enzyme Purification and Assay. Formate dehydrogenase was purified, under anaerobic conditions, as described by Schauer and Ferry (1980) in the presence of dithionite (1 mM), sodium azide (10 mM), and 2-mercaptoethanol (3 mM) and was the generous gift of Dr. Neil Schauer. Enzyme samples were stored at 77 K following anaerobic dialysis against 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, and concentration by pressure filtration. Isotopic substitution of deuterium for hydrogen in enzyme samples was achieved by repeated pressure concentration of FDH under anaerobic conditions from Bicine buffer utilizing ^2H_2O (99.8% enrichment) as solvent. pD_{app} is used to denote the pH in ^2H_2O buffers (Glasoe & Long, 1960).

Formate dehydrogenase activity was assayed by using the formate-dependent reduction of methyl viologen at 603 nm or coenzyme F_{420} at 420 nm (Schauer & Ferry, 1983). One unit of activity was the amount of enzyme that reduced 1 μmol of electron acceptor per minute. Xanthine oxidase (Sigma, St. Louis, MO) assays were carried out in 50 mM potassium phosphate buffer, pH 7.5, and exhibited an AFR of 64 corresponding to 33% functional enzyme (Bray, 1975). The oxidation of xanthine (150 μM) in the presence of air was monitored at 295 nm. One unit of activity was the amount of enzyme that oxidized 1 μmol of xanthine (E_{295} = 12.2 mM^{-1} cm^{-1}) per minute. Both enzyme activities were expressed as units per milligram of protein. Protein was determined by the method of Bradford (1976) using BSA as standard.

Formate dehydrogenase (6.0 and 12.0 mg) in 50 mM potassium phosphate buffer, pH 7.5, was incubated for 1 h at 23 °C in the presence of KCN (5 mM) to liberate thiocyanate. The treated enzyme was taken to dryness by pressure dialysis using a YM-30 membrane (Amicon, Lexington, MA) and the effluent analyzed for the presence of thiocyanate as described by Sorbo (1957). Identical experiments were performed with XO as control.

Potentiometric Titrations. Potentiometric redox titrations and the preparation of controlled reduction enzyme samples were carried out at 25 °C in an anaerobic cell as described by Barber and Salerno (1980). Purified enzyme or crude cell-free extract was equilibrated with the gold-indicating electrode using a series of dye mediators divided into two groups. Group A, comprising the non-viologen mediators, was composed of pyocyanine (E_0' = −60 mV), indigodisulfonic acid (E_0' = −125 mV), 2-hydroxy-1,4-naphthoquinone (E_0' = −137 mV), anthraquinone-2,7-disulfonate (E_0' = −182 mV), anthraquinone-2-sulfonate (E_0' = −225 mV), phenosafranine (E_0' = −255 mV), safranine T (E_0' = −289 mV), and neutral red (E_0' = −325 mV). Group B comprised the viologen mediators, benzyl viologen (E_0' = −311 mV) and methyl viologen (E_0' = −440 mV). Samples were poised at the required potentials using additions of either 0.1 M $Na_2S_2O_4$ or 0.1 M $K_3Fe(CN)_6$ and equilibrated at each potential for a period of 10 min, prior to being withdrawn and transferred into Ar-flushed EPR tubes and frozen in liquid nitrogen. For the experiments involving deuterium substitution, mediators, reductant, and oxidant were prepared by using ^2H_2O as solvent.

Controlled reduction EPR samples of FDH oxidatively inactivated with CN^- were prepared by using the following procedure, performed under anerobic conditions. Buffer and the required mediators were added to the titration cell and reduced to a potential of −100 mV. KCN was added followed by either the purified enzyme or the cell-free extract; the system was incubated at a potential of −100 mV for 10 min, following which either the potential was decreased to its minimum value and the enzyme titrated in an oxidative mode or successive samples were withdrawn during a reductive titration.

Oxidation–reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were obtained by computer fitting of the experimental data points to theoretical Nernst titration curves for the intermediate formed in two, consecutive, n = 1 reduction processes (Clark, 1960). The magnitude of the errors associated with this process is of the order of ±15 mV and has been discussed by Cammack et al. (1976).

Electron Paramagnetic Resonance Spectra. EPR spectra were recorded with a Varien E109 Century Series spectrometer operating at 9-GHz and 100-KHz modulation. Mo(V) spectra were obtained within the temperature range 100–170 K by using a Varian variable-temperature accessory. Quantification of the EPR signals was performed as described by Barber and Siegel (1982) using CuEDTA as an integration standard. Mo(V) spectra from cell-free extract reduced in the presence of CN^- were quantitated by using the amplitude of the g_3 feature, which remained unaffected by the presence of additional, overlapping signals. Low-temperature EPR spectra were recorded at 15 K with an Air Products Heli-Tran liquid helium transfer system.

Computer simulations of experimental EPR spectra were calculated by using a modified version of the program described by Lowe (1978) using a Zenith 150PC microcomputer. Simulations of spectra from samples in which deuterium had been substituted for hydrogen were modified by replacing the doublet splitting (^1H $I = {}^1/_2$) with a triplet splitting (^2H I = 1) and decreasing the superhyperfine coupling constants by 84.6%, respectively (Gutteridge et al., 1978a). g values, measured with respect to a Mn–diphenylpicrylhydrazyl (DPPH) standard, are considered to be accurate to ±0.001.

Results

The high-temperature (173 K) EPR spectrum of FDH purified from *M. formicicum* and reduced with formate in 50 mM Bicine buffer, pH 7.7, is shown in Figure 1A. This signal, ascribed to the formation of Mo(V), exhibited a g_{av} = 2.008 and showed evidence of superhyperfine interaction with two, coupled, protons. Isotopic substitution of ^1H by ^2H in the purified enzyme followed by controlled dithionite reduction yielded the EPR signal shown in Figure 1C. Comparison of this signal with that obtained for the native enzyme revealed the loss of any resolved superhyperfine structure following deuterium substitution, confirming the identity of the coupled nuclei as two, equivalent, exchangeable protons. Computer simulations of both the native and deuterium-substituted enzyme spectra are shown in Figure 1B and Figure 1D, respectively, while the EPR parameters for these species derived from the computer simulations are given in Table I. It should be noted that the incomplete resolution of the g_1 and g_2 features of the deuterium-substituted spectrum (Figure 1C) is due to the presence of an overlapping free radical species that is presumably the result of reduction of the FAD prosthetic group of the enzyme. Figure 1E illustrates the typical EPR spectrum obtained during the course of potentiometric titrations of a crude cell-free extract obtained from *M. formicicum* and is similar to that previously observed for formate-reduced whole cells (Barber et al., 1982).

The inactivation of FDH by cyanide is shown by Figure 2. Incubation of native enzyme, in the oxidized state, at pH 7.5 with excess cyanide resulted in the loss of formate/MV reductase activity with 50% inactivation after approximately 2-min exposure to 20 μM CN^-. Removal of the cyanide by

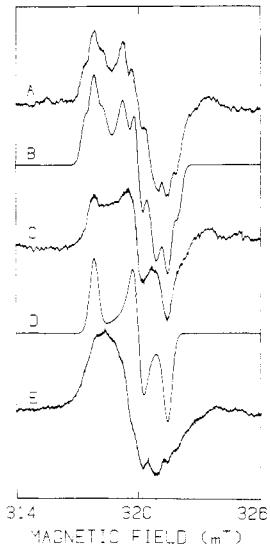

FIGURE 1: Electron paramagnetic resonance spectra of formate dehydrogenase. (A) Purified FDH (14 μM Mo) was reduced, anaerobically, with 5 mM formate for 1 min in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7. (B) Computer simulation of the experimental spectrum shown in (A), using the parameters given in Table I. (C) Purified FDH (4 μM Mo) was extensively dialyzed, anaerobically, against 50 mM Bicine buffer containing 1 mM EDTA using ^2H_2O as solvent, pD_{app} = 7.7. The enzyme was poised at −380 mV for 7 min, using dithionite in the presence of non-viologen mediators (group A, 33 μM each mediator), prior to being frozen in liquid nitrogen. (D) Computer simulation of the experimental spectrum C using the parameters given in Table I with the superhyperfine coupling constants modified as described under Materials and Methods to represent substitution of ^2H for ^1H. (E) *M. formicicum* cell-free extract (11 mg/mL total protein) in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, poised at −400 mV in the presence of non-viologen mediators (35 μM each mediator). All spectra were recorded at 173 K and 0.32-mT modulation amplitude by using the following microwave powers: (A and B) 20 mW; (C) 50 mW. The field scale corresponds to a microwave frequency of 8.987 GHz.

Table I: EPR Spectroscopic Parameters[a] for the Mo(V) Signals from Native and Cyanide-Treated *M. formicicum* Formate Dehydrogenase

	EPR species	
	native	CN treated
g_1	2.020	2.005
g_2	2.006	1.998
g_3	1.997	1.989
A^1_1	0.45	1.05
A^1_2	0.55	0.85
A^1_3	0.50	0.88
A^2_1	0.45	
A^2_2	0.55	
A^2_3	0.50	
D_1	0.19	0.27
D_2	0.19	0.27
D_3	0.19	0.27

[a] Parameters were obtained by comparison of the experimental spectra and computer simulations. Superhyperfine coupling constants and half line widths (D) are given in millitesla.

anaerobic dialysis failed to restore the formate-dependent reduction of either methyl viologen or coenzyme F_{420}, as shown in Table II, while control samples, subjected to identical incubations in the absence of cyanide, showed only marginal decreases in activity. Similar experiments using XO showed no inactivation of xanthine/O_2 activity following exposure to 20 μM cyanide. However, 50% inactivation of XO was obtained following 10-min incubation with 5 mM KCN.

Quantitation of the amount of thiocyanate liberated following treatment of samples of FDH (6 and 12 mg, respectively) with cyanide (5 mM) showed the formation of 7.8 and 8.1 nmol of thiocyanate per milligram of enzyme while control experiments performed with XO resulted in the liberation of 8.2 and 8.5 nmol of thiocyanate per milligram of functional enzyme.

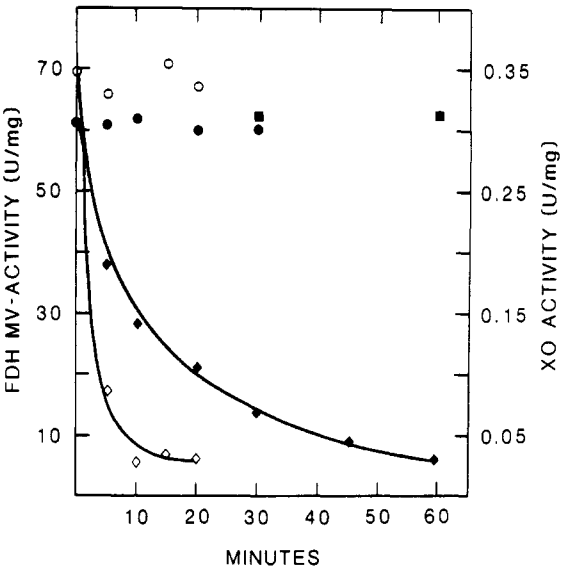

FIGURE 2: Time course of inactivation of formate dehydrogenase and xanthine oxidase by cyanide. Methyl viologen oxidized FDH (1.7 μM) was incubated in the presence (◇) and absence (○) of KCN (20 μM) in 50 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.5, under anaerobic conditions following which aliquots were removed and assayed for formate-dependent reduction of MV as described under Materials and Methods. Xanthine oxidase (2.2 μM) was incubated in the presence of 20 μM KCN (●) and 5 mM KCN (◆) and in the absence of KCN (■) under the same conditions as described for inactivation of FDH and XO activity assayed as described under Materials and Methods.

Table II: Inactivation of Oxidized Formate Dehydrogenase by Cyanide[a]

formate-dependent reduction of MV		formate-dependent reduction of F_{420}	
−KCN	+KCN	−KCN	+KCN
70.7 ± 6.6[b]	6.2 ± 1.1[b]	0.51 ± 0.07[b]	0.12 ± 0.02[b]
67.5 ± 3.1[c]	7.6 ± 1.4[c]	0.40 ± 0.04[c]	0.11 ± 0.06[c]

[a] All values given in units per milligram of protein. [b] Methyl viologen oxidized enzyme (6 μM) was incubated with KCN (20 μM) in 50 mM phosphate buffer, pH 7.5, under anaerobic conditions for 25 min following which samples were removed and assayed as described under Materials and Methods. [c] Cyanide-inactivated and control enzyme samples were anaerobically dialyzed at 4 °C for 24 h against 50 mM phosphate buffer, pH 7.5, following which samples were removed and enzyme activity was determined as described under Materials and Methods.

The results of incubation of cyanide-treated FDH and cyanide-treated XO with sulfide, both in the presence and in the abscence of dithionite, are shown in Figure 3. While reactivation of FDH activity was not observed, significant reconstitution of XO activity was achieved following incubation with sulfide in the presence of dithionite.

The high-temperature EPR signal exhibited by partially reduced cyanide-treated FDH is shown in Figure 4A. Cyanide inactivation of the enzyme resulted in both a change in the line shape of the Mo(V) EPR signal and a shift to higher field when compared to that of native FDH. The rhombic signal, centered at g = 1.997, showed evidence of superhyperfine splitting to a single $s = {}^1/_2$ nucleus with coupling constants of the order of 0.8–1.1 mT. Similar line shapes were observed by using either formate or dithionite as reductant. Generation of this paramagnetic species using enzyme substituted with

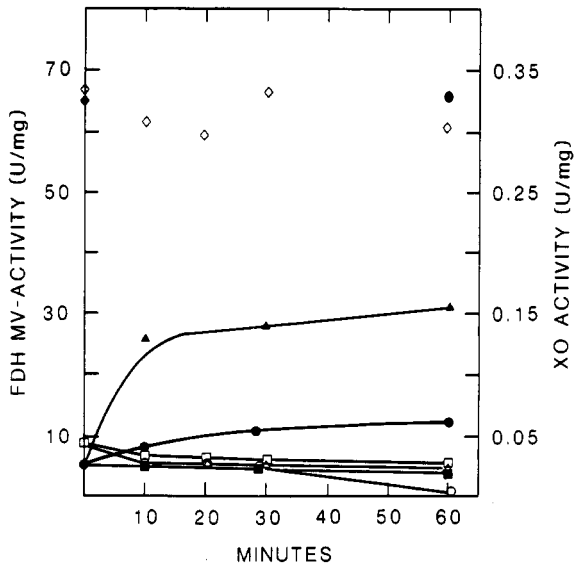

FIGURE 3: Time course of reactivation of cyanide-treated formate dehydrogenase and xanthine oxidase by sulfide. Cyanide-inactivated FDH (see Figure 2) was incubated in the presence of 1 mM dithionite (□), 1 mM Na_2S (○), and 1 mM dithionite plus 1 mM Na_2S (△) in 50 mM potassium phosphate buffer, pH 7.5, under anerobic conditions following which aliquots were removed and assayed for formate-dependent reduction of methyl viologen as described under Materials and Methods. Cyanide-inactivated XO (see Figure 2) was incubated in the presence of 1 mM dithionite (■), 1 mM Na_2S (●), and 1 mM dithionite plus 1 mM Na_2S (▲) under the same conditions as described for cyanide-inactivated FDH. Native FDH (◇) and XO (◆) were incubated under the same conditions with no additions.

^2H resulted in the spectrum shown in Figure 4C. Under these conditions, a simple rhombic line shape was observed without resolved superhyperfine interaction indicative of a single species. The results of computer simulations of this spectrum are shown in Figure 4D while the parameters obtained from the simulations are given in Table I. The simulated spectrum shown in Figure 4B was calculated by using the g values obtained for the ^2H-substituted CN species with the inclusion of superhyperfine coupling to a single, exchangeable proton. The cyanide signal could also be detected following reduction of *M. formicicum* cell-free extract in the presence of CN^- (Figure 4E), although with decreased resolution due to additional, overlapping, paramagnetic species. In contrast, the cyanide signal was not detected in samples of cell-free extract, poised within the potential range of −300 to −350 mV, obtained from *M. formicicum* cells cultured in the presence of WO_4^{2-}.

The low-temperature EPR spectrum obtained from a sample of purified FDH poised at −350 mV following treatment with cyanide is shown in Figure 5. The rhombic signal exhibited g values of $g_1 = 2.047$, $g_2 = 1.948$, and $g_3 = 1.911$ and showed little contamination by overlapping Mo(V) in the central portion of the spectrum.

The results of potentiometric redox titrations of *M. formicicum* cell-free extract in 50 mM Bicine buffer, containing 1 mM EDTA, pH 7.7, in the presence of cyanide are shown in Figure 6. At low potential (<−430 mV), the characteristic Mo(V) CN–FDH signal was absent. However, as the reduced enzyme was reoxidized, the Mo(V) EPR signal was observed to appear, reach a maximum amplitude at approximately −320 mV, and then progressively decrease at higher potentials, becoming absent above −200 mV. The line shape of the EPR signal was unchanged throughout the course of the titration, apart from contributions due to overlapping free radical species due to additional soluble components and the added viologen mediators. The titration curve was fully reversible in that similar behavior of the EPR signal was observed when titrating

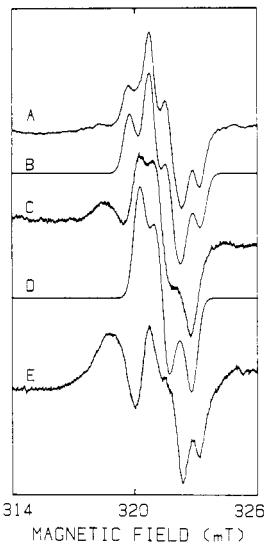

FIGURE 4: Electron paramagnetic resonance spectra of cyanide-treated formate dehydrogenase. (A) Purified FDH (7.3 μM Mo) in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, was incubated in the oxidized form with KCN (5 mM) for 10 min and then poised at −320 mV in the presence of non-viologen mediators (30 μM each mediator) prior to being frozen in liquid nitrogen. (B) Computer simulation of the experimental spectrum A using the parameters given in Table I. (C) FDH (4 μM Mo) was extensively dialyzed against Bicine buffer, using ^2H_2O as solvent, $pD_{app} = 7.78$, incubated with KCN (6 mM) in the oxidized state for 10 min, and then poised at −320 mV in the presence of non-viologen mediators (33 μM each mediator) and frozen in liquid nitrogen. (D) Computer simulation of the experimental spectrum C, using the parameters given in table I, modified to represent the substitution of ^2H for ^1H. (E) *M. formicicum* cell-free extract (13 mg/mL total protein) in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, was incubated with KCN (5 mM) for 10 min and then poised at −330 mV in the presence of non-viologen mediators (35 μM each mediator) prior to being frozen in liquid nitrogen. Spectra were recorded at 173 K and 0.32-mT modulation amplitude by using the following microwave powers: (A and B) 10 mW; (C) 50 mW. The magnetic field scale corresponds to a microwave frequency of 8.983 GHz.

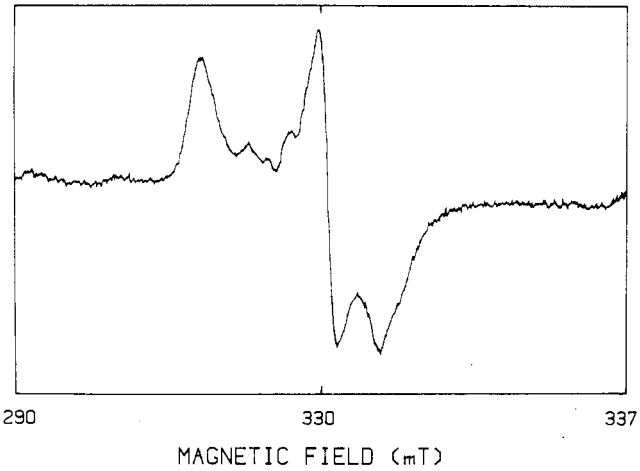

FIGURE 5: Low-temperature electron paramagnetic resonance spectrum of the Fe/S clusters in reduced, cyanide-treated, FDH. Formate dehydrogenase (15 μM Mo) in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, was incubated with KCN (5 mM) in the oxidized state for 10 min and then poised at −350 mV using dithionite in the presence of non-viologen mediator dyes (40 μM each mediator) prior to being frozen in liquid nitrogen. The spectrum was recorded at 15 K by using 20-mW microwave power and a modulation amplitude of 1 mT. The field scale corresponds to a microwave frequency of 9.025 GHz.

the cell-extract in a reductive manner. A similar titration curve was obtained for a sample of cyanide-treated cell-free extract from which excess cyanide was removed by pressure filtration.

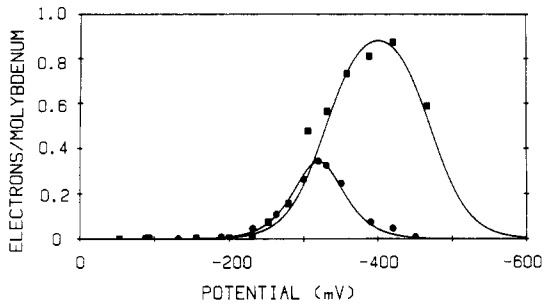

FIGURE 6: Changes in the Mo(V) electron paramagnetic resonance signal during potentiometric titrations of cyanide-treated *M. formicicum* cell-free extract. (●) Cell-free extract (13 mg/mL total protein) in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.7, was incubated with KCN (5 mM) in the oxidized state for 10 min following which mediators (viologen and non-viologen, 35 μM each mediator) were added, the potential was decreased to approximately −460 mV, and samples were removed in an oxidative phase. (■) Behavior of the native Mo(V) EPR signal exhibited by the cell-free extract obtained from *M. formicicum* cultured on $^{95}MoO_4^{2-}$ (Barber et al., 1983) is shown for comparison.

Table III: Oxidation–Reduction Midpoint Potentials for the Mo Center in Formate Dehydrogenase and Xanthine Oxidase

			potential[a]	
enzyme	buffer	pH	Mo(VI)/ Mo(V)	Mo(V)/ Mo(IV)
FDH[b]	Bicine	7.7	−330	−470
CN–FDH	Bicine	7.7	−319	−321
XO[c]	Bicine	7.7	−373	−377
CN–XO[c]	Bicine	7.7	−392	−418

[a] Potentials are given in millivolts relative to the standard hydrogen electrode. [b] Barber et al. (1983). [c] Barber & Siegel (1982).

Potentiometric titration of the native cell-free extract performed in the absence of cyanide showed no evidence for the formation of the cyanide–Mo(V) EPR signal over the potential range −460 to −200 mV. Double integration of a sample of purified FDH, treated with cyanide under anaerobic, oxidized conditions and poised at −320 mV, indicated 35% conversion of the total Mo to the quinquivalent state. In contrast to the spectra obtained with the cell-free extract in the presence of cyanide, at this potential the purified enzyme showed no additional high-temperature paramagnetic species such as that due to a flavin free radical. The titration data points could be readily fit to the Nernst equation, modified to accommodate two, consecutive, one-electron redox processes, corresponding to the scheme:

$$\text{Mo(VI)} \rightarrow \text{Mo(V)} \rightarrow \text{Mo(IV)}$$

with midpoint potentials E_1 and E_2. Computer fitting of the theoretical titration curve using least-squares analysis yielded values of −319 mV for the Mo(VI)/Mo(V) couple (E_1) and −321 mV for the Mo(V)/Mo(IV) couple (E_2), respectively. The corresponding data for the native system, using ^{95}Mo-substituted cell-free extract (Barber et al., 1983) under similar conditions in the absence of CN^-, are shown for comparison. Midpoint potentials calculated for these data points are given in Table III.

Discussion

Previous work, using EPR spectroscopy coupled with ^{95}Mo substitution (Barber et al., 1983), has clearly shown that FDH from *M. formicicum* is a Mo-containing enzyme. Under suitable conditions of oxidation–reduction using either the physiological reducing substrate formate or artificial electron donors, such as dithionite, the Mo center is capable of existing in the intermediate redox state Mo(V) and exhibiting significantly different g values (g_{av} = 2.008) from those previously encountered with other molybdenum hydroxylase enzymes (g_{av} = 1.982–1.964).[2] The EPR spectra of purified FDH containing ^{2}H substituted for ^{1}H have faciliated improved simulation accuracy and the confirmation that the spectrum from the native enzyme includes superhyperfine interaction with two, equivalent, exchangeable protons. The superhyperfine coupling constants for these protons are nearly isotropic (A_{av} = 0.5 mT) and are intermediate in the range so far encountered for Mo-containing enzymes, the maximum and minimum values occurring for the desulfo form of XO (A_{av} = 1.63 and 0.16 mT, respectively) (Bray, 1980). In contrast to XO, native FDH appears to yield comparable Mo(V) EPR spectra (i.e., exhibiting superhyperfine interaction with two, strongly coupled protons) following partial reduction with either the physiological substrate, formate, or artificial electron donors, such as dithionite. The absence of altered spectra in the presence of substrate or product would suggest that FDH does not form spectroscopically distinct complexes with these compounds.

The effects of aerobic cyanide inactivation have been extensively described for the oxidized Mo-containing enzymes, xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase. For XO, cyanide treatment results in the generation of a modified form of the protein, referred to as the "desulfo" species. Both chemical (Massey & Edmondson, 1970) and EXAFS (Bordas et al., 1980) studies have shown that cyanide treatment results in the extraction of a terminal sulfur ligand of the Mo and release as thiocyanate. The alteration in the structure of the Mo center is reflected in an inability of the desulfo enzyme to be reduced by substrate and changes in both the Mo(V) EPR spectra and the oxidation–reduction midpoint potentials. No effects of cyanide treatment have been observed for any of the other redox cofactors of the enzyme.

Incubation of the purified, oxidized, native FDH with cyanide resulted in the irreversible inhibition of catalytic activity. On the basis of a molecular weight of 177 000 for FDH (Schauer & Ferry, 1986), a ratio of 1.4 thiocyanates per cyanide-inactivated enzyme molecule was obtained. However, the *fdh* gene has recently been shown to encode a protein with a calculated molecular weight of 119 652 (Schuber et al., 1986), suggesting that 0.9 thiocyanate was released per enzyme molecule. Analogous experiments carried out using XO showed the liberation of 1.10 and 1.14 thiocyanates per active molybdenum center. The detection of stoichiometric amounts of thiocyanate liberated from cyanide-treated FDH indicates that the enzyme possesses one terminal sulfur ligand to Mo similar to that found for the xanthine-oxidizing molybdenum hydroxylases. However, in contrast to desulfo-XO, we have been unable to reactivate cyanide-treated FDH.

Inactivation of FDH by cyanide altered both the spectroscopic and thermodynamic properties of the Mo center. The perturbation of the Mo(V) spectrum occurs in terms of both a shift in the g values and a reduction of the complexity of the superhyperfine splittings and their magnitude. Overall, the cyanide spectrum is shifted to higher field, yielding a g_{av} of 1.997, a value still significantly higher than that encountered for other Mo-containing enzymes but below the value of the free electron. In addition, the Mo(V)–cyanide species could

[2] The g_{av} values of other molybdenum hydroxylase Mo(V) species have been found to lie within a range with a maximum value of 1.982, determined for the "low-pH" species of dissimilatory nitrate reductase (Vincent & Bray, 1978), and a minimum value of 1.964, determined for the "nitrate complex" of desulfoxanthine oxidase (Gutteridge et al., 1978b).

be generated by using the substrate formate, indicating that cyanide modification of the enzymes does not preclude Mo reduction.

The inability to detect the cyanide–Mo(V) spectrum in the native *M. formicicum* cell-free extract (compare Figures 1E and 4E) may indicate that the modified form of the enzyme is not a naturally occurring degradation product or that isolation conditions, such as strict anaerobiosis and the inclusion of thiols in the media utilized, may effectively prevent conversion to the inactive species.

The low-temperature EPR spectrum of the Fe/S clusters of cyanide-treated FDH yielded the same line shape and g values as the native enzyme, indicating an absence of any spectroscopically detectable effects of cyanide modification on these prosthetic groups.

Cyanide modification of FDH resulted in perturbation of the oxidation–reduction properties of the Mo center. Comparison with the data obtained for the native cell-free extract reveals that these alterations are primarily confined to the Mo(V)/Mo(IV) couple. Thus, cyanide treatment increases the midpoint potential of the Mo(V)/Mo(IV) couple which results in a destabilization of the Mo(V) species and a decreased integrated intensity. We may postulate that in the case of FDH, the observed catalytic inhibition by cyanide results from an inability of the partially reduced enzyme to transfer reducing equivalents from the Mo center to successive redox components, such as flavin or Fe/S. In contrast, cyanide treatment of XO results in a decrease of both the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) redox couples (Barber & Siegel, 1982) and a corresponding inability to be reduced by the physiological substrate, xanthine.

By analogy with xanthine oxidase, we suggest that the form of the enzyme generated following exposure to cyanide in the oxidized state be referred to as "desulfo"-formate dehydrogenase.

Registry No. FDH, 9028-85-7; XO, 9002-17-9; CN^-, 57-12-5; S, 7704-34-9; SCH^-, 302-04-5; Mo, 7439-98-7.

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