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Formylmethanofuran dehydrogenases from methanogenic Archaea

Substrate specificity, EPR properties and reversible inactivation by cyanide of the molybdenum or tungsten iron-sulfur proteins

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Formylmethanofuran dehydrogenases, which are found in methanogenic Archaea, are molybdenum or tungsten iron-sulfur proteins containing a pterin cofactor. We report here on differences in substrate specificity, EPR properties and susceptibility towards cyanide inactivation of the enzymes from *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum* and *Methanobacterium wolfei*.

The molybdenum enzyme from *M. barkeri* (relative activity with *N*-formylmethanofuran = 100%) was found to catalyze, albeit at considerably reduced apparent V_{max}, the dehydrogenation of *N*-furfurylformamide (11%), *N*-methylformamide (0.2%), formamide (0.1%) and formate (1%). The molybdenum enzyme from *M. wolfei* could only use *N*-furfurylformamide (1%) and formate (3%) as pseudosubstrates. The molybdenum enzyme from *M. thermoautotrophicum* and the tungsten enzymes from *M. thermoautotrophicum* and *M. wolfei* were specific for *N*-formylmethanofuran.

The molybdenum formylmethanofuran dehydrogenases exhibited at 77 K two rhombic EPR signals, designated FMD_{red} and FMD_{ox}, both derived from Mo as shown by isotopic substitution with ^{97}Mo. The FMD_{red} signal was only displayed by the active enzyme in the reduced form and was lost upon enzyme oxidation; the FMD_{ox} signal was displayed by an inactive form and was not quenched by O_2. The tungsten isoenzymes were EPR silent.

The molybdenum formylmethanofuran dehydrogenases were found to be inactivated by cyanide whereas the tungsten isoenzymes, under the same conditions, were not inactivated. Inactivation was associated with a characteristic change in the molybdenum-derived EPR signal. Reactivation was possible in the presence of sulfide.

Formylmethanofuran dehydrogenase catalyzes the reversible dehydrogenation of *N*-formylmethanofuran to CO_2 and methanofuran (reaction a), which is a 4-[*N*-(4,5,7-tricarboxyheptanoyl-γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan (DiMarco et al., 1990).

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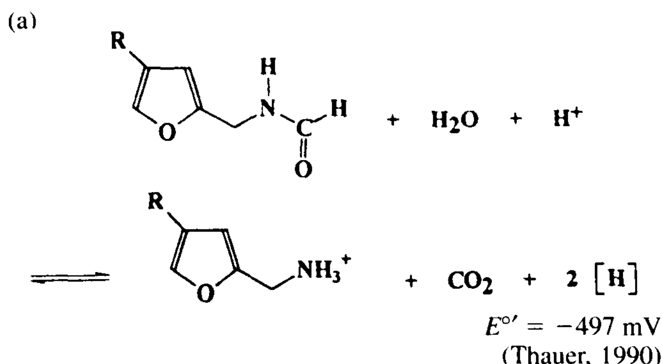
Abbreviations. FMD_{red}, EPR signal attributed to active formylmethanofuran dehydrogenase; FMD_{ox}, EPR signal attributed to inactive formylmethanofuran dehydrogenase; g, Landé splitting factor; g_x, g_y and g_z, Landé splitting factors in the x, y and z directions for anisotropic systems; 1 U = 1 µmol formylmethanofuran dehydrogenated/min.

Enzyme. Formylmethanofuran dehydrogenase (EC 1.2.99.–).

(a)



$E^{\circ\prime} = -497$ mV
(Thauer, 1990)

The physiological electron acceptor/donor is not known. Methylviologen ($E^{\circ\prime} = -446$ mV) can be used as artificial electron acceptor (Börner et al., 1989) and titanium(III)citrate

($E^{o\prime} = -480$ mV; Zehnder and Wuhrmann, 1976) as an artificial electron donor (Bobik and Wolfe, 1989).

The enzyme is found in methanogenic Archaea and in *Archaeoglobus fulgidus*. In the methanogens it is involved in CO_2 reduction to methane and in oxidation of methanol and methylamines to CO_2 (Wolfe, 1991; Ferry, 1993). In the archaeal sulfate reducer it participates in lactate oxidation to 3 CO_2 (Thauer and Kunow, 1993).

Formylmethanofuran dehydrogenases have first been purified and characterized from the following Archaea grown on media supplemented with molybdate: *Methanosarcina barkeri* (Karrasch et al., 1990), *Methanobacterium thermoautotrophicum* (Börner et al., 1991), *Methanobacterium wolfei* (Schmitz et al., 1992c), and *A. fulgidus* (Schmitz et al., 1991). The enzymes were all found to be multisubunit molybdenum iron-sulfur proteins containing molybdopterin guanine dinucleotide as cofactor. The enzyme from *M. thermoautotrophicum* contained in addition molybdopterin adenine dinucleotide and molybdopterin hypoxanthine dinucleotide (Börner et al., 1991).

Recently it was found that *M. thermoautotrophicum* and *M. wolfei* can grow equally well on media supplemented with either tungstate or molybdate (Schmitz et al., 1992a; Bertram et al., 1994). From tungstate-grown cells of both organisms a formylmethanofuran dehydrogenase was isolated which contained tungsten, iron-sulfur centers and molybdopterin guanine dinucleotide and which was shown to be an isoenzyme of the respective molybdenum iron-sulfur protein. Molybdenum-grown *M. thermoautotrophicum* additionally contained the tungsten isoenzyme in which the tungsten was substituted by molybdenum, and tungsten-grown *M. wolfei* the molybdenum isoenzyme in which the molybdenum was substituted by tungsten. Both substituted enzymes were active (Schmitz et al., 1992b; Bertram et al., 1994).

Formylmethanofuran dehydrogenases have been individually reported to use *N*-furfurylformamide as pseudosubstrate (Breitung et al., 1990), to exhibit molybdenum-derived EPR spectra (Schmitz et al., 1992c) and to be inactivated by cyanide (Börner et al., 1989). Comprehensive and detailed investigations of the substrate specificity, the EPR properties and the effect of cyanide, however, were thus far lacking.

MATERIALS AND METHODS

N-Formylmethanofuran and *N*-furfurylformamide were prepared as described by Breitung et al. (1990). *N*-Methylformamide, formamide and sodium formate were obtained from Merck (Darmstadt). Sodium [^{14}C]cyanide (55.6 mCi/mmol) was from Amersham Buchler. Sodium molybdate and sodium tungstate were from Fluka. ^{97}Mo (92.9% enriched) was from Intersales-Holland. (^{97}Mo)Molybdate was prepared by dissolving 48.5 mg of the metal powder in 5 ml 98% H_2SO_4 at room temperature to give a 100 mM stock solution. Sephadex G-25 was from Pharmacia.

M. barkeri strain Fusaro (DSM 804), *M. thermoautotrophicum* strain Marburg (DSM 2133) and *M. wolfei* (DSM 2970) were obtained from the *Deutsche Sammlung von Mikroorganismen* (Braunschweig, Germany). Formylmethanofuran dehydrogenases were purified from these organisms and their specific activities were assayed as previously described (Karrasch et al., 1990; Börner et al., 1991; Schmitz et al., 1992a and c; Bertram et al., 1994). ^{97}Mo-enriched formylmethanofuran dehydrogenases were purified from cells cultured and grown on media containing (^{97}Mo)molybdate (2 μM). Where indicated, the enzymes were isolated from H_2-reduced cells. This refers to cells from cultures sparged with 100% H_2 for 10 min before cooling to 4°C and harvesting. When this sparging with H_2 was omitted, the cells were in an oxidized state because during the cooling period they continued to produce CH_4 from CO_2 present in the culture. These cells are referred to as CO_2-oxidized cells.

EPR spectra were recorded at 77 K using a Varian E-3 EPR spectrometer equipped with a Narda Microline Model 12X1 frequency meter and a Bruker ER035 M NMR gaussmeter. 2,2-Diphenyl-1-picrylhydrazyl (isotropic Landé splitting factor, $g_{iso} = 2.0036$) was used as standard. The EPR spectrometer was interfaced to a personal computer for further off-line data processing (Albracht et al., 1988). Spin concentrations were determined using a solution of 10 mM $CuSO_4 \cdot 5\,H_2O$, 2 M $NaClO_4 \cdot H_2O$ and 10 mM HCl as a reference.

RESULTS

The following experiments were performed with purified formylmethanofuran dehydrogenases from *M. barkeri*, *M. thermoautotrophicum* and *M. wolfei*. In the case of the two *Methanobacterium* species both the molybdenum and the tungsten isoenzyme were investigated. Where indicated, the molybdenum-substituted tungsten isoenzyme from *M. thermoautotrophicum* and the tungsten-substituted molybdenum isoenzyme from *M. wolfei* were also analyzed.

Substrate specificity

The molybdenum enzyme from *M. barkeri* (Breitung et al., 1990) and the molybdenum isoenzyme from *M. wolfei* (Schmitz et al., 1992c) have been reported to catalyze slowly the dehydrogenation of *N*-furfurylformamide. We therefore investigated whether the other formylmethanofuran dehydrogenases can also use this pseudosubstrate and whether other pseudosubstrates can be found. *N*-Furfurylformamide, *N*-methylformamide, formamide and formate were tested. The results are summarized in Table 1.

The molybdenum enzyme from *M. barkeri* was found to catalyze the dehydrogenation of *N*-furfurylformamide, *N*-methylformamide, formamide and formate. The catalytic efficiency (V_{max}/K_m) of the enzyme was, however, much lower (10^5-10^8-fold) with these pseudosubstrates than with *N*-formylmethanofuran. Interestingly, the catalytic efficiency with formate was more than 10-times higher than with formamide. The molybdenum isoenzyme from *M. wolfei* was able to mediate the dehydrogenation of *N*-furfurylformamide and formate but not of *N*-methylformamide and formamide. The molybdenum and tungsten isoenzymes from *M. thermoautotrophicum* and the tungsten isoenzyme from *M. wolfei* proved to be highly specific for *N*-formylmethanofuran; they showed no activity (<0.01 U/mg) with any of the pseudosubstrates in the concentration range tested between 1 mM and 2 M (Table 1).

The finding that formylmethanofuran dehydrogenase from *M. barkeri* can slowly catalyze the dehydrogenation of formate can explain the observation that cell extracts of *M. barkeri* exhibit formate dehydrogenase activity (Mazumder et al., 1985; Bhosale et al., 1989) although this organism can not use formate as a methanogenic substrate.

EPR properties

The molybdenum formylmethanofuran dehydrogenase from *M. thermoautotrophicum*, when isolated from H_2-re-

Table 1. Substrate specificity of formylmethanofuran dehydrogenases from methanogenic Archaea. n.d., not determined.

Enzyme	Substrates used	apparent K_m	apparent V_{max}	V_{max}/K_m
		mM	U/mg	U mg^{-1} mM^{-1}
Mo enzyme from *M. barkeri*	*N*-formylmethanofuran[a]	0.02	175	8800
	N-furfurylformamide[a]	200	20	0.1
	N-methylformamide	5000	0.4	0.00008
	formamide	3000	0.2	0.00007
	formate	1700	1.8	0.001
Mo enzyme from *M. thermoautotrophicum*	*N*-formylmethanofuran	0.03	70	2300
W enzyme from *M. thermoautotrophicum*	*N*-formylmethanofuran	0.03	15	500
Mo-containing W enzyme from *M. thermoautotrophicum*	*N*-formylmethanofuran	0.03	n.d.	n.d.
Mo enzyme from *M. wolfei*	*N*-formylmethanofuran[b]	0.013	37	2800
	N-furfurylformamide[b]	53	0.3	0.006
	formate[b]	35	1.2	0.034
W enzyme from *M. wolfei*	*N*-formylmethanofuran	0.013	11	850
W-containing Mo enzyme from *M. wolfei*	*N*-formylmethanofuran[c]	0.013	27	2100
	N-furfurylformamide[c]	1250	0.1	0.00008
	formate	1100	0.2	0.0002

[a] Data from Breitung et al. (1990).
[b] Data from Schmitz et al. (1992c).
[c] Data from Schmitz et al. (1992b).

duced cells, exhibited a complex EPR spectrum (Fig. 1). Upon oxidation with O_2 the signals were partly quenched. The remaining signal was rhombic with $g_x = 2.004$, $g_y = 1.989$ and $g_z = 1.943$ (Fig. 1). This signal, designated FMD_{ox}, is attributed to an inactive enzyme form since it was exhibited by completely inactive enzyme preparations. Subtraction of the FMD_{ox} signal from the complex spectrum yielded a rhombic signal with $g_x = 2.005$, $g_y = 1.982$ and $g_z = 1.948$ (Fig. 1). This signal, designated FMD_{red}, is attributed to the active enzyme since it was only exhibited by active enzyme. Both the FMD_{red} and FMD_{ox} signals were derived from molybdenum as shown by isotopic substitution with ^{97}Mo: the EPR spectrum of the ^{97}Mo-substituted enzyme (Fig. 1) was very similar to the computed line shape for a 3:1 mixture of the FMD_{red} and FMD_{ox} signals both with hyperfine splitting due to ^{97}Mo (Fig. 1). EPR signals derived from the iron-sulfur center(s) present in the enzyme were not observed at 77 K, the temperature at which the EPR properties were determined. When the molybdenum isoenzyme from *M. thermoautotrophicum* was isolated from CO_2-oxidized cells, the purified enzyme exhibited only the FMD_{ox} signal. Upon reduction with *N*-formylmethanofuran the FMD_{red} signal was induced without altering the FMD_{ox} signal.

The EPR properties of the molybdenum enzymes from *M. barkeri* and *M. wolfei* were very similar to those described for the molybdenum enzyme from *M. thermoautotrophicum*. The enzymes from the two methanogens also exhibited both an FMD_{red} and an FMD_{ox} signal (Table 2).

The relative and absolute intensities of the FMD_{red} and FMD_{ox} signals exhibited by the purified molybdenum enzymes varied from preparation to preparation and changed with time of storage. The spin concentrations (mol/mol enzyme) were between 5% and 10%. The FMD_{red} signal intensity was highest in preparations with high specific activity and decreased upon loss of enzyme activity. The FMD_{ox} signal was found both in active and in inactive preparations but not all inactive preparations showed this signal. During inactivation the FMD_{ox} signal sometimes increased but this was not always the case.

The tungsten isoenzymes from *M. thermoautotrophicum* and from *M. wolfei* were EPR silent at 77 K. Attempts to induce a tungsten-derived signal by reduction or oxidation failed. However, the tungsten substituted molybdenum isoenzyme from *M. wolfei* exhibited after oxidation an EPR signal which was clearly derived from tungsten as indicated by characteristic hyperfine splitting (Schmitz et al., 1992b). This indicates that the redox potentials of the W(VI)/(V) couple and of the W(V)/(IV) couple in the tungsten-substituted molybdenum isoenzyme are less negative and further apart than in the tungsten isoenzymes.

Inactivation by cyanide and reactivation

The molybdenum enzymes from *M. barkeri*, *M. thermoautotrophicum* and *M. wolfei* have been reported to be inactivated by cyanide (Börner et al., 1989; Schmitz et al., 1992c). We determined the rate of cyanide inactivation and whether the cyanide-inactivated enzymes can be reactivated in the presence of sulfide, as this has been described for xanthine oxidase which is also a molybdenum protein containing a pterin cofactor (Wahl and Rajagopalan, 1982; Wahl et al., 1984). The inactivated and reactivated enzymes were analyzed by EPR spectroscopy.

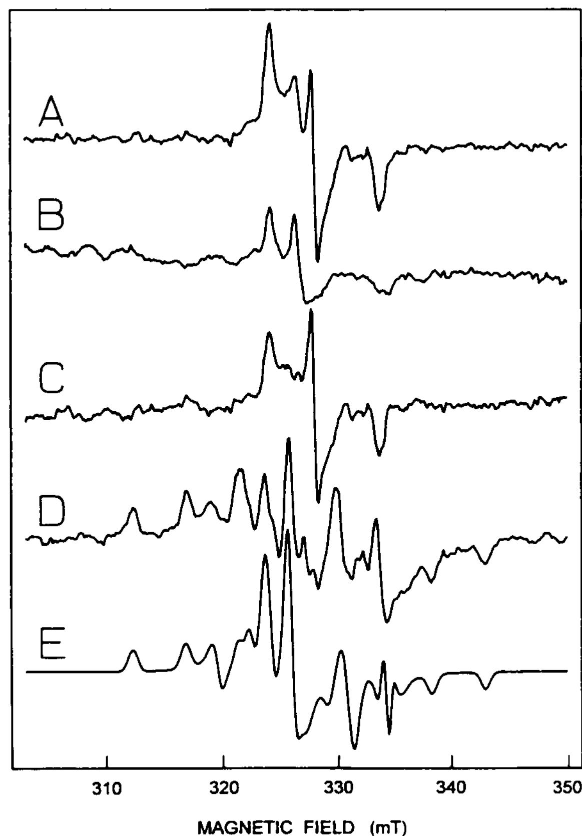


Fig. 1. EPR spectra of molybdenum formylmethanofuran dehydrogenase from *M. thermoautotrophicum*. Enzyme concentration was in the range 40–80 μM. (A) Complex spectrum of purified active enzyme as isolated from H_2-reduced cells. (B) FMD_{ox} signal exhibited by purified enzyme after inactivation by air at 20°C for 30 min. (C) FMD_{red} signal obtained by suitable subtraction of trace B from trace A. (D) EPR spectrum of purified active enzyme enriched in ^{97}Mo (75%). (E) Computer simulation of the spectrum shown in trace D assuming it to be composed of 75% FMD_{red} signal and 25% FMD_{ox} signal. FMD_{red} signal: linewidths (xyz) = 0.37 mT, 0.21 mT, 0.24 mT; coupling constants A_{xyz} (^{97}Mo) = 4.4 mT, 0.5 mT, 4.4 mT; FMD_{ox} signal: linewidths (xyz) = 0.32 mT, 0.27 mT, 0.50 mT; coupling constants A_{xyz} (^{97}Mo) = 0.9 mT, 2.1 mT, 0.01 mT. The *g* values of the EPR signals are given in Table 2. Instrument settings were as follows: microwave frequency, 9100 MHz; modulation frequency, 100 kHz; modulation amplitude, 0.16 mT; microwave power incident to the cavity, 10 mW; temperature, 77 K.

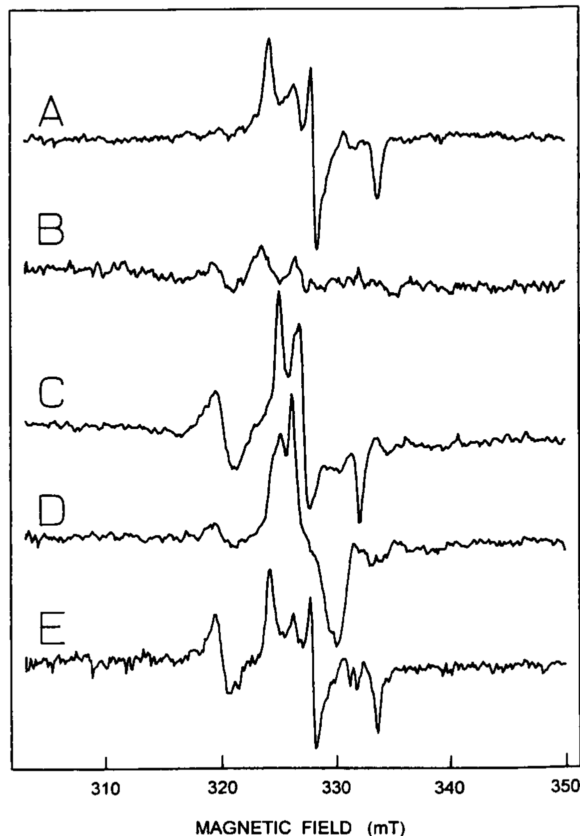


Fig. 2. EPR spectra of molybdenum formylmethanofuran dehydrogenase from *M. thermoautotrophicum* before and after inactivation by cyanide and after reactivation. Enzyme concentration was in the range 30–70 μM. (A) Complex spectrum of purified active enzyme as isolated from H_2-reduced cells. (B) EPR spectrum of purified enzyme after inactivation by 5 mM KCN at 40°C for 20 min. (C) EPR spectrum exhibited by purified enzyme after inactivation by 5 mM KCN at 0°C for 7 h followed by reduction with 2 mM $Na_2S_2O_4$. (D) EPR spectrum exhibited by purified enzyme after inactivation by 5 mM KCN at 40°C for 20 min followed by reduction with 2 mM $Na_2S_2O_4$. (E) EPR spectrum exhibited by purified enzyme first inactivated as described for trace B then reactivated by incubation in the presence of 10 mM Na_2S and 10 mM $Na_2S_2O_4$ at 40°C for 60 min. Prior to reactivation, cyanide was removed by gel filtration on Sephadex G-25. The *g* values of the EPR signals are given in the text. For instrument settings see Fig. 1.

Table 2. *g* values of the EPR signals FMD_{red} and FMD_{ox} exhibited by molybdenum formylmethanofuran dehydrogenases from methanogenic Archaea.

EPR signal	Enzyme source	g_x	g_y	g_z
FMD_{red}	*M. barkeri*	2.005	1.979	1.946
	M. thermoautotrophicum	2.005	1.982	1.948
	M. wolfei[a]	2.003	1.989	1.955
FMD_{ox}	*M. barkeri*	2.006	1.989	1.946
	M. thermoautotrophicum	2.004	1.989	1.943
	M. wolfei[a]	2.000	1.984	1.941

[a] Data from Schmitz et al. (1992c).

Among the formylmethanofuran dehydrogenases investigated, the molybdenum enzyme from *M. barkeri* was the most susceptible to cyanide inactivation (Table 3). The molybdenum isoenzyme from *M. thermoautotrophicum* was less susceptible than that from *M. wolfei*. An effect of cyanide on the activity of the tungsten enzymes was not observed. Interestingly, the molybdenum substituted tungsten isoenzyme from *M. thermoautotrophicum* was inactivated by cyanide.

The following reactivation experiments were performed only with cyanide-inactivated formylmethanofuran dehydrogenase from *M. thermoautotrophicum*. The isoenzyme was found to be reactivated by incubation in the presence of Na_2S under reducing conditions: Purified molybdenum isoenzyme (23 U, 2.9 mg protein) was inactivated by potassium cyanide (5 mM) for 20 min at 40°C in 50 mM Tricine/KOH, pH 8.0. Most of the cyanide was then removed by gel filtration on Sephadex G-25. The inactivated enzyme thus obtained,

Table 3. Susceptibility of formylmethanofuran dehydrogenase from methanogenic Archaea to inactivation by cyanide.

Formylmethanofuran dehydrogenase	Source	Inactivation by cyanide		
		$t_{1/2}$	[KCN]	temperature
		min	mM	°C
Mo enzyme	*M. barkeri*	0.1	0.1	37
	M. thermoautotrophicum	2	5	40
	M. wolfei	1	0.1	20
Mo-containing W enzyme	*M. thermoautotrophicum*	4	5	40
W enzyme	*M. thermoautotrophicum*	∞	5	40
	M. wolfei	∞	1	20

which had 2% of the activity of the control, was then incubated in the presence of 10 mM Na_2S and 10 mM $Na_2S_2O_4$ for 60 min at 40°C and pH 8.0. After this treatment the inactivated enzyme had regained approximately 50% of its initial activity.

Inactivation by cyanide and reactivation was accompanied by characteristic changes in EPR signals. After incubation of the purified molybdenum isoenzyme from *M. thermoautotrophicum* with potassium cyanide (5 mM) for 20 min at 40°C the FMD_{red} signal exhibited before cyanide addition (Fig. 2) was completely lost and the intensity of the FMD_{ox} signal was reduced (Fig. 2). Upon reduction of the inactivated enzyme with 2 mM $Na_2S_2O_4$ a novel rhombic EPR signal was induced with $g_x = 1.998$, $g_y = 1.986$ and $g_z = 1.968$ (Fig. 2). This signal was derived from molybdenum as indicated by isotopic substitution of the enzyme with ^{97}Mo (data not shown). The enzyme exhibiting the novel signal was catalytically inactive. After reactivation of the inactive enzyme in the presence of 10 mM Na_2S and 10 mM $Na_2S_2O_4$ as described above, the main features of the EPR spectrum of the native enzyme (Fig. 2) were restored (Fig. 2).

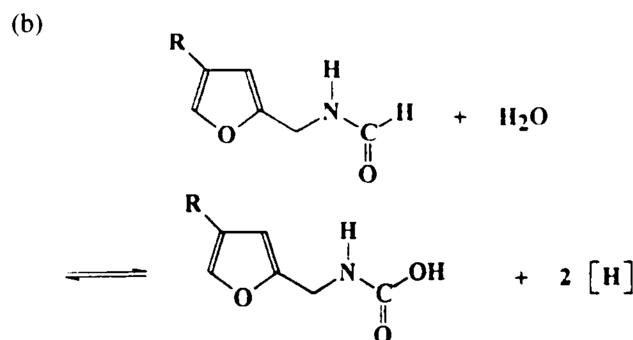
When the molybdenum isoenzyme from *M. thermoautotrophicum* was inactivated by potassium cyanide (5 mM) at 0°C rather than at 40°C, the FMD_{red} signal of the native enzyme (Fig. 2) was also completely quenched. Upon reduction with 2 mM $Na_2S_2O_4$, however, an EPR signal distinct from that observed after inactivation at 40°C was induced with $g_x = 1.996$, $g_y = 1.982$ and $g_z = 1.953$ (Fig. 2). The enzyme exhibiting this signal was also catalytically inactive.

Inactivation of xanthine oxidase and of several other molybdenum enzymes by cyanide is associated with the stoichiometric formation of thiocyanate thought to be generated from a terminal sulfur ligand of molybdenum (Coughlan et al., 1980; Wahl and Rajagopalan, 1982). We therefore determined with formylmethanofuran dehydrogenase from *M. barkeri* whether the molybdenum formylmethanofuran dehydrogenases also contain cyanolyzable sulfur. The enzyme (2.9 mg) in 0.1 M sodium pyrophosphate, pH 8.5, was incubated anaerobically for 2 h at room temperature in the presence of 0.8 mM potassium [^{14}C]cyanide (55.6 mCi/mmol). The products formed were separated from protein and from excess cyanide by gel filtration on Sephadex G-25. The elution profile indicated that thiocyanate had been generated, albeit in substoichiometric amounts. In control experiments with ferredoxin from *M. barkeri* also small amounts of thiocyanate were found although this iron-sulfur protein is devoid of molybdenum. It therefore remains uncertain whether the thiocyanate generated during inactivation of formylmethanofuran dehydrogenase from *M. barkeri* was derived from a terminal sulfur ligand of molybdenum or from an iron-sulfur center of this enzyme. The formylmethanofuran dehydrogenases from the other methanogens were not tested for the formation of thiocyanate after cyanide treatment.

DISCUSSION

Evidence was presented that formylmethanofuran dehydrogenase from *M. barkeri* can catalyze the dehydrogenation of formamide. This finding convincingly demonstrates that *N*-formylmethanofuran can be considered as an *N*-substituted formamide. The higher K_m for formamide and the lower V_{max} (Table 1) indicate that the substituent in formylmethanofuran is required both for effective and correct substrate binding to the enzyme. The finding that two of the investigated formylmethanofuran dehydrogenases can catalyze the dehydrogenation of formate indicates that the amide bond is not a prerequisite for reactivity. This appears to exclude the possibility that the formyl group of *N*-formylmethanofuran is transferred prior to oxidation to an amino or hydroxyl group of the enzyme, since such a transfer starting from free formate is thermodynamically unfavorable. Rather, it suggests that the formyl group is dehydrogenated while still bound to the primary amino group of methanofuran yielding *N*-carboxymethanofuran as product (reaction b) which should break down non-enzymically to CO_2 and methanofuran (Ewing et al., 1980):

(b)



Reaction (b) indicates that formylmethanofuran dehydrogenase belongs to the group of molybdenum enzymes that catalyze an insertion of an oxygen atom derived from H_2O into a C-H bond (Pilato and Stiefel, 1993). Enzymes belonging to this group are xanthine dehydrogenases and xanthine oxidases (Bray, 1988; Wootton et al., 1991), molybdenum-containing formate dehydrogenases (Adams and Mortenson,

1985; Barber et al., 1986; Friedebold and Bowien, 1993), formate-ester dehydrogenase (van Ophem et al., 1992), aldehyde oxidase (Branzoli and Massey, 1974), aldehyde dehydrogenase (Poels et al., 1987), aldehyde oxidoreductase (White et al., 1993), nicotine dehydrogenase (Freudenberg et al., 1988), nicotinate dehydrogenase and 6-hydroxynicotinate dehydrogenase (Nagel and Andreesen, 1990), isonicotinate dehydrogenase and 2-hydroxyisonicotinate dehydrogenase (Kretzer and Andreesen, 1991), quinoline oxidoreductase (Hettrich et al., 1991), quinoline-4-carboxylic acid oxidoreductase (Bauer and Lingens, 1992), quinaldine oxidoreductase (de Beyer and Lingens, 1993), quinaldic acid 4-oxidoreductase (Fetzner and Lingens, 1993), picolinate dehydrogenase (Siegmund et al., 1990), 2-furoyl-coenzyme A dehydrogenase (Koenig and Andreesen, 1990), and pyrimidine oxidase and pyridoxal oxidase (Burgmayer and Stiefel, 1985). Interestingly, one of these enzymes, milk xanthine oxidase, can even catalyze the dehydrogenation of formamide to carbamic acid (Morpeth et al., 1984) which is a reaction also catalyzed by formylmethanofuran dehydrogenase.

Some of the molybdenum enzymes mentioned above have been shown to be reversibly inactivated in the oxidized state by cyanide, with the concomitant generation of thiocyanate and a desulfo enzyme (Massey and Edmondson, 1970; Cramer et al., 1981; Wahl and Rajagopalan, 1982; Wahl et al., 1984; Barber et al., 1986; Nagel and Andreesen, 1990; Fetzner and Lingens, 1993). This feature was also exhibited by the molybdenum formylmethanofuran dehydrogenases. The formation of a desulfo formylmethanofuran dehydrogenase was indicated by the characteristic decrease in the average g value of the EPR signal derived from molybdenum upon inactivation of the enzyme with cyanide followed by reduction with dithionite (Fig. 2). This decrease in the average g value is considered to reflect the replacement of a molybdenum-bound sulfur by oxygen (Bray, 1980; Chang et al., 1990). Different EPR signals were obtained when the desulfo form was generated at 0°C and at 40°C (Fig. 2). Several different desulfo signals have also been obtained from other molybdenum enzymes (Bray, 1980).

The molybdenum enzymes catalyzing the insertion of an oxygen derived from H_2O into a C-H bond undergo molybdenum redox changes during catalysis involving the Mo(VI), Mo(V) and Mo(IV) oxidation states (Pilato and Stiefel, 1993). These enzymes also share the ability to reversibly catalyze half reactions with redox potentials ($E^{\circ\prime}$), probably all more negative than −300 mV. Hence, the redox potential of the uric acid/xanthine couple is −360 mV, that of the CO_2/formate couple is −430 mV, and that of the carboxylic acid/aldehyde couple is −580 mV. The redox potentials of the Mo(VI)/Mo(V) couple and of the Mo(V)/Mo(IV) couple in some of the enzymes have both been shown to be close to −350 mV (Rajagopalan et al., 1990). Upon reduction with substrate or with dithionite the enzymes all exhibit rhombic EPR signals derived from Mo(V) with the average g value generally being below 2.0. The spin concentration of these EPR signals is well below 100% since the redox potentials of the Mo(VI)/Mo(V) couple and of the Mo(V)/Mo(IV) couple are not very far apart. These features are also shared by formylmethanofuran dehydrogenases. Hence, it is concluded that the coordination of molybdenum in formylmethanofuran dehydrogenases is probably similar to that in xanthine oxidase and in the other molybdenum enzymes of this group.

The physiological electron acceptor of the formylmethanofuran dehydrogenases is not yet known. It can be predicted, however, that its redox potential must be near that of the CO_2 + methanofuran/formylmethanofuran couple of −497 mV, since *in vivo* the enzyme functions in both the direction of CO_2 reduction and the direction of formylmethanofuran oxidation. It can also be predicted that the enzymes use a one-electron acceptor rather than a two-electron acceptor since they contain iron-sulfur centers but lack a flavin and thus a one-electron/two-electron switch. The negative redox potential of both the electron donor and the electron acceptor and the presence of one-electron transferring iron-sulfur centers are probably the reason why formylmethanofuran dehydrogenases are rapidly inactivated in the presence of O_2. This assumes that the mechanism of inactivation is autoxidation initiated by the one-electron reduction of O_2 to O_2^- considering that the susceptibility of a one-electron donor towards autoxidation increases the more its redox potential lies below that of the O_2/O_2^- couple (−330 mV). Methanogenic Archaea do contain superoxide dismutase (Takao et al., 1991). Whether the enzyme protects purified formylmethanofuran dehydrogenase from inactivation due to autoxidation has not yet been determined.

Some of the enzymes catalyzing the insertion of oxygen from H_2O into a C-H bond contain tungsten rather than molybdenum and all known tungsten enzymes catalyze this type of reaction. This has been shown for formate dehydrogenase from *Clostridium formicoaceticum* (Leonhardt and Andreesen, 1977), *Clostridium thermoaceticum* (Yamamoto et al., 1983), and *Methanococcus vannielii* (Jones and Stadtman, 1981), for carboxylic acid reductase from *C. thermoaceticum* (White et al., 1989) and *C. formicoaceticum* (White et al., 1991), for aldehyde:ferredoxin oxidoreductase and formaldehyde:ferredoxin oxidoreductase from *Pyrococcus furiosus* and from *Thermococcus litoralis* (Mukund and Adams, 1991 and 1993) and for some of the formylmethanofuran dehydrogenases (Schmitz et al., 1992a; Bertram et al., 1994). In addition to tungsten these enzymes contain a pterin cofactor (Schmitz et al., 1992a; Bertram et al., 1994; Johnson et al., 1993), indicating that the tungsten in these enzymes is coordinated similarly to molybdenum in the respective molybdenum enzymes (Cramer et al., 1985; George et al., 1992). This is also indicated by the finding that the formate dehydrogenase from *C. formicoaceticum* is rapidly inactivated by cyanide (Leonhardt and Andreesen, 1977), although most tungsten enzymes do not appear to exhibit this property. The finding that most tungsten enzymes do not exhibit an EPR signal derived from W(V) upon reduction with substrate or dithionite probably reflects that the redox potentials of the W(VI)/(V) couple and of the W(V)/(IV) couple are predicted to be more negative than those of the respective Mo couples (Rice et al., 1981; Yu and Holm, 1989). This predicition is supported by the observation that all known tungsten enzymes catalyze the dehydrogenation of substrates with redox potentials considerably below −400 mV, whereas molybdenum enzymes can also mediate the dehydrogenation of compounds with more positive redox potentials. For instance, the catalysis of xanthine dehydrogenation to uric acid with a redox potential of −360 mV appears not to be possible with a tungsten enzyme since all xanthine dehydrogenases/oxidases investigated have been shown to be active only with molybdenum (Ljungdahl, 1976; Wagner and Andreesen, 1987; Koenig and Andreesen, 1989 and 1990).

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