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IRON-SULFUR CENTERS INVOLVED IN METHANOGENIC ELECTRON TRANSFER IN METHANOBACTERIUM THERMOAUTOTROPHICUM (DELTA-H)[1]

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SUMMARY: Utilizing a subcellular particulate preparation from Methanobacterium thermoautotrophicum (delta-H) which contains all detectable methanogenic electron transfer activity, we present the results of the effects of the anaerobic addition of oxidized factor F420 and of methyl coenzyme M plus ATP on the EPR signals from reduced iron-sulfur centers and a rapidly-relaxing radical species. Based on these results, we report the existence of a minimum of three iron-sulfur centers which are capable of donating electrons to these cofactors.

Energy-coupled electron transfer in the methanogenic bacteria has been shown to involve several unique carriers of one-carbon intermediates [1], including the cofactors methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (CoM). The identities of the carriers involved in the pathway of electron transfer, however, from H_2 or reduced carbon substrates (e.g., formate, carbon monoxide, methyl groups (methanol, methylamines), or the carboxyl group of acetate) are less well established. Potential candidates include factor F420 (a 5-deazaflavin cofactor which is an obligate two--electron carrier of relatively negative reduction potential) [2], ferredoxin

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[3-5], $NADP^+$ [6-9], 7-mercaptoheptanoylthreonine phosphate (HTP) [10], FAD [11], and possibly corrin [12]. There is no evidence for such a role for NAD^+.

Although the methylcoenzyme M (MeCoM) reductase (MR) system is responsible for the terminal two-electron, methane-yielding step in all methanogens [1], the complete lack of quinones and (except for organisms capable of methyl group oxidation [13,14]) cytochromes apparently rules out a common electron transfer chain analogous to other energy-yielding systems. This makes visible absorption spectroscopy of only limited use for such studies. Low temperature EPR spectroscopy of a particulate preparation from Methanobacterium bryantii has demonstrated the presence of several paramagnetic species, including iron-sulfur centers, Ni(III), and a relatively fast-relaxing radical [15]. Although the EPR characteristics of several isolated enzymes involved in the oxidation of metabolic electron donors have been reported (formate dehydrogenase [16], hydrogenase [17-19], and carbon monoxide dehydrogenase [20]), no studies have reported the presence of EPR-detectable electron transfer centers responsible for delivery of electrons to the MR. An exciting development is the demonstration of an EPR signal from nickel in the +1 or +3 oxidation state in factor F430 in whole cells and also isolated MR, raising the possibility that this cofator is involved in electron transfer [21].

Utilizing the ability to prepare (by extensive ultracentrifugation) a partially resolved subcellular fraction at high protein concentration containing the complete methanogenic electron transfer system (in the species Mb. thermoautotrophicum (delta-H) [22,23]), we report here the results of studies designed to examine the roles of the EPR-visible iron-sulfur and radical species in methanogenic electron transfer, by inducing changes in the EPR spectra of reduced preparations upon addition of physiologically significant electron acceptors. Combining these results with published EPR characteristics of purified methanogenic enzymes, we propose a minimal set of oxidation-reduction centers involved in methanogenic electron transfer in Mb. thermoautotrophicum (delta-H).

EXPERIMENTAL PROCEDURES

EPR Spectroscopy. Magnetic resonance spectra were obtained using a Varian E-109 spectrometer equipped with a 9.5 GHz microwave bridge. 77° K temperatures were maintained using liquid N_2 and a quartz dewar insert. Studies performed at liquid helium temperature were performed using an Air Products liquid helium transfer assembly and an Air Products temperature monitor-controller. Variable instrument parameters are specified in the figure legends. A modulation amplitude of 10 G and modulation frequency of 100 kHz were used for all spectra presented. Scan rates were typically 250 G/min and time constants 0.128 s. Instrument calibration was checked under various running conditions using α-diphenyl-α-picrylhydrazyl (DPPH) powder. Power saturation data are plotted as described by Rupp et al. [24]. A plot of log $S/\sqrt{P}$ vs. log P (S = signal intensity, P = microwave power) was used to determine microwave power saturation.

Preparations from various methanogen species. For the survey of the presence of paramagnetic components in the methanogen species described in Table I, bacteria were obtained as a generous gift from R. S. Wolfe, Univ. Ill. and the pellet fraction from a 150,000 xg 45 min. ultracentrifugation was examined (either air oxidized or reduced with dithionite), as described previously [15].

EPR studies of iron-sulfur centers. Mb. thermoautotrophicum (delta-H) was grown under H_2/CO_2 in a 13 l fermentor and the crude particulate fraction (pellet of a 180,000xg 2 1/2 hr. ultracentrifugation of a French press extract, resuspended in 100 mM Pipes pH 6.9 containing 1 mM EDTA [15]) was prepared under anaerobic conditions. Three samples were placed in serum vials, headspace atmospheres pressurized with H_2 to 10 psi, and samples incubated at 4° C for 12 hr. For substrate oxidations, the H_2 atmospheres in two of the samples were exchanged with N_2. To one of these vials ATP, $MgCl_2$, and methyl-coenzyme M (MeCoM) were added via an anaerobically prepared stock solution to final concentrations of 1 mM, 1 mM, and 10 mM, respectively. Anaerobic, oxidized F420 (1 mM final) was added to the other vial. The samples were then incubated at 40° C for 15 min then transferred anaerobically to EPR tubes and frozen.

RESULTS

Table I shows that the complex signals reported earlier for Mb. bryantii [15] are not present in the particulate fraction of all species of methanogen and in at least one case (Methanococcus voltae) only a relatively small g = 2.01 oxidized species is observed (which is quite possibly due to nonphysiological oxidation [25] by oxygen exposure during sample preparation). We chose Mb. thermoautotrophicum (delta-H) for further EPR studies, due in large part to the ability to obtain highly concentrated preparations of electron transfer components by ultracentrifugation[4]. Table II presents a summary of the various

TABLE I

EPR-Visible Centers in the Crude Particulate Fraction of Representative Methanogens

Methanogen[a]	g=2.01 (oxidized)	Ni(III)	Radical	g=1.94 (reduced)
Mb. bryantii (H_2/CO_2)	+	+	+	+
Mb. thermoautotrophicum (delta-H) (H_2/CO_2)	+	+	+	+
Ms. barkeri (acetate)	+	+	+	+
Ms. barkeri (H_2/CO_2)	+	+	-	+
Mb. ruminantium (H_2/CO_2)	+	-	+	-
Msp. hungatei (H_2/CO_2)	+	-	+	-
Mc. voltae (H_2/CO_2)	+	-	-	-
Mc. voltae (formate)	+	-	-	-

[a] Growth substrate indicated in parentheses.

[4] Such preparations possess the complete, eight-electron transfer complex from H_2 to CO_2 required to produce CH_4, as well as 85% of the coenzyme F420-reducing hydrogenase [23].

TABLE II

EPR-Detectable Iron-sulfur Centers Involved in Methanogenic Electron Transfer in Mb. thermoautotrophicum (delta-H)

Designation	Type	g Values		
		g_1	g_2	g_3
FeS-1	$[Fe_4S_4]^{1+/2+}$	2.056	--	--
FeS-2	$[Fe_4S_4]^{1+/2+}$	2.003	1.936	1.905
FeS-2*	$[Fe_4S_4]^{1+/2+(a)}$	--	1.942	1.896
FeS-3	$[Fe_3S_4]^{1+/0}$	-- 2.015 --		

(a) This signal is proposed to arise from FeS-2, but slightly shifted in g-values due to the presence of ATP/Mg^{2+} + methyl-coenzyme M (see text).

centers observed in this preparation along with the designation for each identifiable center[5], and in the following sections the EPR results documenting these conclusions are presented.

Figure 1A shows an EPR spectrum of the crude particulate fraction after reduction under H_2. Major signal deflections occur at g = 2.056 (peak), g = 2.003 (peak), g = 1.936 (inflection), and g = 1.905 (trough). These signals show temperature and saturation properties typical of reduced-type $[Fe_4S_4]$ iron-sulfur centers, as described below. Differential oxidation-reduction behavior of these signals upon the addition of oxidized electron carriers (vide infra) suggest the presence of at least three iron-sulfur centers, two of which (FeS-1 and FeS-2) are discernable in this spectrum.

Addition of anaerobic oxidized F420 to the hydrogen-reduced crude particulate fraction results in several spectral changes (Fig. 1B). The upward feature at g = 2.056 (assigned to center FeS-1) and the radical feature (visible as shoulders centered at g = 2 and more fully developed in spectrum 1C) disappear, presumably due to oxidation. The observation that the features at g=1.936 and g=1.905 are still present, although diminished in magnitude, suggest that they arise from a different center (i.e. FeS-2) than that responsible for the g=2.056 feature (i.e. FeS-1)[6]. The lineshape of the upward

[5] It is important to note that in the absence of further information this nomenclature designates a minimum set of paramagnetic centers, distinguishable only by the spectral behavior to the experimental manipulations described.

[6] The broad feature at g=2.056 (corresponding to FeS-1) demonstrates saturation and temperature characteristics indicative of a reduced--type Fe_4S_4 iron-sulfur center. The two features predicted to accompany the small and broad g=2.056 feature are probably obscured by the sharper FeS-2 signal in spectrum 1A.

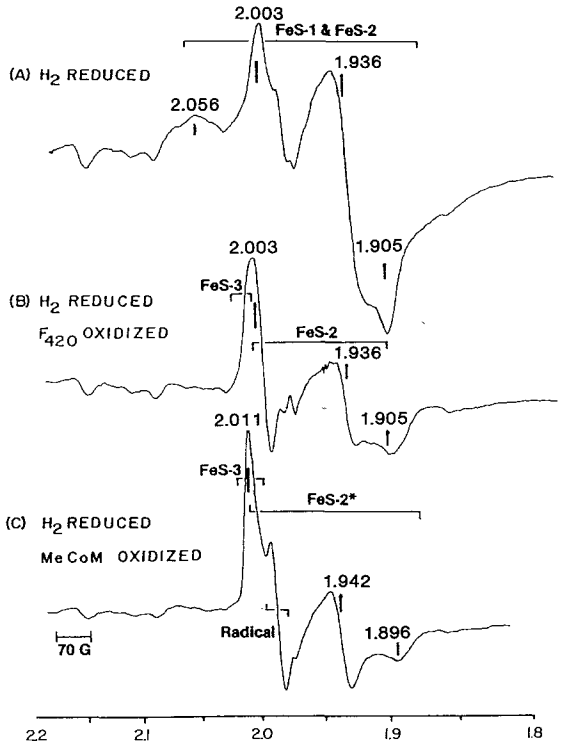


Figure 1. Substrate Oxidations of the Reduced Crude Particulate Fraction from Mb. thermoautotrophicum (delta-H). Reduction with H_2 (A) and subsequent oxidation by factor F420 (B) or by methyl-coenzyme M plus Mg^{2+}/ATP (C) were performed as described in Experimental Procedures. Instrument gains, protein concentrations, and temperatures: (A) 2 x 10^3, 32 mg/mL, 10^o K; (B) 1.23 x 10^3, 38 mg/mL, 11^o K; (C) 2 x 10^3, 26 mg/mL, 9^o K.

feature at g=2.003 in this spectrum suggests that it may result from overlap of the low-field feature of FeS-2 and an additional axial species (perhaps the oxidized center more fully developed by MeCoM addition (FeS-3), as described below). Thus, oxidized F420 appears to oxidize FeS-1, the radical, and, to a lesser extent, FeS-2 and FeS-3.

Upon addition of ATP/Mg^{2+} plus MeCoM to the H_2-reduced preparation (Fig. 1C), the two features previously assigned to FeS-2 (g=1.936 and g=1.905) are diminished in signal intensity and shifted to g=1.942 and g=1.896 (see Fig. 2 for an expansion of this region of the spectrum, which more clearly shows this slight shift in g values; we designate this altered spectrum FeS-2*). The height and sharpness of the feature at g=2.011 we attribute to a superposition of the lowest field feature of the reduced FeS-2* (also shifted, from 2.003) and an oxidized Fe_3S_4 center (FeS-3). The feature assigned to FeS-1 is completely absent. Thus, addition of MeCoM to the H2 reduced crude particulate fraction results in the oxidation of FeS-3 and FeS-1 as well as oxidation of a majority of the FeS-2 centers and a slight perturbation of the signal from those remaining reduced.

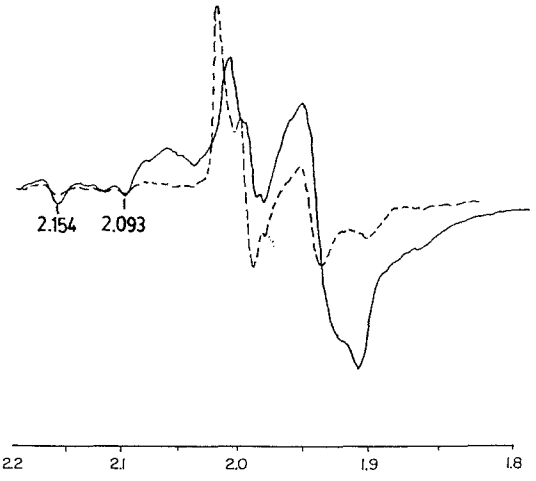
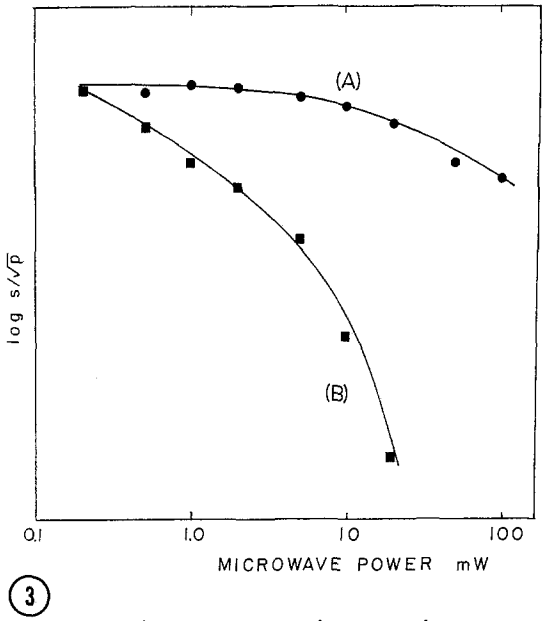


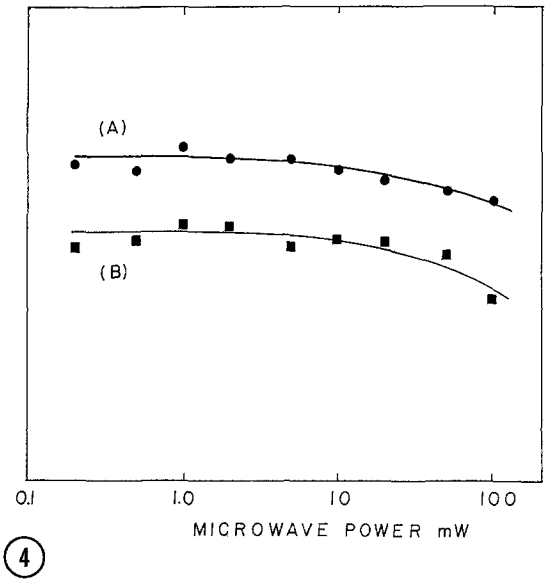
Figure 2. Shift in g-value of FeS-2 Upon addition of Mg^{2+}/ATP plus Methyl-coenzyme M. The solid line is that of Figure 1A and the broken line tracing is that of Figure 1C, with the excursions from Mn^{2+} superimposed.

When observed at 77° K, the only feature present in this magnetic field range is that of the radical at g = 2.0. Since this subcellular preparation contains virtually all of the coenzyme F420 reducing hydrogenase [23] and oxidized F420 addition results in disappearance of this signal (Fig. 1B) we tentatively ascribe this signal to FAD, a component of this purified protein [26] and which is present in a comparable preparation from Mb. bryantii [27]. The 2.0 mT peak to trough linewidth of this signal is characteristic of a neutral rather than anionic species [28].

Shown in Fig. 3A is the microwave power saturation profile for the radical signal at 77° K, determined under conditions where iron-sulfur centers are appreciably reduced (i.e., dithionite addition [15]). A useful comparison can be made between the saturation behavior of this species and that of the flavin radical present in glucose oxidase (Fig. 3B). Whereas significant saturation of the signal of flavin radical not in proximity to another paramagnet (as in the case of glucose oxidase) is generally observed at low power levels, increased relaxation through spin-spin interaction can occur in systems containing one or more nearby iron-sulfur centers (e.g., FAD in succinate dehydrogenase and the binuclear iron-sulfur center S-1 [29]). As shown in Fig. 3A, the glucose oxidase radical saturates much more readily (i.e. the line bends toward the abscissa at a lower power) than the methanogen radical, which suggests that the methanogen radical species is in close proximity to a more rapidly-relaxing iron-sulfur center. Fig. 4 shows the 8° K power saturation profiles for the reduced- type iron-sulfur center FeS-2 (4A; g = 1.936 feature) present in the crude particulate fraction and oxidized-type iron-sulfur center FeS-3 (4B; g = 2.011 feature, see Fig. 1C). The profiles indicate that neither center is appreciably saturated at microwave powers up



(3)



(4)

Figure 3. Comparison of Microwave Power Saturation Behavior of the Radical Species (A) with that of FAD in Glucose Oxidase (B). Instrument gain was adjusted as required by the signal intensities, and microwave power are as specified. (A) crude particulate, reduced with dithionite, 20 mg/mL in 50 mM tris pH 7.5 plus 0.1 mM EDTA; (B) glucose oxidase, reduced with dithionite, 10 mg/mL, in the same buffer. Other conditions are as specified in Experimental Procedures.

Figure 4. Microwave Power Saturation Behavior of Reduced FeS-2 (A) and Oxidized FeS-3 (B). Crude particulate fraction, 32 mg/mL, g=1.94 feature, dithionite reduced (A) or g=2.011 feature, air oxidized (B). Temperature, 8° K. Buffer and instrument conditions as in Fig. 3.

to 100 mW (typical for four-iron (as opposed to two-iron) iron-sulfur centers [24]), and suggest that these centers are good candidates for interaction with the radical species.

DISCUSSION

The demonstration by isotopic labeling studies of intermolecular (and, in the case of acetate, intramolecular) electron transfer indicates the presence of a central "pool" of low potential electron carriers in methanogens which are in relatively free oxidation-reduction communication with the enzymes responsible for the metabolism of various C_1 (or H_2) units [30]. The EPR-identifiable centers reported here could be these putative carriers. It is interesting to note in this regard the striking similarity of the EPR spectral properties of this partially resolved preparation to a purified hydrogenase-containing large molecular weight complex from Ms. barkeri [19].

Assignment of the relative positions of the oxidation-reduction components reported here in terms of an electron transfer sequence is not

straightforward. Nevertheless, several observations suggest possible roles for some of these centers:

The complete disappearance of FeS-1 by the addition of oxidized coenzyme F420 and its resemblance to the low-field feature reported in the purified coenzyme F420-reducing hydrogenase from this species [18] suggests its assignment as part of this enzyme. The disappearance of this signal upon the addition of MeCoM plus ATP suggests further that this enzyme may be able to donate electrons to the MR without added F420; further purification is required to establish this conclusion definitively, however, as well as the relationship of this enzyme to component A1 (which contains coenzyme F420-hydrogenase activity [11]) and also to the coenzyme F420-reducing methylene-tetrahydromethanopterin dehydrogenase [31].

The shift in g-values for the FeS-2 centers remaining reduced after the addition of methyl-CoM suggests functional (if not spatial) proximity to the MR, perhaps associated with a constituent of Component A. We do not know at this point whether the signal perturbation is due to methyl-CoM or instead to ATP, possibly as a consequence of activation by ATP [32]. This preparation has an active ATPase activity [33] and similar EPR spectral perturbations have been observed in nitrogenase for ATP-induced electron transfer in this soluble protein [34].

The linewidth and oxidation-reduction behavior of the radical signal are consistent with assignment as neutral flavin semiquinone. Possible roles for flavin are in the coenzyme F420-reducing hydrogenase (which contains FAD [26]) and/or methyl reductase, which has been shown to require FAD for reconstitution of activity in a purified system [11]. The microwave power saturation characteristics suggest close proximity to another paramagnetic center, perhaps in a protein complex.

Since purified coenzyme F420-reducing hydrogenase contains no oxidized iron-sulfur signal [18] the $[Fe_3S_4]$ center FeS-3 is most probably not associated with this protein. Similar centers are components of many hydrogenases [35], suggesting that it may be associated with a constituent of one (or more) of the components A of the MR [11].

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