SOLUBLE AND MEMBRANE-BOUND PARAMAGNETIC CENTERS IN *METHANOBACTERIUM BRYANTII*

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1. Introduction

The methanogenic bacteria are members of a unique group of organisms (denoted Archaebacteria) which are apparently only distantly related to prokaryotes and eukaryotes [1,2]. Methanogens are characterized by the ability to grow autotrophically solely by the reduction of CO_2 by H_2 to produce methane. Several electron carriers and 'factors' unique to methanogens have been isolated, including coenzymes F_{420} [3,4], F_{342} [5] and F_{430} [5], as well as coenzyme M (2-mercaptoethanesulfonic acid [6,4]). While some light has been shed on the roles of F_{420} and coenzyme M in CO_2 reduction [4,7], virtually nothing is known about the mechanism(s) of energy transduction via electron transport from H_2 to CO_2. By using low-temperature EPR spectroscopy I report here the presence of oxidized and reduced paramagnetic species, both soluble and membrane-bound, in *M. bryantii*. Specifically, the signals observed are:

1. A signal typical of an oxidized iron–sulfur center (either HiPIP-type Fe_4–S_4* or Fe_3–S_3*) in the soluble fraction, possibly hydrogenase.
2. A very unusual membrane-bound rhombic high-temperature oxidized species with all three *g*-values significantly above the free-electron value, which may be either a new type of cluster or a transition metal other than iron, possibly nickel (see section 4).
3. Upon reduction of the membrane fraction, the appearance of at least two '$g = 1.94$'-type iron–sulfur centers as well as a radical signal (which may be a semiquinone-type species or possibly a ligand radical–Ni (II) system, see section 4).

2. Materials and methods

Frozen cells of *M. bryantii* (grown autotrophically on H_2 and CO_2 as in [1]) were generously provided by Dr R. S. Wolfe, Dept. Microbiol., University of Illinois. A thick suspension in buffer (50 mM potassium phosphate (pH 7.7) plus 0.1 mM EDTA) was passed through an Aminco French pressure cell twice (15 000–18 000 lb. in^{-2}). Unbroken cells and debris were removed by centrifugation at 7000 × *g* for 30 min. The pellet was resuspended in buffer and passed through the cell again and spun as before. The supernatants were combined and membranes were pelleted by centrifugation at 150 000 × *g* for 45 min. The supernatant was saved, and the pellet was washed 3 times in buffer by centrifugation. The membranes were finally resuspended in a small volume of buffer to final conc. 67 mg protein/ml, and stored aerobically at −70°C. Ammonium sulfate was added to the first high-speed supernatant to 65% saturation and spun at 27 000 × *g* for 30 min. The pellet was resuspended in a minimal amount of buffer, dialyzed extensively against buffer, and frozen in small aliquots at final conc. 16 mg protein/ml at −70°C. Protein was determined by the microbiuret method [8].

EPR spectra were recorded on a Varian E-9 equipped with an Air Products flowing helium cryostat. Temperature was measured with a thermocouple calibrated by a carbon resistor.

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3. Results

3.1. *Soluble species*

Fig.1A shows an EPR spectrum of the concentrated supernatant fraction at 20 K. Prominent features are a rhombic signal centered at $g = 4.3$ (characteristic of high-spin ferric iron in an anvironment of low symmetry) and a rather isotropic signal appearing just above $g = 2.0$. Fig. 1B shows the $g = 2$ region in more detail at 12 K. The signal intensity is greater at this temperature (note the difference in instrument gain between 1A and 1B) and exhibits a principal upward feature at $g = 2.02$. Addition of dithionite to the sample causes this signal to disappear completely with the appearance of small signals which are probably due to unpelleted membranes (not shown). The shape of the signal, temperature characteristics, and disappearance upon reduction suggest the presence of either a HiPIP-type Fe_4–S_4* center or a Fe_3–S_3* center [9,10]. Very similar signals are exhibited by hydrogenases in the oxidized state, from a variety of bacteria, including facultative aerobes (e.g., *Escherichia coli)*, anaerobic sulfate reducers *(Desulfovibrio desulfuricans),* photosynthetic bacteria *(Chromatium vinosum* and *Rhodospirillum rubrum)* and hydrogen-oxidizing bacteria *(Alcaligenes eutrophus)* [11].

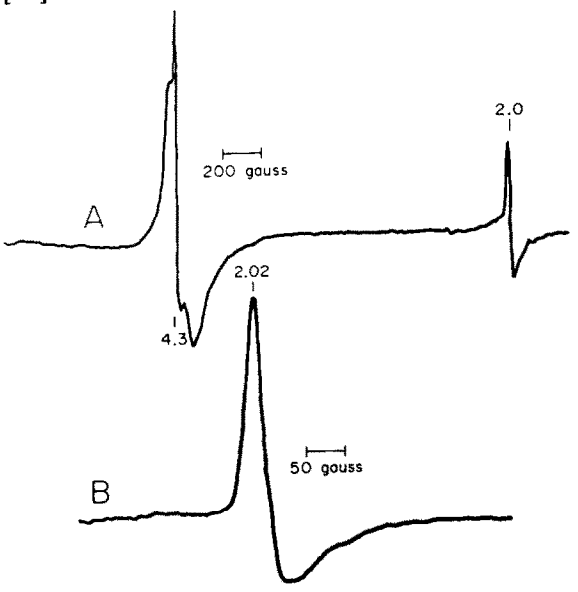

Fig.1. EPR spectra of soluble cell extract of *M. bryantii.* Conditions: 16 mg protein/ml; 9.24 GHz microwave frequency, 12.5 G modulation amplitude, 20 mW power. (A) (Full-field scan, 20 K, 2.5×10^3 gain; (B) $g = 2$ region, 12 K, 2×10^3 gain.

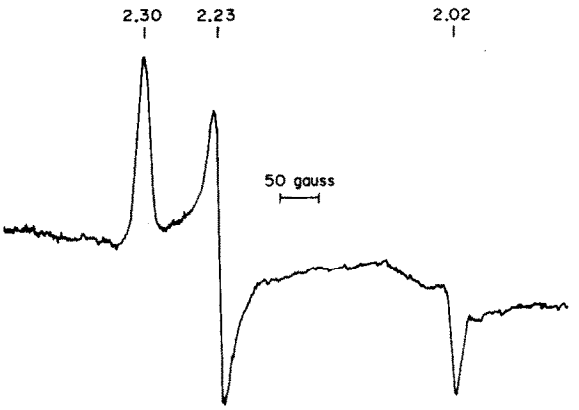

Fig.2. EPR spectrum of oxidized membranes. Conditions: 9.24 GHz frequency, 20 G modulation amplitude, 10 mW power, 24 K, 4×10^3 gain. Field positions calibrated by using a solution of reduced methyl viologen.

3.2. *Membrane-bound species*

Fig.2 shows the spectrum of the major paramagnetic species present in the oxidized methanogen membranes as isolated. This highly unusual signal has all three g-values significantly above 2.0023 (the free electron value), at $g_1 = 2.30$, $g_2 = 2.23$, and $g_3 = 2.02$. The signal is quite intense and the linewidth very sharp up to 77 K (not shown), and begins to saturate at 24 K below 5 mW (not shown). Since the g-values are all significantly above the free electron value this signal is most probably not from oxidized heme, and the redox state of this species argues against a binuclear iron–sulfur center. Signals with the high-field feature near [12,11] and even slightly above [13] $g = 2.0023$ have been reported for HiPIP-type Fe_4–S_4* centers and for the recently discovered Fe_3–S_3* centers [9,10]. However, the large range in g-values (from 2.3–2.02) and sharp rhombic features of the signal as well as the temperature and saturation characteristics [14] argue that if this is the case then this center is very unlike any previously described. An alternate possibility (see section 4) is that this signal is of a transition metal other than iron.

Upon addition of dithionite to the membranes, the signal in fig.2 disappears completely and is replaced by the signals shown in fig.3A. The principal features are a sharp radical-type signal with additional resonances at slightly higher and lower field which are fully developed only at low temperature. When lowered to 8 K and this area is expanded (fig.3B) at least four features are recognizable, at $g = 2.05$, 1.96,

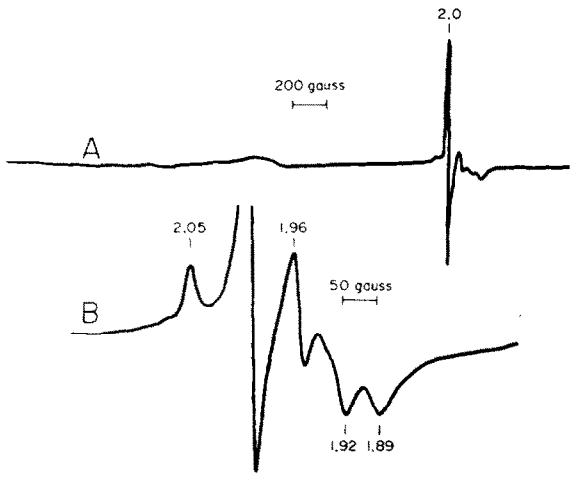

Fig.3. EPR spectrum of reduced membranes. A solution (20 μl) of 10 mg sodium dithionite/ml in 1 M potassium phosphate (pH 7.4) was added to 0.25 ml membranes (67 mg/ml) and immediately frozen. Conditions: 9.24 GHz frequency, 12.5 G modulation amplitude, 10 mW power. (A) Full-field scan, 30 K, 1.25×10^3 gain; (B) $g = 2$ region, 8 K, 2×10^3 gain.

1.92 and 1.89. Additional absorption also occurs at $g \simeq 2$ as evidenced by the difference in magnitude between the upward and downward features of the 'radical' signal at this low temperature. These signals cannot be due to a single species since more than three features are present. This pattern suggests the presence of at least two membrane-bound iron–sulfur clusters.

4. Discussion

This paper presents EPR data reporting the presence of at least five paramagnetic species in *M. bryantii* which are capable of being reduced by dithionite. While no signals corresponding to heme were observed (cf. [15]), this strain of methanogen appears to possess iron–sulfur clusters, both 'HiPIP' (or possibly Fe_3–S_3* [9,10]) and '$g = 1.94$'-type.

With regard to the rhombic signal present in isolated membranes, the fact that all three g-values lie above the free electron value eliminates low-spin heme as a possibility [16], and the oxidation state (paramagnetic in the oxidized state) eliminates an Fe_2–S_2* cluster. The spread of g-values (from $g = 2.3$–2.02), sharp rhombic features, and temperature and saturation characteristics are unlike any reported for known 3 or 4-iron iron–sulfur clusters. It is possible, however, that this signal represents a new cluster signal.

The g-values are similar to those exhibited by a d^7 system in an environment of strong octahedral coordination with both tetragonal and rhombic distortions [17,18]. In such a case, the metal ion is low-spin ($S = 1/2$) with the unpaired electron in an orbital having predominantly d_{z^2} character, resulting in the observed values of $g_\perp > g_\parallel$. Among common d^7 systems, the lack of nuclear hyperfine in this sharp signal eliminates Co(II) as a possibility. Cu(II), which would be expected to give similar g-values [17], can also be eliminated for the same reason. Ni(III), however, with a nuclear spin of the most abundant isotopes (Ni^{58} and Ni^{60}, > 98% abundance) of zero, gives no hyperfine splittings. A low molecular weight (<4000) nickel-containing cofactor (called factor F_{430}) has been reported [19] in heat-treated extracts of the same strain of methanogen used here. The Ni(II)–Ni(III) redox couple is normally very positive in potential, so that Ni(III) would be quite an unlikely species. However, it is known that formation of coordinate complexes of Ni(II) by chelation to a variety of compounds including macrocyclic ligands [20,21] as well as small peptides [22,23] and also bleomycin [23] makes quantitative oxidation possible. EPR examination of these Ni(III) complexes in fact shows spectra very similar to the signal reported here [20,22,23], indicative of low-spin Ni(III) in an octahedral geometry with both tetragonal and rhombic distortions. Nitrogen hyperfine lines in this sharp signal are not observed, as is common for the g_{zz} feature with model complexes having nitrogenous axial ligands [20,22,23]. This, however, does not rule out this possibility, since such splittings are not always seen.

With regard to the radical signal which appears upon dithionite addition, reduction of Ni(II) model complexes containing conjugated systems (e.g., diimines) results in the appearance of a radical-type $g = 2.002$ signal, rather than the anisotropic signal of a Ni(I) d^9 metal complex [20]. This indicates that the electron is highly delocalized and has predominantly ligand character. This suggests the possibility that such a conjugated system is involved in the complexation of Ni in these membranes, and may be involved in CO_2 fixation and/or CH_4 production. In this regard, it is important to note that Ni(III) is isoelectronic (d^7)

with Cob(II)alamin (B_{12r}), which has been shown to be the reaction product of methyl group transfer from methylcobalamin to coenzyme M [24,25] (although it is unclear whether this particular reaction is catalyzed in vivo by methanogens [4]). Indeed, it is postulated that production of an EPR-observable ligand radical signal during turnover is a key event in the mechanism of cobalamin-containing group-transferring enzymatic reactions [26,27]. It is thus possible that a nickel-containing cofactor in the membrane of methanogens (possibly factor F_{430}) is responsible for a key step(s) in CO_2 reduction, either assimilatory or dissimilatory.

Methanogenesis may be described by two steps [28]:

(1) Reduction of electron carrier(s) by hydrogen;
(2) Electron transfer to CO_2 and uptake of water protons.

It is thus possible that the role of the membrane-bound iron–sulfur clusters reported here is in the electron-carrying 'arm' of this process. Such an arrangement may be important in energy metabolism by these bacteria and the mechanism of ATP synthesis and reducing equivalent generation, possibly via transmembrane electrochemical gradients [4,29,31]. Further experimentation is required to define the role(s) of the centers reported here in these processes.

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References

[1] Balch, W. W., Fox, G. E., Magrum, L. J., Woese, C. R. and Wolfe, R. S. (1979) Microbiol. Rev. 43, 260–296.
[2] Woese, C. R., Magrum, L. J. and Fox, G. E. (1978) J. Mol. Evol. 11, 245–252.
[3] Eirich, L. D., Vogels, G. D. and Wolfe, R. S. (1978) Biochemistry 17, 4583–4593.
[4] Wolfe, R. S. and Higgins, I. J. (1979) Int. Rev. Biochem. 21, 267–353.
[5] Gunsalus, R. P. and Wolfe, R. S. (1978) FEMS Microbiol. Lett. 3, 191–193.
[6] Taylor, C. D. and Wolfe, R. S. (1974) J. Biol. Chem. 249, 4879–4885.
[7] Gunsalus, R. P. and Wolfe, R. S. (1980) J. Biol. Chem. 255, 1891–1895.
[8] Zamenhof, S. (1957) Methods Enzymol. 5, 546–557.
[9] Emptage, M. H., Kent, T. A., Huynh, B. H., Rawlings, J., Orme-Johnson, W. H. and Münck, E. (1980) J. Biol. Chem. 255, 1793–1796.
[10] Stout, C. D., Ghosh, D., Pattabhi, V. and Robbins, A. H. (1980) J. Biol. Chem. 255, 1797–1800.
[11] Cammack, R., Maharajh, W. L. and Schneider, K. (1980) in: Interaction Between Iron and Proteins in Oxygen and Electron Transport (Ho, C. ed) Elsevier/North-Holland, Amsterdam, New York, in press.
[12] Yoch, D. C. and Carithers, R. P. (1979) Microbiol. Rev. 43, 384–421.
[13] Chen, J.-S., Mortenson, L. E. and Palmer, G. (1976) in: Iron and Copper Proteins (Yasunobu, K. T. et al. eds) pp. 68–82, Plenum, New York.
[14] Rupp, H., Rao, K. K., Hall, D. O. and Cammack, R. (1978) Biochim. Biophys. Acta 537, 255–269.
[15] Kühn, W., Fiebig, K., Walther, R. and Gottschalk, G. (1979) FEBS Lett. 105, 271–274.
[16] Griffith, J. S. (1957) Nature 180, 30–31.
[17] Maki, A. H., Edelstein, N., Davison, A. and Holm, R. H. (1964) J. Am. Chem. Soc. 86, 4580–4587.
[18] Abragam, A. and Bleaney, B. (1970) in: Electron Paramagnetic Resonance of Transition Ions, pp. 486–487, Clarendon, Oxford.
[19] Whitman, W. B. and Wolfe, R. S. (1980) Biochem. Biophys. Res. Commun. 92, 1196–1201.
[20] Lovecchio, F. V., Gore, E. S. and Busch, D. H. (1974) J. Am. Chem. Soc. 96, 3109–3118.
[21] Busch, D. H. (1978) Acc. Chem. Res. II, 392–400.
[22] Lappin, A. G., Murray, C. K. and Margerum, D. W. (1978) Inorg. Chem. 17, 1630–1634.
[23] Sugiura, Y. and Mino, Y. (1979) Inorg. Chem. 18, 1336–1339.
[24] Frick, T., Francia, M. D. and Wood, J. M. (1976) Biochim. Biophys. Acta 428, 808–818.
[25] Schrauzer, G. N. (1977) Ang. Chem. I E 16, 233–244.
[26] Buettner, G. R. and Coffman, R. E. (1977) Biochim. Biophys. Acta 480, 495–505.
[27] Babior, B. M. and Krouwer, J. S. (1979) CRC Rev. Biochem. 6, 35–102.
[28] Daniels, L., Fulton, G., Spencer, R. W. and Orme-Johnson, W. H. (1980) J. Bact. 141, 694–698.
[29] Doddema, H. J., Hutten, T. J., Van der Drift, C. and Vogels, G. D. (1978) J. Bact. 136, 19–23.
[30] Sauer, F. D., Erfle, J. D. and Mahadevan, S. (1979) Biochem. J. 178, 165–172.
[31] Doddema, H. J., Van der Drift, C., Vogels, G. D. and Veenhuis, M. (1979) J. Bacteriol. 140, 1081–1089.