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MEMBRANE-BOUND FLAVIN ADENINE DINUCLEOTIDE IN METHANOBACTERIUM BRYANTII

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SUMMARY

Noncovalently attached flavin was isolated and partially purified from the membrane fraction of Methanobacterium Bryantii. The flavin was identified as FAD by absorption and fluorescence spectroscopy, effects on the spectra of reduction and of protonation, phenol extractability, behavior in thin layer chromatography in two solvent systems, and ability to reconstitute activity of the FAD-specific enzyme D-amino acid oxidase. These singular organisms thus are capable of synthesizing isoalloxazine-type flavins as well as the unique 5-deazaflavin factor F_{420}. Membrane-bound FAD may thus (in addition to iron-sulfur centers and a nickel species) be involved in energy-coupled methanogenesis.

1. Introduction

The methanogenic bacteria are a unique and diverse group of organisms characterized by the ability to derive all cellular energy requirements from the reduction of CO_2 by H_2 to form CH_4 (1,2). Roberton and Wolfe (3) originally showed a tight coupling between methane production and cellular ATP levels. This demonstrates that, as with all other chemotrophic anaerobes (4), the energy from this oxidation-reduction reaction is stored in the form of the terminal phosphate bond of ATP.

Recent evidence suggests a role for transmembrane ion gradients in Methanogenesis. Sauer, et al. (5) showed that the methane-generating system

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resides in the membrane fraction of cell extracts and that uncoupler has a strong inhibitory effect on methanogenesis. Doddema, et al. (6) originally showed the existence of a membrane-bound ATPase in Methanobacterium thermoautotrophicum and reported data suggesting ATP synthesis by transmembrane ion gradients. Mountfort (7) described uncoupler-sensitive synthesis of ATP by whole cells of Methanosarcina barkeri upon a base to acid shift in medium pH from 8.2 to 4.5. Doddema, et al. (8) recently have reported uncoupler-sensitive synthesis of ATP by subcellular particles of Methanobacterium thermoautotrophicum upon hydrogen addition or imposition of an outwardly-directed potassium gradient.

In an attempt to detect the presence of a membrane-bound electron transfer chain, I recently applied the technique of low-temperature EPR spectroscopy to methanogens (9). I found the membrane fraction of M. bryantii to contain two types of paramagnetic centers capable of undergoing oxidation-reduction using dithionite as electron donor. Signals characteristic of nonheme iron-sulfur centers were present, as well as a very unique species which I attributed to nickel(III) in an environment of octahedral coordination, possibly factor F_{430} (10). In addition, upon reduction by dithionite a radical-type signal appeared.

I report here the additional presence of membrane-bound FAD in M. bryantii. This is the first report of isoalloxazine-type flavin in this unique group of organisms.

2. Material and Methods

Cells (a generous gift of R.S. Wolfe, Department of Microbiology, University of Illinois) were grown and membranes isolated as described previously (9).

For the isolation and partial purification of FAD, membranes (1.5 ml of 70 mg protein/ml in 50 mM potassium phosphate pH 7.7 plus 0.1 mM EDTA) were diluted to 6 mL final volume with 20 mM ammonium acetate and placed on a boiling water bath with stirring for 90 minutes. The suspension was centrifuged at 150,000 x g for 90 minutes and the supernatant lyophilized. The material was dissolved in a minimum amount of distilled water and applied to a Sephadex A-25 anion exchange column, 1 cm x 4 cm pre-equilibrated with 50 mM ammonium acetate. After washing with 50 mL 50 mM ammonium acetate, flavin was eluted with a linear gradient of 50-200 mM ammonium acetate. Fluorescent

fractions were pooled and lyophilized. The sample was dissolved in a minimum amount of distilled water and passed through a Sephadex G-10 column using distilled water. Fluorescent fraction were pooled and lyophilized, and the material was dissolved in distilled water.

The apoprotein of D-amino acid oxidase was prepared as described previously (11). FAD was from Sigma-D-Alanine oxidase activity was assayed by measuring oxygen uptake using a Clark oxygen electrode, in the presence of catalase. Protein was determined by the microbiuret method (12).

3. Results

Fig. 1 shows the absorption spectrum of the partially purified fraction from M. bryantii. The peak at 450 nm is identical in both position and shape to that exhibited by the isoalloxazine ring of FAD and FMN. While there is other absorbing material at lower wavelengths, a slight shoulder does appear at approximately 380 nm.

Fig. 2 shows the fluorescence spectrum of the material at pH 7.7. The equivalence of both the excitation and emission spectra of the sample to that of FMN strongly indicates the presence of an isoalloxazine ring. As with FAD and FMN, the fluorescence and the 450 nm absorbance disappears upon reduction with dithionite. The flavin is also totally extractable into phenol. By comparison of the fluorescence of solutions of identical absorbance at 450 nm, it was clear that the flavin isolated from M. bryantii is greatly quenched relative to FMN at pH 7.7. FAD shows such behavior as a result of internal quenching due to complex formation between the isoalloxazine and adenine rings of FAD (13). As with FAD (14), the fluorescence of the flavin greatly increased upon lowering the pH to approximately pH 1.

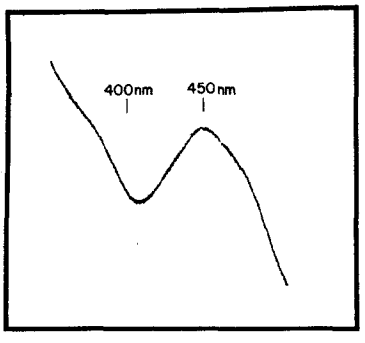

Fig. 1. Absorption spectrum of flavin material from M. Bryantii. Material isolated as described in "Methods" and in water.

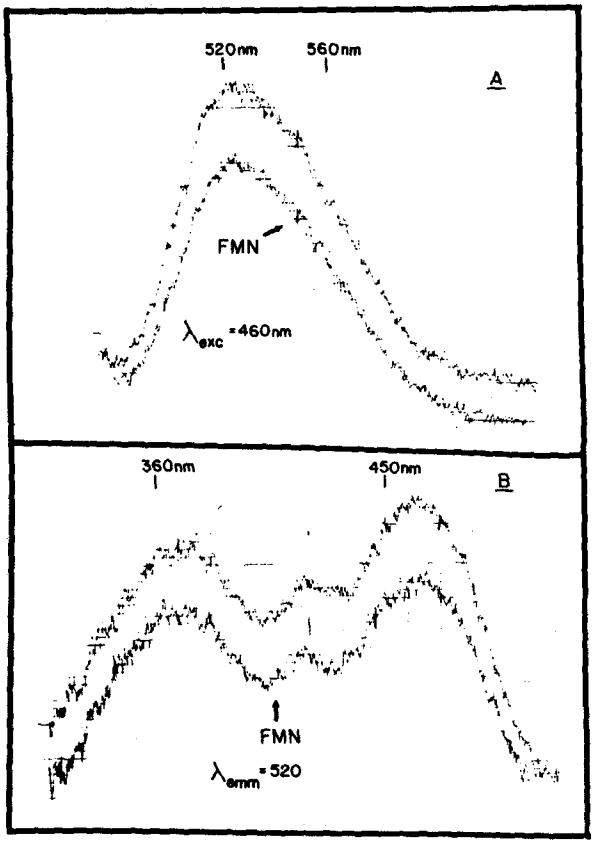

Fig. 2. Fluorescence spectra of flavin. Final concentrations, 1μM flavin isolated from the organism (concentration determined spectrophotometrically assuming ε = 12 mM^{-1}) for the upper tracings, and 0.089 μM FMN for the lower tracings. Buffer is 0.1 M potassium phosphate pH 7.5. A: emission spectrum, with excitation at 460 nm. B: excitation spectrum, with emission measured at 520 nm.

That the flavin isolated from these membranes is FAD is further indicated by the fact that the fluorescent material was indistinguishable from FAD by thin layer chromatography in two different solvent systems, 2% sodium phosphate dibasic and n-butanol-glacial acetic acid-water, 12:3:5 (15).

Finally, the flavin was equally active as FAD in restoring enzymatic activity to the apoprotein of D-amino acid oxidase (Fig. 3), a protein specific for FAD (11).

4. Discussion

The flavin isolated from the membrane fraction of M. bryantii was determined to be noncovalently attached FAD as judged by absorption and

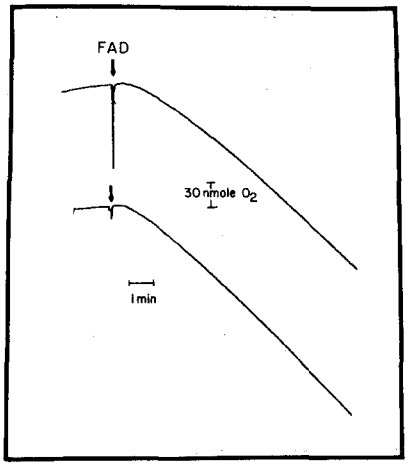

Fig. 3. Reconstitution of activity of D-amino acid oxidase apoenzyme by flavin. Final concentrations (total volume 3 ml): 40 mM sodium pyrophosphate pH 8.5, 12 µg/ml D-amino acid oxidase apoenzyme, 0.8 mg/ml bovine serum albumin, 2.4 mM EDTA, 4 µg/ml catalase, 45 mM DL-alanine. Oxygen uptake was monitored by a Clark oxygen electrode. At the times indicated by the arrows, 1.1 nmole FAD (upper tracing) or flavin isolated from M. Bryantii (lower tracing; concentration determined spectrophotometrically assuming a value for molar absorption of 12 mM^{-1}) was added.

fluorescence spectroscopy, effects on the spectra of reduction and of protonation, phenol extractability, behavior in thin layer chromatography in two solvent systems, and ability to reconstitute activity of the FAD-specific enzyme D-amino acid oxidase.

At least in this species, then, the membrane contains at least three types of electron transport components: FAD, nonheme iron-sulfur centers and a low-spin Ni(III) complex of octahedral symmetry. Assuming quantitative extraction of FAD and a value for the extinction coefficient of 12 mM^{-1} (16), the amount present is roughly equimolar with the Ni(III) species, 0.2 nmole per mg membrane protein.[1]

This amount of electron transport components is similar to that found in other energy-transducing membranes (e.g., 0.15 nmole/mg protein non-covalently attached FAD in Keilin-Hartree beef heart mitochondrial membranes (17)).

[1] The amount of nickel was quantitated by double integration of the EPR signal at 77° compared to a Cu·EDTA standard.

The results reported here show that M. bryantii is able to synthesize both FAD (containing the isoalloxazine ring moiety) and factor F_{420}, which contains the unique ring system 7,8-didemethyl-8-hydroxy-5-deazariboflavin (2). A branch point for the biosynthesis of both flavins from a common precursor considering the pathway of riboflavin synthesis in other microorganisms (18) is not immediately evident.

It has recently been proposed (1) that the methanogenic bacteria are the major representative of a newly recognized but very ancient line of descent, called the archaebacteria. These organisms are apparently only very distantly related to the other bacteria, suggesting that divergence of these two kingdoms occurred very long ago from a common ancestor (called the "progenote"). The finding of FAD, iron sulfur centers (9), and an iron-containing superoxide dismutase which is virtually indistinguishable from the enzyme present in E. coli (19) suggests that these components were present in this early ancestor of both lines of descent. Another possiblity, however, is that the genes for the biosynthesis of these components were obtained by the methanogens by intraspecies transfer at some later time.

This is the first report of the existence of a membrane-bound hydrogen carrier in these organisms, and may be significant in terms of proton gradient generation via a "loop-type" mechanism (20). It is important to note, however, that the flavin moiety in the mitochondrial energy-conserving NADH dehydrogenase complex probably does not function as a hydrogen-carrying "arm" of a loop (21,22).

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