# Redox properties of the flavoprotein of methane monooxygenase

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The redox properties of the flavin and iron-sulfur cluster of the flavoprotein of methane monooxygenase from *Methanobacterium* CRL26 have been determined. The $E_{m7}$ of the flavin/protonated semiquinone = −195 mV, that of the protonated semiquinone/fully reduced form = −250 mV, and that of the $Fe_2S_2$ center = −247 mV. This suggests that the protein cycles between the fully reduced form and the flavin semiquinone form as it shuttles electrons from NADH to the methane hydroxylase.

*Methane monooxygenase* *Flavoprotein* *Redox properties*

## 1. INTRODUCTION

Methanotrophs are a diverse group of organisms that share an appetite for methane, and in some cases methanol, as sole carbon sources for growth [1]. The first enzyme system in the metabolism of methane is methane monooxygenase [1–4], a multicomponent system that catalyses the reaction

$$CH_4 + O_2 + NADH + H^+ = CH_3OH + H_2O + NAD^+$$

The actual oxidation of methane occurs on a protein variously known as the hydroxylase or Component A, while the oxidation of NADH occurs on a protein known as the flavoprotein or Compnent C [1–4]. A third component, Component B [5], may be involved in coupling the oxidation of the reduced flavoprotein by oxygen with the oxidation of methane [2,4], although its mode of action remains obscure. The enzyme system has a range of unique properties, including an ability to oxidize a range of linear alkanes ($C_2$–$C_8$) to the corresponding 1- and 2-alcohols, alkenes ($C_2$–$C_4$) to the corresponding epoxyalkanes, halomethanes to formaldehyde, cyclic alkanes to cyclic alcohols and benzene to phenol [6]. Yet the enzyme remains poorly understood. Dalton and his colleagues have made significant progress in elucidating the components of the enzyme system from *Methylococcus capsulatus* strain Bath (e.g. [2,4,5]) while we have studied the enzyme from a range of organisms, particularly a *Methylobacterium* deposited with the tentative identification CRL26 at the Northern Regional Research Laboratories, Peoria, IL as B-11222 (e.g. [3,6]). Here, we present a characterization of the redox properties of the flavoprotein of methane monooxygenase from CRL26.

## 2. MATERIALS AND METHODS

*Methylobacterium* CRL26 was grown and the flavoprotein purified as described [6,7]. Redox potentiometry followed the method of Dutton [8]. Optical spectra were obtained with a Perkin-Elmer 559A spectrometer, while ESR spectra were obtained with a Varian E109 spectrometer equipped with an Oxford Instruments helium cryostat.

## 3. RESULTS

The flavoprotein contains a single molecule of FAD and an $Fe_2S_2$ iron-sulfur cluster [6]. Fig.1 shows the optical absorption spectrum of the flavoprotein, and the changes caused by the se-

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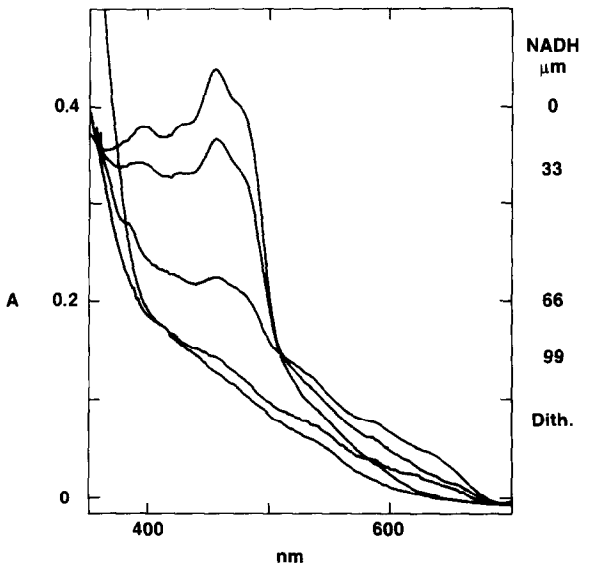


Fig.1. Optical spectra of the flavoprotein of methane monooxygenase. NADH was added to the concentration indicated to a sample of flavoprotein (2.2 mg/ml in 50 mM potassium phosphate buffer, pH 7.0). A slight excess of sodium dithionite was added at the end of the NADH titration.

quential addition of NADH. The prominent feature near 460 nm arises from the oxidized flavin, while the shoulders near 420 and 490 nm arise from the iron-sulfur cluster. All disappear during the reductive titration, while a transient increase in absorption occurs in the red during the titration. Fig.2 shows the same titration examined using ESR spectroscopy. On the left the spectrometer conditions are optimized for observing flavin free radicals, while on the right the conditions are optimized to examine the iron-sulfur cluster. A flavin free radical is seen to appear during the course of the titration, and to disappear upon complete reduction with dithionite. The iron-sulfur cluster, on the other hand, is only partially reduced by concentrations of NADH that yield a maximal flavin radical signal, but is almost completely reduced by excess NADH.

The flavin free radical that appears during the titration absorbs in the red (fig.1), and has a linewidth of approx. 2.0 mT, centered near $g$ 2.003 (fig.2). Together these findings indicate that this is a protonated flavin semiquinone [9–11]. The iron sulfur cluster has $g$ values of 2.04, 1.96 and 1.87,

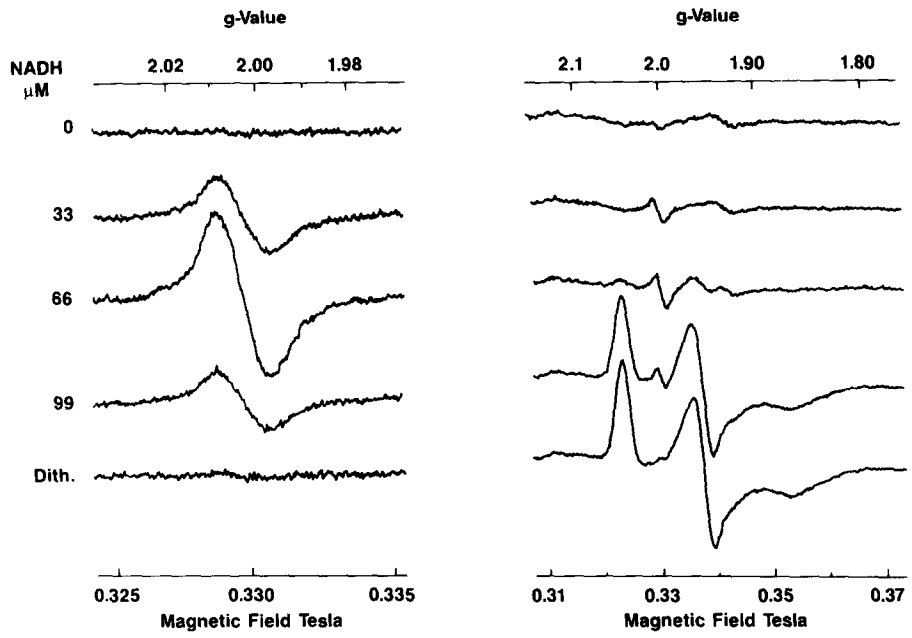


Fig.2. ESR of the flavoprotein of methane monooxygenase. Samples were withdrawn from the experiment of fig.1 and rapidly frozen in liquid nitrogen chilled iso-octane. ESR spectrometer settings: (left) 200 K, 100 $\mu$W applied power, 0.5 mT modulation amplitude; (right) 20 K, 5 mW applied power, 1.25 mT modulation amplitude. All at 9.24 GHz.

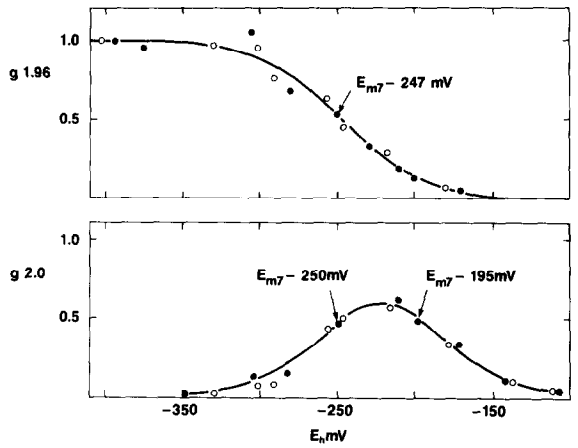


Fig.3. Redox titrations of the redox centers of the flavoprotein of methane monooxygenase. Samples similar to those of figs 1 and 2, with the addition of 40 $\mu$M safranin, indigodisulfonate, 2-hydroxy-1,4-naphtho- and anthraquinones, 1,2- and 1,4-naphthoquinones, 1,2-naphthoquinone-4-sulfonate, 1,4-benzoquinone and duroquinone as redox mediators. These have been shown to not interfere with measurements of quinone free radicals under the conditions used here [13]. ESR spectrometer settings as in fig.2. Open symbols: with the addition of 100 $\mu$M $NAD^+$/NADH.

yielding $g_{av}$ 1.96, characteristic of a $Fe_2S_2$ center [12].

Fig.3 shows redox titrations of the flavin and iron-sulfur cluster, monitored by ESR. The line drawn through the titration of the latter is a Nernst curve, $n = 1$, where

fraction reduced = $[10^{(E_h - E_m)/59} + 1]^{-1}$

The line drawn through the flavin titration is derived from the equations for $n = 2$ couples with stable semiquinones. For the redox couples

flavin + $e^-$ = semiquinone $E_{m1}$

semiquinone + $e^-$ = fully reduced flavin $E_{m2}$

fraction semiquinone =

$$[10^{(E_h - E_{m1})/59} + 10^{(E_{m2} - E_h)/59} + 1]^{-1}$$

The derivation of the latter is discussed in [13]. The midpoint potentials of the redox centers are unaffected by the presence of $NAD^+$/NADH.

## 4. DISCUSSION

Our results are in reasonable agreement with those reported by Lund et al. [4] for the flavoprotein from *Mc. capsulatus* strain Bath. They reported somewhat different midpoint potentials for the flavin couples, with a difference between them of 110 mV, compared to the 55 mV reported here, but this may reflect differences in the treatment of the data. Clearly the two flavoproteins have very similar redox properties, despite coming from taxonomically apparently quite distinct organisms [1]. *Mc. capsulatus* strain Bath belongs to the group that has substantial intracellular membrane structures in the form of discoid vesicles throughout the cell, uses the ribulose monophosphate pathway for carbon assimilation, and has an incomplete tricarboxylic acid cycle lacking 2-oxoglutarate dehydrogenase [1]. *Methylobacterium* CRL26 belongs to the group that has intracytoplasmic membranes aggregated at the cell periphery, uses the serine pathway for carbon assimilation, and has a complete tricarboxylic acid pathway [1].

The redox properties of the flavoprotein, with the midpoint potentials of the iron-sulfur cluster and the semiquinone/hydroquinone couple of the flavin being identical, suggest that the protein probably cycles between the fully reduced and semiquinone state, with the two electrons of NADH going to the semiquinone and the iron-sulfur cluster, and then being delivered, presumably through the iron-sulfur cluster, to the hydroxylase component. The redox properties of the cofactors were unaffected by the presence of excess $NAD^+$/NADH, suggesting no differential binding of these cofactors to the flavoprotein. This is in contrast to the effect of $NADP^+$ on adrenodoxin reductase, which raises the $E_{m7.5}$ of the flavin (which shows no stable semiquinone form) by 100 mV [14]. Whether a ternary complex occurs when the flavoprotein can interact with the hydroxylase enzyme awaits future experimentation.

To date, the only effective way of delivering electrons to the hydroxylase component of methane monooxygenase is to use NADH via the flavoprotein [1–7]. With the elucidation of the potential at which these electrons are delivered to the hydroxylase by the flavoprotein, the discovery of similar potential artificial systems becomes a worthwhile goal.

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