# SHORT COMMUNICATION

## Isolation of a Methanol Dehydrogenase with a Functional Coupling to Cytochrome *c*

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A methanol dehydrogenase containing cytochrome *c* which was reduced on addition of methanol was isolated from *Hyphomicrobium* X. Metal-chelators or oxygen blocked this electron transport. Oxygen changed the coupling irreversibly, transforming the enzyme in the complex into the already known, $NH_4^+$-requiring enzyme form. This 'classical' methanol dehydrogenase differs in several respects from the more natural enzyme reported here.

### INTRODUCTION

Methanol dehydrogenase is a $NAD(P)^+$-independent enzyme which is thought to be coupled to the electron-transport chain at the level of cytochrome *c* because (i) cytochrome *c* becomes reduced on addition of methanol to membrane preparations without reducing cytochrome *b* (Bamforth & Quayle, 1978*a*; Netrusov & Anthony, 1979); (ii) antimycin A (an inhibitor blocking electron transport between cytochromes *b* and *c*) inhibits the oxidation of substrates other than methanol (Netrusov *et al.*, 1977; Van Verseveld & Stouthamer, 1978; Netrusov & Anthony, 1979); and (iii) a mutant of *Pseudomonas* AM1, which lacks cytochrome *c* but contains methanol dehydrogenase, cannot oxidize methanol (Anthony, 1975).

However, electron transfer could not unequivocally be demonstrated with pure methanol dehydrogenase and cytochrome *c* (Anthony, 1975). In addition, in partially purified methanol dehydrogenase, known to be closely associated with cytochrome *c* (Anthony & Zatman, 1967; Duine *et al.*, 1978), the link is not functional (after cytochrome *c* in the preparation has been oxidized, methanol is not able to reduce it). Therefore, although experiments with whole cells and membranes point to electron transfer between methanol dehydrogenase and cytochrome *c*, until now, experiments with the purified components have failed to support this.

As we observed that the methanol-oxidizing capacity of lysozyme-permeabilized cells could only be maintained in the absence of oxygen, we have investigated the possibility that anaerobic conditions might preserve the functionality of the coupling between methanol dehydrogenase and cytochrome *c*.

### METHODS

*Preparation of the methanol dehydrogenase complex.* Frozen *Hyphomicrobium* X (10 g), cultured and stored as previously described (Duine *et al.*, 1978), was suspended in 14 ml 36 mM-Tris/39 mM-glycine buffer, pH 9·0. The suspension was flushed with $N_2$ (containing less than 5 p.p.m. $O_2$) for 10 min in a French pressure cell. The bacteria were disrupted with a pressure of 110 MPa and passed through a hypodermic needle into a centrifuge tube, capped with a rubber seal. The centrifuge tube was kept anaerobic by flushing with $N_2$ through hypodermic needles. After removing the needles, the capped tube was centrifuged at 48000 *g*

for 15 min at 4 °C. All subsequent manipulations were performed under $N_2$ in a glove box at room temperature. The supernatant was applied to a DEAE-Sephacel column ($1\cdot2 \times 8$ cm) which had previously been equilibrated with 36 mM-Tris/39 mM-glycine buffer. The column was washed with similar buffer until the effluent became colourless. Methanol dehydrogenase activity eluted as a red band with 36 mM-Tris/21 mM-$H_3PO_4$ buffer, pH 6·5.

*Oxidation and reduction.* First, 12 µl 0·1 M-potassium ferricyanide was added anaerobically to 0·6 ml methanol dehydrogenase, having the same concentration as used for the scan in Fig. 1, and the absorption spectrum was recorded about 2 min after mixing. The cuvette was then transferred to the glove box and, after addition of 72 µl 1 M-methanol solution, the absorption spectrum was again recorded. Owing to the presence of ferricyanide/ferrocyanide, spectra could only be measured above 450 nm. The spectra had exactly the same shape as depicted in Fig. 1(b), but, owing to dilution, the absorbance was somewhat lower.

*Analytical measurements.* The enzyme assay was based on measuring the discoloration time of Wurster's Blue, referred to as compound II in previous experiments (Duine *et al.*, 1978). Absorption spectra were recorded on a Beckman UV 5260 spectrophotometer. Electron spin resonance spectra were measured as previously described (Duine *et al.*, 1978; Westerling *et al.*, 1979).

## RESULTS AND DISCUSSION

The DEAE-Sephacel purification step under anaerobic conditions yielded a methanol-oxidizing preparation (referred to as the complex) with an absorption spectrum having maxima at 415·5, 521·6 and 550·1 nm (Fig. 1). These maxima lie in the range of those reported for cytochrome *c* in *Hyphomicrobium* (Tonge *et al.*, 1974; Large *et al.*, 1979).

After adding potassium ferricyanide to the complex anaerobically, the $\alpha$ and $\beta$ bands of cytochrome *c* were absent but addition of methanol restored them to their original value (Fig. 1b). This reduction was, however, not possible in the presence of 0·01 M-EDTA or 0·20 M-potassium phosphate. EDTA is known to be an inhibitor of methanol oxidation in whole cells (Anthony & Zatman, 1964) and high phosphate concentrations were used in order to accumulate methanol during oxidation of methane in methane-grown *Pseudomonas* (*Methylomonas*) *methanica* by Higgins & Quayle (1970). Anthony (1975) reported that cytochrome *c* reduction by methanol in whole cells of *Pseudomonas* AM1 was abolished by high phosphate concentrations. As we found that these agents did not inhibit the 'classical' methanol dehydrogenase in the assay with artificial electron acceptors (J. A. Duine & J. Frank, Jzn, unpublished results), it is clear that the electrons in the complex are transferred in another way. Probably a metal is involved in the coupling of the methanol dehydrogenase to the cytochrome *c* in the complex.

Oxidation of cytochrome *c* in the complex was also possible using $O_2$ (Fig. 1), but in this case addition of methanol did not produce the original, reduced spectrum (even after flushing with $N_2$). Thus, $O_2$ transforms the complex into a form which shows no electron flow from methanol dehydrogenase to cytochrome *c*. The oxidation of cytochrome *c* by $O_2$ was unexpected because the isolation procedure under aerobic conditions yielded a preparation which contained reduced cytochrome *c*. This preparation had different chromatographic properties and, as mentioned in the Introduction, a non-functional coupling.

Besides oxidizing cytochrome *c*, $O_2$ also induced a maximum at 350 nm and an increase in the absorbance at about 400 nm. These spectral features are comparable with those found for 'classical' methanol dehydrogenase in this organism (Duine *et al.*, 1978). There is also a striking resemblance in other respects between the complex after reaction with $O_2$ and the 'classical' methanol dehydrogenase. Thus the free radical content increased threefold and the electron spin resonance signal had the same properties as found previously (Duine *et al.*, 1978; Westerling *et al.*, 1979). Furthermore, the ratio of the activity of the complex in the presence of $NH_4^+$ to the activity in its absence increased to the normal value of about 20 (Table 1). From Table 1 it appears that this ratio for the untransformed complex is about 2, which implies some stimulation by $NH_4^+$. This most probably reflects the presence of a small amount of transformed complex, due to a slight contamination by $O_2$, because the preparation contained some free radical and a slight shoulder could be seen in the spectrum at 350 nm (Fig. 1a).
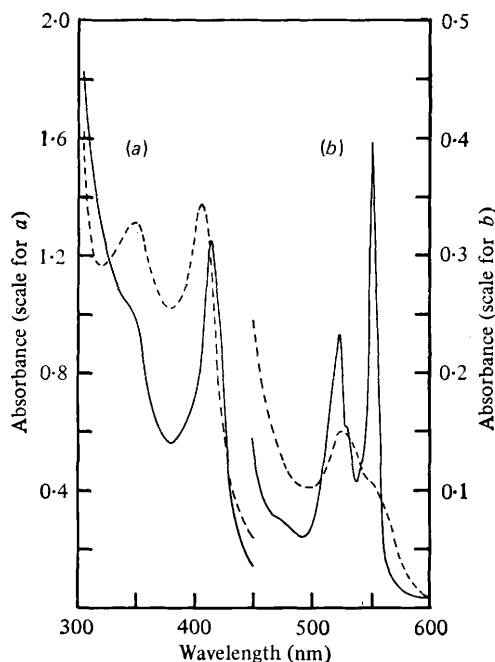
Fig. 1. Absorption spectra of the methanol dehydrogenase complex: ——, spectrum of the complex eluted from the DEAE-Sephacel column, measured in a stoppered cuvette; – – –, spectrum after 1 h exposure to air. The left-hand scale refers to (a) and the right-hand scale to (b). The spectra above 450 nm (b) also depict the effect on the complex of anaerobic oxidation with potassium ferricyanide (– – –) and subsequent reduction with methanol (——), see Methods.

Table 1. *Effect of $NH_4^+$ on enzyme activity during the transformation of methanol dehydrogenase by oxygen*

Either 0·2 ml 0·1 M-$Na_4P_2O_7$ or 0·2 ml 0·1 M-$Na_4P_2O_7$ with 50 mM-$NH_4Cl$, adjusted to pH 9·0 with conc. HCl, and containing 1 mM-methanol was mixed with 0·2 ml 0·3 mM-Wurster's Blue in water. The complex eluted from the DEAE-Sephacel column was exposed to air with slow stirring. Samples of 0·05 ml, with the same concentration as used for the scan in Fig. 1, were taken at the indicated time and added to the test mixture. If not indicated otherwise, all manipulations were performed anaerobically with $N_2$-flushed solutions at room temperature.

| | Discoloration time (s) | |
| --- | --- | --- |
| Incubation time in air (min) | With $NH_4^+$ | Without $NH_4^+$ |
| 0 | 10 | 24 |
| 1 | 12 | 50 |
| 11 | 16 | 120 |
| 20 | 16 | 150 |
| 30 | 18 | 170 |
| 60 | 18 | 200 |
| 120 | 18 | 340 |

In view of this spectral change and the formation of free radical, we conclude that there is a change in the redox-state of the prosthetic group in methanol dehydrogenase. As we do not know how many components in the complex are affected by $O_2$, the relationship between the need for $NH_4^+$, the non-functional coupling with cytochrome $c$ and the different redox-state is unclear. In this context it should be noted that this transformation by $O_2$ is not unique; it has been reported that $O_2$ transforms succinate dehydrogenase extracted from mitochondria into a form which has no reconstitutive ability in the electron-transport chain (Ackrell *et al.*, 1977).

It has been suggested that methanol dehydrogenase *in vivo* needs an activator (Anthony & Zatman, 1967; Bamforth & Quayle, 1978*b*). However, we did not succeed in detecting a stimulatory effect of $NH_4^+$ on the methanol oxidation of washed lysozyme-permeabilized cells and, as far as we know, no natural activator has yet been found. As already discussed, the slight stimulatory effect of $NH_4^+$ on the isolated complex may be ascribed to a partial transformation by $O_2$. Therefore, we may conclude that the enzyme *in vivo*, unlike the 'classical' enzyme form, needs no activator.

An alternative explanation is that the enzyme *in vivo* contains a bound activator which is destroyed by $O_2$ yielding the 'classical' enzyme form. In any case, it is clear that the 'classical' methanol dehydrogenase differs in several respects from the anaerobic preparation, which probably reflects the *in vivo* functional form of the enzyme.

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