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Methanol oxidation under physiological conditions using methanol dehydrogenase and a factor isolated from *Hyphomicrobium* X

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Anion-exchange chromatography of a cell-free extract from *Hyphomicrobium* X yielded a cytochrome c_H-containing methanol dehydrogenase (MDH) preparation only when all manipulations were carried out under anaerobic conditions. The properties of MDH in this preparation were unusual since significant activity could be detected in the absence of an artificial activator (NH_4^+-salts) and the absorption spectrum was clearly different from that of MDH isolated in the usual way (aerobically). After gel filtration, the activator independency was lost, but reappeared when the front fractions (containing activator-dependent MDH) were combined with the low molecular mass fractions. Although the compound in these fractions (factor X) could replace the artificial activator in an assay at pH 9.0, it is clearly not an NH_4^+-salt. In addition, it could not be replaced by coenzyme A, tetrahydrofolate, reduced glutathione, ubiquinone Q-9 or a number of divalent metal ions. Inactivation of factor X occurred in the presence of oxygen and cytochrome *c*. Using ferricytochrome c_L and MDH at pH 7.0, it was shown that factor X stimulates the methanol oxidation rate under this physiological condition.

Methanol dehydrogenase; Pyrroloquinoline quinone; Cytochrome c_L; Methanol oxidation; (*Hyphomicrobium* X)

1. INTRODUCTION

It is generally accepted that methanol dehydrogenase (MDH) donates reduction equivalents derived from methanol oxidation to the respiratory chain at the level of cytochrome *c* [1]. However, using purified MDH and cytochrome c_L, the natural electron acceptor for MDH in methylotrophic bacteria, only very low reaction rates were observed, compared to methanol oxidation rates of whole bacteria or to the rates observed in the normal 'unphysiological' assay at high pH with NH_4^+-salt as the activator and a dye as the artificial electron acceptor [2,3].

In earlier studies we found that anaerobically isolated preparations of MDH were able to transfer electrons to cytochrome *c*, a property which was lost after exposure of the preparation to oxygen [4]. Concomitantly, the activity in an assay with artificial electron acceptor became activator-dependent. These observations suggested that either a mediator or the components themselves changed on O_2 exposure, resulting in the inactivation of the physiological electron transfer mechanism. In order to discriminate between these possibilities, anaerobically prepared preparations from *Hyphomicrobium* X were chromatographed under exclusion of O_2. This revealed the existence of a factor enabling the assay of MDH at physiological conditions. Moreover, it was shown that MDH in these preparations has an absorption spectrum different from that of any other redox form reported for this enzyme.

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Abbreviations: PQQ, pyrroloquinoline quinone; Mops, 4-morpholinepropanesulphonic acid; MDH_{red}, reduced methanol dehydrogenase; MDH_{sem}, MDH in the semiquinone state; THF, tetrahydrofolate; WB, Wurster's blue (the free radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine)

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2. MATERIALS AND METHODS

2.1. *Growth of cells*

Hyphomicrobium X was grown at 30°C on a mineral medium [5], supplemented with methanol (0.4%, v/v). Cells were harvested at the end of the exponential growth phase, transferred into an anaerobic glove box, washed once with 0.05 M potassium phosphate buffer (pH 7.0) and stored anaerobically at −20°C. Cells were used within one week after harvesting.

2.2. *Fractionation of cell-free extract*

All manipulations were carried out at room temperature in a glove box kept anaerobic by a mixture of 97.5% N_2 and 2.5% H_2 in the presence of a catalyst (RO-21, BASF) removing traces of O_2 [6]. All buffers were made anaerobic by degassing on a vacuum line, followed by flushing with N_2 (containing less than 1 ppm O_2). Chromatographic fractions were tested for MDH activity with the dye/activator assay (see section 2.3).

Frozen cells (10 g wet wt) were suspended in 15 ml of 36 mM Tris/39 mM glycine buffer (pH 9.0) and disrupted once in a French pressure cell at 110 MPa. The suspension coming from the cell passed through a hypodermic needle into a centrifuge bottle, capped with a rubber seal and kept anaerobic by flushing with N_2. The suspension was centrifuged at 48000 × *g* for 15 min at 4°C. The resulting cell-free extract (16 ml, activity 439 U) was applied to a DEAE-Sepharose column (40 × 2.2 cm) which was equilibrated with 36 mM Tris/39 mM glycine buffer (pH 9.0). The column was washed with the same buffer. Part of the MDH activity was not adsorbed but eluted as a red band (21 ml, activity 288 U). This preparation was designated MDH_I. After the column effluent became colourless, the column was washed with 36 mM Tris/21 mM H_3PO_4 buffer (pH 6.5). A second fraction containing MDH activity was eluted and designated MDH_{II} (14 ml, activity 120 U). MDH_{II} was further purified (aerobically) as described elsewhere [5].

The cytochrome *c* adsorbed to the DEAE-Sepharose column was eluted with 36 mM Tris/21 mM H_3PO_4 buffer (pH 6.5), containing 0.1 M NaCl. This cytochrome *c* was further purified (aerobically) as described elsewhere [7]. Cytochrome c_L and cytochrome c_H, used as a reference, were purified aerobically as described previously [7].

Fractions containing MDH_I were pooled and 1.0 ml samples were applied to a PD-10 gel filtration column (Pharmacia) equilibrated with 0.02 M potassium phosphate buffer (pH 7.0) and the eluate collected in 1 ml portions. MDH_I activity (factor X free) eluting in the void volume was concentrated anaerobically under pressure over an Amicon YM-2 membrane. Fractions containing factor X (assayed with MDH_I, factor X free, in the dye/factor assay (see section 2.3)) were also pooled and stored anaerobically at 4°C. The MDH_I (factor X free) preparation was applied to a silica gel column (8 × 1 cm, 70–230 mesh, no.60, Merck) equilibrated with 0.02 M potassium phosphate buffer (pH 7.0). MDH activity passed through the column. Active fractions were pooled, concentrated anaerobically under pressure over a Millipore membrane (cut-off level 10000) and stored anaerobically at 4°C. The cytochrome *c* adsorbed to the silica gel was eluted with 0.2 M potassium phosphate buffer (pH 7.0), containing 10% (w/v) polyethylene glycol 6000. It was further purified (aerobically) by a procedure developed for cytochrome c_H [7].

2.3. *Methanol dehydrogenase assays*

The assays were performed anaerobically, unless stated otherwise, in quartz cuvettes which were sealed with subaseal stoppers (Hellma). The cuvettes were filled in the glove box with the assay mixtures (1 ml). The reactions were started by injecting anaerobic MDH samples with a Hamilton syringe.

2.3.1. The dye/activator assay

This is the usual assay for MDH, using a high pH (pH 9.0), NH_4^+-salts (64 mM) as activators, WB (100 μM) as the electron acceptor and methanol (5 mM) as the substrate. Reduction rates of WB were measured as described [8].

2.3.2. The dye/factor assay

In order to test the presence or absence of factor X in MDH preparations, the same mixture was used as in the dye/activator assay, except that NH_4Cl was omitted. The same assay was used to test samples for the presence of factor X, except that a factor X free MDH_I preparation, prepared by gel filtration as described in section 2.2, was used.

2.3.3. The physiological assay

To test the capability of MDH preparations to oxidize methanol under physiological conditions, the natural electron acceptor, cytochrome c_L, was used and a buffer of pH 7.0. The assay mixture contained: 50 mM Mops buffer (pH 7.0), 7.6 μM ferricytochrome c_L and 1.0 mM methanol. To test the effect of factor X, a factor X preparation was used which had been obtained by gel filtration of MDH_I, as described in section 2.2. In this case, the assay mixture contained 0.22 μM purified MDH_{II}. Ferricytochrome c_L was prepared by oxidizing ferrocytochrome c_L with a slight excess of potassium ferricyanide.

Ferricytochrome c_L reduction rates were measured at 550 nm, using absorption coefficients of 21.6 $mM^{-1} \cdot cm^{-1}$ for ferrocytochrome c_L and 6.7 $mM^{-1} \cdot cm^{-1}$ for ferricytochrome c_L [7]. The concentration of MDH_{II} was determined using an absorption coefficient of 38 $mM^{-1} \cdot cm^{-1}$ at 343 nm (unpublished).

2.4. *Determination of ammonia*

The detection of ammonia was based on the enzymatic conversion of ammonium and α-ketoglutarate by glutamate dehydrogenase [9]. The assay mixture (1 ml) contained 0.2 M potassium phosphate buffer (pH 7.6), 6 mM α-ketoglutarate, 0.42 mM NADH and 6 units of glutamate dehydrogenase (Boehringer). The assay was calibrated with NH_4Cl (0–0.1 mM).

2.5. *Ubiquinone extraction*

Ubiquinones were extracted from whole cells of *Hyphomicrobium* X as described by Yamada et al. [10]. Q-9, the major ubiquinone present in *Hyphomicrobium* strains [11], was obtained from Sigma. The concentration of Q-9 was determined at 275 nm using an absorption coefficient of 14.7 $mM^{-1} \cdot cm^{-1}$ [12].

2.6. *Spectrophotometry*

Absorption spectra were taken and steady-state kinetics were performed with a Hewlett-Packard HP 8450A photodiode array spectrophotometer at 20°C. Multicomponent analysis was carried out with the software supplied with the spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. *Properties of MDH_I and MDH_{II}*

Anion-exchange chromatography gave two MDH fractions, MDH_I and MDH_{II}. The MDH_I preparation showed activity in the dye/factor assay (table 1) and had the spectral characteristics reported in [4]. It should be noted however, that in contrast to the earlier observation [4], MDH_I was not eluted with the buffer of pH 6.5, but during the washing with Tris/glycine buffer (pH 9.0). MDH_I represented approx. 70% of the activity in the cell-free extract measured with the dye/activator assay. It should be stressed that MDH_I is only found if anaerobic conditions are applied. Under aerobic conditions all MDH activity is retained by the column material and eluted as MDH_{II} on applying the Tris/phosphate buffer (pH 6.5).

The MDH_{II} fraction (approx. 30% of the activity in the cell-free extract) was eluted at pH 6.5 and appeared to be in the reduced form (MDH_{red}) [13]. This preparation did not show activity in the dye/factor assay (table 1).

The major cytochrome *c* coeluting with MDH_I appeared to be cytochrome c_H, whereas the cytochrome *c* which was eluted with 36 mM Tris/21 mM H_3PO_4 buffer (pH 6.5), containing 0.1 M NaCl, was cytochrome c_L. This was established by polyacrylamide gel electrophoresis using genuine cytochrome c_L and cytochrome c_H as standards (not shown).

Exposure of the MDH_I preparation to O_2 induced the same spectral changes as described in [4]. An absorption maximum around 350 nm was induced and the cytochrome c_H became oxidized. Concomitantly, the activity in the dye/factor assay was lost. However the activity measured with the dye/activator assay remained constant.

Although the MDH_I preparation showed activity in the dye/factor assay, after chromatography on the PD-10 gel filtration column, none of the fractions were active in the dye/factor assay, but the fractions corresponding to the void volume showed activity in the dye/activator assay. Incubating these fractions (MDH_I, factor X free) with the low molecular mass fraction (factor X preparation) restored the activity in the dye/factor assay (table 1, fig.1). Aerobic gel filtration of the MDH_I preparation after exposure to air, yielded low molecular mass fractions which were unable to restore the dye/factor activity. Exposure to O_2 of the MDH_I preparation (factor X free) did not induce the absorption maximum at 350 nm, but the

Table 1

The effect of factor X on MDH activity

Preparations	MDH activity (μmol WB reduced · ml^{-1} · min^{-1})	
	Dye/activator assay	Dye/factor assay
Cell-free extract	3.6	3.4
DEAE-Sepharose		
MDH_I	3.7	3.6
MDH_{II}	3.5	0.6
Gel filtration		
MDH_I (factor X free)	3.0	0.01
Factor X (fraction 9)	n.d.	n.d.
MDH_I (factor X free) + factor X (fraction 9)	3.0	1.7

n.d., not detectable. MDH preparations were tested with the dye/factor assay. To indicate the amounts of MDH used, the values of the dye/activator assay are also shown. Information on the preparations used is given in the legend of fig.1

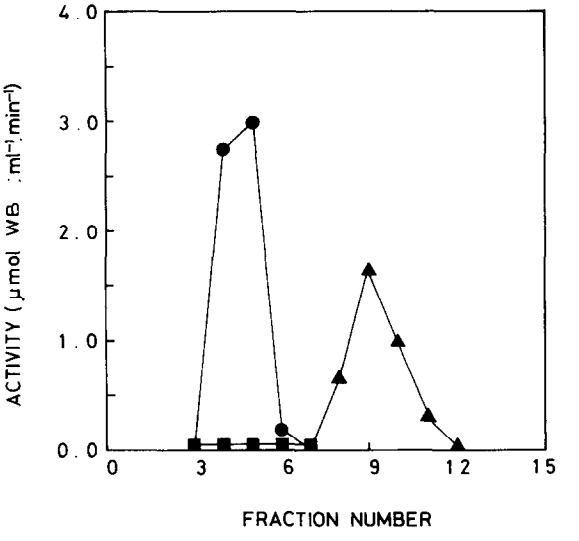

Fig.1. Distribution of MDH_I activity and factor X on anaerobic gel filtration. MDH_I activity in the fractions was assayed by testing samples (50 μl) using the dye/activator assay (●) and the dye/factor assay (■). The distribution of factor X was measured by the dye/factor assay, using MDH_I (factor X free) from fraction number 5 (50 μl) and adding 100 μl of a fraction to test the presence of factor X (▲).

cytochrome c_H became oxidized. The absorption spectrum of MDH in the MDH_I preparation was calculated via multicomponent analysis (fig.2). The amounts of reduced and oxidized cytochrome c_H in the preparation were estimated by performing a multicomponent analysis in the wavelength region 492–566 nm, assuming a negligible contribution of MDH in that region. The spectrum appeared to be similar to that of the MDH_I preparation eluted from the silica gel column. The spectrum differed from those reported for all redox forms of MDH observed so far [13], suggesting that the cofactor PQQ in a MDH_I preparation exists in an unusual form. One of the possibilities already suggested [14], is that the pyrrole ring of PQQ is in its open form. Ring closure could happen when MDH_I is transformed by O_2 into MDH_{sem}, the semiquinone form of MDH, having an absorption maximum at 350 nm (fig.2). Similarly, ring closure might also occur when the cofactor is extracted from the enzyme. In view of the unique properties of MDH_I and its natural behaviour compared to MDH_{II}, it is proposed to refer to this enzyme form as MDH_{iv} in the future, this in view of its assumed high degree of relatedness to MDH in vivo.

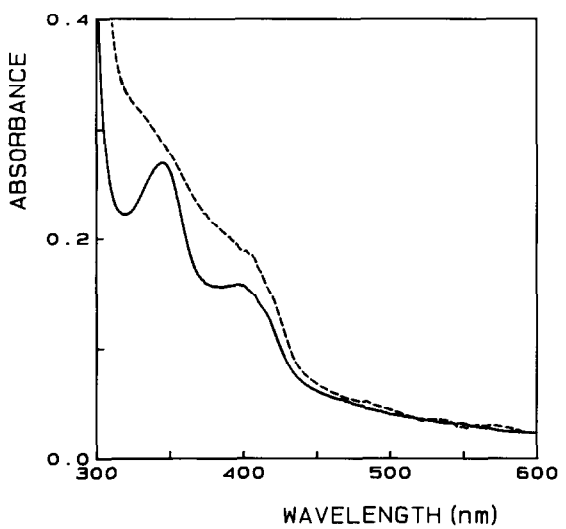

Fig.2. Absorption spectra of MDH forms. Spectra were taken of the MDH_I preparation (10.8 μM MDH) in 0.02 M potassium phosphate buffer (pH 7.0) before (– – –) and after (——) exposure to O_2, followed by gel filtration. The spectra shown were corrected for the presence of oxidized and reduced cytochrome c_H in the MDH_I preparation by multicomponent analysis, using the genuine spectrum of cytochrome c_H [7].

3.2. *Properties of factor X*

As shown in fig.1, factor X was separated from MDH on gel filtration. The factor X preparation was stable against O_2 exposure, that is, it was still capable of activating MDH_I (factor X free) and MDH_{II} preparations in the dye/factor assay under anaerobic conditions.

On incubating the ferrocytochrome c_H fraction obtained after the DEAE-Sepharose step with factor X in the presence of oxygen, cytochrome c_H became oxidized and factor X became inactivated. A similar inactivation probably occurs in the presence of ferrocytochrome c_L since factor X was inactive in the physiological assay under aerobic conditions, as shown in table 2. This inactivation by O_2 and the fact that the factor X preparation obtained by gel filtration contained only 0.04 mM NH_3 (an amount far too low to account for the stimulating effect, table 2), exclude that factor X is an NH_4^+-salt.

Factor X could not be replaced in the dye/factor assay by divalent cations such as Mg^{2+}, Ca^{2+}, Cu^{2+} or Mn^{2+} (0.05–0.5 mM), coenzyme A (24 μM), THF (24 μM), GSH (10 μM), Q-9 (0.6 μM) or ubiquinones extracted from whole cells of *Hyphomicrobium* X (these compounds were chosen on the basis of their possible involvement with methanol or formaldehyde oxidation). Factor X was heat stable (10 min, 100°C), was not retained by a dialysis membrane and did not bind to DEAE-Sepharose, CM-Sepharose (pH 7.0) or to a Seppak C_{18}-silica cartridge (pH 2.0). Stimulation of the overall reduction rate in the physiological assay was more effective with cytochrome c_L (fac-

Table 2

Methanol oxidation under physiological conditions

Additions	MDH_{II} activity (nmol c_L reduced (nmol MDH_{II})$^{-1}\cdot s^{-1}$)	
	Anaerobically	Aerobically
None	0.057	0.061
Factor X (10 μl)	0.155	0.061
NH_4Cl (0.04 mM)	0.066	0.066

Homogeneous MDH_{II} was tested using the physiological assay with and without factor X and 0.04 mM NH_4Cl (the latter concentration corresponding with the amount of NH_3 present in the factor X sample). Factor X was prepared as indicated in section 2.2

tor 2.7) than with cytochrome c_H (factor 1.2) as the electron acceptor.

The properties of factor X suggest that it is a low molecular mass compound with a structure that is oxidized by O_2 in the presence of cytochromes. Due to the lack of knowledge about its identity, the concentrations of factor X in the preparations are unknown. Therefore, although the rates of the physiological assay increase by a factor of 3 on addition of factor X, the rates are still low compared with those obtained in the dye/activator assay possibly because of the low amounts of factor X in the preparation.

Arguments have been provided in the past that NH_4^+-salts are not activators for methanol oxidation in vivo [13]. Although factor X stimulates the activity of MDH_I (factor X free) as well as MDH_{II} in the physiological and the dye/factor assays, just like NH_4^+-salts do in the dye/activator assay, it is clear that factor X is not an NH_4^+-salt. Therefore, factor X might be the 'natural' activator for MDH, while the variable results for cytochrome *c* as the electron acceptor in an in vitro assay might be explained by partial removal of factor X from the preparation and/or its inactivation by O_2 with cytochrome *c* functioning as a catalyst. Although the finding of factor X throws light on this long standing problem, further studies are necessary to elucidate its structure and specific role in the catalytic cycle of MDH.

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