NAD-dependent, PQQ-containing methanol dehydrogenase: a bacterial dehydrogenase in a multienzyme complex

J.A. Duine, J. Frank and M.P.J. Berkhout

Laboratory of Biochemistry, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Received 24 January 1984

Cell-free extracts of methanol-grown *Nocardia* sp. 239 only show significant dye-linked methanol-oxidizing activity when NAD^+ is added to the assay mixture. This activity resides in a multienzyme complex which could be resolved into 3 components, namely the methanol dehydrogenase, NAD-dependent aldehyde dehydrogenase and NADH dehydrogenase. In its dissociated form, the methanol dehydrogenase no longer shows dye reduction and although rises in the absorbance values around 340 nm are seen on addition of methanol plus NAD^+ to the enzyme, this is not due to NADH production. However, dye reduction (NAD dependent) could be restored on incubating methanol dehydrogenase with the corresponding NADH dehydrogenase, obtained from the enzyme complex. It is concluded that this novel methanol dehydrogenase transfers the reducing equivalents, derived from methanol, directly to its associated NADH dehydrogenase via a mechanism in which NAD^+ and PQQ are involved.

Methanol dehydrogenase *NAD-dependent aldehyde dehydrogenase* *NADH dehydrogenase* *Multienzyme complex* *Pyrroloquinoline quinone* Nocardia *sp. 239*

1. INTRODUCTION

Methylotrophic, Gram-negative bacteria metabolize methanol by means of an NAD(P)-independent dehydrogenase, the activity of which is measured at high pH in the presence of NH_4^+ salts via dye-reduction [1]. This 'classical' methanol dehydrogenase (EC 1.1.99.8) is a quinoprotein since pyrroloquinoline quinone (PQQ), is its prosthetic group [2,3].

However, this enzyme may not be universally involved in bacterial oxidation of methanol. For example, in some Gram-positive methylotrophic bacteria, classical methanol dehydrogenase could not be detected [4,5]. Cell-free extracts of *Streptomyces* sp. 239 have been reported [5] to oxidize methanol (although at a low rate) via a dye-linked dehydrogenase at pH 7.0 in the absence of NH_4^+ salts. Reinvestigations [6] confirmed that in this organism, which is most probably a *Nocardia* sp., the presence of the classical methanol dehydrogenase cannot be demonstrated. Since it was observed [6] that this methanol-grown organism excretes PQQ (just like Gram-negative methanol utilizers) the question arose whether an unusual quinoprotein methanol-oxidizing enzyme might be present in this organism.

Abbreviations: PQQ, pyrroloquinoline quinone; DCIP, 2,6-dichlorophenolindophenol

2. MATERIALS AND METHODS

2.1. *Cultivation of the organism*

Nocardia sp. 239, LMD 80.32 (originally described [5] as *Streptomyces* sp. 239), was grown aerobically at 37°C with 0.5% ethanol plus 0.5% methanol in a mineral salt medium [6]. During growth, the pH of the culture was maintained at 6.6 with concentrated ammonia. As described in [6], the organism first utilizes ethanol and after a lag phase, methanol. Cells were harvested when all methanol had disappeared from the culture.

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

2.2. *Isolation of the multienzyme complex*

Frozen bacterial cell paste was thawed and mixed with an equal volume of 0.02 M potassium phosphate buffer (pH 7.2). The bacteria were disrupted in a French pressure cell at 110 MPa. To lower the viscosity, DNase was added. The suspension was centrifuged at 48000 × *g* for 20 min at 4°C.

The supernatant was applied to a column of DEAE–Sephacel, equilibrated with 0.02 M potassium phosphate (pH 7.2), and the column washed with the same buffer. The multienzyme complex was eluted with 0.02 M potassium phosphate (pH 7.2), containing 1 M KCl and 2% sorbitol. Further purification was obtained by gel filtration on a Fractogel HW 55 column in 0.2 M sodium phosphate (pH 7.0), containing 2% sorbitol.

2.3. *Isolation of the methanol, aldehyde and NADH dehydrogenases*

The cell-free extract was applied to DEAE–Sephacel, as described above. After washing the column, NADH-dehydrogenase was eluted with 0.5 M potassium phosphate (pH 6.8). The methanol dehydrogenase, together with the aldehyde dehydrogenase, was subsequently eluted with 0.02 M potassium phosphate (pH 7.2), containing 1 M KCl and 2% sorbitol. The methanol dehydrogenase was separated from aldehyde dehydrogenase by HPLC gel filtration on a TSK G3000 SW column in 0.2 M potassium phosphate buffer (pH 7.0).

NADH-dehydrogenase from *Bacillus subtilis* was partly purified as in [7].

2.4. *Enzyme assays*

Dehydrogenase activities of the multienzyme complex were measured in 0.1 M tetrasodium pyrophosphate (pH 9.0), with or without 0.12 M NH_4Cl (as indicated), by following dye reduction at 600 nm in the presence of 40 μM DCIP plus 2.5 mM NAD^+ or by following NADH production at 340 nm in the presence of 2.5 mM NAD^+. Methanol, aldehyde, and NADH dehydrogenase activities were measured in the presence of 2 mM methanol, 1 mM formaldehyde, and 280 μM NADH, respectively.

After removal of NADH dehydrogenase from the complex, methanol and aldehyde dehydrogenase activities could only be measured by following the rise in absorbance at 340 nm, using the same buffer, substrate and NAD concentrations as indicated above. For methanol dehydrogenase activity, it is essential to use the buffer with 0.12 M NH_4Cl.

2.5. *Analytical procedures*

For analysis of PQQ, enzyme-containing fractions were denatured in a boiling water bath for 2 min. After cooling and centrifugation, the supernatants were analyzed for PQQ as in [8]. Conversion of ethanol and methanol during cultivation of the organism was followed by gas chromatography on a Porapak Q column.

3. RESULTS AND DISCUSSION

3.1. *Methanol oxidation by cell-free extract*

Despite many attempts, the presence of classical or an NAD(P)-dependent methanol dehydrogenase could not be demonstrated in the cell-free extract. Occasionally, a low (too low to have physiological significance) activity of dye-linked methanol oxidation was observed at pH 7.0. This low activity was lost upon short dialysis, suggesting that a low-M_r compound might be essential for activity. On testing several compounds it appeared that the presence of NAD^+ (which cannot be replaced by $NADP^+$) was required to obtain significant dye-linked methanol oxidation (table 1). In contrast, formaldehyde oxidation could be measured via NADH production, although it was also possible

Table 1

Dehydrogenase activities in the cell-free extract

Substrate	Addition of		DCIP reduction[a]	NADH production[b]
	NAD^+	DCIP		
Methanol	−	+	3	
Methanol	+	−		2
Methanol	+	+	31	
Formaldehyde	−	+	6	
Formaldehyde	+	−		48
Formaldehyde	+	+	57	
NADH	−	+	181	

[a] nmol DCIP reduced · min^{-1} · mg^{-1} protein

[b] nmol NADH produced · min^{-1} · mg^{-1} protein

to measure it via dye reduction on addition of NAD^+ plus DCIP. The latter observation is not surprising in view of the fact that the cell-free extract also showed dye-linked NADH oxidation activity (table 1).

3.2. *The multienzyme complex*

The methanol-, formaldehyde- and NADH-oxidizing activities adsorbed to DEAE ion exchangers. Elution of methanol-oxidizing activity was only possible with buffers containing compounds like sorbitol, glycerol or poly(ethylene glycol). Curiously, the total formaldehyde and NADH oxidation activities were also found in this eluate and all 3 activities showed the properties described above for the cell-free extract. Although sorbitol was necessary for elution of the methanol-oxidizing activity, it did not function as an enzyme stabilizer since the oxidation activity for methanol (but not for NADH or formaldehyde) was lost on standing. This inactivation appeared to be related to the presence of O_2, a situation which also exists in the case of anaerobically isolated classical methanol dehydrogenase where a very rapid transformation occurs to an enzyme form, requiring NH_4^+ salts in the assay, on admission of O_2 [9]. It was indeed found in this case that addition of NH_4^+ salts raised the activity for methanol (but not for formaldehyde or NADH), as shown in table 2 for a partly transformed preparation. This indicated that, in contrast to classical methanol dehydrogenase, this enzyme oxidizes methanol but not formaldehyde. Furthermore, it is clear that from this stage on, it is necessary to add NH_4Cl to the assay for methanol dehydrogenase activity.

The fact that methanol oxidation by the eluate did not produce NADH but the dye-linked activity nevertheless required the presence of NAD^+ can be explained by assuming that:

(i) Either the NADH produced cannot dissociate from the enzyme but has to be oxidized by a dye-linked NADH dehydrogenase;

(ii) or the methanol oxidation is essentially dye-linked but the formaldehyde produced has to be removed by an NAD-dependent formaldehyde dehydrogenase.

Both hypotheses assume a close physical interaction between the enzymes, which is in agreement with the fact that all 3 activities are present in one fraction. Further confirmation for the existence of a multienzyme complex was obtained from gel filtration experiments with a buffer containing sorbitol, showing that the 3 activities elute together (fig.1).

Table 2

The effect of NH_4Cl and NAD^+ addition on the dehydrogenase activities of the multienzyme complex, purified by gel filtration on Fractogel (fig.1)

Substrate	Addition of		DCIP reduction[a]
	NH_4Cl	NAD^+	
Methanol	−	−	0
Methanol	−	+	12
Methanol	+	+	88
Ethanol	+	+	1
Formaldehyde	−	+	142
Formaldehyde	+	+	135
NADH	−	−	459
NADH	+	−	458

[a] nmol DCIP reduced · min^{-1} · mg^{-1} protein

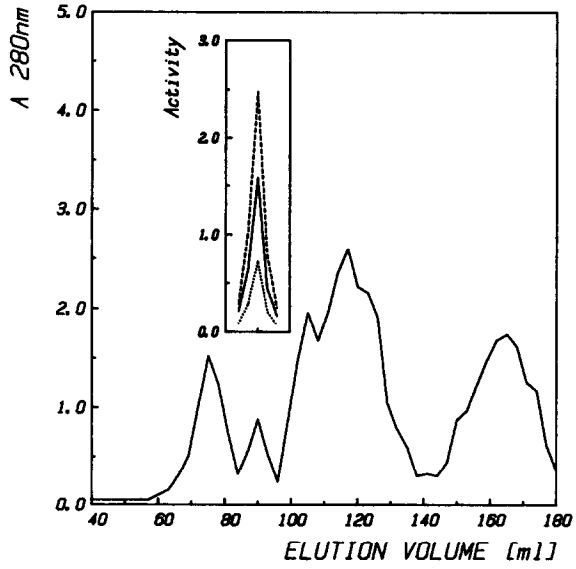

Fig.1. Gel filtration of multienzyme complex on a Fractogel HW-55 column in 0.2 M sodium phosphate buffer (pH 7.0), containing 2% sorbitol. The sample used was multienzyme complex, purified by DEAE–Sephacel chromatography. Dehydrogenase activities were measured with the dye-reduction assay: (···) methanol dehydrogenase, (——) aldehyde dehydrogenase, (– – –) NADH dehydrogenase.

3.3. *Dissociation of the multienzyme complex*

NADH dehydrogenase could be dissociated from the multienzyme complex, adsorbed to DEAE–Sephacel, by eluting with 0.5 M potassium phosphate (pH 6.8). Subsequently, the usual elution buffer was applied. In the fraction obtained, no dye-linked formaldehyde oxidation was found, although formaldehyde dehydrogenase was present, as shown by NADH production. Even better activities were found with acetaldehyde and propionaldehyde, indicating that formaldehyde oxidation is due to an aspecific NAD-dependent aldehyde dehydrogenase.

When the fraction was tested for methanol oxidation, no dye-linked methanol dehydrogenase activity was found. Surprisingly, in contrast to the multienzyme complex, a rise in absorbance at 340 nm was observed on adding methanol plus NAD^+ to the fraction. However, as is apparent from fig.2, the rise soon leveled off (not observed with formaldehyde plus NAD^+). This phenomenon is not due to the attainment of an equilibrium, as further addition of methanol and NAD^+ had no effect. Furthermore, when an attempt was made to estimate NADH at this point by using NADH dehydrogenase from *B. subtilis*, having a low K_m value for NADH [7], no activity was found. Hence the rise is due to a change in the absorption spectrum of the enzyme and free NADH is not produced, in accordance with hypothesis (i). To substantiate this further, the fraction was incubated with the NADH dehydrogenase, obtained from the DEAE–Sephacel column. As is obvious from fig.3, after incubation for 20 min, the original NAD-dependent, dye-linked methanol dehydrogenase activity reappeared while the 'absorbance rise activity at 340 nm' concomitantly disappeared.

The fact that methanol and aldehyde dehydrogenase activities behaved differently in so many respects indicated that they did not originate from one enzyme. HPLC gel filtration of the fraction indeed separated the two activities. Fractions active for methanol were not so for formaldehyde, ethanol, propanol, isopropanol and $NADP^+$ could not replace NAD^+. Cyclopropanol, which is a

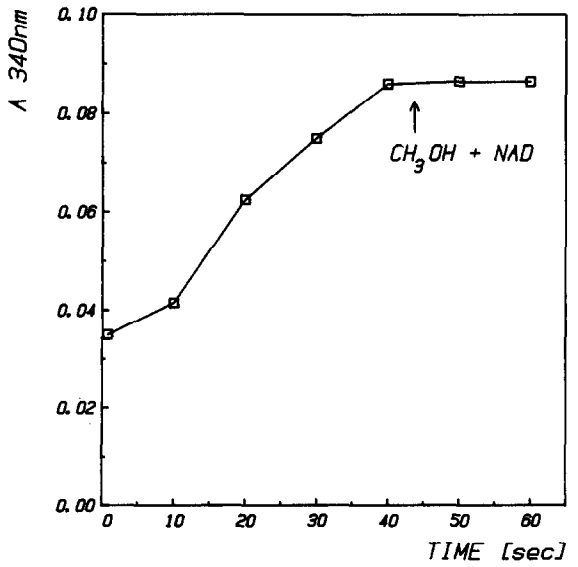

Fig.2. The rate of absorbance increase at 340 nm in methanol dehydrogenase after adding methanol (2 mM) and NAD^+ (2.5 mM) to the enzyme in 0.1 M tetrasodium pyrophosphate (pH 9.0), containing 0.12 M NH_4Cl. The enzyme preparation used was the fraction eluted from DEAE–Sephacel, after removal of NADH dehydrogenase.

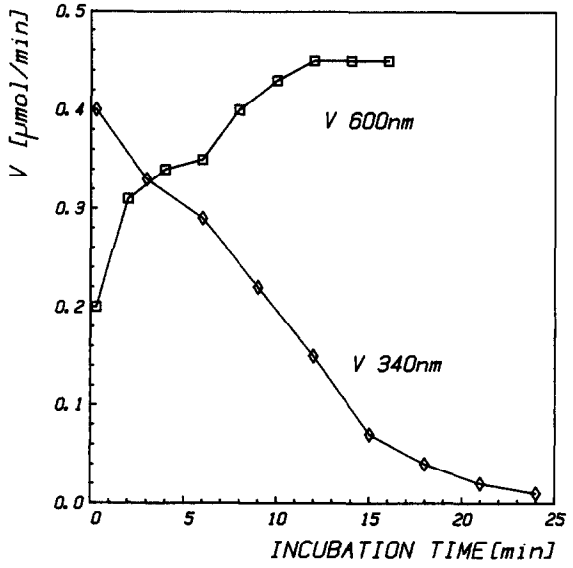

Fig.3. Methanol dehydrogenase activities during the incubation of the methanol dehydrogenase-containing fraction with the NADH dehydrogenase-containing fraction. NAD-dependent DCIP reduction (v 600 nm) and the rate of absorbance increase at 340 nm (v 340 nm) were measured as described in section 2. The fractions used were obtained from the DEAE–Sephacel column.

suicide substrate for classical methanol dehydrogenase [10], does not react with this enzyme. After denaturation, amounts of PQQ proportional to the methanol dehydrogenase activities were found in the fractions. The fact that no activity was found with formaldehyde or other substrates again indicates that hypothesis (i) is correct and that the enzyme is an NAD-dependent quinoprotein dehydrogenase, specific for methanol.

3.4. *Comparison with classical methanol dehydrogenase*

So far oxidation of methanol has been ascribed to an NAD(P)-independent quinoprotein dehydrogenase which has been purified from a large number of widely differing methylotrophic bacteria [1]. The enzyme described here is a novel one, as it is specific for methanol, is NAD-dependent, occurs in a multienzyme complex with aldehyde and NADH dehydrogenase, and is not inhibited by cyclopropanol. There are, however, similarities between the novel and the classical enzyme: both contain PQQ and a free radical ($PQQH^{\cdot}$) (not shown) and O_2 transforms them into an enzyme form, requiring NH_4^+ salts in the assay.

In view of the peculiar test conditions, the question can be raised whether the presence of novel methanol dehydrogenase has been overlooked in other cases. As a similar enzyme has now been found in a methanotroph (unpublished), the discovery of the novel enzyme will have important consequences for the view on the bioenergetics and methane oxidation of methylotrophic bacteria.

ACKNOWLEDGEMENTS

The authors are grateful to Dr W. Hazeu for advice on the cultivation of the organism, to Dr K. Hellingwerf for providing an NADH preparation from *B. subtilis*, and to ICI, Agricultural Division, Billingham, England, for financial support.

REFERENCES

[1] Anthony, C. (1982) The Biochemistry of Methylotrophs, pp.167–186, Academic Press, New York.
[2] Duine, J.A., Frank, J. and Verwiel, P.E.J. (1980) Eur. J. Biochem. 108, 187–192.
[3] Salisbury, S.A., Forrest, H.S., Cruse, W.B.T. and Kennard, O. (1979) Nature 280, 843–844.
[4] Loginova, N.V. and Trotsenko, Yu.A. (1981) FEMS Microbiol. Lett. 5, 239–243.
[5] Kato, N., Tsuji, K., Tani, J. and Ogata, K. (1975) in: Microbial Growth on C_1-compounds, pp.91–98, The Society of Fermentation Technology, Japan.
[6] Hazeu, W., De Bruyn, J.C. and Van Dijken, J.P. (1983) Arch. Microbiol. 135, 205–210.
[7] Bergsma, J., Van Dongen, M.B.M. and Konings, W.N. (1982) Eur. J. Biochem. 128, 151–157.
[8] Duine, J.A., Frank, J. and Jongejan, J.A. (1983) Anal. Biochem. 133, 239–243.
[9] Duine, J.A., Frank, J. and De Ruiter, L.G. (1979) J. Gen. Microbiol. 115, 523–526.
[10] Dijkstra, M., Frank, J., Jongejan, J.A. and Duine, J.A. (1984) Eur. J. Biochem., in press.