Alternative NAD\(^+\)-dependent formate dehydrogenases in the facultative methylotroph *Mycobacterium vaccae* 10

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Received 12 February 1991
Accepted 1 March 1991

Key words: Formate dehydrogenase; Methylotroph; Molybdenum; *Mycobacterium vaccae*

1. SUMMARY

*Mycobacterium vaccae* 10 growing in methanol medium synthesizes two inducible alternative NAD\(^+\)-dependent formate dehydrogenases (FDH). In the presence of molybdenum, the dominating form of the enzyme is FDH I with *M* \(_{r}\) 440 kDa and *K* \(_{m}\) 0.32 mM for sodium formate. FDH I reduced ferricyanide as well as NAD\(^+\), and it was reversibly inactivated by formate. NAD\(^+\) stabilized FDH I against this inactivation. Under conditions of artificial molybdenum deficiency (tungsten in the medium), the second enzyme (FDH II) appeared with *M* \(_{r}\) about 93 kDa and *K* \(_{m}\) 8.3 mM for sodium formate, and no FDH I activity was detected. FDH II did not reduce ferricyanide and was not inactivated by formate. The activity of FDH I was restored in tungsten-grown cells by pulse addition of molybdenum under conditions of blocked protein synthesis, suggesting the pre-existence of inactive apo-FDH I.

2. INTRODUCTION

NAD\(^+\)-dependent formate dehydrogenases (FDH, E.C.1.2.1.2.) are widely distributed among methanol oxidizing microorganisms. Homodimeric FDHs were isolated and studied from methylotrophic yeasts [1,2] and two methylotrophic bacteria, *Pseudomonas* sp. 101 [3] and *Moraxella* sp. C-1 [4]. The common properties of these enzymes are: absence of artificial dye-reduction activity, low affinity to formate (*K* \(_{m}\) values from 10 to 15 nM) and absence of any prosthetic groups in their active centers.

The other type of NAD\(^+\)-dependent FDH is represented by that from *Pseudomonas oxilaticus* [5], which is characterized by a complex subunit structure (\(\alpha_2\beta_2\)), ability to reduce artificial dyes, high affinity to formate (*K* \(_{m}\) 0.135 mM) and by the presence of organic and metallic prosthetic groups. FDHs from *Methylobacterium extorquens* AM1 [6] and *Methylobacterium methylica* [7] may also belong to this group of NAD\(^+\)-dependent FDHs.

At present only one bacterium, *Pseudomonas* sp. 101, is known to possess both types of NAD\(^+\)-dependent FDH, the syntheses of which...
are regulated by the presence or absence of molybdenum [8]. In this study we present evidence for the existence of two different NAD⁺-dependent FDHs in the facultative methylotrophic bacterium Mycobacterium vaccae 10.

3. MATERIALS AND METHODS

3.1. Organism and cultivation

The strain M. vaccae 10 was obtained from the Microbiology Department, Moscow University. Cultures were grown aerobically in shaken flasks at 28°C for 48 h in medium (pH 7.2) containing (g/l); KH₂PO₄ (2.0), (NH₄)₂SO₄ (2.0), NaCl (0.5), MgSO₄ • 7H₂O (0.025), CaSO₄ • 2H₂O (0.005), FeSO₄ • 7H₂O (0.005), MnCl₂ • 4H₂O (0.0025), D-biotin (30 mg/l) and methanol (0.5% v/v). Where indicated, 100 μM Na₂MoO₄ • 2H₂O or 100 μM Na₂WO₄ • 2H₂O was added to the medium.

3.2. Preparation of cell-free extract

Cells at the end of the exponential growth phase were collected by centrifugation, suspended in buffer A (0.1 M KH₂PO₄, pH 7.5) and disrupted by 0.1-mm diameter glass beads at 2°C. Cells and large debris were removed by centrifugation (8000 x g, 30 min) and the supernatant was used as cell-free extract.

3.3. Enzyme activity

NAD⁺-dependent FDH activity was measured spectrophotometrically at 340 nm and 20°C in a reaction mixture containing buffer A, 1 mM NAD⁺ and cell-free extract (80–400 μg of protein/ml). Ferricyanide-reducing FDH activity was determined in the same buffer at 420 nm with 1 mM ferricyanide instead of NAD⁺. Reactions were started by addition of 240 μM of sodium formate. Specific activity was expressed as nmol of reduced accepter/min per mg of protein. Protein was determined by the method of Bradford [9].

3.4. Analytical gel-filtration

Cells (2 g wet weight) were suspended in 15 ml of phosphate buffer (20 mM KH₂PO₄, pH 7.0) containing 200 mM KCl and 10 mM KNO₃ (buffer B) and cell-free extract was prepared. Nucleic acids were precipitated by streptomycin sulfate treatment (1% final concentration) and removed by centrifugation. Supernatant (3 ml) was applied to the column (1.6 x 91 cm) with Toyopearl HW-55(F) equilibrated with buffer B. Elution was performed with buffer B at 4°C and 2.5 ml fractions were collected. For determination of M, the col-

### Table 1

Effects of varying carbon source, and molybdenum and tungsten content, of culture media on growth of M. vaccae 10, and specific activities of FDH

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Metal anion (100 μM)</th>
<th>Final biomass A₆₆₀</th>
<th>FDH (nmol min⁻¹ mg⁻¹)</th>
<th>NAD⁺-dependent</th>
<th>Ferricyanide-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (0.5%)</td>
<td>None</td>
<td>2.55</td>
<td>108</td>
<td>368</td>
<td></td>
</tr>
<tr>
<td>Methanol (0.5%)</td>
<td>MoO₄²⁻</td>
<td>2.66</td>
<td>275 (4) a</td>
<td>806</td>
<td></td>
</tr>
<tr>
<td>Methanol (0.5%)</td>
<td>WO₄²⁻</td>
<td>2.93</td>
<td>1582 (98) a</td>
<td>309</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%)</td>
<td>None</td>
<td>2.88</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%)</td>
<td>MoO₄²⁻</td>
<td>2.66</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%)</td>
<td>WO₄²⁻</td>
<td>2.93</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%) plus formate (0.1%)</td>
<td>None</td>
<td>2.45</td>
<td>28</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%) plus formate (0.1%)</td>
<td>MoO₄²⁻</td>
<td>2.57</td>
<td>46</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Glucose (0.4%) plus formate (0.1%)</td>
<td>WO₄²⁻</td>
<td>2.89</td>
<td>506</td>
<td>127</td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses represent activity inhibition by 16 μM NaN₃.
umn was calibrated with the following markers ($M_r$): ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), bovine serum albumin (67 000), carbonic anhydrase (29 000) and cytochrome c (12 400).

3.5. Experiments with molybdate pulse in vivo

Cells were grown in methanol medium with 100 μM $WO_3^{2-}$ for 44 h (the middle of exponential growth phase) and then chloramphenicol (50 μg/ml) was added to culture. Cells were collected by centrifugation and were resuspended in the initial volume of fresh medium containing 50 μg/ml chloramphenicol, 0.05% methanol and 200 μM $MoO_4^{2-}$. The suspension was incubated in a rotary shaker at 28°C for 30 min, then cells were precipitated by centrifugation and disrupted in buffer B as described above. Cell-free extract was analysed by gel-filtration.

4. RESULTS

During screening of the collection of methylotrophic strains of the Microbiology Department, Moscow University, it was found that NAD$^+$-dependent FDH activity in $M. vaccae$ 10 was very sensitive to the presence of tungsten or molybdate in the growth media. As a rule, tungsten highly stimulated NAD$^+$-dependent activity (15-fold) without affecting the ferricyanide-dependent activity. Molybdate increased both NAD$^+$ and ferricyanide-dependent FDH activities (2.5- and 2.1-fold, respectively) (Table 1). NAD$^+$-dependent FDH activity in an extract of W-grown cells was very sensitive to inhibition by 16 μM of sodium azide. In extracts of Mo-grown cells, this activity was highly resistant to the same concentration of this competitive inhibitor. This result suggested the existence of two NAD$^+$-dependent FDHs with distinct substrate/inhibitor specificities in the two different cell-free extracts of $M. vaccae$ 10. In glucose-grown cells, all FDH activities were totally repressed, and neither $MoO_4^{2-}$ nor $WO_3^{2-}$ per se induced any FDH. The true inducer was formate added in combination with glucose. Again, the specific activities depended on the identity of heavy metal anion (Table 1).

The existence of at least two NAD$^+$-dependent FDHs (FDH I in Mo-containing and FDH II in W-containing media) in $M. vaccae$ 10 was confirmed by the measurements of $K_m$ values for formate in two types of extracts: 0.32 mM for FDH I and 8.3 mM for FDH II. The concentration of NAD$^+$ during these experiments was 2 mM.

![Figure 1](image-url)

Fig. 1. Typical gel-filtration patterns of methanol-grown $M. vaccae$ 10 cell-free extracts on Toyopearl HW-55. Growth medium contained (A), 100 μM $MoO_4^{2-}$ or (B) and (C), 100 μM $WO_3^{2-}$. In variant (C) 200 μM $MoO_4^{2-}$ were pulse-added after 44 h of cultivation (see METHODS). Inserted tables represent changes of total FDH activity in different stages of extract manipulation: 1, initial extract; 2, extract after treatment with streptomycin sulfate; 3, extract after gel-filtration.
In extracts of Mo-grown cells, FDHI was one of the longest proteins, with an $M_r$ of approximately 440 kDa. This enzyme was also able to reduce ferricyanide (Fig. 1A). Very low but detectable NAD$^+$-dependent FDH activity appeared also in fractions with a lower $M_r$, and this enzyme was completely inhibited by ferricyanide. A distinct elution pattern of FDH activity was found in extracts of W-grown cells (Fig. 1B), where only one enzyme (FDHII) with an $M_r$ of 93 kDa, which did not reduce ferricyanide, was detected.

Ferricyanide-reducing FDH activity, which was relatively high in cell-free extracts of W-grown cells, was totally inactivated during gel-filtration on Toyopearl HW-55 (Fig. 1B) or Sephadex G-25 (not shown).

The absence of FDHII in W-grown cells may be a result of an antagonistic tungsten effect on molybdate transport and metabolism in *M. vaccae* 10. This effect has been described for many bacterial Mo-containing enzymes [10–12]. Putative Mo-containing FDHI from *M. vaccae* 10 is probably synthesized as an inactive (or partially active) apo-enzyme in the presence of tungsten. This hypothesis was confirmed by pulse addition of molybdate to the suspension of W-grown cells in the presence of chloramphenicol; both alternative NAD$^+$-dependent FDHs were detected during gel-filtration (Fig. 1C). These results were interpreted as being direct physiological evidence for the existence of Mo in FDHI.

It was shown earlier that NAD$^+$-dependent FDH in Mo-grown cells of *Pseudomonas* sp. 101 is inactivated during incubation with formate [8]. FDHI from *M. vaccae* 10 is also rapidly inactivated by formate (Fig. 2). This inactivation was prevented by adding NAD$^+$ to the reaction mixture prior to formate. Furthermore, mainly inactivated FDHI could be restored to partial activity by incubation with NAD$^+$, although the reactivation was never complete under the conditions employed here. Partial reactivation of formate-inactivated FDHI was also achieved by removing excess formate by gel-filtration through Toyopearl HW-55 (Table 2). Both NAD$^+$- and ferricyanide-reducing activities in extracts of W-grown cells of *M. vaccae* 10 were absolutely resistant to formate (not shown).

![Graph](image_url)

**Table 2**

Reactivation of formate-inactivated FDHI from extract of Mo-grown *M. vaccae* 10 cells by gel-filtration

<table>
<thead>
<tr>
<th>Stage of treatment</th>
<th>Total FDHI activity (nmol min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD$^+$-dependent</td>
</tr>
<tr>
<td>Initial extract</td>
<td>831</td>
</tr>
<tr>
<td>Extract after</td>
<td></td>
</tr>
<tr>
<td>treatment with formate</td>
<td>13</td>
</tr>
<tr>
<td>Extract after gel-filtration</td>
<td>307</td>
</tr>
</tbody>
</table>

Extract (3 ml) in buffer B was treated by 100 mM of sodium formate for 30 min at 20°C and then subjected to gel-filtration through Toyopearl HW-55.
5. DISCUSSION

The ability to synthesize two different NAD+-dependent FDHs, demonstrated earlier in *Pseudomonas* sp. 101 [8], has now also been shown in *M. vaccae* 10. When active Mo-containing FDHI cannot be synthesized due to the absence of Mo, FDHII, the other (‘spare’) NAD+-dependent FDH, is induced.

Preliminary experiments on the nature of FDHII from *M. vaccae* 10 have shown that it is a dimer of 46 kDa-subunits, contains no prosthetic groups or metals and accounts for at least 10% of soluble protein in W-grown cells (V.I. Tishkov, personal communication). This FDHII is an analogue of well-known NAD+-dependent FDHs from methylotrophic yeasts [1,2] and two methylotrophic bacteria [3,4]. Although FDHII has a low affinity for formate compared to FDHI, the high intracellular level of the former enzyme may thus compensate for the loss of the latter. On the other hand, FDHI is an analogue of the novel, presumably Mo-containing, NAD+-dependent FDH from *Pseudomonas* sp. 101 [8]. Both these enzymes are large proteins and are inactivated by formate. This effect of formate was also reported for FDHs from *P. oxalaticus* [5] and from *M. extorquens* AM1 [6]. Reversibility of formate-induced inactivation, demonstrated for FDHI from *M. vaccae* 10 (Fig. 2, Table 2), suggests the existence of a regulatory mechanism mediated by intracellular concentrations of formate and NAD+. The reason for the ‘switch off’ effect of FDHI in the absence of native oxidizer (NAD+) is not known at present time.

The nature and physiological role of the third ferricyanide-reducing FDH, which appears in *M. vaccae* 10 growth on tungsten-containing medium, are now under investigation. Presumably, this enzyme is the partially active apo-enzyme of FDHI, defective in Mo-cofactor. This enzyme (apo-enzyme) was very unstable during gel-filtration (Fig. 1B) suggesting that the putative Mo-cofactor plays not only a catalytic but also a structural role in FDHI. Earlier, an analogues, artificial acceptor-specific FDH detected in cell extracts of *Pseudomonas* sp. 101 grown without added molybdenum, was shown to be inactivated during practically all kinds of chromatography [13].

REFERENCES