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Dijkhuizen, L.; Timmerman, J.W.C.; Harder, W.

Published in:
FEMS Microbiology Letters

DOI:
[10.1016/0378-1097\(79\)90186-1](https://doi.org/10.1016/0378-1097(79)90186-1)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1979

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Citation for published version (APA):

Dijkhuizen, L., Timmerman, J. W. C., & Harder, W. (1979). A Pyridine Nucleotide-Independent Membrane-Bound Formate Dehydrogenase in *Pseudomonas Oxalaticus* OX1. *FEMS Microbiology Letters*, 6(1), 53-56. [https://doi.org/10.1016/0378-1097\(79\)90186-1](https://doi.org/10.1016/0378-1097(79)90186-1)

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A PYRIDINE NUCLEOTIDE-INDEPENDENT MEMBRANE-BOUND FORMATE DEHYDROGENASE IN *PSEUDOMONAS OXALATICUS* OX1

L. DIJKHUIZEN, J.W.C. TIMMERMAN and W. HARDER *

Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 25 March 1979

1. Introduction

In the metabolism of oxalate and formate in *Pseudomonas oxalaticus*, elucidated by Quayle and coworkers [1], an important role was attributed to a soluble NAD-dependent formate dehydrogenase (NAD-FDH; EC 1.2.1.2) [2]. The reaction catalysed by this enzyme was reported to be the major (if not the only) energy-yielding reaction in the metabolism of both substrates. Circumstantial evidence has been obtained that apart from the soluble NAD-FDH another formate-oxidizing enzyme, located in the cytoplasmic membrane, may be present in this organism during growth in the presence of formate or oxalate [3,4]. The occurrence of such a membrane-bound formate-oxidizing enzyme was also reported in a strain of *P. oxalaticus* which differs from the organism studied in our laboratory in that it was unable to grow on formate as the sole source of carbon and energy [5,6]. In subsequent work, the ability of cytoplasmic membrane vesicles, isolated from oxalate-grown cells, to oxidize formate was demonstrated [7]. This activity was detected spectrophotometrically with the artificial electron acceptors phenazine methosulphate (PMS)/2,6-dichlorophenol-indophenol (DCPIP) and was also present in cell-free extracts prepared from *P. oxalaticus* grown on formate, oxalate or mixtures of formate with other substrates [8]. This raised the question whether in addition to the NAD-FDH, the membrane-bound enzyme is also involved in the generation of energy, by feeding electrons from formate oxidation into the respiratory chain, or that it functions merely as an oxidase,

by reacting directly with oxygen. The latter could be of advantage to the organism as a detoxification mechanism at higher concentrations of formate, which causes substrate inhibition of growth [9]. Therefore, we decided to study the properties of this enzyme, particularly with respect to its association with the electron transport chain in order to assess its role in the metabolism of oxalate and formate in *P. oxalaticus*.

2. Materials and Methods

2.1. Growth conditions and isolation of membrane vesicles

P. oxalaticus OX1 was grown in batch culture in a fermenter with a working volume of 2 l [10] on a mineral medium [9] with 20 mM sodium formate as the carbon source. During growth the pH was kept constant at pH 7.5 by the controlled addition of 8 N formic acid. Cells were harvested before the end of the logarithmic growth phase at a density of 0.5–0.7 g of dry weight/l. Membrane vesicles were isolated as described by Kaback [11], except that 10 mM formate was added to all the buffer solutions, and spheroplast lysis was performed under a nitrogen atmosphere. Vesicles were stored in liquid nitrogen in 50 mM potassium phosphate buffer, containing 10 mM formate, at a protein concentration of 2–3 mg/ml. Enzyme properties were studied after centrifugation and washing to free the vesicles from formate.

2.2. Assays of enzyme activity

The NAD-linked FDH activity was assayed as described previously [8]. The NAD-independent

* To whom reprint requests should be sent.

FDH activity was assayed routinely with a mixture of PMS (0.29 mM) and DCPIP (0.20 mM) under anaerobic conditions in the system described previously [8]. Tests for the electron acceptor specificity of the enzyme were performed under the same assay conditions. Molar extinction coefficients ($M^{-1}cm^{-1}$) and concentrations of the respective electron acceptor used were: DCPIP, 0.20 mM, $\epsilon_{600} 16.3 \cdot 10^3$; benzyl viologen, 1.0 mM, $\epsilon_{600} 14.7 \cdot 10^3$; methyl viologen, 1.0 mM, $\epsilon_{600} 11.3 \cdot 10^3$; methylene blue, 0.05 mM, $\epsilon_{650} 89 \cdot 10^3$; $K_3[Fe(CN)_6]$, 2.0 mM, $\epsilon_{420} 0.90 \cdot 10^3$; NAD, NADP, 1.0 mM, $\epsilon_{340} 6.22 \cdot 10^3$; FAD, 0.1 mM, $\epsilon_{450} 11.3 \cdot 10^3$; FMN 0.1 mM, $\epsilon_{420} 0.90 \cdot 10^3$; NAD, NADP, 1.0 mM, $\epsilon_{340} 6.22 \cdot 10^3$; FAD, 0.1 mM, $\epsilon_{450} 11.3 \cdot 10^3$; FMN 0.1 mM, $\epsilon_{450} 12.2 \cdot 10^3$. The oxidation of formate with oxygen as the electron acceptor was measured with a Clark-type oxygen electrode [9] in 3.0 ml of air-saturated 50 mM potassium phosphate buffer, pH 6.7. The reaction was started by the addition of formate (10 mM).

2.3. Inhibition studies

The inhibitors Amytal, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) and Antimycin A were dissolved in ethanol, and potassium cyanide in water. Enzyme and inhibitor were preincubated for 5 min and then the reaction was started by the addition of formate (10 mM). Ethanol itself, in concentration as in the presence of inhibitor (less than 1%), did not inhibit the enzyme.

2.4. Cytochrome spectra

Anaerobic reduction of components of the electron transport chain in membrane vesicles was measured with an Aminco split-beam spectrophotometer (Aminco DW2 UV/VIS) at room temperature. The oxidized/oxidized base-line was adjusted for the membrane suspension (10. ml) containing 50 mM potassium phosphate buffer, pH 6.7, and 0.97 mg protein in both sample and reference cells. Formate (10 mM) was added to the sample cell and ferri-cyanide (5 mM) to the reference cell. The reduced/oxidized difference spectra were recorded when absorbance changes were no longer observed and an anaerobic steady state was reached.

2.5. Protein determination

Protein was determined with the method of Lowry et al. [12], using bovine serum albumin as a standard.

3. Results

Attempts to isolate the membrane-bound FDH from formate-grown cells of *P. oxalaticus* by ultrasonic treatment or disruption in a French pressure cell were not satisfactory since they resulted in low recoveries and yielded preparations which rapidly lost activity. An additional disadvantage of these procedures was the high degree of contamination of the membrane-bound FDH with NAD-linked FDH. This NAD-linked FDH reacts with many dyes, including PMS/DCPIP, as shown by Müller et al. [13]. For these reasons it was decided to use a more gentle method and isolate cytoplasmic membrane vesicles. These vesicles contained high activities of the PMS/DCPIP-linked FDH but no detectable activity of NAD-linked FDH. Also ultrasonic treatment of the vesicles, in order to unmask any soluble NAD-FDH activity enclosed in the vesicles during their preparation, did not result in the appearance of NAD-linked FDH activity. During the isolation of vesicles, the enzyme could be protected against inactivation by including formate (10 mM) in the buffer solutions and performing spheroplast lysis under a nitrogen atmosphere. Enzyme preparations obtained via the above procedure showed activities between 2.6–9.8 $\mu\text{mol DCPIP reduced/min} \cdot \text{mg}^{-1}$ of protein. Most of the enzyme (>90%) could be solubilized and separated from the membranes by incubation with 0.75% (w/v) Triton X-100 for 30 min at 4°C and centrifugation at 100 000 $\times g$ for 1.5 h. The pH-activity profile of the solubilized enzyme showed an optimum at pH 6.7. In determining this profile, the variation of the molar extinction coefficient of DCPIP at 600 nm with pH [14] has been taken into account. However, the solubilized enzyme preparation was not suitable for further purification since it rapidly lost its activity and could no longer be stabilized with formate. Further properties of the FDH, described in this paper, were therefore studied using the vesicle preparations.

Table 1 shows the specificity of the membrane-

TABLE 1

Electron acceptor specificity of formate dehydrogenase in membrane vesicles from formate-grown cells of *P. oxalaticus* OX1

No activity was observed with NAD, NADP, FAD, FMN, benzylviologen and methylviologen.

Acceptor	Relative activity of formate dehydrogenase ^a
PMS + DCPIP	100 ^b
DCPIP	31
methylene blue	7.5
ferricyanide	36
oxygen	116 ^c

^a Activities were calculated on the basis of a two-electron transfer.

^b Specific activity was: 9.8 $\mu\text{mol}/\text{min} \cdot \text{mg}^{-1}$ of protein.

^c Activity was calculated assuming a stoichiometry: $\text{HCOOH} + \frac{1}{2} \text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$.

bound FDH for various electron acceptors. Apart from PMS/DCPIP and oxygen, enzyme activity was observed with DCPIP alone, methylene blue and ferricyanide. The solubilized enzyme showed the same pattern of activity with these electron acceptors except that hardly any activity was observed with oxygen. This indicates that the reaction between formate and oxygen observed with the vesicles did not result from a direct transfer of electrons from the enzyme to oxygen, but was probably mediated by the electron transport chain. It seemed therefore likely that in vivo the enzyme does not function as an oxidase but requires membrane-bound carriers to transfer electrons from formate to oxygen.

To investigate the nature of these electron carriers, the effect of inhibitors of the respiratory chain on the "formate oxidase" activity was studied. The results (Table 2) show that this activity was very sensitive towards inhibition by Antimycin A and cyanide, whereas HQNO was less inhibitory and Amytal did not inhibit at all. The PMS/DCPIP-linked FDH activity was 100% inhibited by potassium cyanide (1 mM) but for less than 10% by Antimycin A (100 μM) and HQNO (33 μM) (results not shown). Since the activity measured with PMS/DCPIP was not dependent on the presence of membranes (see above), this indicates that the membrane-bound FDH itself is

TABLE 2

Effect of various inhibitors on the "formate oxidase" activity in membrane vesicles from formate-grown cells of *P. oxalaticus*

Inhibitor	Concentration (μM)	Relative activity of "formate oxidase"
None	—	100 ^a
Amytal	1000	100
Antimycin A	33	46
	100	13
HQNO	10	75
	33	54
	100	54
KCN	100	4
	330	0

^a Specific activity was 5.1 $\mu\text{mol O}_2/\text{min} \cdot \text{mg}^{-1}$ of protein.

sensitive towards inhibition by cyanide.

Reduced minus oxidized cytochrome spectra of membrane vesicles (Fig. 1) showed the ability of formate to reduce cytochromes of the *c*-(522 and 551 nm), *b*-(530 and 558 nm) and, possibly, *a*-type (590–600 nm). Addition of small amounts of ferricyanide to the sample cuvette resulted in reoxidation of the cytochromes. In the presence of excess formate a rapid reduction of cytochrome *b* and a slower reduction of cytochrome *c* was subsequently ob-

Fig. 1. Effect of formate on the reduction of cytochromes in membrane vesicles from formate-grown cells of *Pseudomonas oxalaticus* OX1. Formate (10 mM) reduced minus ferricyanide (5 mM) oxidized (I). Base line (II). Spectra were recorded with a scan speed of 1 nm/s and a band pass of 3 nm.

served. This indicates that the membrane-bound FDH probably donates electrons to the respiratory chain at the level of cytochrome *b* or before, since ferricyanide accepts electrons at the oxidase site of this chain [15,16].

The apparent K_m of the enzyme for formate, measured with the PMS/DCPIP system, was 0.28 mM. This is close to the K_m for formate (0.135 mM) determined for purified NAD-linked FDH [13].

4. Discussion

The properties of the membrane-bound FDH from *P. oxalaticus* described in this paper closely resemble those of a membrane-bound FDH present in *Escherichia coli* during aerobic and anaerobic (with nitrate) growth in the presence of formate [17]. The latter enzyme is a molybdo-protein, which contains cytochrome *b* and transfers electrons via ubiquinone into the respiratory chain [17,18]. The data presented in Table 2 and Fig. 1 indicate that the enzyme from *P. oxalaticus* is also able to transfer electrons at the same level (ubiquinone or cytochrome *b*) into the respiratory chain. Since the affinity constant (K_m) for formate of the NAD-linked FDH and the membrane-bound FDH in this organism are of the same order of magnitude, it has to be expected that both enzymes compete for formate inside the cell if the membrane-bound FDH is located at the inside of the membrane as it is in *E. coli*. Physiologically, generation of reducing power for biosynthesis and membrane energization for ATP synthesis and other energy requiring processes may thus be accomplished by the two separate enzymes. A comparable situation has been reported for the hydrogen bacterium *Alcaligenes eutrophus*, which contains a soluble NAD-hydrogenase and a respiratory chain-linked membrane-bound hydrogenase [19]. Evidence has been presented [20] that site 1 phosphorylation is absent in *P. oxalaticus*. Therefore, any oxidation of NADH, produced in the NAD-linked FDH reaction, via the electron transport chain will yield the same amount of energy as from formate oxidation via the membrane-bound FDH.

Acknowledgements

The investigations were supported (in part) by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). We are indebted to Dr. J.P. van Dijken for his help in preparing this manuscript.

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