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The role of the MxaD protein in the respiratory chain of *Methylobacterium extorquens* during growth on methanol

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Abstract

The largest of the gene clusters coding for proteins involved in methanol oxidation is the cluster *mxaFJGIR(S)ACKLDEHB*. Disruption of most of these genes leads to lack of growth on methanol. The previous results showed that the mutant lacking MxaD grows on methanol although at a low rate. This is explained by the low rate of methanol oxidation by whole cells. The specific activity of methanol dehydrogenase (MDH) is higher in the mutant but its electron acceptor (cytochrome c_L) is unchanged. Using the purified proteins, it was shown that the rate of interaction of MDH and cytochrome c_L was higher in the wild-type MDH containing some MxaD proteins, which was absent in the mutant MDH. It is suggested that the gene *mxaD* codes for the 17-kDa periplasmic protein that directly or indirectly stimulates the interaction between MDH and cytochrome c_L; its absence leads to a lower rate of respiration with methanol and therefore a lower growth rate on this substrate.

Keywords: Quinoprotein; Pyrroloquinoline quinone; Methanol dehydrogenase; Cytochrome; Electron transport

1. Introduction

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph, which, during growth on methanol, produces the quinoprotein methanol dehydrogenase (MDH) in the periplasm [1–3]. MDH is composed of two sets of two subunits, α and β, and contains pyrroloquinoline quinone, PQQ, as its prosthetic group. Its three-dimensional structure has been solved and PQQ has been shown to be located with a bound calcium ion in the center of the β-barrel structure of the α subunit [4]. Electrons extracted by MDH are passed to cytochromes c_L and c_H sequentially, and finally to molecular oxygen by the terminal oxidase, cytochrome aa_3 [5].

More than 25 genes are required for MDH synthesis including seven *pqq* genes. The biggest of the gene clusters includes the sequence *FJGIR(S)ACKLDEHB* [2,6]. The genes *mxaF*, *mxaI* and *mxaG* code for the α subunit, the β subunit and the electron acceptor cytochrome c_L, respectively, and *mxaAKL* are involved in the insertion of calcium ions into MDH. To study the functions of other *mxa* genes, we have constructed several mutants by homologous recombination with the kanamycin-resistance gene inserted, including mutant D11 which has a mutation in the *mxaD* gene but is still able to grow (poorly) on methanol [7]. In this paper, we focus on the *mxaD* gene. The MxaD protein is likely to be periplasmic because it has a periplasmic signal sequence in its N terminus [7]. No homologous proteins with known functions are present in database. This paper presents some evidence that the MxaD protein plays some role in the periplasmic electron transport chain for oxidation of methanol.

2. Materials and methods

2.1. Growth of bacteria, and measurement and analysis of proteins and enzymes

Methylobacterium extorquens AM1 was grown at 30 °C containing methanol (0.5% v/v), succinate (0.2% w/v) or

Abbreviations: MDH, Methanol dehydrogenase

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methylamine–HCl (0.2% w/v) as described previously; for growth of the *mxaD* mutant, D11, all growth media contained 25 μg/ml kanamycin [7]. Cells grown to the end of the logarithmic phase were broken through a French pressure cell press with 20 mM Tris–HCl buffer, pH 8.0, and a cell-free extract was prepared by removal of whole cells and debris by low-speed centrifugation. The cell-free extract was further ultracentrifuged at 120,000 × *g* for 90 min at 4 °C to obtain the soluble fraction.

Protein was measured by the improved Lowry method [8] using bovine serum albumin standard. Methods for measurement of MDH and cytochrome c_L concentrations, SDS-PAGE, protein staining with Coommassie blue and staining for heme, were as described previously [9,10].

All enzyme assays were performed at 25 °C. One unit of enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 μmol of substrate per minute. MDH was routinely assayed spectrophotometrically at pH 9 using a dye-linked assay containing phenazine methosulfate and 2,6-dichlorophenolindophenol [9]. The methanol:cytochrome c_L oxidoreductase activity of MDH was assayed using 2,6-dichlorophenolindophenol as terminal electron acceptor as described in Ref. [11].

Whole-cell respiration in intact cells was performed polarographically with a Clark-type oxygen electrode in 0.1 M Tris–HCl (pH 8.5). Bacteria (100 mg wet cells/ml), grown on methanol to end-log phase, were suspended in 0.1 M Tris–HCl (pH 8.5), and shaken for 1 h. After that, 20 μl of the cell suspension was used for assay in 0.1 M Tris–HCl (pH 8.5), in a total volume of 600 μl. For the 'methanol oxidase' assay, the reaction was started by the addition of 6 μl of 1 M methanol. In the case of the TMPD oxidase assay, ascorbate was first added to a final concentration of 3.3 mM, followed by addition of TMPD to a final concentration of 1 mM. When the sensitivity to cyanide was examined, KCN was added at several concentrations after the addition of TMPD.

2.2. Purification of MDH and cytochrome c_L

The soluble protein fraction was passed down a column of DEAE-cellulose equilibrated with 20 mM Tris–HCl (pH 8.0). MDH and cytochrome c_H were not adsorbed on this column. The active MDH fractions were pooled, concentrated by an ultrafiltration with a UK-10 or UK-20 membrane (Advantec Toyo), and then applied onto a Superdex S-200 column equilibrated with 25 mM MES buffer (pH 5.5). The purified MDH was stored at 4 °C for short times and at − 80 °C for longer periods.

Cytochrome c_L, which had absorbed to the DEAE column, was eluted with a gradient of 0–250 mM NaCl in the same buffer. Without dialysis, solid ammonium sulfate was added to give 40% saturation at 4 °C, and the precipitate collected after centrifugation for 10 min at 10,000 × *g* was discarded. The supernatant was applied to a Phenyl-Sepharose column equilibrated with 20 mM MOPS buffer containing 2 M ammonium sulfate (pH 7.0), and cytochrome c_L was eluted with a decreasing gradient of 2–0 M ammonium sulfate in the same buffer. The cytochrome c_L was desalted on a Sephadex G-25 column equilibrated in 20 mM Tris–HCl (pH 8.0), and then applied to a Q-Sepharose column equilibrated with the same buffer. Cytochrome c_L was eluted with a gradient of 0–200 mM NaCl in the same buffer, and applied on a Superdex S-200 column equilibrated with 20 mM Tris–HCl buffer (pH 8.0). The purified cytochrome c_L was stored at − 80 °C.

2.3. The redox titration of cytochrome c_L

Purified cytochrome c_L (5 μM) was dissolved in 20 mM MOPS–NaOH, pH 7.0. Redox mediators used were 100 mM ferrocyanide, 20 μM 2-hydroxyl-1,4-naphtoquinone, 10 μM PMS, 10 μM PES, 20 μM DAD and 20 μM duroquinone. The titration was carried out by the successive addition of ferricyanide under anaerobic conditions with streaming O_2-free Ar gas in a redox cuvette, and monitoring of redox potential by an electrode. The oxidation–reduction level of the heme was monitored by the absorbance difference at 550 versus 539 nm.

3. Results and discussion

It was confirmed that, unlike the other *mxa* mutants, the *mxaD* mutant can grow on methanol, but at about 30% of the growth rate measured with wild type (WT). On other substrates, such as methylamine and succinate, the growth rate was unaltered in the mutant. Remarkably, the specific activity of MDH was almost three times higher than the wild-type value when measured in the dye-linked assays system in crude soluble extracts of methanol-grown cells (0.6 U/mg protein) or methylamine-grown cells (0.2 U/mg protein). The mutation had little effect on the MDH activity when grown on succinate (0.07 U/mg protein).

SDS-PAGE of the cell-free extracts showed that the amount of protein due to MDH (both subunits) was at least three times higher in the mutant than in WT bacteria, as expected from the specific activity measurements. The amounts of cytochrome c_H and cytochrome c_L (measured by heme staining) were similar to those in WT bacteria.

Although the amounts of the three soluble proteins involved in methanol oxidation were at least as high as in WT bacteria, the rates of methanol oxidation by whole cells was only 20% of the WT rates, which is sufficient to explain the lower growth rate of the mutant. The rate of oxidation of ascorbate/TMPD by whole cells (and its sensitivity to cyanide) was unaffected by the mutation, indicating that the cytochrome *c* oxidase activity had not been altered. These results indicate that interaction between MDH and cytochrome c_L is disturbed in the mutant, or that some other unknown component might be required for the efficient operation of the 'methanol oxidase' respiratory chain.

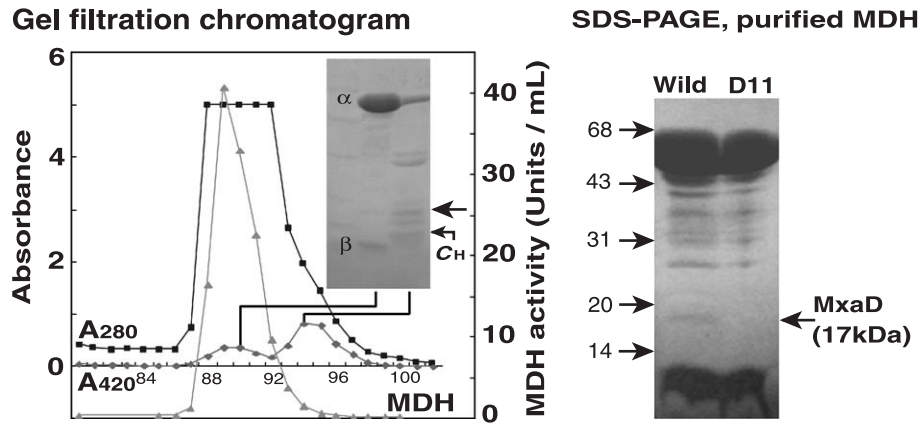

Fig. 1. The gel filtration step in the purification of MDH from wild-type bacteria. The experiment is described in Section 2. In the left panel, squares and circles indicate absorbance at 280 and 420 nm (to show cytochrome), respectively; the triangles show MDH activity measured in the dye-linked assay system. The small SDS-PAGE insert shows the presence of the MxaD protein in the cytochrome c_H fraction. The larger SDS-Page gel shows the result using a larger amount of the purified MDH (50 μg) in order to see more clearly the MxaD protein.

To investigate this further, MDH and cytochrome c_L were purified from the wild-type and mutant cells. The methods used (see Section 2) were slightly simpler than those reported previously [9,10], but the proteins were almost pure when analyzed by SDS-PAGE (Fig. 1). Fig. 1 shows the gel filtration used in the second step of purification from the wild-type cells; MDH was in the earlier fractions, followed by the red fractions containing cytochrome c_H. These fractions also contained a 17-kDa protein (Fig. 1), which was absent during gel filtration of the proteins from the mutant (data not shown). The N-terminal sequence of this protein (HGPTPQKVSQ) matched perfectly that of the MxaD protein predicted from the nucleotide sequence of *mxaD* after removal of the signal sequence [12]. When larger amounts of the MDH were applied to the column, several minor protein bands were seen in the purified MDH including some MxaD protein (confirmed by N-terminal sequencing). Most of these minor proteins were also present in the MDH purified from the mutant, but the 17-kDa protein was absent. (Fig. 1). These results confirm that the *mxaD* gene codes for a 17-kDa periplasmic protein and suggest that it may be able to bind to MDH. However, this interaction appears to be weak because, from the observation in SDS-PAGE analysis, the amount of MxaD was quite low compared with those of the two subunits of MDH.

The absorption spectra of cytochrome c_L purified from both the wild-type and the *mxaD*-mutant cells were similar to each other in both the oxidized and reduced forms (data not shown). The midpoint redox potential of the cytochrome c_L from the mutant (252 mV) was almost identical to that measured for the cytochrome from WT bacteria (248 mV). These results indicate that the cytochrome c_L was not altered by the *mxaD* mutation.

Preliminary kinetic experiments using the purified MDH and cytochrome c_L suggested that the maximum reaction rate (V_{max}) of MDH purified from the mutant cells was about 25% of that measured with the wild-type MDH, regardless of whether the cytochrome c_L was from the wild-type or from the *mxaD*-mutant cells. The two MDHs were purified to the same extent and the only observable difference was that the MDH preparation from the mutant lacked the MxaD protein. This result hints therefore that the presence of the MxaD protein may lead to a higher rate of interaction between MDH and cytochrome c_L.

It has long been known that the rates of electron transfer measured between pure MDH and cytochrome c_L are too low to account for the whole cell respiration rates and it has often been proposed that other proteins or cofactors might be involved (for a discussion of this, see Refs. [5,13]). Clearly the preliminary results presented here suggest that the MxaD protein or a product of its activity might be important in increasing the activity of this part of the electron transport chain. This might be by affecting the initial docking process which involves electrostatic interactions between MDH and cytochrome c_L [5,11] or by affecting the subsequent re-orientation to give the active electron transfer complex [14], or by increasing the rate of electron transfer between the reduced PQQ in MDH and the heme of cytochrome c_L.

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