Calcium in Quinoprotein Methanol Dehydrogenase Can Be Replaced by Strontium

Osa0 Adachi, Kazunobu Matsushita, Emiko Shinagawa and Minoru Ameyama

Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

Received April 25, 1990

Formation of quinoprotein methanol dehydrogenase (MDH) in Methylobacillus glycogenes, an obligate methylotroph, was strictly controlled by calcium (Ca) in the culture medium. Both the growth of the organism and the total enzyme activity of MDH were also repressed at less than 1 ppm of Ca, although specific activity of MDH showed a similar level. Ca in MDH was replaced with other metals during cultivation of M. glycogenes. When strontium (Sr) chloride was fed instead of CaCl2, Ca in MDH was completely replaced by Sr and showed a specific activity over ten times higher than Ca-containing MDH, although there was no appreciable increase in the MDH content in cells cultured in Sr medium. Methanol oxidase activity was also elevated in the cells that were cultured in the medium with Sr.

In our previous report on quinoprotein methanol dehydrogenase (MDH), we indicated that MDH is a metalloprotein containing one atom of Ca per mole of MDH.1) Binding of Ca is so tight that it is difficult to remove Ca completely from MDH without denaturation of the enzyme protein. A partial resolution of Ca was made by a metal chelator and exogenous addition of Ca led to the complete reactivation of the enzyme to the original level. In the course of study to elucidate the mechanism of pyrroloquinoline quinone (PQQ) accumulation by methylotrophs, an interesting phenomenon was observed, that is, when Ca, one of the inorganic ingredients in the culture medium, was replaced by other metal ions, the corresponding metal-containing MDH was produced. For this paper, we studied the effects of various divalent metals, especially of Sr, on the formation of quinoprotein MDH.

Materials and Methods

Microorganisms and culture. An obligate methylotroph, M. glycogenes, was used throughout. The composition of the culture medium was essentially the same as described previously,2) except that all ingredients were dissolved in twice distilled water. The medium (100 ml) was prepared in a 500-ml shaking flask to which a Klett tube was attached, and sterilized at 120°C for 20 min. Methanol was added to 1% (v/v) under sterile conditions. The inoculum suspension, which had been prepared under sterile conditions was added to the medium and incubation was done at 30°C with shaking. Growth of the microorganism was measured periodically by reading turbidity, without opening the stopper, through the branching Klett tube of the shaking flask by a Klett-Summerson photoelectric colorimeter equipped with a filter (No. 808) covering wavelengths from 640 to 700 nm.

Assay of enzyme activity. A cell-free extract was prepared with 50 mM Tris–HCl, pH 8.0, containing 3 mM methanol unless otherwise stated. MDH activity was measured essentially by the method described by Yamanaka and Matsumoto.3) The enzyme activity of a dye-linked aldehyde dehydrogenase (ALDH) was measured by the method described by Patel et al.4–5) One unit of both enzyme activities was defined as the amount of enzyme catalyzing one micromole of substrate oxidation per min under the conditions used. Protein was measured by the method of Lowry et al.6) with bovine serum albumin as a standard. Methanol oxidase was assayed with a cell suspension of M. glycogenes using a conventional oxygen electrode apparatus. The reaction mixture contained 300 μmol of...
potassium phosphate, pH 6.5, and 10 μmol of methanol in a total volume of 2.9 ml. The reaction was started by the addition of a cell suspension (0.1 ml), the optical density of which at 660 nm had been adjusted to 2.50, and done at 25°C. One unit of methanol oxidase activity was defined as the amount of cells that consumed 1 μatom of O2 per min under these assay conditions.

Measurement of PQQ. PQQ in the culture medium was measured with a quinoprotein apo-glucose dehydrogenase essentially by the method described before.7)

SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was done as described by Laemmli.8) A slab gel (7 x 8 cm) composed of a 5% stacking gel and a 13.5% separating gel was used. Samples were pretreated in 2% SDS at 30-40°C for 30 min before they were put on a slab gel. The molecular weight markers used were phosphorylase b (mol. wt. 93,000), bovine serum albumin (mol. wt. 68,000), ovalbumin (mol. wt. 43,000), carbonic anhydrase (mol. wt. 30,000), and lysozyme (mol. wt. 14,000).

Analytical ultracentrifugation. Estimation of the sedimentation rate of the purified MDH was done with a Hitachi model SCP55H ultracentrifuge at 60,000 rpm with schlieren optics.9)

Metal analysis. Metals in the culture medium and the purified MDH were analyzed with a conventional atomic absorption spectrophotometer.

**Results and Discussion**

**Course of growth of M. glycogenes**

*M. glycogenes* was grown on the basal medium with 1% methanol as the sole carbon source (Fig. 1). Bacterial growth, enzyme activities of MDH and ALDH, PQQ in the culture medium, and methanol during cultivation were monitored periodically. The maximal levels of MDH, ALDH, and PQQ were indicated in the figure. It was obvious that MDH appeared and disappeared in response to the methanol concentration in the culture medium and became almost undetectable after prolonged cultivation. On the other hand, ALDH was maintained at a constant level even at late stationary phase. These results suggested that accumulation of PQQ in the culture medium might be a result of degradation of MDH but not ALDH.

![Fig. 1](image1.png)

**Fig. 1.** Course of the Growth of *M. glycogenes* on Methanol.

*M. glycogenes* in 1% methanol was cultured at 30°C under shaking for the period indicated in culture medium (100 ml) in a 500-ml shaking flask. The bacterial growth was measured by the optical density at 660 nm. Methanol content in the culture medium (MeOH) was measured with a purified MDH essentially by the same method as described in Materials and Methods, except that a limited volume of the culture medium was added to the assay medium and then incubated at 25°C until the extent of reduction of 2,6-dichlorophenol indophenol reached a constant level. The maximal level of specific activity of MDH and ALDH, and PQQ content are indicated.

![Fig. 2](image2.png)

**Fig. 2.** Effects of Ca on the Growth of *M. glycogenes*.

Various concentrations of Ca was initially mixed into the culture medium as shown in ppm. Courses of each batch were monitored for the period indicated by measuring the turbidity with a Klett-Summerson photoelectric colorimeter. At the end of culture, supernatant solution was separated from cells and PQQ contents were assayed. MDH and ALDH activities were also assayed with cell-free extracts prepared from individual cells.

**Effects of Ca on the growth of M. glycogenes**

*M. glycogenes* was grown on limited concentrations of Ca to see the effects of Ca
Ca in Methanol Dehydrogenase Can Be Replaced by Sr

Fig. 3. Effects of Replacement of Ca with Other Divalent Metals on the Growth of *M. glycogenes.*

Ca (15 ppm) was replaced with other divalent metals (15 ppm each) as indicated. The course of bacterial growth was monitored by reading the turbidity by a Klett-Summerson photoelectric colorimeter. At the end of culture, supernatant solution was separated from cells and PQQ contents were assayed. MDH and ALDH activities were also assayed with cell-free extracts prepared from individual cells.

on the bacterial growth. When Ca was present at over 1 ppm, the bacterial growth was not affected (Fig. 2). When the Ca concentration was further limited, repression of the bacterial growth was apparent and no actual growth occurred unless a trace of Ca was in the culture medium. Specific activities of MDH and ALDH, and the amount of PQQ accumulated in the culture medium were found at similar levels when compared among the cells grown at the initial Ca concentrations of 0.25 ppm, 1.0 ppm, and 28 ppm, though the total enzyme activities of MDH and ALDH were different due to the mass of cells grown.

*Growth of M. glycogenes in the presence of various metals*

Ca was replaced by other divalent metals and the corresponding bacterial growth was measured (Fig. 3). Ca gave the best growth among the metals tested. When Ca was replaced by Sr, a similar level of growth occurred with some delay in reaching early stationary phase. With other metals, such as Ba, Ce, La, and Y, the bacterial growth was further delayed. Levels of MDH, ALDH, methanol oxidase (MOX), and PQQ were compared between Ca-grown and Sr-grown cells in Table I. It was clear that the specific activities of MDH and MOX were elevated in Sr-grown cells. When examined by SDS-polyacrylamide gel electrophoresis, there was no difference in MDH content in the cell-free extracts between Ca-grown cells and Sr-grown cells, judging from the intensity of stained protein bands (data not shown). As described later, MDH with an elevated specific activity can be obtained when the organism is grown in a culture medium containing Sr instead of Ca, although the reason is not known. MOX was assayed in the same cell populations by adjusting the cell density used.

**Table I. Comparison of Enzyme Activities between Ca-Grown Cells and Sr-Grown Cells of *M. glycogenes***

* *glycogenes* was grown on 1% methanol medium containing Ca or Sr at 15 ppm. Ca-grown cells were harvested after 120 hr of cultivation and Sr-grown cells after 160 hr of cultivation, when both were at the early stationary phase. A cell-free extract was prepared from individual cells and MDH and ALDH were assayed. A portion of intact cells was suspended to make a homogeneous cell suspension with 50 mM potassium phosphate, pH 6.5, and the optical density at 660 nm was adjusted to 2.5. Methanol oxidase (MOX) was assayed with the cell suspensions under identical assay conditions using a conventional oxygen electrode apparatus. PQQ contents in the culture medium were measured in the supernatant obtained by centrifugation.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Growth (<em>A</em>&lt;sub&gt;660&lt;/sub&gt; nm)</th>
<th>PQQ (µg/ml)</th>
<th>MDH (U/mg)</th>
<th>ALDH (U/mg)</th>
<th>MOX (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>2.55</td>
<td>25.0</td>
<td>0.20</td>
<td>2.53</td>
<td>0.01</td>
</tr>
<tr>
<td>Sr</td>
<td>2.35</td>
<td>25.0</td>
<td>1.10</td>
<td>3.49</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Purification of MDH from Sr-grown cells of *M. glycogenes***

* *glycogenes* was harvested from the late exponential phase and MDH was purified from the cells by the method of Yamanaka and Matsumoto. After MDH was passed through a DEAE-cellulose column, the enzyme solution was put on a CM-Sephadex C-50 column. MDH was eluted by a linear gradient of KCl to 0.35 M. Fractions containing MDH activity over 5.0 units per mg protein were pooled as the purified MDH. The purified enzyme showed a single sedimentation peak in an
The purified MDH (19.0 mg per ml) in 50 mM Tris-HCl, pH 8.0, containing 3 mM methanol and 1% sucrose was analyzed. Centrifugation was done at 60,000 rpm and the temperature was kept at 20°C throughout measurements. Photographs were taken at 35 min, 43 min, and 60 min (from left to right) after reaching the maximal speed.

Analytical ultracentrifugation giving 7.2 s as the apparent sedimentation velocity (Fig. 4). The purified MDH showed a specific activity of 6.0 to 10.0 units per mg protein, several times higher than that of MDH from Ca-grown cells. In Fig. 5, some of the purification steps are shown by SDS polyacrylamide gel electrophoresis. It should be noted that MDH from Sr-grown cells is also composed of two different subunits making an $\alpha_2\beta_2$ structure as reported by Anthony et al. [10] One atom of Sr was contained in the purified MDH and no appreciable contamination of Ca was found in the MDH.

The ratio of Ca to Sr in the culture medium was varied to see whether Sr has a novel effect to MDH activity. As illustrated in Fig. 6, all flasks were shaken until the organism reached the late exponential phase and MDH and ALDH were assayed. Since no appreciable differences were observed in ALDH and PQQ contents, only the specific activity of MDH was shown. The background of Ca in the culture medium to which no Ca was added was estimated to be less than 0.1 ppm. In the absence of Sr, the metal in the MDH formed was Ca, while Sr-rich MDH was formed when Ca was absent or present but restricted. Judging from these results, the affinity for Ca in the MDH formation is higher than Sr and thus Ca seems to be the intrinsic element spontaneously incorporated into MDH when the organism is grown on a usual medium. In this sense, Sr-containing MDH may be artificially formed. No appreciable differences in ALDH content under the various culture conditions mentioned above suggested the absence of any essential metals in ALDH. PQQ in all culture flasks were also at similar levels around 25 μg per ml.

Acknowledgments. We wish to express our sincere thanks to Mr. T. Takeda, Mr. T. Matsumoto, and Mr.
M. Hokazono for the skillful technical assistance during this study. In the metal analysis by atomic absorption spectrometry we are indebted to the sincere efforts of Dr. H. Shindoh, of this department, and Mr. K. Aritomi, Industrial Technology Institute, Yamaguchi Prefectural Government.

References