NADP-Specific Isocitrate Dehydrogenase of Mycobacterium phlei
ATCC 354: Purification and Characterization

By KULDEEP R. DHARIWAL*† AND T. A. VENKITASUBRAMANIAN
Department of Biochemistry, Vallabhbhai Patel Chest Institute, University of Delhi,
Delhi 110007, India

(Received 9 March 1987; revised 24 April 1987)

NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) from Mycobacterium phlei
ATCC 354 was purified to homogeneity by ammonium sulphate fractionation, followed by
DEAE cellulose and Sephadex G-200 chromatography. The pH optimum of the enzyme was 8.5.
The $K_m$ values for isocitrate and NADP were 74 and 53 $\mu$M, respectively. Mn$^{2+}$ was essential for
enzyme activity. The enzyme lost all activity on incubation at 70 °C for 15 min; isocitrate and
NADP protected against this thermal inactivation. $p$-Chloromercuribenzoate inhibited the
enzyme; pre-incubation of enzyme with isocitrate + Mn$^{2+}$ prevented this inhibition. The
purified enzyme showed concerted inhibition by glyoxylate + oxaloacetate and was inhibited by
oxalomalate.

INTRODUCTION

Isocitrate dehydrogenase [threo-D,-isocitrate:NADP oxidoreductase (decarboxylating); EC 1.1.1.42] has been purified from a number of bacterial and mammalian sources. The bacterial enzyme, with a few exceptions, is NADP-dependent (Burchall et al., 1964; Ragland et al., 1966). On the other hand, the mammalian enzyme is both NAD- as well as NADP-dependent. NADP-specific isocitrate dehydrogenase of mycobacteria is inducible, and is present at high activity compared with other dehydrogenases of mycobacteria (Ragland et al., 1966; Dhariwal & Venkitasubramanian, 1975). This paper describes for the first time the purification and properties of this enzyme from a mycobacterium (Mycobacterium phlei ATCC 354).

METHODS

Organism and growth conditions. M. phlei ATCC 354 was grown as a surface culture at 37 °C in a medium (pH 7.0) consisting of asparagine, 5 g; potassium dihydrogen phosphate, 5-9 g; potassium sulphate, 0.5 g; citric acid, 1-5 g; magnesium carbonate, 0-6 g; glycerol, 20 ml and water to 1 litre. The cells were harvested during the late exponential phase of growth (5th day) by centrifugation and washed three times with glass distilled water to remove traces of medium.

Enzyme assay. The activity of isocitrate dehydrogenase was determined at room temperature (25–30 °C) from the rate of increase in $A_{340}$ (Goldman, 1956). The reaction system (3 ml) contained the following (pmol): Tris/HCl buffer (pH 7.3), 30; MnCl$_2$, 10; NADP, 1; isocitrate, 9; and enzyme protein.

Enzyme purification. Cell suspensions in 0-05 M-Tris/HCl buffer (pH 7-4) were placed in a beaker surrounded by ice and sonicated for 2 min in an MSE 100 W ultrasonic disintegrator at a frequency of 20 kHz. The sonoicated material was centrifuged at 10000 g for 30 min to obtain a cell-free extract. Solid ammonium sulphate was added with constant stirring to the cell-free extract to bring it to 65% saturation. The precipitated proteins were discarded and the supernatant was brought to 95% saturation. After 2 h, it was centrifuged and the precipitate was dissolved in 4 ml 0-05 M-Tris/HCl buffer (pH 7-4). It was dialysed against the same buffer (1 litre) for about 16 h with at least three changes. The dialysed enzyme obtained at 65-95% ammonium sulphate saturation was applied

† Present address: Department of Molecular and Cellular Biology, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206, USA.

0001-14067 © 1987 SGM
to a DEAE cellulose column (2 × 24 cm) equilibrated with 0.05 M-Tris/HCl, pH 7.4. Enzyme was eluted by using a linear gradient of KCl (0-05-0-50 M) in 0.05 M-Tris/HCl (pH 7-4). The flow rate was 30 ml h⁻¹; 3 ml fractions were collected and assayed for enzyme activity and protein content. Fractions containing maximum activity of the enzyme were pooled, concentrated to 3 ml, dialysed and loaded on to a Sephadex G-200 column (2.2 × 60 cm). The enzyme was eluted with Tris/HCl buffer (pH 7.4) at a flow rate of 10 ml h⁻¹. Fractions (2.5 ml) were collected and assayed for enzyme activity and protein. Fractions containing maximum enzyme activity were pooled and concentrated.

Gel electrophoresis. Samples containing 50-200 μg protein were subjected to polyacrylamide disc gel electrophoresis (PAGE) in gels made with 7% (w/v) acrylamide (Davis, 1964). Electrophoresis was done for 90 min in Tris/glycine buffer, pH 8.3 (6 g Tris and 28.8 g glycine in 1 litre), with a current of 4 mA per tube. The gels were stained with Amido Schwarz and destained mechanically with 7% (v/v) acetic acid. SDS-PAGE was done by the method of Weber & Osborn (1968). The protein samples were denatured in 0.01 M-potassium phosphate buffer, pH 7.0, containing 2% (w/v) SDS and 1% 2-mercaptoethanol at 37 °C for 2 h. Polyacrylamide gels (7% w/v) contained 0.2% SDS. The proteins were subjected to electrophoresis for 2 h in 0.01 M-potassium phosphate buffer, pH 7.0, containing 0.1% SDS at a current of 4 mA per tube. The gels were stained with Coomassie blue for 4 h and destained mechanically in a mixture of methanol (5%, v/v) and acetic acid (7.5%, v/v).

Enzyme activity staining. Isocitrate dehydrogenase activity was detected in polyacrylamide gels after electrophoresis by washing gels once, for 15 min, in ice-cold 0.5 M-Tris/HCl buffer (pH 8.5), then staining in the dark for 10 min at 25 °C. The staining solution (4 ml) contained the following (μmol): Tris/HCl buffer, pH 8.5, 150; DL-isocitrate, 5; NADP, 1; MnCl₂, 5; nitro blue tetrazolium, 0.35 and phenazine methosulphate, 0.10. After staining, gels were washed sequentially with water and 7.5% (v/v) acetic acid (Gabriel, 1971).

RESULTS AND DISCUSSION

Homogeneity of the preparation

Isocitrate dehydrogenase of M. phlei ATCC 354, purified 247-fold from a cell-free extract (Table 1), ran as a single band in PAGE. Only one band could be seen when the protein concentration was varied from 50 to 200 μg or when PAGE was done at different pH values (8.0, 8.3 and 9.0). In a separate experiment, PAGE of the purified enzyme was done in duplicate, staining one gel for protein and the other for enzyme activity. The enzyme activity staining on the gel revealed only one band which corresponded to the protein band. A single symmetrical peak was obtained on rechromatography of the purified enzyme in Tris/HCl buffer (0.05 M, pH 7.4) on a Sephadex G-200 column. In addition, the purified enzyme ran as only one band in SDS-PAGE. Thus, the purified preparation of isocitrate dehydrogenase was homogeneous with respect to molecular size and charge.

Properties of the purified enzyme

The effect of pH on the enzyme was determined over a range of pH values (6.0-10.0). For oxidation of isocitrate, the optimum pH was 8.5. The enzyme was highly specific for isocitrate and NADP. There was no activity in the presence of NAD (3.3 mM). K_m values for isocitrate and NADP were 74 and 53 μM, respectively.

The enzyme showed an absolute requirement for Mn²⁺, with an optimum concentration of 0.16 mM. When no divalent cations were added to the mixture, enzyme activity was only 10% of that in the presence of Mn²⁺. Mg²⁺, Zn²⁺ and Na⁺ could partially replace Mn²⁺ giving about 50, 40 and 35% of original enzyme activity respectively. No activity was detected when Co²⁺, Ni²⁺, Fe²⁺, Cu²⁺, NH₄⁺, Hg²⁺ or Ca²⁺ replaced Mn²⁺. The enzyme from other bacterial sources requires either Mn²⁺ or Mg²⁺ for its activity.

Incubation of enzyme for 15 min at 70 °C led to 100% loss of activity and at 40 °C, 40%. This loss of enzyme activity depended on protein concentration, diluted enzyme preparations (0.1 mg protein ml⁻¹) losing more compared to undiluted ones (1 mg protein ml⁻¹). Isocitrate (33 mM) and NADP (3.33 mM) protected slightly against thermal inactivation. Similarly, the inactivation of 3-hydroxybutyrate dehydrogenase from this bacterium by incubation for 15 min at 37 °C was also dependent on protein concentration. Ca²⁺, NADH and to some extent Mn²⁺, but not substrate, protected that enzyme from inactivation (Dhariwal & Venkitasubramanian, 1978).

The thiol inhibitors, p-chloromercuribenzoate (PCMB) in particular, are well-known inhibitors of isocitrate dehydrogenase from other micro-organisms. Their effect on the enzyme

2458  K. R. DHARIWAL AND T. A. VENKITASUBRAMANIAN
Isocitrate dehydrogenase from M. phlei

Table 1. Purification of isocitrate dehydrogenase

Preparation of cell-free extract and purification of enzyme were as described in Methods. One unit (U) of enzyme is the amount of enzyme that converts 1 μmol of NADP to NADPH min⁻¹ at 37 °C.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity [U (mg protein)⁻¹]</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>3050</td>
<td>1353</td>
<td>0.44</td>
<td>–</td>
</tr>
<tr>
<td>65–95% (NH₄)₂SO₄ fraction</td>
<td>213.6</td>
<td>869</td>
<td>4.06</td>
<td>9.2</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>37.4</td>
<td>386</td>
<td>10.32</td>
<td>23.4</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>0.862</td>
<td>94.2</td>
<td>109-00</td>
<td>247.0</td>
</tr>
</tbody>
</table>

from M. phlei ATCC 354 was studied by pre-incubating the enzyme with inhibitor (0.33 mM) for 5 min. In these conditions, PCMB completely inhibited activity whereas iodoacetate and mercuric chloride were partially inhibitory (results not shown). However, the enzyme, when pre-incubated additionally with both isocitrate and Mn²⁺, was not inhibited by PCMB. Over 50% protection was also achieved when the enzyme was pre-incubated with NADP and Mn²⁺ and the reaction was started with isocitrate. Pre-incubation with isocitrate, NADP or Mn²⁺ separately gave no protection. Barrera & Jurtshuk (1970) suggested that the isocitrate–Mn²⁺ complex binds with thiols associated with isocitrate dehydrogenase activity and protects it from inhibition by PCMB. The NADP–Mn²⁺ complex can also bind, though to a much lesser extent than the isocitrate–Mn²⁺ complex, and thus gives little protection. This suggestion could explain our results and similar results obtained with isocitric dehydrogenase from Bacillus stearothermophilus (Howard & Becker, 1970).

Dithiothreitol, 2-mercaptoethanol and glutathione (3-33 mM) all inhibited enzyme activity by around 25%. Mono, di and triphosphates of various nucleotides were tested as possible inhibitors of enzyme. Inhibition was in the order triphosphates > diphosphates > monophosphates (results not shown).

The effect of glyoxylate, oxaloacetate and several other acids on enzyme activity is shown in Table 2. Considerable inhibition was observed only when both glyoxylate and oxaloacetate were present. Such a concerted inhibition has been observed for isocitrate dehydrogenase from Brevibacterium flavum (Shio & Ozaki, 1968), Azotobacter vinelandii (Barrera & Jurtshuk, 1970), Crithidia fasciculata (Marr & Weber, 1969), Thiobacillus novellus (Charles, 1970) and several other organisms. Several hypotheses have been put forward to explain the mechanism of this inhibition. Ruffo et al. (1967) reported that oxalomalate, a condensation product of glyoxylate and oxaloacetate, inhibits the enzyme. Others concluded that this probably was not true because of the long time required for the formation of oxalomalate (Shio & Ozaki, 1968; Charles, 1970).

Table 2. Concerted inhibition of isocitrate dehydrogenase

Enzyme specific activity in the absence of inhibitors was 15 U (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Addition (0-33 mM)</th>
<th>Percentage inhibition in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glyoxylate (0-33 mM)</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>95</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>0</td>
</tr>
<tr>
<td>Citrate</td>
<td>4</td>
</tr>
<tr>
<td>Succinate</td>
<td>15</td>
</tr>
<tr>
<td>Fumarate</td>
<td>10</td>
</tr>
<tr>
<td>Malate</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>12</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0</td>
</tr>
</tbody>
</table>
Nimmo (1986) studied the mechanism of inhibition of *Escherichia coli* isocitrate dehydrogenase by glyoxylate and oxaloacetate and showed that oxalomalate is a potent inhibitor of the enzyme. The failure to detect oxalomalate by other workers was probably because oxalomalate is spontaneously decarboxylated to 4-hydroxy-2-oxoglutarate (Nimmo, 1986). The addition of oxalomalate (0.33 mM or 10 μM) to the reaction mixture resulted in nearly 90% inhibition of the enzyme in the present investigation. The enzyme was inhibited by 70% even at 1 μM oxalomalate. Though the commercially obtained (Sigma) oxalomalate used in this study was shown by HPLC to be contaminated by other compounds (Nimmo, 1986), it is believed that the inhibition observed is due to oxalomalate and not to the impurities present.

REFERENCES


