Enzymes of Malate Oxidation in *Mycobacterium leprae* Grown in Armadillo Livers

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A NAD-dependent malate dehydrogenase is the principal enzyme for malate oxidation by *Mycobacterium leprae*, FAD-dependent malate-vitamin K reductase was detected at about 1% the level of the NAD-dependent activity. Both enzyme activities were detected in extracts from *M. leprae* treated with NaOH to abolish host-derived activities which might be adsorbed to the bacteria and the NAD-dependent enzyme was shown to be electrophoretically distinct from the host-tissue enzyme, thus establishing that these were both authentic bacterial enzymes. *Mycobacterium leprae* does not possess malic enzyme.

INTRODUCTION

*Mycobacterium leprae*, in sufficient quantity for metabolic studies, is only available from experimentally infected *Dasypus novemcinctus* L. (nine-banded armadillo). Although only milligram quantities of the leprosy bacilli can be purified from armadillo tissues, metabolic pathways for carbon sources have been demonstrated in work with crude extracts and whole organisms (Wheeler, 1982, 1983). Previously, Wheeler (1982) suggested that the tricarboxylic acid cycle might be operating in *M. leprae*, since succinate was oxidized to CO2 by suspensions of bacilli. We now report the first attempt to identify any of the enzymes of that cycle in *M. leprae*.

The enzymes for malate oxidation were of primary interest because mycobacteria divide broadly into two groups with respect to these enzymes. Generally, slow-growing mycobacteria have malate dehydrogenase (EC 1.1.1.37; l-malate: NAD⁺ oxidoreductase) but little or no 'malic enzyme' [EC 1.1.1.40; l-malate: NADP⁺ oxidoreductase (oxaloacetate decarboxylating)]. This group includes *M. tuberculosis*, *M. avium*, *M. scrofulaceum*, *M. kansasii*, and *M. phlei* – which is not regarded as a slow grower (Seshadri et al., 1978a). *Mycobacterium lepraemurium*, grown in vivo, has malate dehydrogenase activity (Mori et al., 1971). Fast-growing mycobacteria (e.g. *M. smegmatis* and *M. fortuitum*) possess mainly malic enzyme (Seshadri et al., 1978a). When purified, malic enzyme from *M. smegmatis* was NADP specific and lacked reducing activity towards oxaloacetate, demonstrating that it is an authentic malic enzyme (Seshadri et al., 1978b). A possible explanation (Seshadri et al., 1978a) for this difference is that the fast-growing organisms have a greater requirement than the slow-growers for NADPH for biosynthetic purposes. Malic enzyme would be the main enzyme for generating NADPH; where the requirement for NADPH is less in slow-growers, malate dehydrogenase would be the main enzyme for malate oxidation, and NADPH could be generated by other enzymes. Certainly, the fast-growing mycobacteria would have a high demand for NADPH in order to synthesize rapidly their lipid-rich cell walls.
In addition, mycobacteria possess a malate-vitamin K reductase [EC 1.1.99.1; L-malate (receptor) oxidoreductase]. This FAD-dependent enzyme is present at higher levels in fast growing mycobacteria (Kimura & Tobari, 1963), in which it is the principal or only enzyme for oxidation of malate to oxaloacetate, since malate is converted to pyruvate with malic enzyme. In slow-growing mycobacteria, there are, therefore, two enzymes present for conversion of malate to oxaloacetate (Reddy et al., 1975).

The study of malate oxidation in \textit{M. leprae} enabled us to place the organism in one of these 'growth-rate groups' and to detect for the first time enzymes involved in oxidative metabolism.

\section*{METHODS}

\textit{Bacterial cell-free extracts}. Extracts of \textit{Mycobacterium leprae} and \textit{M. phlei} were prepared from sonicates as described by Wheeler et al. (1982). The \textit{M. leprae} organisms were harvested from armadillos by a scheme including separation on a density-gradient and in an aqueous two-phase system designed to exploit the relatively hydrophobic nature of the bacteria. Host debris was essentially absent from the final suspension of \textit{M. leprae} (Report, 1979; Wheeler & Gregory, 1980).

\textit{Armadillo liver extracts}. In this work, cell-free extracts of armadillo liver were prepared from liver tissue homogenized with EDTA (Wheeler & Gregory, 1980), MgSO\textsubscript{4} and no EDTA or from infected liver tissue (Wheeler et al., 1982). The bacteria were removed from the infected liver homogenate by centrifugation (Wheeler et al., 1982).

\textit{NaOH treatment of \textit{M. leprae}}. NaOH treatment of leprosy (Wheeler & Gregory, 1980; Wheeler et al., 1982) and tuberculosis (Kanai, 1967) bacilli abolished adsorbed host-derived activity. Such treatment does not affect \textit{M. leprae} viability (A. C. R. E. Lowe, personal communication). Suspensions of \textit{M. leprae} were incubated in 1 M-NaOH for 1 h at 25 °C, as described by Wheeler et al. (1982).

\textit{Desalting of extracts}. Desalted extracts from \textit{M. leprae} were prepared with an Amicon CF25 centrifuge ultrafiltration cone (700 g, 4 °C: molecular weight cut off 25000). After desalting, the salt concentration was 1% (w/v) of the level in extracts that were not desalted.

\textit{Enzyme assays}. Modifications of the methods of Seshadri et al. (1978a) were used. The incubation volume was 500 µl and the temperature 22 °C, for all enzyme assays. Extracts were stored at −80 °C with no noticeable loss of activity.

Extracts were assayed for malate dehydrogenase with 60 mM-glycine/NaOH; pH 10-0, 2 mM-malate, 0-33 mM-NAD, 0-05 units diaphorase (Sigma; from porcine heart) and 0-6 mM-p-iodonitrotetrazolium violet – the modification of this assay to read (at 500 nm; e\textsubscript{500} = 12-6) reduced p-iodonitrotetrazolium violet minimized the interfering effect of other oxidoreductases and eliminated interference by NADH oxidase in NAD dependent enzymes assays with crude extracts (Hinman & Blass, 1981). This enzyme was also assayed in the direction of NADH oxidation with 2 mM-oxaloacetate, 0-17 mM-NADH or NADPH and 60 mM-glycine/NaOH, pH 10-0.

Malic enzyme was assayed with 2 mM-malate, 0-33 mM-NADP and 1 mM-MnCl\textsubscript{2} buffered with 25 mM-Tris/HCl (pH 7-4). To detect malate-vitamin K reductase (Seshadri et al., 1978a), extracts were incubated with 33 mM-Tris/HCl (pH 7-4), 16 mM-KCl, 5 mM-MgCl\textsubscript{2}, 8 mM-malate, 5 µM-FAD and 0-6 mM-p-iodonitrotetrazolium violet (read at 500 nm).

All enzyme activities were measured against controls with no malate or oxaloacetate and all incubations were started with cell free extracts unless otherwise stated.

\textit{PAGE. Mycobacterium leprae} and armadillo liver extracts (25 µg each) were applied (volume of extracts: 1–30 µl) with 2 vol. glycerol and 5 µl 0-1% (w/v) aqueous bromophenol blue, directly to cylindrical (70 × 5 mm diam.) small pore gels of 7-5% (w/v) acrylamide. The gel system was that described by Davis (1964). After electrophoresis gels were incubated in 0-6 M-glycine/NaOH (pH 10-0) for 15 min then incubated in 60 mM-glycine/NaOH, 8 mM-malate, 0-17 mM-NAD, 0-6 mM-phenazine methosulphate and 0-08 mM-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) until bands of staining were resolved.

\section*{RESULTS}

NAD-dependent malate dehydrogenase activity was present in three different extracts (one from NaOH-treated bacteria) of \textit{M. leprae} at almost identical levels (Table 1). This enzyme was also present in extracts of \textit{M. phlei} (strain NCTC 10266, grown at 37 °C in Headley–Wright broth with Tween-80; see Wheeler & Gregory, 1980) and in extracts of armadillo liver (homogenized with MgSO\textsubscript{4}, no EDTA): rates of malate oxidation were 60 nmol min\textsuperscript{−1} (mg protein)\textsuperscript{−1} and 30 nmol min\textsuperscript{−1} (mg protein)\textsuperscript{−1}, respectively. The level of malate dehydrogenase measured in \textit{M. phlei} broadly agreed with that of Seshadri \textit{et al.} (1978a). The reaction in all extracts, proceeded rapidly for up to 30 s, before continuing at the highly reproducible rates quoted above.
Table 1. Malate oxidation in extracts of *M. leprae*

When NAD or NADP were included as co-enzymes, malate at 2 mM and non-desalted extracts were used. When FAD was included as co-enzyme, malate was at 8 mM; the rates of reaction were not markedly different for desalted and non-desalted extracts so a mean figure is quoted, the mean values are from at least three assays except where indicated.

<table>
<thead>
<tr>
<th>Co-enzyme</th>
<th>pH</th>
<th><em>M. leprae</em> (untreated)</th>
<th><em>M. leprae</em> (NaOH-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>7-4</td>
<td>0.18</td>
<td>0.16</td>
</tr>
<tr>
<td>NAD</td>
<td>10-0</td>
<td>46-0*</td>
<td>54-3</td>
</tr>
<tr>
<td>NADP</td>
<td>7-4</td>
<td>ND</td>
<td>0.019</td>
</tr>
<tr>
<td>NADP</td>
<td>10-0</td>
<td>ND</td>
<td>0.048</td>
</tr>
<tr>
<td>FAD</td>
<td>7-4</td>
<td>0.52*</td>
<td>0.59</td>
</tr>
</tbody>
</table>

ND, Not detectable (less than 0.004).

* Mean value from two extracts.

for up to 30 min. When NADP was substituted for NAD, reaction rates at pH 10, or pH 7-4, were very low (Table 1).

With oxaloacetate, a steady rate of oxidation of NADH or NADPH was observed between 2 and up to 20 min after starting the incubation. Initially faster, steady rates of oxidation with extract from NaOH-treated *M. leprae* were: for NADH, 136 nmol min⁻¹ (mg protein)⁻¹; for NADPH 0.52 nmol min⁻¹ (mg protein)⁻¹.

Extracts from *M. leprae* and armadillo liver were electrophoresed on polyacrylamide gels and stained for NAD-dependent malate dehydrogenase (Fig. 1). When NADP was substituted for NAD in the staining solution, faint bands, in the same position as when NAD was used, were detected in both gels to which *M. leprae* and armadillo liver extracts were applied. To observe the bands with NADP, 750 μg cell-free extract (in both cases) was applied to gels.

A problem in looking for malate-vitamin K reductase in crude extracts is, since it is FAD dependent, that if NAD is present FAD would be used as an intermediate electron carrier between NAD and p-iodonitrotetrazolium violet. In such a case malate-vitamin K reductase activity could in fact be due to NADH generation by malate dehydrogenase. Indeed, 1–3 μM-NAD was present in μg extracts of *M. leprae* (unpublished data). Therefore, extracts of *M. leprae* were desalted and (as well as extracts not desalted) assayed for FAD-dependent malate dehydrogenase (Table 1). Further experiments were done with the desalted extract from NaOH-treated *M. leprae* to confirm that the activity observed with FAD was not due to the NAD-dependent, malate dehydrogenase. When FAD was omitted or replaced by 0.05 units diaphorase, no activity was observed. When the assay mixture including extract, but with no malate (reaction was started with malate) was pre-incubated with 0.1 unit NADase (Sigma; from Neurospora crassa) for 15 min at 22 °C, malate was oxidized at 0.5 nmol min⁻¹ (mg protein)⁻¹. When 1 μM-NAD was included in addition to the assay mixture, malate was oxidized at 0-7 nmol min⁻¹ (mg protein)⁻¹. The addition of a vitamin K₁ (2-methyl-3-phytyl-1,4-napthoquinone; Sigma) suspension (in 3.3 mM-Tris/HCl, pH 7.4; sonicated for 2 min) to 0-25 mM, had no effect on FAD-dependent malate oxidation in extracts (desalted or not) of *M. leprae*.

**DISCUSSION**

NAD-dependent malate dehydrogenase is the main enzyme for malate oxidation in *M. leprae*. Electrophoretic studies, and studies with extracts from NaOH-treated *M. leprae* demonstrated that this was an authentic *M. leprae* enzyme. There is also a relatively low level of FAD-dependent activity. In desalted extracts of *M. leprae*, NADase did not abolish this activity, and diaphorase could not replace FAD, confirming that FAD was participating in the enzyme
Fig. 1. PAGE of extracts from *M. leprae* and armadillo liver. Gels (Davis, 1964) were stained for malate dehydrogenase (NAD-dependent) activity. Malate was omitted from the staining mixture in gels 2 and 3. No extract was applied to gel 1; extracts applied to other gels were from: *M. leprae* (untreated) gels 2, 5 and 6; NaOH-treated *M. leprae* (gel 4); infected armadillo liver (gels 3 and 8); and armadillo liver extracts with MgSO₄ and no EDTA (gels 6 and 7) or EDTA (gel 9). Staining was complete within 1 h at 22°C. M, denotes enzyme from *M. leprae*; A, denotes enzyme from armadillo liver; bromophenol blue marker ran approx. 5 mm from end of gel. The origin is at the top of the photograph.

reaction. The FAD reduction was not an artefact resulting from reduction by NADH generated by the presence of malate dehydrogenase. This indicated the presence of low levels of malate-vitamin K reductase, which is part of a pathway of electron-transport characteristic of mycobacteria (Brodie & Adelson, 1965). The product of malate oxidation by both enzymes in *M. leprae*, is oxaloacetate, the next step from malate in the tricarboxylic acid cycle.

NADP was reduced very slowly in extracts of *M. leprae*, but (a) the increase in activity from pH 7-4 to pH 10-0, (b) the shared position on electrophoresis of the NADP and NAD reducing activity in the presence of malate, and (c) oxaloacetate dependent NADPH oxidation suggested that this activity was due to the NAD-dependent malate dehydrogenase using NADP at a low rate. Examples of NAD-dependent malate dehydrogenase which can reduce NADP at relatively slow rates have been purified from beef-heart; both mitochondrial (Seigel & Egland, 1961) and extra-mitochondrial (Egland & Breiger, 1962). The only mycobacterial malate dehydrogenase purified, from *M. tuberculosis* H₃₇ Ra, is NAD-specific (Goldman, 1956).

These findings place *M. leprae* amongst the broadly slow-growing group of mycobacteria with malate dehydrogenase. NADPH for biosynthetic purposes in *M. leprae* could be generated through the hexose (monophosphate pathway which is known to operate in *M. leprae* (Wheeler, 1983). Further studies of tricarboxylic acid cycle enzymes could identify further NADPH-generating enzymes similar to the situation in *M. tuberculosis* H₃₇ Rv (Murthy et al., 1962).

A comparison of the rate of malate oxidation in *M. leprae* with that in other mycobacteria (Seshadri et al., 1978a), suggests that the level of malate dehydrogenase in *M. leprae* is unusually high, but attribution of any significance to this observation awaits further study of the tricarboxylic acid cycle enzymes in *M. leprae*. 
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REFERENCES


