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Superoxide Dismutase, Peroxidatic Activity and Catalase in *Mycobacterium leprae* Purified from Armadillo Liver

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Superoxide dismutase has been identified and peroxidatic activity demonstrated in *Mycobacterium leprae*. The superoxide dismutase, shown indirectly to be a manganese-containing enzyme, was present at low activity in the cell-free extract. Peroxidatic activity was detected in a haemoprotein on polyacrylamide gels, but quantitative assay was not possible. Catalase, although present in a cell-free extract, appeared to be a host-derived enzyme, thus emphasizing the importance of establishing the authenticity of enzyme activities in host-derived *M. leprae*. The implications for the growth of *M. leprae in vivo* and its non-cultivability are discussed in the light of these findings.

INTRODUCTION

Until recently, very little material for biochemical study of *Mycobacterium leprae* has been available. However, it is now possible to prepare about 50 mg (dry wt) of pure bacteria from 50 g (wet wt) of liver from experimentally infected armadillos, representing an increase of 2 to 3 orders of magnitude over the yield from human biopsy.

Initially, it was decided to study superoxide dismutase (EC 1.15.1.1; superoxide: superoxide oxidoreductase), catalase (EC 1.11.1.6; hydrogen-peroxide:hydrogen-peroxide oxidoreductase) and peroxidase (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) since these enzymes may play a role in the survival of *M. leprae* inside host phagocytes, which produce superoxide radical (O_2^-) and H_2O_2 (Karnovsky *et al.*, 1975; Segal & Allison, 1979). Superoxide dismutase has been observed in all mycobacteria so far studied (Kusunose *et al.*, 1976*a*, *b*; Ichihara *et al.*, 1977); this enzyme appears to be a pre-requisite for aerobiosis (Fridovich, 1975). Catalase and peroxidase are present in most mycobacteria (Tirunarayanan & Vischer, 1957), but are notably absent in isoniazid-resistant strains of *M. tuberculosis*. Catalase-negative strains were found to be more susceptible to H_2O_2 and this was one of the factors associated with low virulence (Jackett *et al.*, 1978). Wayne & Diaz (1976, 1979) have used mycobacterial catalases in taxonomic studies.

Since bacteria were harvested from host tissue, it was important to establish that enzyme activities in the cell-free extracts of *M. leprae* were, in fact, due to bacterial enzymes. A rigorous scheme of purification of the bacteria was followed before certain properties of the enzymes, particularly electrophoretic mobility, were compared with similar enzymes from a cell-free extract of armadillo liver.

Mycobacteria other than *M. leprae* were used for comparison and also for establishing suitable methods without using valuable *M. leprae* cell-free extract.

METHODS

Organisms used. Mycobacterium leprae was obtained from heavily [$> 5 \times 10^9$ bacilli (g wet wt tissue)$^{-1}$] infected liver (either fresh or stored at $-80\,^{\circ}C$) of experimentally infected nine-banded armadillos (*Dasypus*

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novemcinctus, Linn.) by the methods described in 'Report' (1979). The infected liver was homogenized in Tris base and centrifuged. The fraction rich in bacteria was then treated with DNAase (DNAase 1, Worthington Biochemical Corporation) followed by density gradient centrifugation using Percoll (Pharmacia), and partitioning in a dextran/polyethylene glycol aqueous two-phase system. The upper phase was then centrifuged and the pellet, which consisted of *M. leprae*, was washed. The bacteria obtained were free of contaminating tissue, as judged by counterstaining with soluble blue (Wheeler & Draper, 1980).

A sample of pure *M. leprae* (10 mg dry wt) was treated with 5 ml 1 M-NaOH at 25 °C. The suspension was neutralized after 1 h with 1 M-citric acid and centrifuged at 10000 *g* for 10 min. The pellet was washed three times and resuspended in 1·5 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES; adjusted to pH 7·2 with KOH). The supernatant was desalted and concentrated to 2 ml using an Amicon CF25 centrifuge ultrafiltration cone (700 *g*, 4 °C); the final concentration of citrate was calculated to be 10 mM.

Mycobacterium lepraemurium was grown in mice and harvested from liver (Rees *et al.*, 1960). Bacteria were obtained free from contamination by differential centrifugation and partition in an aqueous two-phase system [5 % (w/w) polyethylene glycol (PEG) 6000/7 % (w/w) Dextran T500/0·01 M-NaCl/0·01 M-potassium phosphate, pH 6·9]. *Mycobacterium phlei* NCTC 10266 was grown in Headley–Wright nutrient broth with 0·05 % (v/v) Tween 80 at 37 °C.

Preparation of bacterial cell-free extracts. Bacteria (10 to 60 mg dry wt) were suspended in a total volume of 10 ml 1·5 mM-HEPES (adjusted to pH 7·2 with KOH) and ultrasonically treated for periods no longer than 3 min for a total of 15 min for *M. phlei* and *M. lepraemurium* and 11 min for *M. leprae* at 100 W in a Dawe Soniprobe type 7532A, cooled on wet ice. The probe was completely enclosed in a Dawe Sealed-Atmosphere Treatment Chamber type 7530-5A in order to contain the aerosol formed. The disrupted material was centrifuged at 20000 *g* for 10 min and the supernatant was re-centrifuged for 10 min to remove remaining bacteria and debris. The final supernatant (cell-free extract), containing 0·25 to 1·5 mg protein ml^{-1} was stored at −80 °C. It was not possible to detect acid-fast bacteria in a pellet (obtained by centrifuging at 20000 *g* for 10 min) from the cell-free extract.

Preparation of cell-free extract of armadillo liver. A slight modification of the early stages of purification of manganese-superoxide dismutase from liver (Crapo *et al.*, 1978) was used. Liver from an armadillo which had been inoculated, but had not become infected, was used. The liver was suspended at 4 °C in 3·5 vol. buffer containing 20 mM-HEPES (adjusted to pH 7·8 with KOH) plus 10 μM-EDTA, and homogenized for 3 × 2 min in a Sorvall Omnimix. Debris was removed by centrifugation at 14000 *g* for 30 min. The supernatant material was dialysed against distilled water for 24 h, lyophilized and then dissolved in distilled water to at least 10 mg protein ml^{-1} when required. Liver extract, either lyophilized or in solution, was stored at −20 °C.

Enzyme assays. Superoxide dismutase was assayed indirectly by following the inhibition of the reduction by O_2^- of nitroblue tetrazolium ('NBT assay' of Beauchamp & Fridovich, 1971) or of ferricytochrome *c* (Crapo *et al.*, 1978), at 25 °C. These methods were modified so that the reaction volumes were 500 μl, and 20 μM-KCN was included in all assays. It was necessary to remove superoxide dismutase-like activity present in the cytochrome *c* (Koch-Light) by Sephadex G-75 chromatography and then to assay the cytochrome *c* by reduction with sodium dithionite (Crapo *et al.*, 1978) before it was used in the assay for superoxide dismutase.

Catalase was assayed by a modification of the method described by Winder (1960). The reaction mixture (total volume 150 μl) contained 10 mM-H_2O_2, 1·33 mg bovine serum albumin ml^{-1}, 17 mM-NaH_2PO_4/K_2HPO_4, pH 7·0, cell-free extract and inhibitors. The mixture was incubated at 25 °C and the reaction was stopped, usually after 30 min, by adding 4·4 ml $TiCl_4$ reagent (0·33 mg ml^{-1} in 1·5 M-H_2SO_4).

Peroxidatic activity was assayed by the method of Putter (1974) except that *o*-dianisidine hydrochloride was used instead of guaiacol.

Polyacrylamide gel electrophoresis. Cylindrical (70 mm × 5 mm diam.) small pore gels including *N,N'*-methylene bisacrylamide as 3 % of the acrylamide (with no stacking gel) were used. Aqueous bromophenol blue was used as the tracking dye in each gel. Superoxide dismutase and peroxidatic activity were detected using the methods of Misra & Fridovich (1977), except that 8 % (w/v, total acrylamide) gels were used. It was possible to stain for peroxidatic activity, wash the gels for 15 min in distilled water, then stain for superoxide dismutase (omitting cyanide), thus demonstrating superoxide dismutase-positive areas in the same gels. Cyanide was always used in gels stained for superoxide dismutase only. Mn- and Fe-superoxide dismutases were differentiated by the method of Britton *et al.* (1978).

The method for detecting catalase was based on that used by Diaz & Wayne (1974) using 5 % (w/v, total acrylamide) gels (Clarke, 1964). Initially, we used starch in our assay but this was subsequently replaced by a more satisfactory system employing a stain based on ferricyanide (Woodbury *et al.*, 1971).

Table 1. *Comparison of superoxide dismutase from M. leprae extracts and other cell-free extracts*

Percentage inhibition of NBT or cytochrome *c* reduction was plotted against the concentration of protein (in cell-free extracts) and the specific activity in units (mg protein)$^{-1}$ was calculated (Beauchamp & Fridovich, 1971; Crapo *et al.*, 1978). Since the incubation mixture was 500 μl, 50 % inhibition was said to be caused by 0·167 units. Except for *M. leprae* an incubation was carried out in which about 50 % inhibition actually occurred. Cell-free extracts were then applied to 8 % (w/v) polyacrylamide gels. Some bands were identified as Fe-superoxide dismutase by inhibition by H_2O_2. A slight difference in the specific activity of superoxide dismutase was noted for two preparations of *M. leprae* cell-free extracts; the values for both are given.

	Superoxide dismutase specific activity [units (mg protein)$^{-1}$]			
Cell-free extract	NBT assay	Cyt *c* assay	Relative mobility	Bands inhibited by H_2O_2
M. leprae 1	0·043	ND	0·68	None
2	0·075	0·17		
M. lepraemurium	7·7	14·3	0·36	None
M. phlei	7·8	5·9	0·34, 0·78	0·78
Armadillo liver	16·6	9·3	0·27, 0·50	0·50

ND, Not determined.

RESULTS

Superoxide dismutase

The activity of superoxide dismutase in extracts from *M. leprae* was low in comparison with that in extracts from other mycobacteria (Table 1). The assay involving cytochrome *c* was more sensitive than the NBT assay for *M. leprae* and *M. lepraemurium*, but the NBT assay was more sensitive for armadillo liver and *M. phlei* superoxide dismutases.

It was important to include 20 μM-KCN in this assay to prevent interference by peroxidative reactions, which also inhibited reduction of NBT and cytochrome *c*, resulting in higher apparent specific activities of superoxide dismutase. For instance, a 2·4-fold overestimation with the NBT assay and a 1·7-fold overestimation with the cytochrome *c* assay occurred for *M. lepraemurium* cell-free extract. With *M. leprae* cell-free extract, a 2·2-fold overestimate occurred using the NBT assay if cyanide was omitted. However, if the KCN concentration was increased up to 2 mM, there was no further inhibition of cytochrome *c* or NBT reduction in the presence of any of the cell-free extracts tested.

It is unlikely that the method of isolation of pure *M. leprae* affected superoxide dismutase activity, since the cell-free extract of *M. phlei* suspension which had been exposed to the same purification procedure had a specific activity [7·6 units (mg protein)$^{-1}$] not significantly different from that of an extract of *M. phlei* suspension which had simply been washed free of incubation medium. Polyacrylamide gel electrophoresis followed by staining for superoxide dismutase showed a single band for *M. leprae* cell-free extract, in a different position from the two armadillo liver superoxide dismutase bands (Table 1). Well-defined bands could be obtained with 200 μg liver protein and 600 μg *M. leprae* protein. It was possible to include liver and *M. leprae* cell-free extracts on the same gel and show the three superoxide dismutase bands exhibiting the expected mobilities.

Catalase

Catalase was detected in *M. leprae* cell-free extracts, but this catalase could not be distinguished from armadillo liver catalase (Table 2). Pre-incubation of extracts for 20 min at 25 °C with 3-amino-1,2,4-triazole (at 1·5 × concentrations in Table 2) followed by addition of H_2O_2 and incubation for 30 min failed to demonstrate any differences in inhibition between *M. leprae* and armadillo liver enzyme activity (*M. phlei* catalase appeared

Table 2. *Comparison of catalase from M. leprae extracts and other cell-free extracts*

The assay is described in Methods. One unit of catalase activity is defined as $(1/t) \times \ln (S_0/S_t)$, where t is the incubation time (s), and S_0 and S_t are the concentrations of H_2O_2 at zero time and at the end of the incubation, respectively (Winder, 1960). For inhibition studies, $1{\cdot}4 \times 10^{-4}$ units catalase was pre-incubated for 20 min with 3-amino-1,2,4-triazole before H_2O_2 was added. The reaction was stopped after 30 min. Cell-free extracts were applied to 5 % (w/v) polyacrylamide gels.

Cell-free extract	$10^3 \times$ Catalase specific activity [units (mg protein)$^{-1}$]	Inhibition (%) by 3-amino-1,2,4-triazole 1 mM	10 mM	100 mM	Relative mobility
M. leprae	1·35 ± 0·30*	46	81	100	0·27
Armadillo liver	400	47	79	98	0·27
M. phlei	10·6 ± 3·1*	51	69	90	0·77, 0·86
M. lepraemurium	8·4	ND	ND	ND	0·70, 0·75

ND, Not determined.
* ± Standard error, four separate extracts.

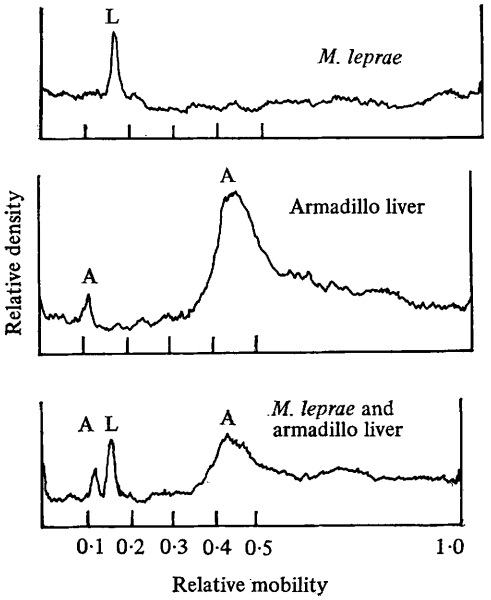

Fig. 1. Peroxidatic activity in *M. leprae* extracts and in cell-free extract of armadillo liver. Polyacrylamide gels loaded with *M. leprae* and armadillo liver extract, run both separately and combined, were stained for peroxidatic activity and scanned by transmitted light (slit width 0·06 mm) on a Joyce Loebl microdensitometer Mk III: A, bands from cell-free extract of armadillo liver; L, bands from *M. leprae* extract. No bands were observed if H_2O_2 was omitted from the staining procedure.

to be slightly less susceptible to higher concentrations of 3-amino-1,2,4-triazole). After polyacrylamide gel electrophoresis, only a single band of catalase activity was detected for both liver (3·2 μg protein) and *M. leprae* (100 μg protein) extracts, both with a relative mobility of 0·27. This could not be resolved into further bands by increasing the *M. leprae* cell-free extract protein applied to the gel up to 1 mg, or by combining *M. leprae* and liver cell-free extracts on the same gel. The relative mobility was different from that of bands from other mycobacteria: *M. phlei*, *M. lepraemurium* (Table 2) and *M. tuberculosis* (Diaz & Wayne, 1974) bands with catalase activity all had relative mobilities of 0·70 to 0·86.

Peroxidatic activity

Attempts to assay peroxidatic activity in *M. leprae* cell-free extracts to estimate specific activity were unsuccessful, but peroxidatic activity could be demonstrated on a polyacrylamide gel (Fig. 1). In a gel loaded with *M. leprae* cell-free extract containing 750 μg protein, a thin, brown band was observed after electrophoresis; after staining (H_2O_2 followed by *o*-dianisidine) as described in Methods, a very thin orange band was observed in the same place. The stained gel could be stored in distilled water for several weeks at room temperature without loss of intensity or diffusion of the stained band, since the oxidized *o*-dianisidine product is highly insoluble in water. The unstained coloured protein would have diffused during such storage. The band of peroxidatic activity did not stain in a control in which H_2O_2 was omitted. A band with similar properties (before and after staining) was observed for liver cell-free extract, but this was shown to be a different protein by running liver extract (400 μg protein) and *M. leprae* cell-free extract (700 μg protein) on the same gel. Two bands, before and after staining, were observed in the same positions where they had been observed when run separately (Fig. 1). An additional band of peroxidatic activity, more intense and diffuse, was detected from liver extracts.

Gels stained for peroxidatic activity could be washed for 15 min in distilled water immediately after the bands corresponding to peroxidatic activity had developed, then stained for superoxide dismutase. Bands with the characteristic mobilities (Table 1) of liver and *M. leprae* superoxide dismutases were observed (as well as the bands of peroxidatic activity) on such gels.

Effect of NaOH treatment on enzyme activities

It was possible to detect catalase in suspensions of whole *M. leprae*. By calculating the protein equivalent (protein obtained in the cell-free extract from the bacteria), it could be shown that the specific activity was $1{\cdot}1 \times 10^{-3}$ units (mg protein equivalent)$^{-1}$, a value in the range of specific activities of extracts (Table 2). This activity was inhibited completely by 100 mM-3-amino-1,2,4-triazole. Treatment with NaOH, as described in Methods, completely abolished any catalase activity that could be detected in whole bacteria. Very little protein (25 μg from 10 mg bacteria) and no catalase activity was released by the NaOH treatment.

The cell-free extract prepared from NaOH-treated *M. leprae* contained no catalase activity [the minimum specific activity that could be detected was 10^{-5} units (mg protein)$^{-1}$] and 0·050 units superoxide dismutase (mg protein)$^{-1}$ using the NBT assay. Peroxidatic activity and superoxide dismutase from the NaOH-treated bacteria could both be detected on polyacrylamide gels.

DISCUSSION

The results obtained for catalase activity emphasize the importance of establishing that any enzyme activity detected in cell-free extracts or in cell suspensions of tissue-grown bacteria is, in fact, a bacterial enzyme. It was not possible, either by investigation of the pattern of inhibition by 3-amino-1,2,4-triazole or by polyacrylamide gel electrophoresis (even with heavy loading of the bacterial cell-free extract), to show any difference between the host liver and the '*M. leprae*' enzymes. There was no evidence for the existence of an *M. leprae* catalase. On the contrary, the evidence suggested that the enzyme found in the *M. leprae* cell-free extract was, in fact, armadillo liver catalase; its electrophoretic mobility contrasted sharply with those of all other mycobacterial catalases on 5 % polyacrylamide gels (results presented here, and by Diaz & Wayne, 1974).

Authentic *M. leprae* superoxide dismutase and peroxidatic activity were, however, detected. Neither of the corresponding enzymes from the liver were present in *M. leprae*

cell-free extracts. NaOH treatment has been shown to abolish acid phosphatase activity attached superficially to the surface of *M. tuberculosis* (Kanai, 1967). Such treatment of a suspension of pure *M. leprae* (which does not affect its viability; C. Lowe, unpublished results) abolished catalase activity, while superoxide dismutase and peroxidatic activity were unaffected. These results are further evidence for *M. leprae* superoxide dismutase and peroxidatic activity being of bacterial origin and for catalase being host-originated. Kanai (1967) has suggested that tubercule bacilli become coated with host-material as they become established in host tissues and this may be seen as an adaptation to intracellular growth. *Mycobacterium leprae* may similarly become coated with host-material during its growth in the liver, and this would account for the presence of catalase. However, we cannot exclude that the host-material is adsorbed on to the surface of the leprosy bacteria during isolation.

Peroxidatic activity could only be detected on polyacrylamide gels, possibly because it was concentrated into a sharp band. In the absence of such concentration, the assay described by Putter (1974) was probably not sensitive enough to detect peroxidatic activity in extracts of *M. leprae*. The peroxidatic nature of this band was confirmed by omitting H_2O_2 from the stain, when no colour developed. A single band, which was brown prior to staining, was observed suggesting that it was a haemoprotein. It was not clear whether this represented a true peroxidase: for instance, non-specific peroxidatic activity of a respiratory pigment may have been observed.

Mycobacterium leprae superoxide dismutase activity was not inhibited by pre-incubation in 5 mM-H_2O_2 (Britton *et al.*, 1978) or by assaying with 2 mM-KCN (Crapo *et al.*, 1978). These results are consistent with it being a manganese-containing enzyme. Such an enzyme has been purified from *M. lepraemurium* (Ichihara *et al.*, 1977). We have further shown in this paper that *M. leprae* and *M. lepraemurium* both possess a superoxide dismutase more easily detected at pH 7·8 than pH 10. Generally, the reverse is true (Crapo *et al.*, 1978). This suggests that for these mycobacterial enzymes the ratio of rate constants for O_2^- at pH 10 compared with that at pH 7·8 are rather lower (Klug *et al.*, 1972) than for other superoxide dismutases. The cell-free extract from which Ichihara *et al.* (1977) prepared superoxide dismutase had a specific activity 13 times higher than the extract which we prepared from whole *M. lepraemurium* (grown *in vivo*) by a method similar to that used by Ichihara *et al.* (1977). The difference in specific activities may reflect a difference between the organism grown in culture medium and in mice. Using the NBT assay, the specific activity of *M. leprae* superoxide dismutase was less than 1 % of the specific activity in other mycobacterial cell-free extracts (and also cell-free extract from armadillo liver).

The specific activity of superoxide dismutase is proportionately lower than certain hydrolytic enzymes (acid phosphatase, some glycosidases), where the specific activity in *M. leprae* cell-free extracts is about 5 % of the specific activity in cell-free extracts from other mycobacteria (*M. phlei*, *M. lepraemurium*; unpublished results). In view of the importance of superoxide dismutase (Gregory & Fridovich, 1973) and catalase (Gifford, 1968) in the survival and growth of bacteria, the low activity of superoxide dismutase and absence of catalase might be one of the reasons for the slow growth of *M. leprae in vivo*.

It has been shown that peroxides form in bacteriological media (Barry *et al.*, 1956). If *M. leprae* cannot produce catalase and has low superoxide dismutase and peroxidatic activities, the effect of toxic anions in media may contribute to the failure of the organism to grow in such conditions.

The method of preparation of pure bacteria (at least when carried out using *M. phlei*) does not affect the superoxide dismutase activity so it seems unlikely that loss of activity during purification was a reason for low activity of superoxide dismutase. Generally, the specific activities of enzymes in *M. leprae* might be expected to be low since only a small proportion (about 10 %) of the bacteria are viable (McDougall *et al.*, 1979).

It has been suggested (Jackett *et al.*, 1978) that unsaturated lipids in the lipid-rich cell-wall

of *M. tuberculosis* might act as a defence against H_2O_2 and O_2^- by offering a substrate for harmless lipoperoxidation. Such cell-wall lipids might offer some protection in *M. leprae* where the enzymological defence against toxic anions appears to be incomplete.

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