Peroxidatic Activity of Mycobacteria and Relation to Catalase

FRANK G. WINDER
Department of Biochemistry, Trinity College, Dublin, Ireland

Received for publication 4 May 1966

ABSTRACT

WINDER, FRANK G. (Trinity College, Dublin, Ireland). Peroxidatic activity of mycobacteria and relation to catalase. J. Bacteriol. 92:413-417. 1966.—Catalase from Mycobacterium smegmatis was purified about 50-fold. All fractions showed a ratio of peroxidatic activity to catalatic activity approximately the same as that of the crude extract, a ratio only about four times that given by catalase from Micrococcus lysodeikticus. This and other evidence strongly suggest that the peroxidatic activity of M. smegmatis is due to its catalase. Less complete evidence suggests that this is true in the case of Mycobacterium tuberculosis also. It is suggested that in the context of the mycobacteria the term "peroxidatic activity" should replace the term "peroxidase" unless evidence is found that a true peroxidase exists in these organisms.

Mycobacteria possess catalase and, also, peroxidatic activity towards certain phenols such as pyrogallol. Both activities are usually lacking in isoniazid-resistant strains of Mycobacterium tuberculosis (1, 11). It is usually assumed that the catalatic and peroxidatic activities of these organisms are due to two distinct enzymes. Some species of mycobacteria and some strains of M. tuberculosis are negative to a qualitative test for peroxidatic activity but give a positive catalase test, but a very few strains are reported to give opposite results (4, 13, 14).

However, catalase from various sources has some peroxidatic activity toward phenols (9, 12), and this raises the possibility that the peroxidatic activity of mycobacteria is due to their catalase. Further, the evidence that strains of M. tuberculosis highly resistant to isoniazid usually arise by single-step mutation, together with the fact that such strains usually lack both catalatic and peroxidatic activities, suggests that both activities are governed by a single gene and hence to the same enzyme in this organism (17). Other evidence from this and other mycobacteria points in the same direction (7, 16, 19). The work reported in this paper provides further evidence, particularly in the case of M. smegmatis, that the peroxidatic activity is due to catalase.

MATERIALS AND METHODS

Organisms used. Micrococcus lysodeikticus (Staphylococcus aureus) was strain NCTC 2665 from the National Collection of Type Cultures. Mycobacterium smegmatis was a strain maintained in this laboratory (18). M. tuberculosis strain BCG was obtained from Glaxo Laboratories Ltd.

Growth of organisms. M. lysodeikticus was grown in submerged culture in CCY liquid medium (6) dispersed in 830-ml lots in 2-liter conical flasks which were shaken on a rotary shaker at 220 oscillations per min, amplitude, 1 inch, for 40 hr at 35°C. The cells were harvested by centrifugation. M. smegmatis was grown for 5 days at 37°C on the surface of modified Proskauer & Beck medium supplemented with ferric and zinc ions (18). M. tuberculosis was grown for 28 days at 37°C on the surface of modified Sauton method (20). Both mycobacteria were harvested by filtration, sucked dry, and frozen at −15°C.

Extraction and purification of catalase. Extraction and purification of catalase from M. lysodeikticus was carried out as described by Herbert (8) as far as the end of step 5 (first ammonium sulfate fractionation), except that twice the amount of lysozyme (L. Light & Co., Coinbrook, Bucks., England) was used and lysis was allowed to continue for 24 hr at 30°C.

Cells of mycobacteria were disintegrated by grinding with Ballotini beads (no. 10) in a large chilled mortar. This material was suspended in 0.5% (w/v) NaCl and centrifuged. The supernatant fluids were used for the preparation of catalase, as described by Herbert (8), starting at step 2 and proceeding as far as the end of step 5.

The partially purified catalase from M. smegmatis was chromatographed on diethylaminoethyl (DEAE) cellulose powder (Whatman DE50). The DEAE cellulose was suspended in 0.01 M tris(hydroxyethyl)-amino methane (Tris) buffer (pH 7.8), washed twice by decantation, and poured into columns 0.8 cm in diameter to give a bed depth of about 20 cm. Dialyzed
solutions of the catalase fractions (about 10 mg of protein) were transferred to the columns, and step elution with 0.05, 0.1, 0.2, 0.3, and 0.8 M NaCl in 0.01 M Tris buffer (pH 7.8) was used. The eluate was collected in 120 fractions of 2 ml each. The temperature was maintained at about 5°C.

Assays. Catalase and peroxidase were generally assayed as described by Winder and O'Hara (19), after suitable dilution. A qualitative test for the presence of catalase (Galasinski, Wołosowicz, and Tysarowski, 1962) was done on all fractions from the columns; those fractions showing activity were assayed quantitatively. When the effect of catechol on catalase activity was examined, activity was assayed by a manometric method (3). Protein was assayed on all fractions from the columns by reading the absorption at 280 μg. These columns fractions which had catalase activity were also assayed for protein by the Folin-Ciocalteau method (10), and this method was employed for protein estimation in all fractions obtained by other procedures. Crystalline bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was employed as a protein standard.

RESULTS

Catalase from _M. lysodeikticus_ was purified only in order that the behavior and peroxidatic activity of _M. smegmatis_ catalase might be compared with these properties of the well-known _M. lysodeikticus_ enzyme. The yield of catalase obtained from _M. lysodeikticus_ was poor. This was largely due to the fact that the cells, as grown by us, had an unexpectedly low catalase content and were very resistant to lysozyme action; even after prolonged lysozyme treatment only about 25% of the cells were lysed. However, about a 30-fold purification of the catalase was achieved, and during this purification the ratio of peroxidatic to catalatic activity remained constant (Table 1).

For the purification of catalase from _M. smegmatis_, the method of Galasinski, Wołosowicz, and Tysarowski (5), which was developed specifically for the purification of catalase from this organism, was first tried. However, in our hands, the original method of Herbert (8) was more satisfactory. In fact, we got a higher recovery of catalase from _M. smegmatis_ than from _M. lysodeikticus_ during the same purification steps (Table 2). The ratio of peroxidatic and catalatic activity was about four times higher in this organism than in _M. lysodeikticus_. The ratio of activities remained close to constant during the purification process (Table 2); there was no systematic change in this ratio during the purification, the small departures from constancy being random, and mostly due to pH effects.

To test further whether the peroxidatic and catalatic activities were associated with each other, several batches of partially purified catalase were chromatographed on DEAE cellulose. When this was done on the 30 and 40% saturation ammonium sulfate fractions, more than a dozen protein peaks were obtained in each case; yet all of the catalatic and peroxidatic activities recovered were associated with a single peak, in which they occurred with increased specific activity and in the same ratio as before (Table 3).

Attempts were made to purify the catalase from _M. tuberculosis_ BCG by the same procedure. Each time this was tried, the major part of the activity disappeared on treatment with ethyl alcohol-ethyl alcohol partition.

### Table 1. Catalatic and peroxidatic activities during purification of catalase from Micrococcus lysodeikticus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol</th>
<th>Total protein</th>
<th>Specific catalatic activity</th>
<th>Recovery of catalase</th>
<th>Specific peroxidatic activity</th>
<th>Ratio of peroxi-</th>
<th>Catalatic-catalatic activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme-treated cell suspension</td>
<td></td>
<td></td>
<td>5.13</td>
<td>100</td>
<td>0.295</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>After ethyl alcohol-chloroform treatment</td>
<td>275</td>
<td>4,490</td>
<td>5.13</td>
<td>100</td>
<td>0.295</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>After first ammonium sulfate-ethyl alcohol partition</td>
<td>420</td>
<td>546</td>
<td>3.37</td>
<td>8.24</td>
<td>0.202</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>After second ammonium sulfate-ethyl alcohol partition</td>
<td>120</td>
<td>136</td>
<td>5.50</td>
<td>3.44</td>
<td>0.347</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Fractionation with ammonium sulfate</td>
<td></td>
<td></td>
<td>13.3</td>
<td>1.73</td>
<td>0.805</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>Precipitated between 30 and 40% saturation...</td>
<td>10</td>
<td>1.07</td>
<td>167.4</td>
<td>0.83</td>
<td>10.98</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>Precipitated between 40 and 50% saturation...</td>
<td>10</td>
<td>2.83</td>
<td>20.9</td>
<td>0.28</td>
<td>1.38</td>
<td>0.066</td>
<td></td>
</tr>
</tbody>
</table>

^a_ Expressed as milliliters per milligram of protein per minute.

^b_ Expressed as micromoles of purpurogallin per milligram of protein per minute.
alcohol-chloroform, and all remaining activity was lost during ammonium sulfate-ethyl alcohol partition (Table 4). Examination of the fractions normally discarded showed that this loss of activity was not due to its having gone into a different fraction from usual at these steps, and, hence, destruction of activity was involved. The ratio of peroxidatic to catalatic activity was similar to—if anything slightly higher than—that observed in the case of M. smegmatis (Table 4). The simultaneous disappearance of both activities was in keeping with their both belonging to one enzyme in this organism also.

The properties of the purified M. smegmatis enzyme were not studied extensively, owing to the difficulty of getting sufficient material. Like catalase from M. lysodeikticus, it showed peroxidatic activity toward catechol in addition to pyrogallol, and no measurable activity toward guaiacol. The effect of pH on both catalases was similar, and was similar to its effect on their peroxidatic activities.

Further evidence that the peroxidatic activity of M. smegmatis extracts was due to catalase was given by the fact that substances which act as hydrogen donors for peroxidase reduced the catalatic activity of the preparation. For example, 15 mM catechol reduced by 50% the rate at which a crude preparation released oxygen from 12 mM hydrogen peroxide.

Peroxidatic activities of extracts of M. smegmatis and M. tuberculosis were also determined at pH 4 with catechol as hydrogen donor (14). Extracts of both organisms, whether crude or partially purified, showed much lower peroxidatic activity under these conditions than under the normal conditions of assay. Further, the ratio of peroxidatic activity determined by this method to the activity as normally determined was not affected by whether the extract was derived from M. tuberculosis or from M. smegmatis or by the degree of purification of the extract. This suggests that the peroxidatic activity observed with the use of catechol at pH 4 is due to the same enzyme that gives the reaction with pyrogallol at pH 7.

**DISCUSSION**

The results suggest strongly that the peroxidatic activity of M. smegmatis is due to its catalase. All catalases show peroxidatic activity (12), and it is only necessary to accept that the M. smegmatis enzyme has a three- to fourfold higher ratio of peroxidatic to catalatic activity than the M. lysodeikticus enzyme. This contrasts with typical peroxidases; for example, horse-radish peroxidase has at least a 1 million-fold higher ratio of peroxidatic to catalatic activity, as measured by the methods described in this paper, than M. lysodeikticus catalase. There is no evidence for the presence in M. smegmatis of an enzyme with such a high ratio of peroxidatic to catalatic activity, or with a different specificity toward hydrogen donors from that displayed by catalase.

Owing to our failure to purify the enzyme from M. tuberculosis, the evidence for this organism is

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol</th>
<th>Total protein</th>
<th>Specific catalatic activity</th>
<th>Recovery of catalase</th>
<th>Specific peroxidatic activity</th>
<th>Ratio of peroxidatic-catalatic activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude cell-free extract</td>
<td>300</td>
<td>3,260</td>
<td>3.06</td>
<td>100</td>
<td>0.534</td>
<td>0.17</td>
</tr>
<tr>
<td>After ethyl alcohol-chloroform treatment</td>
<td>430</td>
<td>411</td>
<td>10.3</td>
<td>42.5</td>
<td>2.02</td>
<td>0.20</td>
</tr>
<tr>
<td>After first ammonium sulfate-ethyl alcohol partition</td>
<td>130</td>
<td>130</td>
<td>23.05</td>
<td>30.0</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td>After second ammonium sulfate-ethyl alcohol partition</td>
<td>18</td>
<td>38.6</td>
<td>29.6</td>
<td>17.4</td>
<td>8.34</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Expressed as milliliters per milligram of protein per minute.

*Expressed as micromoles of purpurogallin per milligram of protein per minute.
The greater destruction of \textit{M. tuberculosis} catalase than of the \textit{M. smegmatis} enzyme during the purification procedure may reflect a greater instability of the former enzyme, or it may be due to the presence of a component in the former extract which combines irreversibly with the enzyme during purification.

The only definite argument in favor of a peroxidase in \textit{M. tuberculosis} independent of its catalase is that certain tests for peroxidatic activity (particularly those employing catechol at pH 4), when applied to unbroken cells, give positive results with most strains of \textit{M. tuberculosis}, while these tests give weaker or negative results with other mycobacteria, even though they contain catalase, and they give apparently negative results also with a few catalase-containing strains of \textit{M. tuberculosis} (4, 13, 14). However, the facts that these tests are only qualitative and that they are performed on unbroken cells greatly limit the conclusions that should be drawn from them (2, 17). Our findings that \textit{M. smegmatis} extracts have as much peroxidatic activity as \textit{M. tuberculosis} extracts, whether this is assayed with the use of pyrogallol or catechol, at pH 4 or 7, indicate that the different responses of whole cells of these two organisms to peroxidase tests result from a difference in some other property of the cells; in both organisms, the catalase is largely but not entirely intracellular (16), and a difference in penetration of substrates or in escape or further metabolism of products could obviously affect the response to the test.

\begin{table}
\centering
\caption{Chromatography of partially purified \textit{Mycobacterium smegmatis} catalase on DEAE cellulose: activities of peak tubes}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Fraction$^a$ & Tube no. & Total protein & Specific catalatic activity$^b$ & Recovery of catalase & Specific peroxidatic activity$^c$ & Ratio of peroxidatic-catalatic activities \\
\hline
A & 42 & 0.75 & 169 & 17 & 37.6 & 0.22 \\
 & 43 & 0.8 & 177 & 20 & 38.2 & 0.22 \\
 & 44 & 0.8 & 149 & 11 & 37.1 & 0.25 \\
 & 45 & 0.6 & 134 & 5.4 & 34.6 & 0.26 \\
B & 53 & 2.7 & 87.5 & 8.3 & 16.0 & 0.18 \\
 & 54 & 3.6 & 124 & 24 & 24.8 & 0.20 \\
 & 55 & 3.2 & 88.1 & 11 & 19.8 & 0.22 \\
 & 56 & 4.0 & 32.2 & 4.9 & 5.7 & 0.18 \\
\hline
\end{tabular}
\begin{flushleft}
$^a$ Fraction A was precipitated by 30\% ammonium sulfate saturation; fraction B, by 40\% ammonium sulfate saturation.

$^b$ Expressed as milliliters per milligram of protein per minute.

$^c$ Expressed as micromoles of purpurogallin per milligram of protein per minute.
\end{flushleft}
\end{table}

\begin{table}
\centering
\caption{Catalatic and peroxidatic activities during attempted purification of catalase from \textit{Mycobacterium tuberculosis} BCG}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Fraction & Vol & Total protein & Specific catalatic activity$^a$ & Recovery of catalase & Specific peroxidatic activity$^c$ & Ratio of peroxidatic-catalatic activities \\
\hline
Crude cell-free extract & \textit{ml} & mg & \% & & & \\
After ethyl alcohol-chloroform treatment & 155 & 557 & 2.56 & 100 & 1.00 & 0.39 \\
After first ammonium sulfate-ethyl alcohol partition & 270 & 41.4 & 4.83 & 14 & 1.35 & 0.28 \\
 & 103 & 4.38 & 0 & 0 & 0 & -- \\
\hline
\end{tabular}
\begin{flushleft}
$^a$ Expressed as milliliters per milligram of protein per minute.

$^c$ Expressed as micromoles of purpurogallin per milligram of protein per minute.
\end{flushleft}
\end{table}
Hence, as a result of these qualitative tests performed on whole cells, it is undesirable that terms such as "peroxidase-positive" or "peroxidase-negative" should be applied to bacteria. They should be referred to as "positive (or negative) to the catechol-hydrogen peroxide test at pH 4" (or to whatever test is used). Even when peroxidatic activity is determined by use of cell-free extracts of the organism, the activity should be referred to as "peroxidatic activity towards pyrogallol" (or whatever substrate is used). It should not be referred to as "peroxidase," unless the activity is shown to have properties which distinguish it from catalase and from nonspecific hemoprotein catalases, such as a very high ratio of peroxidatic to catalatic activity and a different specificity towards hydrogen donors.

An unfortunate terminology was suggested by G. Vanunaryanan, Vischer, and Bruhin (15), who used the term "peroxidase" to refer to peroxidatic activity determined with whole cells and "pseudoperoxidase" to refer to peroxidatic activity determined on cell-free filtrates.

ACKNOWLEDGMENTS

I thank Vanne Cowdy, Hilary Watchorn, and May Eaton for carrying out most of the work described in this paper. This investigation was supported by a grant from the Medical Research Council of Ireland.

LITERATURE CITED