Extracellular and Cytosolic Iron Superoxide Dismutase from Mycobacterium bovis BCG

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Two forms of iron superoxide dismutase (SOD) were purified from cell extract (CE) and culture filtrate (CF) of Mycobacterium bovis BCG, respectively. The molecular weight of both enzymes was estimated to be approximately 84,000 by gel filtration, whereas that of their subunits was 21,500, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that each of purified enzymes is composed of four identical subunits. The specific activities of CE SOD and CF SOD were 3,850 and 4,040, respectively. The purified enzymes were not joined by disulfide bonds and were, to some extent, resistant to sodium dodecyl sulfate. Their activities were lost by H2O2 but not by azide and cyanide, indicating iron SODs. Enzyme activities were detectable over a broad range of pHs, from 5.0 to 9.0, and were stable for 6 months at −20°C. Each value of pH was 4.5. In Western blots, both enzymes reacted with sera of tuberculosis patients, but not with normal sera. The N-terminal amino acid sequences of CE SOD and CF SOD were the same, suggesting that there is no N-terminal signal sequence.

Superoxide dismutases (SODs), which catalytically scavenge the superoxide radical (O2−) to hydrogen peroxide and molecular oxygen, serve a protective role against oxygen toxicity in all aerobic organisms (13, 37). Because the superoxide radical is a normal product of the univalent reduction of molecular oxygen, SODs are thought to be the primary defense against its potential cytotoxicity (13). There are three common forms of SODs, which differ in the metal ion cofactor at the active site. Manganese-containing SODs (MnSODs) are found in bacteria and mitochondria, while iron-containing SODs (FeSODs) are mainly found in the cytosol of prokaryotes, in primitive eukaryotes, and in some green plants (4, 5). In contrast, copper-zinc SODs (Cu-ZnSODs) are mostly found in the cytosol of eukaryotes, surprisingly in an increasing although limited number of bacteria (10, 18).

Until now, numerous papers have reported the distribution, characterization, and biological significance of SODs in mycobacteria (17, 19, 23). However, only a few bacteria have been shown to secrete SOD during growth. It was shown previously that Mycobacterium tuberculosis, a human pathogen, secreted iron SOD, while the nonpathogenic species, Mycobacterium smegmatis and Mycobacterium phlei, did not (19). Similarly, it was shown previously that the virulent strain Nocardia asteroides GUH-2 secretes the enzyme into the growth medium (7).

SODs could also be involved in the pathogenicity of Mycobacterium leprae (33), although the secretion of the enzyme has not been demonstrated and remains controversial.

Members of the mycobacteria have emerged as major opportunistic pathogens in humans, with the advent of the AIDS epidemic (16). Recently, such a case was also reported in a human immunodeficiency virus-infected individual who displayed reactivation of Mycobacterium bovis BCG 30 years after vaccination (3), although M. bovis BCG has been used in many countries for vaccination to prevent tuberculosis. BCG vaccination may cause disseminated mycobacterial infection, an illness caused by the vaccine itself in patients suffering from severe immune deficiency.

In this study, we explored the production and secretion of SOD by M. bovis BCG by investigating the purification and some properties of M. bovis BCG SOD along with evidence of the association of this enzyme with the cytosol and the growth medium of the organism.

MATERIALS AND METHODS

Bacterial strain and culture condition. The M. bovis BCG strain used in this study was Pasteur strain 1173P2 and was obtained from the Korea Institute of Tuberculosis. The bacterium was cultured at 37°C in Sauton’s medium (30) without Triton.

Preparation and fractionation of CEs and CFs. Cells were collected on Whatman filter paper, washed three times with phosphate-buffered saline, and suspended with sterilized distilled water. After homogenization, cells were disrupted with a French press (Aminco, Rochester, N.Y.) at a pressure of 18,000 lb/in2 and centrifuged at 15,000 rpm for 30 min at 4°C. Culture filtrates (CFs) were filtered through a 0.2-μm-pore-diameter membrane (Gelman, Ann Arbor, Mich.) and concentrated by an Amicon concentrator with a YM-10 membrane (Amicon, Beverly, Mass.). Each of the cell extracts (CEs) and CFs was precipitated by 70 to 90% ammonium sulfate, suspended with D.W., lyophilized, and stored at −20°C.

Enzyme assay and protein determination. The activity of the enzyme was assayed at 37°C for 30 min. The assay mixtures (0.5 ml) contained 50 μl of 0.5 M potassium phosphate (pH 7.5), 25 μl of 16% Triton X-100, 2.5 μl of 10 mM EDTA, 75 μl of 1.2 mM NTC, 2.5 μl of xanthine oxidase (1.0 U), the sample, 25 μl of 4 mM hypoxanthine, and distilled water. The A540 was monitored (Shimadzu UV-200) after addition of 0.5 ml of a solution containing 1 M formate buffer (pH 3.5), 10% Triton X-100, and 40% formaldehyde. An enzymatic unit was defined as the amount of the enzyme required to cause a 50% inhibition in the rate of reduction of NTC under the assay conditions (27). The standard protein used in unit determination was SOD from bovine kidney (Sigma, St. Louis, Mo.).

The protein concentration was determined by the Lowry method (21) with bovine serum albumin as a standard.

Purification of CE SOD and CF SOD. Crude CE and CF extracts were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 0.5 M ammonium sulfate, briefly centrifuged, and chromatographed on phenyl Sepharose 4B (1.6 by 15 cm (Sigma)) at room temperature in the same buffer. The gradient procedure was performed with D.W. at a flow rate of 30 ml/h. SOD fractions were dialyzed against D.W. (pH 7.0) and lyophilized (Labconco, Kansas City, Mo.). The lyophilized material was dissolved with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl prior to chromatography at a flow rate of 20 ml/h on a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) gel filtration (1.6 by 85 cm) system calibrated with standard proteins (Pharmacia) at 4°C.
TABLE 1. Purification of SOD from CEs of M. bovis BCG

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Fold purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>75.31</td>
<td>9,635.6</td>
<td>127.9</td>
<td>1.00</td>
<td>100.0</td>
</tr>
<tr>
<td>HIC</td>
<td>2.17</td>
<td>6,766.9</td>
<td>3,118.4</td>
<td>24.38</td>
<td>70.0</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>1.34</td>
<td>5,159.0</td>
<td>3,850.0</td>
<td>30.10</td>
<td>55.5</td>
</tr>
</tbody>
</table>

* A unit is defined as the amount of SOD needed to cause a 50% inhibition in the rate of oxidation of neotetrazolium chloride at 37°C.
* Seventy to 90% ammonium sulfate precipitation.
* Sepharose CL-6B hydrophobic interaction chromatography.
* Sephacryl S-200 superfine molecular sieve chromatography.

RESULTS

Purification and properties of CE and CF SODs. The SODs were effectively purified by 70 to 90% ammonium sulfate precipitation and underwent hydrophobic interaction chromatography with phenyl Sepharose CL-6B and Sephacryl S-200 gel filtration. The results showed the SDS-PAGE pattern of purified CE SOD (20 μg) and CF SOD (20 μg) (Fig. 1a). A single band with a molecular weight of about 21,500 was stained by Coomassie brilliant blue. The specific activity of CE-SOD was 3,850 U/mg of protein, representing a purification of 30.1-fold with 53.5% recovery. CF SOD was purified approximately 28.57-fold with a specific activity of 4,040 U/mg of protein and 29.1% recovery (Tables 1 and 2). The activities of purified enzymes were stable at –20°C for 6 months (data not shown). The molecular weights of the native enzymes, as determined by gel filtration on Sephacryl S-200 (Fig. 2b) and native electrophoresis (Fig. 1b), were about 84,000. These results indicated that each of the purified SODs is a tetramer composed of identical polypeptides with a molecular weight of about 21,500.

The stability of both enzymes was detectable over a broad range of pHs, 5.0 to 9.0, but CF SOD was a little more stable than CE SOD at pH 4.0 (Fig. 3). For unknown reasons, the specific activity and stability of CF SOD are higher than those of CE SOD.

The pI value of both enzymes was 4.5, and those areas of activity were visualized in the same bands by zymography (Fig. 4).
Subunit molecular weight. The enzymes preincubated with sample buffer for 2 h at 37°C in the presence and absence of 2-mercaptoethanol in sample buffer were subjected to SDS-PAGE (4, 37). Comparison of their mobility to that of molecular weight of the standards yielded a subunit with a molecular mass of 21,500 Da in both the absence and presence (Fig. 5) of mercaptoethanol, indicating that the enzymes are not affected by mercaptoethanol. Even though a 66,000-Da protein band was also detected on the electrophoresis gel, it was suggested that the native enzyme structures are resistant to SDS because...
of their partial disruption by SDS treatment. From this result, it is clear that the enzymes are composed of four subunits of equal size and that these subunits are not joined by interchain disulfide bonds.

**Effect of inhibition.** In Fig. 6, both of the purified CE and CF SODs were inhibited completely by 1 mM H$_2$O$_2$ (CE and CF in lane 4), but not by 10 mM azide (CE and CF in lane 3) and 3 mM cyanide (CE and CF in lane 2), as shown by zymography. This indicated that the purified enzymes are iron-SOD (4, 35) and that CF SOD may be a secreted form of CE SOD. To confirm this hypothesis, when a 3-day culture filtrate was precipitated by 85% ammonium sulfate and dialyzed with D.W., a protein band with the same molecular weight of the purified CF SOD appeared by activity-staining electrophoresis (data not shown).

**N-terminal amino acid sequence comparison.** When purified enzymes were subjected to Edman degradation and determination of N-terminal amino acid sequences, the N-terminal amino acid sequence of CE SOD was AEYTLPDLIXDYGAL and that of CF SOD was AEYTLPDLIDYGAL. Ten of the 14 amino acids are identical, implying that there are no N-terminal signal peptides responsible for secretion. Although an unidentified sequence existed, it was predicted that the X of CE SOD is glutamic acid (E), as judged from Edman degradation data.

**Western blot analysis.** To examine whether the purified enzymes are working as antigenic molecules in tuberculosis patients, Western analysis was carried out with sera from the patients. Both CE SOD (Fig. 7, CE, lanes 1 to 8) and CF SOD (Fig. 7, CF, lanes 1 to 4) were reacted with sera of tuberculosis patients, but were not reacted with normal sera (Fig. 7, CE, lanes 9 to 12, and CF, lanes 5 to 8). Even though only one result is presented, the Western blotting data strongly indicated that both of the purified SODs have the same antigenic determinant, as shown by antigenicity against the sera of tuberculosis patients.

**DISCUSSION**

Iron-SOD has been purified from CE and CF of *M. bovis* BCG grown in Sauton's medium. Purified enzymes showed similarities with respect to molecular weight, pH profile, subunit structure, substrate specificity, sensitivity to inhibitors, and antigenicity to tuberculosis patients. The molecular weight and tetrameric form of the enzymes (Fig. 1) were similar to those of SODs of *M. tuberculosis* (19), *Mycobacterium avium* (12), and *N. asteroides* (7). However, there is a slight difference from those of *M. leprae*, *M. smegmatis*, *Mycobacterium leprae*um, *Mycobacterium intracellulare*, and *Mycobacterium duvalii*, even with BCG (22). Since adoption of a tetrameric form is thought to confer the stability of the enzyme in cellular and extracel-
lular fluids (15), the purified SODs showed a broad pH stability (Fig. 3). These results were similar to those with many other SODs (24, 28, 31).

CE SOD and CF SOD shared strong N-terminal amino acid sequence homologies with SODs from mycobacteria, such as M. tuberculosis (93%), Mycobacterium fortuitum (93%), M. avium (87%), M. leprae (87%), and N. asteroides (87%), but low homologies with those from other bacteria, such as Escherichia coli (60%), Pseudomonas aeruginosa (40%), and Bacteroides fragilis (33%) (Table 3). The correspondence in N-terminal amino acid sequence between CE SOD and CF SOD indicated that the SOD of M. bovis BCG is not preceded by signal peptides. Thus, it was suggested that the structural gene of the enzyme from M. tuberculosis is not preceded by a signal peptide sequence (38). Although M. bovis BCG iron SOD appeared to be cytosolic as well as a secreted protein, it was not the product of autolysis, because it was found in CF within 3 days of growth under identical culture conditions. SODs from CE and CF showed identical molecular weights, pIs, metal cofactors, N-terminal amino acid sequences, and Western blots, suggesting that CF SOD should be due to direct secretion from CE SOD. How SOD is exported in mycobacteria remains unknown, since there are no possible signal peptides (12, 38). Probably, some specific systems for protein exportation exist in mycobacteria. Further investigation is in progress.

It has been reported that superoxide enhances formation of hydroxyl radical (OH·), a highly reactive molecule that will react with various biomolecules, including lipids, proteins, and DNA, both by reducing Fe3+ to Fe2+ and by serving as a source of H2O2 (26). Most bacteria contain SODs and catalase as means of eliminating superoxide and H2O2, respectively. Pathogenic microorganisms are exposed to exogenous superoxide and H2O2 generated by host neutrophils and other phagocytes (26). The secreted SOD has been documented to occur in a few virulent mycobacteria (2, 7, 12), showing that it could be involved in pathogenicity (6, 12, 19). The secretion of SOD in virulent forms of N. asteroides, as well as its association with the outer cell envelope, could provide protection against killing by superoxide radicals (6), which are produced during active phagocytosis. More direct evidence for such a protective role had been obtained previously via administration of a monoclonal antibody specific for the SOD of N. asteroides (8). The fact that exogenously added SOD has protected bacteria against phagocytic attack (36) also illustrates the importance of SOD. Therefore, the SOD secreted by mycobacteria is more important, because it could function in the bacteria as a first line of defense against oxygen-mediated killing.

It has been also reported that the SOD in the purified protein derivative of M. tuberculosis used for the skin test is a general antigen (29). In this study (Fig. 7), both M. bovis BCG CE SOD and CF SOD obviously showed antigenicity against sera from patients with tuberculosis and had the same antigenic determinant. This result showed that this SOD could be used as a marker protein for diagnosis of tuberculosis. However, the specificity and sensitivity need to be determined with a larger number of sera from patients with different stages of tuberculosis and other mycobacterial infections and from BCG-vaccinated or nonvaccinated individuals.

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REFERENCES