Identification and subcellular localization of a novel Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis*

C.H. Herbert Wu[a], Jyy-Jih Tsai-Wu[b], Yung-Tzung Huang[a], Ching-Yi Lin[a], Gunn-Guang Lioua[a,c], Feng-Jen S. Lee[a,*]

[a] *Institute of Molecular Medicine, School of Medicine, National Taiwan University, 7 Chung Shan South Road, Taipei 100, Taiwan*
[b] *Department of Clinical Medicine, National Taiwan University Hospital, Taipei 100, Taiwan*
[c] *Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan*

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Abstract Periplasmic copper, zinc superoxide dismutases (Cu,ZnSOD) of several Gram-negative pathogens have been shown to play an important role in protection against exogenous superoxide radicals and in determining virulence of the pathogens. Here we report the cloning and characterization of the *sodC* gene, encoding Cu,ZnSOD, from the Gram-positive *Mycobacterium tuberculosis*. The predicted protein sequence contains 240 amino acids with a putative signal peptide at the N-terminus and shows ~25% identity to other bacterial *sodC*. Recombinant proteins of a full-length *sodC* and a truncated form lacking the putative signal peptide were overexpressed in *Escherichia coli* and affinity purified. Renatured recombinant *M. tuberculosis sodC* protein possessed characteristics of a Cu,ZnSOD. Immunoblotting with an antiserum against the recombinant *M. tuberculosis* Cu,ZnSOD allowed detection of a single polypeptide in the lysate of *M. tuberculosis*. This polypeptide has a similar size as the recombinant protein without the putative signal peptide indicating that the endogenous Cu,ZnSOD in *M. tuberculosis* might be processed and secreted. Furthermore, immunogold electron microscopic image showed that Cu,ZnSOD is located in the periphery of *M. tuberculosis*. The enzymatic activity and subcellular localization of this novel Cu,ZnSOD suggest that it may play a role in determining virulence of *M. tuberculosis*.

Key words: Cu,Zn superoxide dismutase; Periplasmic; Gram-positive bacterium

1. Introduction

Superoxide dismutase catalyzes the conversion of the superoxide radicals (O_2^-) into oxygen molecule (O_2) and hydrogen peroxide (H_2O_2) in the first step of a series of protective reactions to remove free radicals generated during the reduction of molecular oxygen [1–3]. Three types of superoxide dismutases (SOD) exist: those cofactored by manganese (MnSOD), iron (FeSOD), and copper and zinc (Cu,ZnSOD). MnSODs and FeSODs share sequence and structural similarities whereas Cu,ZnSODs fall into a distinct phylogenic family [1–3].

MnSODs are found in mitochondria and prokaryotes, whereas FeSODs are found in prokaryotes, primitive eukaryotes and some plants. Cu,ZnSODs were originally found in eukaryotes and later also in several Gram-negative bacteria including *Escherichia coli* [4], *Haemophilus* species [5,6], *Caulobacter crescentus* [7], *Paracoccus denitrificans* [8], *Pseudomonas* species [9], *Brucella abortus* [10], and *Photobacterium leiognathi* [11,12].

In contrast to the cytoplasmic localization of MnSOD and FeSOD, Cu,ZnSOD is found in the periplasm of bacteria [5,10,13–18]. Since superoxide is not able to cross the cytoplasmic membrane [19], the periplasmically localized Cu,ZnSOD can provide protection to the periplasmic or membrane constituents from superoxide generated outside of the cells. Since generation of reactive oxygen species in the macrophage is an important component of the microcidal response of the host, protection conferred by the peripherally localized Cu,ZnSOD is particularly important for the survival of intracellular pathogens like *Salmonella typhimurium* or *Mycobacterium tuberculosis* whose primary host is the macrophage. In fact, *Salmonella* defective in Cu,ZnSOD showed enhanced sensitivity to the microcidal activity of superoxide, reduced lethality in a mouse model of oral infection, and lower persistence in the liver and spleen after intraperitoneal injection [20]. It is conceivable that Cu,ZnSOD of the Gram-positive bacterium *M. tuberculosis* may play a similar role in determining the bacterial virulence. As the first step in analyzing the importance of Cu,ZnSOD in the pathogenesis of *M. tuberculosis*, we have cloned the *sodC* gene, characterized the recombinant proteins, and determined its subcellular localization.

2. Materials and methods

2.1. Bacteria and plasmid construction

E. coli strains XL1 blue (Stratagene) and BL21(DE3) (Novagen) were used for cloning and overexpression of the recombinant proteins, respectively. *M. tuberculosis* H37Rv was used for the electron microscopic analysis.

Most of the cloning procedures were carried out according to standard protocols [21]. Fragments of the *sodC* gene were PCR amplified from a genomic DNA of *M. tuberculosis* using oligonucleotide pairs 5′-CATATGTCTACAGTTCCGGGTACCA-3′ and 5′-GGATCCAAGCTAGCCGGAACCAATGA-3′ for the full-length clone and 5′-CATATGCCAAAGCCCGCCGATCA-3′ and 5′-GGATCCAAGCTAGCCGGAACCAATGA-3′ for the truncated form. The PCR products were cloned into the T-vector pT7-Blue (Novagen) and subsequently subcloned into the *Nde*I and *Bam*HI sites of the expression vector pET15b (Novagen). Both strands of the cloned fragments were sequenced with the ABI BigDye (Perkin Elmer Applied Biosystems) fluorescence sequencing chemistry according to the protocols provided by the vendor on an ABI Prism 310 Genetic Analyzer automatic sequencer (Perkin Elmer Applied Biosystems).

2.2. Overexpression, affinity purification, and renaturation of the sodC protein

Overexpression and affinity purification of the recombinant proteins were according to the standard protocol [21]. Expression of the recombinant proteins was induced by incubating the BL21(DE3) bacte-

*Corresponding author. Fax: (886) (2) 2395-7801.
E-mail: fengjen@ha.mc.ntu.edu.tw

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rium carrying the recombinant *sodC* plasmid in LB broth containing 0.5 mM IPTG at 37°C for 100 min. The bacteria were harvested by centrifugation and lysed with sonication in 10 mM phosphate buffer pH 7.4, 10 mM imidazole. The recombinant proteins in the inclusion bodies were denatured and solubilized in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 8 M urea, 50 mM imidazole and purified with an affinity nickel column Hi-Trap-Chelating (Pharmacia) according to the procedure provided by the vendor. The purified recombinant proteins were renatured with dialysis against 50 mM Tris-HCl pH 7.8, 1 mM $CuSO_4$, 1 mM $ZnSO_4$ at 4°C for several changes and finally stored in the same buffer supplemented with 20% glycerol. The recombinant proteins which were soluble in the cell-free extracts were purified with the same column under native condition (i.e. without dialysis against $CuSO_4$).

2.3. In situ SOD activity assay

Bacteria were resuspended in 50 mM phosphate buffer pH 7.8, 0.1 mM EDTA, sonicated, and centrifuged to obtain bacterial extracts. Activities of the SOD isozymes were assayed by separating samples on a 10% non-denaturing polyacrylamide gel [22] and visualized by the nitroblue tetrazolium activity stain method [23]. Potassium cyanide (1 mM) was used to selectively inhibit Cu,ZnSOD activity.

2.4. Antisera production and Western analysis

Procedures for antiserum production and Western blot analysis were based on the standard protocols [21,24].

M-sodC used for antiserum production was prepared by fractionating the affinity purified M-sodC in SDS-PAGE, stained with Coomassie brilliant blue R-250, and sliced out of the gel. Purified M-sodC protein in the acrylamide gel were mixed with complete Freund adjuvant to immunized 3-month-old New Zealand White rabbits followed by three boosts with proteins mixed with incomplete Freund adjuvant. Antisera were collected at a 10-day interval starting from the last boost.

Protein samples to be analyzed were fractionated on a SDS-PAGE gel, electrotransferred to an Immobilon-P membrane (Millipore), and subjected to detection with rabbit antiserum followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham). Target bands were detected with the enhanced chemiluminescence kit (ECL, Amersham) and recorded on a Hyperfilm-MP film (Amersham).

2.5. Immunogold electron microscopic image

Preparation of 15 nm colloidal gold-IgG complex and immunogold labeling were performed as described with little modification [25,26]. Briefly, several *M. tuberculosis* colonies were scraped from the agar slant and fixed in 1% formaldehyde, 0.1 M phosphate-citrate buffer pH 7.2, at 4°C overnight. The fixed samples were neutralized with 0.1 M ammonium chloride, 0.1 M phosphate-citrate buffer pH 7.2 at 0°C for 30 min followed by dehydration through a series of methanol (50% at 0°C for 15 min, 75%, 90%, and two consecutive 100% at −20°C for 1 h each). The dehydrated samples were infiltrated with a graded series of the embedding agent LR Gold (25% in 10% PVP-6000 at −20°C for 1 h, 50% in 10% PVP-6000 at −20°C for 2 h, 75% at −20°C for 4 h, 100% at −20°C overnight). Polymerization of the embedding plastics was initiated with longwave UV irradiation at −20°C for 24 h and hardened at room temperature for 24 h. Ultrathin sections (100 nm) of the LR Gold embedded samples were mounted on 200 mesh nickel grids covered with carbon-backed collodion film. Sections on the grids were blocked with 3% normal goat serum in PBS for 10 min, incubated with the rabbit anti-Cu,ZnSOD antibody for 15 min, washed with 1% normal goat serum in PBS, and finally incubated with the colloidal gold-IgG complex for 10 min. The grids were washed extensively with triple glass-distilled water before contrasted with uranyl acetate and lead citrate. The sections were examined with a Zeiss EM109 electron microscope (Zeiss, Germany).

3. Results and discussion

3.1. Cloning of the M. tuberculosis sodC gene

The periplasmically localized *sodC*, encoding Cu,ZnSOD, has been shown to be an important factor affecting virulence of *S. typhimurium* and *Neisseria meningitidis* [20,27]. As the first step to determine if *sodC* could also be a virulence factor

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1/1                                                 31/11
ATG CCA AAG CCC GCC GAT CAC CGC AAT CAC GCA GCT GTC AGC ACG TCG GTC CTG TCC GCG
TAC GGT TTC GGG CGG CTA GTG GCG TTA GTG CGT CGA CAG TCG TGC AGC CAG GAC AGG CGC
M   P   K   P   A   D   H   R   N   H   A   A   V   S   T   S   V   L   S   A
61/21                                               91/31
TTG TTT CTG GGC GCC GGT GCC GCG CTG CTG AGC GCA TGC TCG TCG CCG CAG CAC GCG TCT
AAC AAA GAC CCG CGG CCA CGG CGC GAC GAC TCG CGT ACG AGC AGC GGC GTC GTG CGC AGA
L   F   L   G   A   G   A   A   L   L   S   A   C   S   S   P   Q   H   A   S
121/41                                              151/51
ACA GTT CCG GGT ACC ACG CCG TCG ATT TGG ACC GGA TCG CCC GCG CCG TCG GGA CTT TCG
TGT CAA GGC CCA TGG TGC GGC AGC TAA ACC TGG CCT AGC GGG CGC GGC AGC CCT GAA AGC
T   V   P   G   T   T   P   S   I   W   T   G   S   P   A   P   S   G   L   S
181/61                                              211/71
GGT CAC GAC GAG GAG TCG CCC GGT GCG CAG AGC CTG ACC AGT ACC CTG ACG GCG CCC GAC
CCA GTG CTG CTC CTC AGC GGG CCA CGC GTC TCG GAC TGG TCA TGG GAC TGC CGC GGG CTG
G   H   D   E   E   S   P   G   A   Q   S   L   T   S   T   L   T   A   P   D
241/81                                              271/91
GGC ACG AAG GTA GCG ACC GCG AAG TTC GAG TTC GCC AAC GGC TAT GCC ACC GTC ACG ATC
CCG TGC TTC CAT CGC TGG CGC TTC AAG CTC AAG CGG TTG CCG ATA CGG TGG CAG TGC TAG
G   T   K   V   A   T   A   K   F   E   F   A   N   G   Y   A   T   V   T   I
301/101                                             331/111
GCG ACG ACC GGC GTC GGT AAG CTC ACG CCC GGC TTC CAC GGC CTA CAC ATC CAC CAG GTG
CGC TGC TGG CCG CAG CCA TTC GAG TGC GGG CCG AAG GTG CCG GAT GTG TAG GTG GTC CAC
A   T   T   G   V   G   K   L   T   P   G   F   H   G   L   H   I   H   Q   V
361/121                                             391/131
GGT AAG TGT GAG CCC AAC TCG GTT GCC CCC ACC GGC GGT GCG CCC GGC AAC TTT CTG TCC
CCA TTC ACA CTC GGG TTG AGC CAA CGG GGG TGG CCG CCA CGC GGG CCG TTG AAA GAC AGG
G   K   C   E   P   N   S   V   A   P   T   G   G   A   P   G   N   F   L   S
421/141                                             451/151
GCC GGC GGC CAC TAC CAC GTG CCA GGG CAT ACC GGC ACC CCC GCC AGC GGC GAC CTG GCC
CGG CCG CCG GTG ATG GTG CAC GGT CCC GTA TGG CCG TGG GGG CGG TCG CCG CTG GAC CGG
A   G   G   H   Y   H   V   P   G   H   T   G   T   P   A   S   G   D   L   A
481/161                                             511/171
TCG CTG CAG GTA CGC GGT GAC GGT TCG GCG ATG CTG GTG ACC ACC ACC GAC GCC TTC ACC
AGC GAC GTC CAT GCG CCA CTG CCA AGC CGC TAC GAC CAC TGG TGG TGG CTG CGG AAG TGG
S   L   Q   V   R   G   D   G   S   A   M   L   V   T   T   T   D   A   F   T
541/181                                             571/191
ATG GAC GAC CTG CTG AGC GGC GCG AAA ACC GCG ATC ATC ATT CAC GCC GGC GCC GAC AAC
TAC CTG CTG GAC GAC TCG CCG CGC TTT TGG CGC TAG TAG TAA GTG CGG CCG CGG CTG TTG
M   D   D   L   L   S   G   A   K   T   A   I   I   I   H   A   G   A   D   N
601/201                                             631/211
TTT GCC AAC ATT CCG CCA GAA CGC TAC GTC CAG GTC AAT GGG ACT CCG GGT CCC GAC GAG
AAA CGG TTG TAA GGC GGT CTT GCG ATG CAG GTC CAG TTA CCC TGA GGC CCA GGG CTG CTC
F   A   N   I   P   P   E   R   Y   V   Q   V   N   G   T   P   G   P   D   E
661/221                                             691/231
ACG ACG TTG ACC ACC GGC GAC GCC GGC AAG CGG GTG GCG TGC GGT GTC ATT GGT TCC GGC
TGC TGC AAC TGG TGG CCG CTG CGG CCG TTC GCC CAC CGC ACG CCA CAG TAA CCA AGG CCG
T   T   L   T   T   G   D   A   G   K   R   V   A   C   G   V   I   G   S   G
721/241                                             751/251
TAG ccg aat tcc agc aca ctg gcg gcc gtt act agt gga tcc ggc tgc taa
ATC ggc tta agg tcg tgt gac cgc cgg caa tga tca cct agg ccg acg att
*   P   N   S   S   T   L   A   A   V   T   S   G   S   G   C   *
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Fig. 1. Nucleotide and amino acid sequences of the *sodC* gene of *M. tuberculosis*. L-sodC starts from 1A and ends at 723G encoding a polypeptide of 240 residues. S-sodC starts from 118T and ends at 723G encoding a polypeptide of 201 residues. M-sodC starts from 118T and has a T to A point mutation at nucleotide 721. This mutation causes loss of the original stop codon and produces an extra 16 residues at the C-terminus. The putative signal peptide is underlined.

in *M. tuberculosis*, we sought to clone this gene. A BLAST homology search using Cu,ZnSODs from several different organisms as queries identified a potential *sodC* homologue in the *M. tuberculosis* DNA database (accession number: Z84724 28 803–29 700). The full-length open reading frame of *M. tuberculosis sodC* encodes a protein of 240 amino acids (Fig. 1). Alignment of the deduced amino acid sequence of *M. tuberculosis sodC* and other *sodC* revealed that *M. tuberculosis sodC* is ∼25% identical to bacteria *sodC*. Using the PSORT program analysis (http://psort.nibb.ac.jp/), a putative signal peptide was found at the N-terminus of this polypeptide (Fig. 1). Analysis of the *sodC* gene of *C. crescentus* and *Haemophilus ducreyi* showed the presence of a leader sequence suggesting that these proteins are secreted and localized to the periplasmic space [13,28]. This notion was confirmed with experiments preparing spheroplast and analyzing the Cu,ZnSOD activity in the periplasmic and cytoplasmic fractions in *C. crescentus* [13]. Full-length (L-sodC), a truncated form lacking the putative signal peptide (S-sodC), and a clone (M-sodC) containing extra nucleotides at the 3′ end were cloned from *M. tuberculosis* genomic DNA with PCR strategy. Sequences of the cloned fragments, which matched the documented sequences in the database, were confirmed with fluorescence sequencing on both strands.

3.2. Expression and characterization of the recombinant Cu,ZnSOD

Recombinant proteins were purified, and antibodies were prepared as described in Section 2. All three sodC DNA frag-

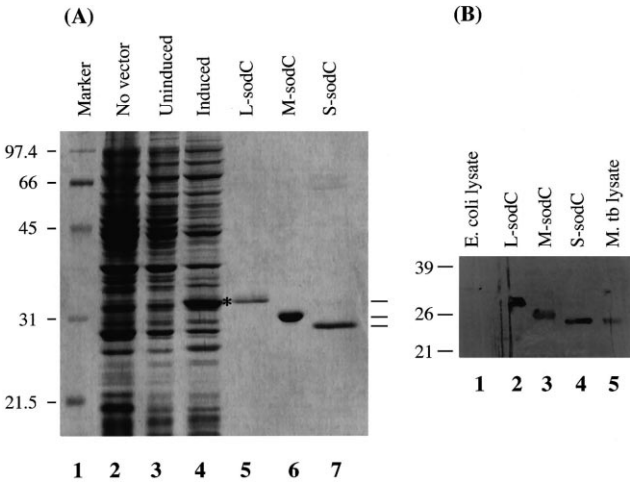

Fig. 2. Coomassie brilliant blue R-250 staining (A) and Western blot (B) of the bacterial lysates and purified recombinant *M. tuberculosis sodC* proteins. A: Lane 1: molecular weight markers; lane 2: bacterial extract of *E. coli* BL21(DE3); lanes 3, 4: bacterial extracts of *E. coli* BL21(DE3) carrying the *sodC* plasmid without and with a 0.5 mM IPTG induction; * marks the position of the overexpressed L-sodC; lanes 5–7: purified L-sodC, M-sodC, and S-sodC, respectively. Positions of the three recombinant proteins are marked on the right. B: Lane 1: bacterial extract of *E. coli* BL21(DE3); lanes 2–4: purified L-sodC, M-sodC, and S-sodC, respectively; lane 5: bacterial extract of *M. tuberculosis* H37Rv.

ments were subcloned into the expression vector pET15b and overexpressed in the bacterium BL21(DE3). The majority of the overexpressed proteins were present in the insoluble inclusion body upon IPTG induction. Recombinant proteins of all three forms in the inclusion body were denatured and solubilized with urea and purified to near homogeneity (Fig. 2A). A minor fraction of these proteins was also purified in a soluble form from the cell lysates.

Rabbit polyclonal antiserum against the purified recombinant M-sodC protein recognized all three forms of the purified proteins while it showed no cross-reactivity to *sodC* of *E. coli* (Fig. 2B, lane 5). More significantly, this antiserum recognized a single polypeptide in the lysate of *M. tuberculosis* suggesting that the *sodC* sequence is indeed expressed in this pathogen (Fig. 2B). Furthermore, this polypeptide shows a similar size as the recombinant S-sodC, which is the *sodC* protein without the putative signal peptide (Fig. 2B, lane 4 and 5). This finding strongly suggests that the *sodC* protein in *M. tuberculosis* was processed into a mature form and secreted. This hypothesis is further supported by the subcellular localization of this protein as demonstrated in the following section.

Unlike the yeast Cu,ZnSOD, which can be overexpressed in a soluble form in the cytoplasm of *E. coli* and shows its enzymatic activity (Fig. 3A, lane 2), the majority of the overexpressed *M. tuberculosis* Cu,ZnSOD in *E. coli* was found in the insoluble inclusion body and did not possess any enzymatic activity (data not shown).

To reconstitute the enzymatic activity of *M. tuberculosis* Cu,ZnSOD, purified recombinant proteins prepared from the inclusion body were denatured with urea and renatured with dialysis in the presence of Cu^{2+} and Zn^{2+}. The cytoplasmic FeSOD and MnSOD form homo- and hetero-dimers in *E. coli* and presented three distinct bands in in situ SOD activity gel (Fig. 3B, lane 1). The renatured recombinant M-sodC protein purified from the inclusion body formed multiple bands in the SOD in situ activity gel (Fig. 3B, lanes 2–4). It is assumed that multiple conformations of the active recombinant proteins are formed during the renaturation process, but do not necessarily reflect the authentic conformation.

Since a minor fraction of the overexpressed recombinant *M. tuberculosis* Cu,ZnSOD in *E. coli* was in the cytoplasm soluble form, we wanted to test whether the soluble form possesses SOD activity. Recombinant Cu,ZnSOD (S-Sodc) purified from this fraction showed a single band in the activity gel

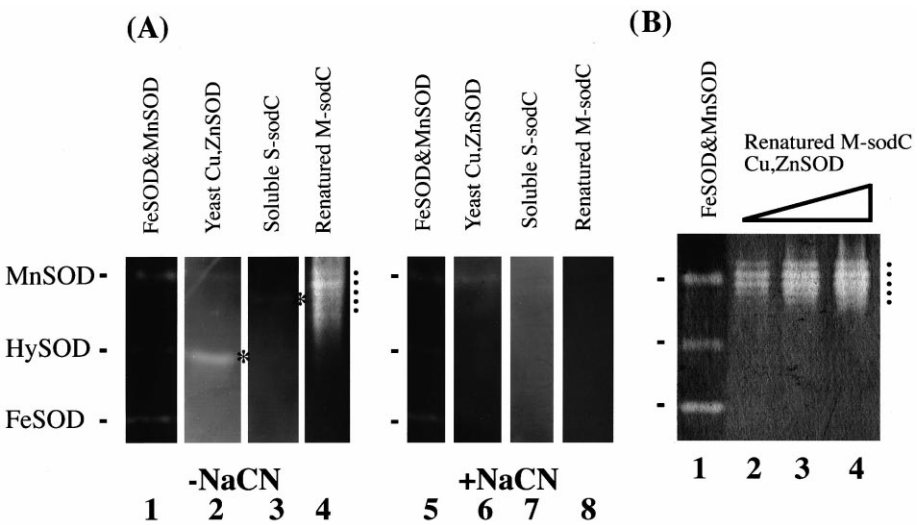

Fig. 3. A: In situ enzymatic activities of recombinant SODs. Lanes 1–4 and lanes 5–8: activity assay in the absence and in the presence of 1 mM cyanide, respectively; lanes 1 and 5: bacterial extract of *E. coli* BL21(DE3); lanes 2 and 6: *E. coli* BL21(DE) lysate containing a recombinant yeast Cu,ZnSOD; lanes 3 and 7: purified recombinant S-sodC in a native conformation; lanes 4 and 8: 16.2 μg of the purified renatured M-sodC. B: Lane 1: bacterial extract of *E. coli* BL21(DE3) showing MnSOD, hybrid SOD, and FeSOD activities; lanes 2–4: 5.4 μg, 10.8 μg, and 16.2 μg of renatured M-sodC protein. Multiple conformations of the purified renatured recombinant protein as marked on the right. * marks the site of the corresponding enzyme.

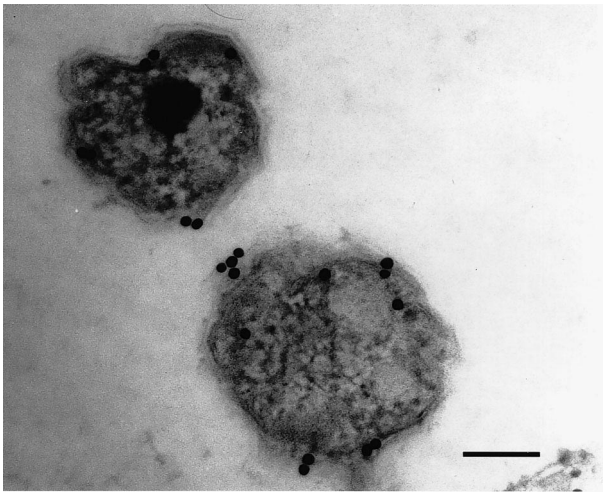

Fig. 4. Immunogold electron microscopic image showing the subcellular localization of the Cu,ZnSOD in *M. tuberculosis*. Bar = 0.1 μm.

assay (Fig. 3A, lanes 3). The activities of *M. tuberculosis* Cu,ZnSOD (S-Sodc), yeast Cu,ZnSOD and renatured forms of *M. tuberculosis* Cu,ZnSOD (M-Sodc) can be inhibited by a cyanide treatment which is a characteristic of Cu,ZnSOD (Fig. 3B, lanes 5–8)[8]. This demonstrates that the *M. tuberculosis sodC* sequence indeed possesses a bona fide Cu,Zn-cofactored superoxide dismutase activity.

The soluble form enzyme migrates between the MnSOD dimer (46 kDa) and the hybrid of MnSOD and FeSOD (44 kDa). With reference to the molecular weight of the monomeric Cu,ZnSOD of *M. tuberculosis* (28 kDa), it is still not clear if this protein exists in the bacteria as a monomer in *E. coli* [29] or a dimer in *P. leiognathi* [30].

3.3. Subcellular localization of the M. tuberculosis Cu,ZnSOD

Cu,ZnSODs of many Gram-negative bacteria possess a signal peptide sequence and are secreted to the periplasmic space [5,10,13–18]. Localization of the Cu,ZnSOD was achieved with immunogold labeling electron microscopy in *E. coli* [16]. Since the periplasmic space is not present in the Gram-positive *M. tuberculosis*, we sought to determine if the Cu,ZnSOD was secreted to the periphery of the bacterium. In an immunogold labeling and electron microscopic experiment, Cu,ZnSOD was shown to be predominantly localized to the periphery of *M. tuberculosis* supporting that this protein is a secreted protein (Fig. 4). This is consistent with the fact that the *M. tuberculosis* Cu,ZnSOD possesses a putative signal peptide sequence. Since a putative signal peptide sequence was found in Cu,ZnSOD of *M. tuberculosis* (Fig. 1) and the endogenous protein had the same size as the recombinant protein without the putative signal peptide (Fig. 2B), it is expected that the protein is a secreted protein. However, it is not clear if the secreted Cu,ZnSOD is solely deposited in the periphery of the bacteria or is secreted to the environment.

Superoxide molecules generated outside the cytoplasm are unable to pass the inner membrane of *E. coli* and, by extension, other Gram-negative bacteria [19]. Superoxide generated from the cytoplasmic metabolism is expected to remain in the cytoplasm, and FeSOD and MnSOD are responsible for neutralizing the intracellular superoxide. This is supported by the fact that *E. coli* lacking cytoplasmic FeSOD and MnSOD are more sensitive than the wild type *E. coli* to paraquat [31], a drug that generates elevated levels of cytoplasmic superoxide [32]. In contrast, the absence of Cu,ZnSOD in *C. crescentus* had no effect on its sensitivity to paraquat or chlorpromazine [18]. Mutations in Cu,ZnSOD caused elevated sensitivities to the exogenous superoxide in *S. typhimurium, N. meningitidis,* and *H. ducreyi* suggesting that the primary role of Cu,ZnSOD was to protect bacteria from the exogenous oxyradicals [20,27,33,34].

Although sources of the exogenous superoxide to damage the periplasmic or membrane constituents were not clear, it is conceivable that the host phagocytic cells could generate a high level of free radicals to inactivate the pathogens. The presence of Cu,ZnSOD in the periplasm of these pathogens could provide a defense mechanism against the hostile environment in the host cells and hence maybe required for virulence [34]. Overexpression of a hydrogen peroxide-resistant periplasmic Cu,ZnSOD protected *E. coli* from macrophage killing [35] and Cu,ZnSOD mutants of *S. typhimurium, N. meningitidis,* and *H. ducreyi* were shown to be less virulent in killing mice infected with the pathogens [20,27,33,34]. By analogy, Cu,ZnSOD of *M. tuberculosis* may play a similar role in determining virulence. More efforts will be put into studying the biological function of Cu,ZnSOD and virulence of *M. tuberculosis*.

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