

## Purification and Characterization of Extracellular Superoxide Dismutase in Mouse Lung

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**Extracellular superoxide dismutase (EC-SOD) is the major isozyme of SOD in arteries, but is also abundant in lungs. In particular, mouse lungs contain large amounts of EC-SOD compared to lungs in other mammals. This suggests that EC-SOD may have an amplified function in the mouse lung. This study describes the purification and characterization of mouse EC-SOD as well as its localization in mouse lung. Mouse EC-SOD exists primarily as a homotetramer composed of a pair of dimers linked through disulfide bonds present in the heparin-binding domains of each subunit. In addition, mouse EC-SOD can exist in active multimeric forms. We developed and utilized a polyclonal antibody to mouse EC-SOD to immunolocalize EC-SOD in mouse lung. EC-SOD labeling is strongest in the matrix of vessels, airways, and alveolar septa. This localization suggests that EC-SOD may have important functions in pulmonary biology, perhaps in the modulation of nitric oxide-dependent responses.**

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**Key Words:** antioxidants; arteries; hypertension; nitric oxide.

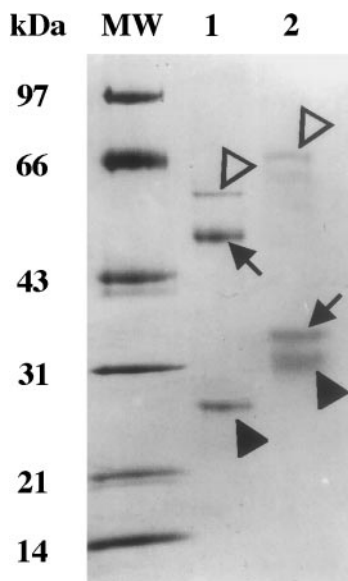
Superoxide dismutases (SOD) are antioxidant enzymes that catalyze the dismutation of superoxide into hydrogen peroxide and oxygen. There are three isozymes of SOD in mammals including the cytoplasmic and nuclear CuZn SOD (1), the mitochondrial Mn SOD (2), and the Cu/Zn containing extracellular SOD (EC-SOD), which is found predominantly in the extracellular matrix of tissues and to a lesser extent in extracellular fluids (3–6). Most studies concerning this class of enzymes have focused on CuZn and Mn SOD, and therefore less is known about the biochemical properties of EC-SOD.

Mice contain high levels of EC-SOD in their lungs as compared to other mammals. In fact, the activity of EC-SOD in the mouse lung is 3- to 10-fold higher than that found in most mammals, and is 30-fold higher than that found in rat lungs (4). The high level of this antioxidant enzyme in the mouse lung suggests an important role for extracellular superoxide scavenging in this organ, possibly as protection against the high oxygen to which the lung is continually exposed. In addition, EC-SOD may also play an important role in modulating airway resistance in the lungs. EC-SOD is known to exist in high concentrations in arteries in all tissues (6–8) where it is believed to be important in modulating nitric oxide-dependent vasorelaxation (9). The uniquely high levels of EC-SOD in the mouse lung suggest this animal is ideal for investigating the importance of this antioxidant enzyme in pulmonary pathobiology. Also, the ease of genetic manipulation in the mouse makes this an ideal species in which to study the function of this antioxidant in the lung. The major limitations in studying the importance of this enzyme in the mouse is the difficulty in obtaining the purified protein and the inability of antibodies directed against EC-SOD from other species to cross-react with mouse EC-SOD. In this study we developed a purification procedure for mouse EC-SOD and created antibodies to both the protein and a synthetic peptide derived from the mouse EC-SOD sequence. The purified protein and the antibodies were used to characterize the structure, function and localization of EC-SOD in the mouse lung to better understand its physiologic function.

### MATERIALS AND METHODS

*Materials.* Xanthine oxidase was purchased from Boehringer Mannheim (Germany). Heparin Sepharose, concanavalin A Sepharose, 3,4-dichloroisocoumarin, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 1,10-phenanthroline, xanthine, equine cyto-

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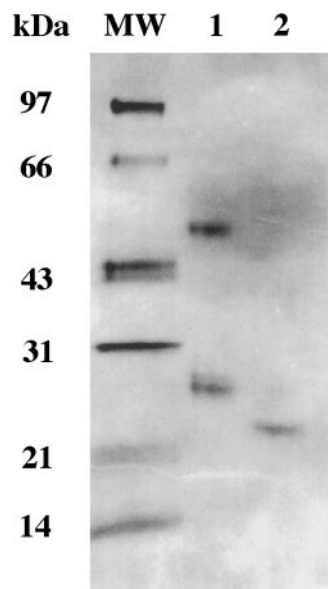
**FIG. 1.** SDS-PAGE of purified mouse EC-SOD. Lane 1 contains 5  $\mu\text{g}$  of nonreduced EC-SOD. Lane 2 contains 5  $\mu\text{g}$  of EC-SOD reduced with 30 mM dithiothreitol prior to SDS-PAGE. Note the disappearance of dimeric EC-SOD (lane 1, arrow) and the appearance of the full-length monomeric EC-SOD (lane 2, arrow) in addition to the proteolysed monomer (lanes 1 and 2, solid arrowhead) with the treatment of dithiothreitol in lane 2 indicating the presence of an intersubunit disulfide bond in EC-SOD. Amino terminal sequence analysis of the dimer revealed the same amino terminal sequence as that of the monomers. An additional higher molecular weight band (open arrowhead) was observed in both the reduced and unreduced lanes. Amino terminal sequence analysis revealed this protein to be albumin. Also note that both the EC-SOD monomer (closed arrowhead) and albumin (open arrowhead) migrated slower after reduction with dithiothreitol. This decreased mobility indicates that there are intrasubunit disulfide bonds in EC-SOD and albumin.

chrome *c*, and bovine trypsin were purchased from Sigma (St. Louis, MO).

**Purification of mouse EC-SOD from mouse lungs.** Mouse lungs were obtained from Pelfreeze. During a typical purification, 200 pairs of mouse lungs (~120 g) were homogenized with a polytron at 4°C in 600 ml of 50 mM potassium phosphate, pH 7.4, 0.3 M potassium bromide. A proteinase inhibitor cocktail composed of 3,4-dichloroisocoumarin (0.1 mM), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (0.2 mM), and 1,10-phenanthroline (1 mM) was added to limit adventitious proteolysis (10). The homogenate was centrifuged at 20,000*g* for 30 min. Polyethylene glycol was added to the supernatant to a final concentration of 15% followed by centrifugation at 5000*g* for 20 min. The pellet was resuspended in 50 mM Tris-Cl, 50 mM NaCl, pH 7.5, and dialysed into 50 mM Tris-Cl, 50 mM NaCl, pH 7.5.

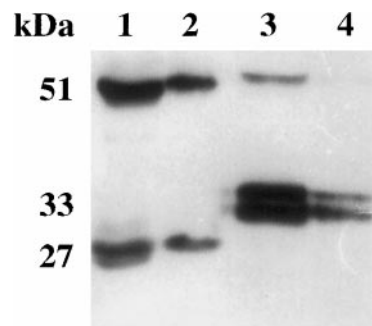
**Heparin-Sepharose chromatography.** After dialysis the sample was applied to a 200 ml heparin-Sepharose (Sigma) open column (2.5 cm  $\times$  20 cm). The heparin-Sepharose was washed with Tris-Cl, pH 7.5 (50 mM Tris-Cl, pH 7.5, 50 mM NaCl) until the  $A_{280}$  of the flowthrough was zero. Heparin binding proteins were eluted with a linear gradient from 50 mM to 1 M NaCl in 50 mM Tris-Cl, pH 7.5 (total volume of 2 liters) at a flow rate of 40 ml/hour. Fractions containing EC-SOD were analyzed by reducing SDS-PAGE. The fractions containing the least amount of contaminants were pooled.

**Q-Sepharose chromatography.** The heparin Sepharose pool was dialysed into 50 mM Tris-Cl, pH 7.5. The sample was applied to a 25

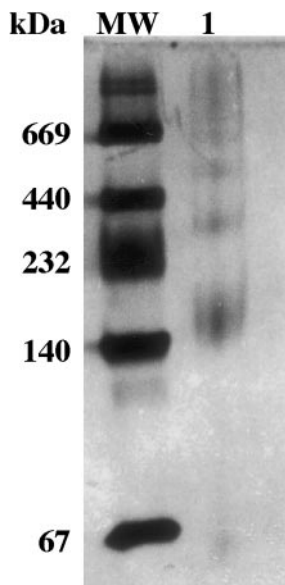


**FIG. 2.** SDS-PAGE of EC-SOD proteolysed with trypsin. 5  $\mu\text{g}$  of EC-SOD was titrated with increasing amounts of trypsin. Lane 1 contains 5  $\mu\text{g}$  of nonreduced EC-SOD. Lane 2 contains 5  $\mu\text{g}$  of nonreduced EC-SOD treated with trypsin at an EC-SOD:trypsin molar ratio of 1:0.1. Note the loss of the dimer with trypsin treatment. Amino terminal sequence analysis of the remaining lower molecular weight EC-SOD monomer in lane 2 is identical to the amino terminal sequence of nonproteolyzed EC-SOD indicating that the proteinase cleaved EC-SOD in the carboxy terminal end of the protein.

ml Q-Sepharose column connected to a FPLC system (Pharmacia). The column was washed with 50 mM Tris-Cl, pH 7.5, and eluted with a linear gradient of NaCl (0–1 M) at 1%/min with a flow rate of 1 ml/min. Fractions containing EC-SOD activity were analyzed with reducing SDS-PAGE and fractions with the least amount of contaminants were pooled.



**FIG. 3.** Western blot analysis of mouse EC-SOD. Lane 1 contains 0.02  $\mu\text{g}$  of purified nonreduced mouse EC-SOD. Lane 2 contains 20  $\mu\text{g}$  of nonreduced protein from a mouse lung homogenate. Lane 3 contains 0.02  $\mu\text{g}$  of pure mouse EC-SOD reduced with 20 mM dithiothreitol. Lane 4 contains 20  $\mu\text{g}$  of protein from a mouse lung homogenate reduced with 20 mM dithiothreitol. The blot was labeled with a polyclonal antibody to a peptide containing the sequence of the first 20 amino acids of mouse EC-SOD. This antibody is specific for mouse EC-SOD and binds both the monomers and the dimer in the lung homogenate.



**FIG. 4.** Nondenaturing gel electrophoresis of EC-SOD. A Coomassie blue-stained gel is shown, with molecular weight standards (MW), and 25  $\mu$ g EC-SOD (lane 1). Native EC-SOD is a tetramer of MW 165 kDa and larger aggregates of tetramers of MW 340 and 520 kDa.

**Concanavalin A-Sepharose chromatography.** The Q-Sepharose pool was dialyzed in 50 mM Tris-Cl, pH 7.5. The sample was applied to a 25 ml concanavalin A-Sepharose column (Sigma). The column was washed with 200 ml of 50 mM Tris-Cl, 100 mM NaCl, 2 mM MnCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, pH 7.5. Binding protein was then eluted with 300 ml of 200 mM methyl- $\alpha$ -D-glucopyranoside in the same buffer as the wash.

**Mono Q-chromatography.** The concanavalin A eluent was dialyzed into 50 mM Tris-Cl, pH 7.5. The sample was applied to a HR 5/5 Mono Q-Sepharose column connected to a FPLC system (Pharmacia). The column was washed with 50 mM Tris-Cl, pH 7.5, and eluted with a linear gradient of NaCl (0–1 M) at 0.5%/min using a flow rate of 1 ml/min. Fractions containing EC-SOD activity were analyzed with SDS-PAGE and fractions with the least amount of contaminants were pooled.

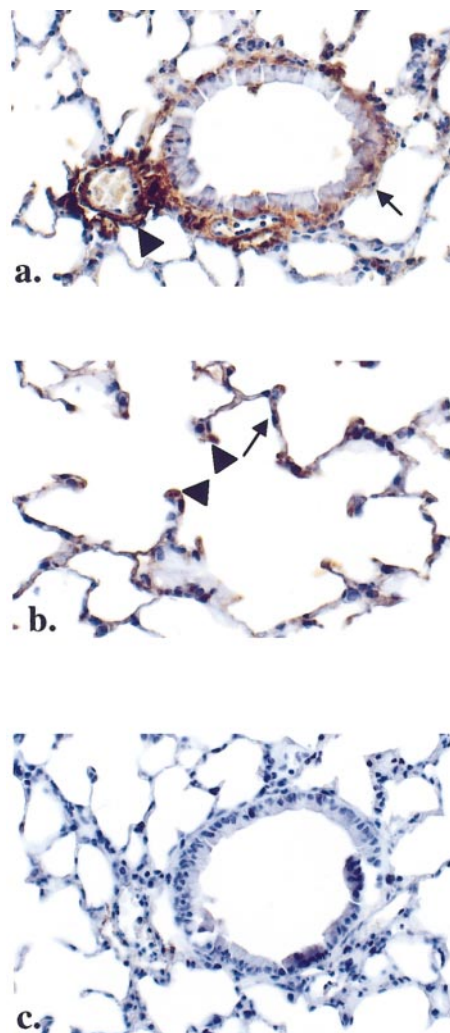
**Mono S-chromatography.** The EC-SOD pool from the mono Q column was dialyzed into 50 mM sodium acetate, pH 4.8, and concentrated to 100 ml using an Amicon concentrator (100,000 dalton cutoff). Two 50 ml applications of this concentrate were applied to a HR 5/5 Mono S-Sepharose column attached to a FPLC system (Pharmacia). The column was eluted at 1 ml/min using 50 mM sodium acetate, pH 4.8, and a linear 1%/min gradient of NaCl (0–1 M). Fractions containing EC-SOD were identified by activity analysis and SDS-PAGE and pooled.

**Analysis of EC-SOD activity.** During the purification of EC-SOD, its activity was measured by inhibition of cytochrome *c* reduction at pH 10.0 as previously described (11). Total protein was determined by measurement of the absorption at 280 nm.

The activity of EC-SOD in the original lung homogenate was measured by the same method after separation of EC-SOD from CuZn SOD and Mn SOD. This separation was accomplished by passing the tissue homogenates over a concanavalin A-Sepharose column as described (12). Total protein in this homogenate was determined by the Coomassie Plus protein assay (Pierce, Rockford, IL).

**Polyacrylamide gel electrophoresis (PAGE).** SDS-PAGE was performed with 5–15% gradient gels using a glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury (13). For reducing SDS-PAGE, samples were incubated for 30 min with 30 mM dithiothreitol and 1% SDS followed by incubation with 60 mM iodoacetic acid for 30 min in the dark. The samples were then boiled for 10–30 min. For nonreducing SDS-PAGE the dithiothreitol/iodoacetic acid incubations were omitted from the samples. Nondenaturing PAGE was performed in the same system except SDS was omitted from all buffers and the samples were not boiled prior to electrophoresis.

**SOD activity stain.** Following nondenaturing PAGE, the gel was soaked in 50 ml of a solution containing 10.5 mg nitro-blue tetrazolium, 0.6 mg riboflavin, and 110  $\mu$ l TEMED in water for 40 min in the dark. After incubation the gel was transferred to water and developed over a fluorescent light.



**FIG. 5.** Immunohistochemical localization of EC-SOD in the mouse lung. Sections of mouse lung were labeled with the antibody to mouse EC-SOD or nonimmune serum and detected using a biotin/streptavidin-horseradish peroxidase technique. (a) The extracellular matrix surrounding bronchioles (arrow) and vessels (arrowhead) have high labeling densities for EC-SOD as depicted by the brown labeling. (b) Labeling for EC-SOD was also found on the surface of alveoli (arrow) and in the alveolar septal tips (arrowhead). (c) No labeling is present when nonimmune serum is substituted for the antibody to EC-SOD.

TABLE 1  
Purification of EC-SOD from Mouse Lung

|                      | Total protein<br>(mg) <sup>a</sup> | EC-SOD<br>total units | EC-SOD<br>units/mg protein | Purification<br>factor | % Yield |
|----------------------|------------------------------------|-----------------------|----------------------------|------------------------|---------|
| 1. Lung homogenate   | 6,541                              | 97,000                | 14.8                       | 1                      | 100     |
| 2. 15% PEG cut       | 4,510                              | 90,510                | 20.1                       | 1.35                   | 93.0    |
| 3. Heparin Sepharose | 241.5                              | 37,905                | 157                        | 10.6                   | 39.0    |
| 4. Q-Sepharose       | 50                                 | 21,000                | 420                        | 28.4                   | 21.6    |
| 5. Concanavalin A    | 15.4                               | 19,975                | 1,295                      | 87.5                   | 20.0    |
| 6. Mono Q            | 9.2                                | 14,457                | 1,571                      | 106                    | 14.9    |
| 7. Mono S            | 0.702                              | 10,500                | 15,000                     | 1013                   | 10.8    |

<sup>a</sup> Protein concentration determined by measurement at  $A_{280\text{nm}}$  except for lung homogenate and PEG cut which were determined using Coomassie Plus Protein Reagent.

**Amino acid sequence analysis of EC-SOD.** Purified and proteolytic fragments of EC-SOD were subjected to SDS-PAGE in the presence of 10 mM dithiothreitol and then transferred to Immobilon-P transfer membranes (Millipore) for amino terminal sequence analysis (14). Samples were then analyzed by automated Edman degradation using an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis employing an Applied Biosystems Model 120A HPLC. Samples were applied to Porton peptide or protein sample support discs, and sequenced utilizing the modified cycles PI-BGN and PI-1 recommended by Porton Instruments.

**Proteolysis of EC-SOD.** Samples of EC-SOD were incubated with trypsin (1:0.2 molar ratios of EC-SOD to active site titrated trypsin) for 30 min at 37°C. Trypsin was then inhibited with 0.1 mM 3,4-dichloroisocoumarin (DCI) for 30 min at 25°C. The samples were analyzed by SDS-PAGE without reduction and stained with Coomassie brilliant blue.

**Antibody production.** A peptide with the sequence of the first 19 amino acids in mouse EC-SOD and containing a cysteine residue at position 20 (SSFDLADRLDPVEKIDRLDC) was synthesized and conjugated to Keyhole Limpet Hemocyanin (KLH) via the MBS method by Multiple Peptide Systems (San Diego, CA). Specific pathogen-free New Zealand White rabbits were injected intramuscularly at four-week intervals with the peptide/KLH conjugate (0.2 mg of peptide) for a total of four injections. A final bleed was done two weeks after the last injection. An IgG fraction of the last bleed was obtained by passage of serum through a protein-G column. IgG was eluted with 0.2 M glycine, pH 2.7, and collected in 4 ml fractions into tubes containing 200  $\mu$ l of 2 M Tris-Cl, pH 8. Fractions containing IgG were determined by measuring the absorbance at 280 nm and pooled. The IgG was then dialysed into 20 mM sodium phosphate, 50 mM NaCl, pH 7.4. An antibody to purified mouse EC-SOD was also made using an identical protocol with injections of 0.3 mg of purified EC-SOD for each injection.

**Western blot analysis.** Following SDS-PAGE, proteins were electrophoretically transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were blocked overnight at 4°C with 5% milk in PBS (20 mM potassium phosphate (pH 7.4), 0.15 M potassium chloride), 0.01% antifoam A, 0.001% merthiolate. The membrane was then incubated with rabbit anti-mouse EC-SOD antibody in PBS with 0.3% Tween 20, followed by incubation with HRP conjugated goat anti-rabbit IgG. The antibody to EC-SOD was then detected using an ECL detection system (Amersham, England).

**Immunohistochemistry.** Mouse lungs were fixed with 2% paraformaldehyde/0.2% glutaraldehyde in 0.05 M phosphate-buffered saline (PBS; 1.2 g  $\text{NaH}_2\text{PO}_4$ , 8 g NaCl, 0.2 g KCl, pH 7.3) for 1 h followed by overnight fixation in 2% paraformaldehyde at 4°C. After fixation the tissues were processed for paraffin embedding.

Serial sections of the paraffin-embedded tissues, 4–8  $\mu$ m, were cut (2 sections/slide). Sections were then labeled for EC-SOD using an indirect immunoperoxidase method (15, 16) with a biotinylated goat anti-rabbit IgG and streptavidin-horseradish peroxidase. To reduce background staining the sections were incubated in 1%  $\text{H}_2\text{O}_2$  in methanol to inactivate endogenous peroxidases, 10 mM borohydride to block aldehydes, and nonspecific binding was blocked by incubation with 5% normal goat serum (NGS), 5% milk, and 1% BSA in PBS. Primary and secondary antibody dilutions were determined empirically and made in PBS with 1% milk plus 1% BSA (milk was not included in the streptavidin solution). The slides were developed using diaminobenzidine (10 mg diaminobenzidine, 50 ml 0.05 M Tris-Cl, pH 7.6, 100  $\mu$ l 3%  $\text{H}_2\text{O}_2$ ) and counterstained with hematoxylin. As a control one serial section on each slide was labeled with antibody specific for mouse EC-SOD or pre-immune rabbit serum. As a further control, a lung from a knockout mouse which does not express EC-SOD (17) was labeled with the antibody to mouse EC-SOD.

## RESULTS

**Purification of EC-SOD from mouse lung.** Table 1 summarizes the protocol utilized for the purification of mouse EC-SOD. Polyethylene glycol precipitation of the lung homogenate resulted in a substantial reduction in the total protein (~2 grams) without a significant loss of EC-SOD activity. Heparin-Sepharose affinity chromatography was an effective purification step, although only 39% of the original activity was recovered. This loss of activity was likely due to a reduction in heparin affinity, resulting from proteolysis of the heparin-binding domain (18). Mono Q-Sepharose chromatography resulted in substantial reduction of total protein (~5-fold) with a moderate loss of enzymatic activity (~2-fold). Concanavalin A-Sepharose chromatography provided a considerable level of purification, resulting in a 3-fold purification (overall 87.5-fold) with only a 1.6% loss in enzymatic activity. The final purification steps involved a combination of Mono Q- and Mono S-Sepharose chromatography. This effective purification step resulted in a final specific activity of EC-SOD of 15,000 units/mg protein with an overall yield of 10.8% of the starting activity.

*EC-SOD exists as a tetramer composed of two disulfide-linked dimers.* After the final Mono S-Sepharose purification step, the protein was shown to be essentially pure as determined by SDS-PAGE (Fig. 1). Analysis of purified EC-SOD under reducing conditions revealed two bands, 35 and 31.5 kDa, respectively, which correspond to mouse EC-SOD as determined by amino terminal sequence analysis (Fig. 1, lane 2). This suggests that the difference in molecular weights of the EC-SOD bands is due to truncation of the carboxyl-terminal region of EC-SOD as has previously been shown for human EC-SOD (19). A third minor band of higher molecular weight also noted, which was determined by amino terminal sequence analysis to represent mouse albumin (open arrowheads).

Under non-reducing conditions, denatured EC-SOD was shown to migrate primarily as a 51 kDa dimer, although a small amount was found in monomeric form (Fig. 1, lane 1). Notably, prolonged boiling of the protein for 30 min did not alter the electrophoretic pattern noted for nonreduced EC-SOD, indicating that a covalent interaction was present. Incubation with 10 mM DTT resulted in dissociation of the dimeric EC-SOD with the appearance of additional full length monomeric EC-SOD (Fig. 1, lane 2) indicating that the covalent interaction of the dimeric EC-SOD is due to an interchain disulfide bond. It is also apparent that the molecular weight of the monomer in the nonreducing lane (Fig. 1, lane 1, 27 kDa) is lower than that of the monomer in the reducing lane (Fig. 1, lane 2, 31.5 kDa). This indicates that there is an intrasubunit disulfide bond in addition to the intersubunit disulfide bond. These results are also observed in Western blots of total lung homogenates probed with an antibody to mouse EC-SOD (Fig. 3), indicating that the interchain and intrachain disulfide bonds are not artifacts of purification.

*Dissociation of EC-SOD dimers by limited proteolysis.* Trypsin has previously been demonstrated to cleave human EC-SOD only within the heparin-binding domain located near the carboxyl-terminus of the protein (20). Figure 2 illustrates that incubation of mouse EC-SOD with trypsin resulted in the disappearance of the dimeric protein and the appearance of a single monomeric band (~23 kDa). It is interesting to note that this monomer produced by trypsin is approximately 4 kDa smaller than the naturally proteolysed EC-SOD subunit, indicating that trypsin cleaves mouse EC-SOD at a point amino terminal to the native site of proteolysis.

*Demonstration of EC-SOD multimer formation.* Analysis of EC-SOD with nondenaturing PAGE revealed that the majority of native EC-SOD migrates as a tetramer with a molecular weight of 165 kDa (Fig. 4). However, many higher molecular weight bands were

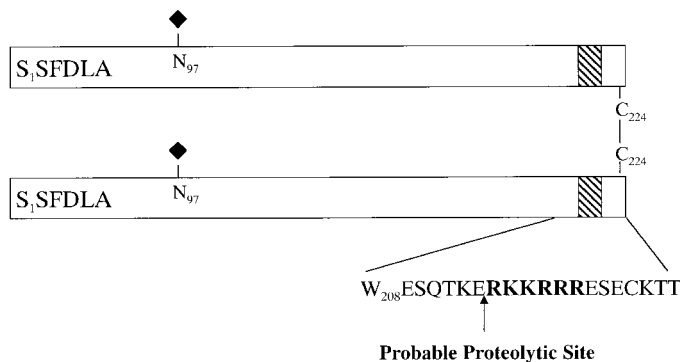
also apparent which increase in size by approximately the tetrameric molecular weight (340 kDa = two tetramers, 520 kDa = three tetramers etc.). An SOD activity stain revealed all bands identified with Coomassie staining contained SOD activity (not illustrated). These results indicate that mouse EC-SOD exists as tetramers and larger multimers.

*EC-SOD is primarily found in vessels and airways in the mouse lung.* Immunocytochemical localization of EC-SOD in mouse lung was performed using an antibody generated to a 20 amino acid peptide corresponding to the amino terminal sequence of mouse EC-SOD. Figure 3 illustrates that the antibody is specific for mouse EC-SOD in lung homogenates.

Figure 5 demonstrates the localization of EC-SOD in mouse lung. Note intense labeling for EC-SOD in matrix of pulmonary vessels (Fig. 5a). Intense labeling was also noted in the matrix of airways (Fig. 5b) and in the alveolar septa, especially in the septal tips (Fig. 5a, arrows). However, the mesothelium does not contain EC-SOD (not illustrated). In addition, inflammatory cells, primarily macrophages and neutrophils also label strongly for EC-SOD (not illustrated). Labeling with preimmune serum resulted in complete absence of labeling (Fig. 5c). Finally, a lung from a mouse that does not make EC-SOD (17) was labeled with the antibody to mouse EC-SOD and resulted in a complete absence of labeling (not illustrated). These results indicate that the labeling observed was specific for EC-SOD.

## DISCUSSION

Purification of EC-SOD from mouse lung is more difficult and results in poorer yields than similar purifications from human aortas (19). Similar to the purification of mouse EC-SOD performed by Ookawara *et al.*, the purification from mouse lung required six steps, although in the present study, the EC-SOD was still contaminated with albumin (Fig. 1) (21). This is in contrast to the three-step purification from human aortas, which resulted in a completely pure product (19). In addition, only a 10.8% yield of EC-SOD was obtained from mouse lung compared to a 46% yield from human aortas. This increased difficulty in purification is likely due to the large amount of blood and the significantly lower specific activity of EC-SOD in the mouse lung compared to human aorta. In addition, significantly more protein was lost in the heparin-Sepharose chromatography step, compared to the small loss of protein in the purification of human EC-SOD. This may indicate that a larger percentage of EC-SOD in the mouse lung has reduced affinity to heparin, due to proteolysis of the heparin-binding domain, as compared to the human aorta. Addition of a polyethylene glycol precipitation and concanavalin



**FIG. 6.** Schematic diagram of mouse EC-SOD. The illustration depicts the amino terminal sequence of the protein. Mouse EC-SOD has a single N-linked glycosylation at asparagine 97 ( $N_{97}$ ,  $\blacklozenge$ ) which gives the enzyme its affinity for concanavalin A. There is also an intersubunit disulfide bond between cysteine 224 of one subunit and cysteine 224 of the second subunit. The heparin-binding domain is shown in bold letters and contains a series of positively charged amino acids consisting of arginine (R) and lysine (K) residues, which gives the enzyme its affinity to negatively charged heparin. The complete EC-SOD protein consists of two of these disulfide-linked dimers which associate noncovalently to form a tetramer. Tetramers also associate noncovalently to form larger multimers. Finally, the presumed proteolytic cleavage site is shown (arrow). Cleavage of this site results in a loss of the interchain disulfide linkage and a loss of the heparin-binding domain.

A-Sepharose chromatography to the procedure utilized for the purification of human EC-SOD resulted in a significant augmentation to the yield of the mouse protein.

Protein sequence analysis indicated that the amino terminal sequence of EC-SOD is SSFDLADRLDPVEK. This is contrary to predicted signal peptide cleavage sites, which would predict an amino terminal sequence of MSNP or NPGE (22). This suggests that mouse EC-SOD contains a signal peptide and a propeptide, or that an artifactual cleavage occurred in the amino terminal region of mouse EC-SOD during purification. However, Western blot analysis of the purified protein and lung homogenate show no differences in mobility on SDS-PAGE (Fig. 3) suggesting the amino terminal sequence of the purified protein is not an artifact of purification. Nevertheless, the amino terminal sequence observed in this study would indicate that each subunit of mouse EC-SOD is 217 amino acid residues long (Fig. 6).

Analysis of mouse EC-SOD revealed that it contains a disulfide-linked dimer. This intersubunit disulfide bond is located between  $Cys_{224}$  of two subunits and links the heparin-binding domains together. The location of the disulfide bond is similar to the location of the interchain disulfide bond in human EC-SOD (19). Like human EC-SOD, proteolysis with trypsin results in a loss of the covalent dimeric association of the EC-SOD subunits. Analysis by SDS-PAGE revealed that mouse EC-SOD exists as tetramers and larger multimers and

that all multimeric form of the enzyme are active. Therefore, mouse EC-SOD and human EC-SOD appear to share similar biochemical properties.

Immunohistochemical localization of EC-SOD in mouse lung was performed using antibodies generated to synthetic peptides containing a sequence corresponding to the amino terminal 19 amino acids of mouse EC-SOD. Mouse EC-SOD was found to be located predominantly in pulmonary vessels. Ookawara *et al.* also found strong vascular labeling for EC-SOD in the mouse tissues they examined (35). The high activity of EC-SOD in blood vessels is intriguing since one speculated function for EC-SOD is as a modulator of the activity of endothelium derived relaxing factor (EDRF) (9), a nitric oxide related species. EDRF is important in the maintenance of low vascular resistance and is inactivated by the superoxide anion (23–27). Using transgenic mice, EC-SOD has been shown to modulate the activity of nitric oxide, or nitric oxide related species, in the brain (28, 29). EC-SOD has also been shown to protect EDRF from superoxide *in vitro* (30). The high labeling density surrounding pulmonary vessels also suggests that the utilization of mouse arteries may have resulted in a similar ease in the purification of EC-SOD as was found with human EC-SOD in human aortas. The only limitation of using mouse arteries is of course the amount of starting material that can be easily obtained.

While most tissues contain high levels of EC-SOD in arteries (6–8, 31), the lung is unique in that in addition to its high vascular content, it also contains airways. Notably, in addition the strong vascular labeling, EC-SOD was also found to have strong labeling densities in the airways of the mouse lung. This may explain why the lungs have such high activity compared to most other organs.

The airway labeling revealed that EC-SOD is present in both the alveolar septa and the matrix of larger airways. Within the alveoli, labeling was especially strong in the septal tips, presumably in association with type I collagen as was noted in human alveolar septal tips (6). The localization in the alveoli is consistent with the finding that alveolar type II cells synthesize EC-SOD in mice (22). The association of EC-SOD with type I collagen has been speculated to be important in preventing superoxide-mediated destruction of collagen (6, 9). The strong labeling for EC-SOD in the matrix of larger airways suggests that EC-SOD may also have a role in modulating airway tone.

It is also interesting to note that EC-SOD labeling was found in alveolar macrophages, where it has been shown to be synthesized (22). This is consistent with other findings in rat that demonstrate strong labeling for EC-SOD in macrophages and neutrophils after LPS stimulation (32). Localization in alveolar macrophages suggests that these cells may serve to regulate EC-SOD levels by first synthesizing and then secreting

EC-SOD into the extracellular space. However, the exact role of EC-SOD in these cells remains to be determined.

In conclusion, mouse EC-SOD shares many biochemical characteristics with human EC-SOD (33). Both are predominantly tetramers composed of two disulfide-linked dimers. The immunochemical findings point to several potentially important roles for EC-SOD in pulmonary pathobiology. The high labeling density in the pulmonary vasculature may play an important role in the maintenance of low pulmonary vascular resistance. The high labeling of EC-SOD in large airways suggest a possible role for EC-SOD in asthma where inflammation and oxidants are known to contribute to airway hyperreactivity. Finally, the strong labeling of EC-SOD in alveolar septa may play a role in modulating inflammatory responses to the large number of inhaled particulates that reach this space and thus help maintain an anti-inflammatory environment in this region. The production of an antibody to mouse EC-SOD and a number of genetically altered mice which express excess or depleted levels of EC-SOD (17, 34) will allow for further studies into the importance of this antioxidant enzyme in pulmonary pathophysiology.

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