Human extracellular superoxide dismutase is a tetramer composed of two disulphide-linked dimers: a simplified, high-yield purification of extracellular superoxide dismutase

Tim D. OURY*†, James D. CRAPO*‡, Zuzana VALNICKOVA* and Jan J. ENGHILD*

*Department of Pathology,and ‡Department of Medicine, Duke University Medical Center, P.O. Box 3712, Durham, NC 27710, U.S.A.

Studies examining the biochemical characteristics and pharmacological properties of extracellular superoxide dismutase (EC SOD) have been severely limited because of difficulties in purifying the enzyme. Recently EC SOD was found to exist in high concentrations in the arteries of most mammals examined and it is the predominant form of SOD activity in many arteries. We now describe a three-step, high-yield protocol for the purification of EC SOD from human aorta. In the first step, the high affinity of EC SOD for heparin is utilized to obtain a fraction in which EC SOD constitutes roughly 13 % of the total protein compared with only 0.3 % of that of the starting material. In addition, over 80 % of the original EC SOD activity present in the aortic homogenate was retained after the first step of purification. EC SOD was further purified using a combination of cation- and anion-exchange chromatography. The overall yield of EC SOD from this purification procedure was 46 %, with over 4 mg of EC SOD obtained from 230 g of aorta. Purified EC SOD was found to exist predominantly as a homotetramer composed of two disulphide-linked dimers. However, EC SOD was also found to form larger multimers when analysed by native PAGE. It was shown by urea denaturation that the formation of multimers increased the thermodynamic stability of the protein. Limited proteolysis of EC SOD suggested that there is one interchain disulphide bond covalently linking two subunits. This disulphide bond involves cysteine-219 and appears to link the heparin-binding domains of the two subunits.

INTRODUCTION

Three isoenzymes of superoxide dismutase (SOD) have been identified in mammals. These are CuZn-SOD, which is found primarily in the cytoplasm and nucleus of cells [1], mitochondrial Mn-SOD [2] and 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]. Much is known about the physical properties of the CuZn- and Mn-SODs and both enzymes have been crystallized [7,8]. However, detailed characterization of native EC SOD has been limited due to difficulties in purification of the enzyme.

Previous studies suggest that EC SOD is a tetrameric glycoprotein composed of at least four identical 30 kDa subunits [9–11]. EC SOD in extracellular fluids is heterogeneous in its affinity for heparin [12,13] and three subtypes exist: type C with high affinity; type B, with intermediate affinity; and type A, without affinity for heparin. The heparin-binding domain is believed to be a cluster of positively charged amino acids in the C-terminal region of the protein [14], and the heterogeneity is believed to result from partial proteolysis of this binding domain resulting in a sequential loss of heparin-binding affinity [5]. There is also some loss of heparin affinity secondary to non-enzymic glycosylation of lysine residues within the heparin-binding domain [15]. The heterogeneity of the final tetrameric protein (types A, B and C EC SOD) is a result of the variable amounts of type C (full-length) and type A (proteolysed) subunits in the final tetramer [14]. While all three types of EC SOD exist in plasma, virtually all of the EC SOD in the extracellular matrix of tissues is type C, or high-affinity EC SOD [5]. The proteinases responsible for the cleavage of the heparin-binding domain *in vivo* are not known.

One possible physiological function for EC SOD is to modulate the activity of endothelium-derived relaxing factor (EDRF), putatively identified as a nitric oxide-related species. EDRF is important in the maintenance of low vascular resistance and is inactivated by the superoxide anion [16–20]. Using transgenic mice, EC SOD has been shown to modulate the activity of nitric oxide, or nitric oxide-related species, in the brain [21,22]. EC SOD has also been shown to protect EDRF from superoxide *in vitro* [23]. In addition, EC SOD may be important in protecting cell surfaces and extracellular matrix proteins from superoxide-mediated damage. Biochemical studies have shown that EC SOD exists in high concentrations in both systemic and pulmonary blood vessels and in airways [24], and, in contrast with tissues examined previously, EC SOD was found to be the predominant form of SOD in most arteries examined. Immunocytochemical localization of EC SOD revealed it to be present both in the adventitia and in smooth-muscle cells surrounding blood vessels and airways [6]. The localization of EC SOD around smooth-muscle cells correlates with its speculated function in modulating EDRF-dependent smooth-muscle relaxation.

A simplified, high-yield method for the purification of EC SOD is described in the present study. The purification scheme takes advantage of the known properties of EC SOD and should prove useful in allowing large quantities of this protein to be easily purified for further biochemical, physiological and possible pharmacological studies. Moreover, we show that EC SOD

Abbreviations used: DCI, 3,4-dichloroisocoumarin; DTT, dithiothreitol; EC SOD, extracellular superoxide dismutase; ERDF, endothelium-derived relaxing factor; SOD, superoxide dismutase.

† To whom correspondence should be addressed.

exists as a covalently linked dimer which further associates into tetramers and larger aggregates. The dimer is stabilized by one interchain disulphide bond formed by the C-terminal cysteine-219 of each subunit.

MATERIALS AND METHODS

Materials

Xanthine oxidase, N-glycosidase F and endoproteinase Lys-C were purchased from Boehringer-Mannheim (Germany). Heparin–Sepharose, concanavalin A–Sepharose, 3,4-dichloroisocoumarin (DCI), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 1,10-phenanthroline, xanthine, equine cytochrome *c* and bovine trypsin were purchased from Sigma (St. Louis, MO, U.S.A.).

Purification of EC SOD from human aorta

Human aorta was obtained at autopsy within 10 h *post mortem*. During a typical purification procedure, approx. 230 g of aorta was homogenized in a Waring blender at 4 °C in 2 litres of 50 mM potassium phosphate (pH 7.4)/0.3 M potassium bromide. A proteinase-inhibitor cocktail composed of DCI (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 [25]. The homogenate was filtered through cheesecloth and centrifuged at 18000 ***g*** for 30 min. The supernatant was concentrated to 500 ml using an MP-2 dual hollow-fibre system (Spectrum) equipped with two 50 kDa molecular-mass cut-off cartridges and dialysed in 50 mM Tris/HCl/50 mM NaCl, pH 7.5.

Heparin–Sepharose chromatography

Following dialysis, the sample was applied in batches to 200 ml of heparin–Sepharose (Sigma). The heparin–Sepharose was washed with 500 ml of buffer [50 mM Tris/HCl (pH 7.5)/50 mM NaCl] using a Buchner funnel with a fibrous glass frit and then poured into a column (2.5 cm × 20 cm). The column bed was washed with Tris buffer until the A_{280} of the eluate was zero. Heparin-binding proteins were eluted with a linear gradient of NaCl (50 mM–1 M) in 50 mM Tris/HCl, pH 7.5 (total volume 2 litres), at a flow rate of 40 ml/h. Fractions containing EC SOD activity were analysed by reducing SDS/PAGE. The least contaminated fractions were pooled.

Mono S chromatography

The EC SOD pool from the heparin–Sepharose column was dialysed in 50 mM sodium acetate, pH 4.8, and concentrated to 100 ml using an Amicon concentrator (100 kDa cut-off). Two 50 ml portions of this concentrate were applied to a HR 5/5 Mono S Sepharose column attached to an 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 then pooled.

Mono Q chromatography

The pooled EC SOD fractions from Mono S chromatography were dialysed in 50 mM Tris/HCl, pH 7.5. The resulting sample was applied to an HR 5/5 Mono Q Sepharose column connected to an FPLC system (Pharmacia). The column was washed with 50 mM Tris/HCl, pH 7.5 and eluted with a linear gradient of NaCl (0–1 M) at 0.5 %/min with a flow rate of 1 ml/min. Fractions containing EC SOD were identified by SDS/PAGE and pooled.

Analysis of EC SOD activity

During the purification procedure, EC SOD activity was measured by inhibition of cytochrome *c* reduction at pH 10.0 as described previously [26]. Total protein was determined by measurement of the absorption at 280 nm. The absorption coefficient of recombinant human EC SOD is 1.73 $ml \cdot mg^{-1} \cdot cm^{-1}$ [27]. The activity of EC SOD in the original aortic homogenate was measured by the same method following separation of EC SOD from CuZn-SOD and Mn-SOD. This separation was accomplished by passing the tissue homogenate through a concanavalin A–Sepharose column as described [28]. Total protein in the homogenate was determined by Coomasie Plus protein assay (Pierce, Rockford, IL, U.S.A.).

PAGE

SDS/PAGE was performed in 5–15 % gradient gels using a glycine/2-amino-2-methyl-1,3-propanediol/HCl system described by Bury [29]. For reducing SDS/PAGE, samples were boiled for 10 min in the presence of 30 mM dithiothreitol (DTT)/1 % SDS before electrophoresis. For non-reducing SDS/PAGE, the DTT was omitted from the samples.

Non-denaturing PAGE was performed in the same system except that SDS was omitted from all buffers and the samples were not boiled before electrophoresis. Transverse urea-gradient PAGE was performed using 7 % acrylamide gels containing a horizontal, linear gradient of 0–8 M urea [30]. Electrophoresis of the samples was carried out in the same buffer system as for native PAGE.

Western-blot analysis

EC SOD samples from SDS and transverse urea-gradient polyacrylamide gels were electrophoretically transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membranes were blocked overnight with 5 % milk in PBS [20 mM potassium phosphate (pH 7.4)/0.15 M KCl], containing 0.01 % antifoam A/0.001 % merthiolate at 4 °C. The membrane was then incubated with rabbit anti-(EC SOD) antibody [24] in PBS with 0.3 % Tween-20, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. The antibody to EC SOD was detected using an enhanced chemiluminescence detection system (Amersham International).

SOD activity stain

Following non-denaturing PAGE, the gel was soaked, in the dark, in water containing 10.5 mg of Nitro Blue Tetrazolium, 0.6 mg of riboflavin and 110 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (50 ml final vol.) for 40 min. The gel was then transferred to water and developed over a fluorescent light.

Amino-acid-sequence analysis of EC SOD

Proteolytic fragments of purified EC SOD were subjected to SDS/PAGE in the presence of 10 mM DTT and then transferred to Immobilon-P transfer membranes (Millipore) for N-terminal-sequence analysis [31]. Samples were analysed by automated Edman degradation using an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis by HPLC

(Applied Biosystems Model 120A). The samples were applied to Porton peptide or protein sample support discs and sequenced using the modified cycles, PI-BGN and PI-1, recommended by Porton Instruments.

Treatment of EC SOD with N-glycosidase F

EC SOD (25 μg) was incubated overnight with 1 unit of N-glycosidase F at pH 7.5 and at 37 °C, according to the manufacturer's instructions. The product was analysed using SDS/PAGE.

DTT titration of EC SOD

Samples of EC SOD were incubated, in the dark, with varying concentrations of DTT (0 to 30 mM) in 50 mM Tris/HCl/50 mM NaCl, pH 7.5, for 30 min at 25 °C and were then alkylated with a two-fold molar excess of iodoacetic acid for 30 min at room temperature. All samples were placed in boiling water for 10 min, subjected to SDS/PAGE and stained with Coomassie Brilliant Blue.

Proteolysis of EC SOD

Samples of EC SOD were incubated with various concentrations of trypsin (1:0 to 1:0.2 molar ratios of EC SOD to active-site-titrated trypsin) at 37 °C for 30 min. Trypsin was inhibited with 0.1 mM DCI at 25 °C for 30 min. The samples were analysed by non-reducing SDS/PAGE and the gels were stained with Coomassie Brilliant Blue. A further sample of EC SOD (225 pmol) was incubated with 4.5 pmol of trypsin as described above. The sample was subjected to non-reducing SDS/PAGE and transferred to Immobilon-P transfer membranes for N-terminal-sequence analysis as described above. Purified EC SOD was incubated at 37 °C for 1 h with Lys-C endoproteinase at EC SOD/proteinase ratios between 1:0.02 and 1:0.8 (w/w) respectively. The proteinase was inhibited by the addition of 0.1 mM DCI. The samples were separated and identified with non-reducing SDS/PAGE.

Tissue homogenization in the presence of iodoacetic acid

Human aorta (2 g) was homogenized in 20 ml of 50 mM potassium phosphate (pH 7.4)/0.3 M KBr. A proteinase-inhibitor cocktail composed of DCI (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 [25]. Free cysteine residues in the homogenate were alkylated by the addition of 30 mM iodoacetic acid to prevent artifactual cysteine dimerization.

RESULTS

Purification of EC SOD from human aorta

Figure 1(A) illustrates the results of heparin–Sepharose chromatography. EC SOD exhibited high affinity for heparin and was eluted at a higher salt concentration than the majority of other heparin-binding proteins. This step produced a significant purification of EC SOD, and SDS/PAGE enabled identification of EC SOD subunits (Figure 1A, fraction 250). A high recovery of the total EC SOD (81 %, Table 1) was obtained.

Mono S chromatography (Figure 1B) also resulted in substantial purification of EC SOD. Precipitation of protein during dialysis of the sample in sodium acetate buffer, pH 4.8, was found to be non-EC SOD protein, as the activity of EC SOD in the supernatant was unchanged. Following Mono S chromatography, EC SOD was found to be the major protein isolated by SDS/PAGE (Figure 1B, fraction 40). This step resulted in a 69 % recovery (56 % overall recovery) of EC SOD (Table 1).

The purity of EC SOD was determined by SDS/PAGE following Mono Q chromatography (Figure 1C) and it was found to be uncontaminated. The recovery of EC SOD activity in this step was 82 % (46 % overall recovery). Two bands with molecular masses of 32 kDa and 29.5 kDa were identified by SDS/PAGE (Figure 1C). N-terminal-sequence analysis of each of these bands revealed sequences corresponding to the N-terminal sequence of EC SOD, suggesting that the difference in the molecular masses of the EC SOD subunits was a result of either carbohydrate heterogeneity or truncation of the C-terminal region of EC SOD.

Deglycosylation of EC SOD

Treatment of EC SOD with *N*-glycosidase F to remove *N*-linked carbohydrate resulted in precipitation of the protein. The insolubility of deglycosylated EC SOD has been reported previously [32]. However, the protein was soluble in SDS sample buffer. Electrophoresis of the solubilized protein resulted in the persistence of two bands with a lower molecular mass than the glycosylated EC SOD (not shown). These results suggest that the difference in mobility of the two EC SOD bands is not a result of carbohydrate heterogeneity, which is in accordance with previous studies [33]. The difference in mobility of the two EC SOD bands, therefore, most likely results from C-terminal truncation of the EC SOD subunits, which has been speculated previously, with the higher-molecular-mass subunit consisting of the full-length EC SOD protein.

Stability of native EC SOD dimers to reduction

Figure 2 shows that non-reduced, denatured EC SOD migrates as a dimer of 50 kDa with some C-terminal truncated monomer also present. N-terminal sequence analysis of the 50 kDa band confirmed that the protein was EC SOD. Notably, prolonged boiling of the samples for 30 min did not alter the electrophoretic pattern seen with non-reduced samples, indicating that a covalent interaction was present. Incubation with increasing concentrations of DTT resulted in a loss of the dimeric EC SOD and the appearance of additional full-length monomeric EC SOD, indicating that the covalent interaction of the dimeric EC SOD was due to an interchain disulphide bond. The monomer was seen to have decreased mobility after extensive reduction (most obvious between lanes 5 and 6 in Figure 2), indicating the presence of intra-subunit disulphide bonds.

To rule out the possibility that the interchain dimer was formed artifactually during the purification of the protein, a small fragment of aorta was homogenized in the presence of 30 mM iodoacetic acid to alkylate any free cysteine residues in the homogenate. A sample of this homogenate was subjected to non-reducing SDS/PAGE. A Western blot of the gel probed with an antibody for EC SOD showed that dimeric EC SOD was still present (Figure 3, lane 1). Reduction of the aortic homogenate with 60 mM DTT resulted in a loss of the dimer (Figure 3, lane 3). This indicates that the interchain disulphide bond of EC SOD is part of the native molecule and is not an artifact of the purification process.

Dissociation of EC SOD dimers by limited proteolysis

Previously, trypsin has been shown to cleave EC SOD only within the heparin-binding domain near to the C-terminus of the

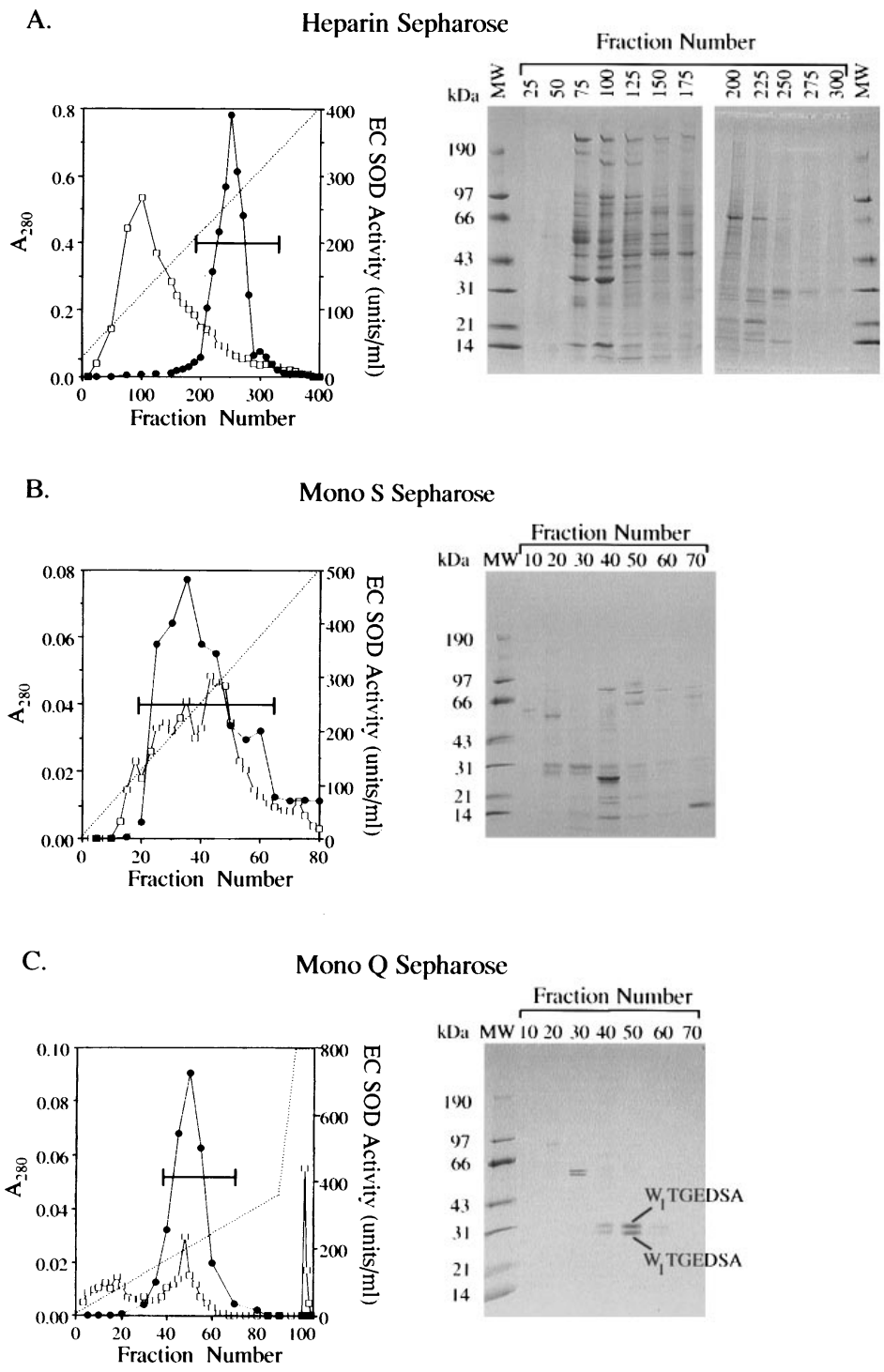

Figure 1 Purification of EC SOD from human aorta

(A) Heparin–Sepharose chromatography of human aorta homogenate. Heparin-binding proteins were eluted using a linear 0–1 M NaCl gradient and collected in 4 ml fractions. The left panel shows the elution profile of EC SOD activity (●) and the A_{280} (□) of the heparin-binding proteins in relation to the NaCl gradient (dotted line). The fractions which were pooled for further purification are indicated by the horizontal bar. The right panel shows reducing SDS/PAGE of 50 μl aliquots of fractions 25–175, and 100 μl aliquots from fractions 200–300. **(B)** Mono S Sepharose chromatography of EC SOD pool of heparin–Sepharose column eluates. The proteins were eluted using a linear 0–1 M NaCl gradient and collected in 1 ml fractions. The left panel shows the elution profile of EC SOD activity (●) and the A_{280} (□) of the binding proteins in relation to the NaCl gradient (dotted line). The fractions pooled for further purification are indicated by the horizontal bar. The right panel shows reducing SDS/PAGE of 50 μl aliquots of the fractions indicated. **(C)** Mono Q Sepharose chromatography of the EC SOD pool of the mono S Sepharose column eluates. The proteins were eluted using a linear 0–0.5 M NaCl gradient followed by a step to 1 M NaCl and were collected in 1 ml fractions. The left panel shows the elution profile of EC SOD activity (●) and the A_{280} (□) of the binding proteins in relation to the NaCl gradient (dotted line). The fractions pooled for further purification are indicated by the horizontal bar. The right panel shows reducing SDS/PAGE of 50 μl aliquots of the fractions indicated. N-terminal sequence analysis of bands indicated revealed the N-terminal sequence of EC SOD as shown. Both sequences correspond to the N-terminal sequence of EC SOD as predicted by cDNA analysis [36]. MW: molecular-mass in kDa.

Table 1 Purification of EC SOD from human aorta

Step	Volume (ml)	EC SOD (units/ml)	EC SOD (total units)	EC SOD (units/A_{280})	Purification factor	Yield (%)
1. Aortic homogenate	2750	63.3	174075	63*	1	100
2. Heparin–Sepharose	460	308	141680	2406	38	81
3. Mono S Sepharose	70	1400	98000	7000	111	56
4. Mono Q Sepharose	43.7	1839	80364	18853	299	46

* Protein concentration determined with Coomasie Plus protein reagent; all other values in this column are expressed per A_{280}.

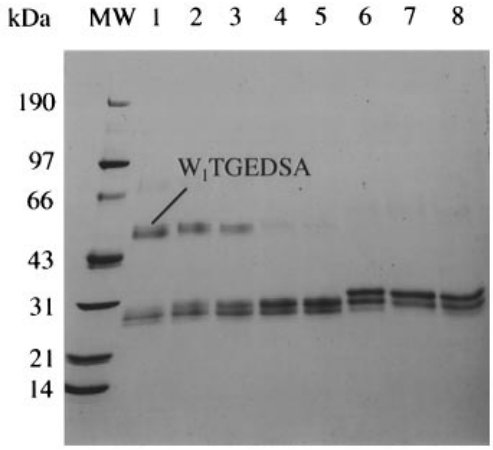

Figure 2 Titration of EC SOD with DTT

EC SOD (5μg) was titrated with increasing concentrations of DTT and then subjected to SDS/PAGE. Lanes 1–7 contain 5 μg of EC SOD treated with 0, 0.05, 0.1, 0.5, 1, 10 or 20 mM DTT respectively. Note the disappearance of dimeric EC SOD and the increase in full-length monomeric EC SOD with increasing concentration of DTT. N-terminal sequence analysis of the dimer is shown and corresponds to the N-terminal sequence of EC SOD as predicted by cDNA analysis [36]. MW: molecular-mass in kDa.

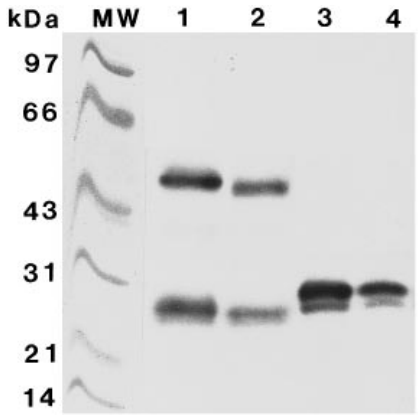

Figure 3 Western-blot analysis of non-reduced EC SOD in an alkylated aortic homogenate

Lane 1 contains 15 μg of non-reduced protein from an aorta homogenate which was alkylated with 30 mM iodoacetic acid. Lane 2 contains 0.01 μg of non-reduced purified EC SOD. Lane 3 contains 15 μg of protein from the alkylated aorta homogenate reduced with 60 mM DTT. Lane 4 contains 0.01 μg of purified EC SOD reduced with 30 mM DTT. Note that dimeric EC SOD is present in the alkylated aorta homogenate (upper band, lane 1), but can be reduced with DTT (lane 3), indicating that the interchain disulphide bond is not an artifact of the purification procedure. MW: molecular-mass in kDa.

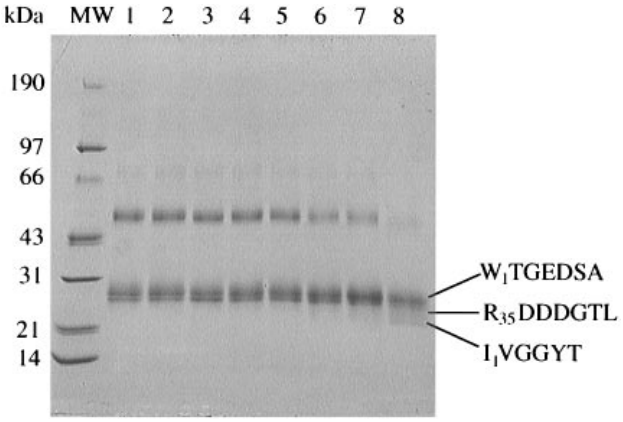

Figure 4 Titration of EC SOD with trypsin

EC SOD (5 μg) was titrated with increasing amounts of trypsin. Lanes 1–8 contain EC SOD/trypsin molar ratios of 1:0, 1:0.003, 1:0.005, 1:0.008, 1:0.01, 1:0.03, 1:0.05, and 1:0.2 respectively. Note the loss of the dimer and the higher-molecular-mass monomer with increasing trypsin concentration. N-terminal-sequence analysis of the remaining lower-molecular-mass EC SOD monomer in lane 8 is shown (top sequence) and corresponds to the N-terminal sequence of EC SOD as predicted by cDNA analysis [36]. N-terminal sequence analysis of the two lowest molecular-mass bands appearing in lane 8 are also shown. The middle sequence corresponds to the sequence of EC SOD beginning at arginine-35. The bottom sequence is that of trypsin. MW: molecular-mass in kDa.

protein [34]. Figure 4 illustrates that incubation of EC SOD with increasing concentrations of trypsin resulted in the disappearance of the dimeric protein. In addition, the separation of the upper and lower monomeric subunits also diminished with trypsin treatment leaving a broader band (approx. 27 kDa). N-terminal sequence analysis of this band revealed only the N-terminal sequence of EC SOD. After treatment with the highest concentration of trypsin (Figure 4, lane 8), two bands of lower molecular mass appeared below the EC SOD monomeric subunits. N-terminal sequence analysis of these bands indicated that the upper band (approx. 24 kDa) was EC SOD cleaved by trypsin between arginine-34 and arginine-35. The lower band was identified as trypsin.

The localization of the cysteine residues involved in the interchain disulphide bond was identified using the results of the trypsin digestion. Trypsin cleaved EC SOD primarily in the heparin-binding domain (Figure 5) [34], resulting in the dissociation of the dimer. There is only one cysteine residue (cysteine-219) C-terminal to the trypsin cleavage site in the heparin-binding domain. These results indicate that the site of the interchain disulphide bond is at cysteine-219 (Figure 5) and demonstrate that EC SOD is highly resistant to trypsin digestion. The cleavage of EC SOD arginine-34 (Figure 5) occurred only with high concentrations of trypsin. Proteolysis at this site cannot contribute to the disruption of the dimeric interaction because of the lack of cysteine residues N-terminal to arginine-34.

Previously, endoproteinase Lys-C also has been shown to cleave EC SOD only in the heparin-binding domain [34]. Incubation of EC SOD with endoproteinase Lys-C dissociated the dimeric EC SOD in non-reducing SDS/PAGE (results not shown). These results confirm that the cysteine residue C-terminal

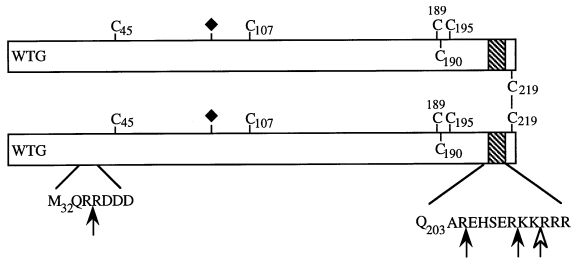

Figure 5 Schematic representation of the primary structure for the EC SOD subunit showing the inter-subunit disulphide bond

Cysteine residues, the location of the heparin-binding domain (▨) and the N-linked carbohydrate (◆) of the EC SOD subunit are indicated. The proteolytic sites of trypsin (closed arrows)- and endoproteinase Lys-C (open arrow)-treated EC SOD are also shown.

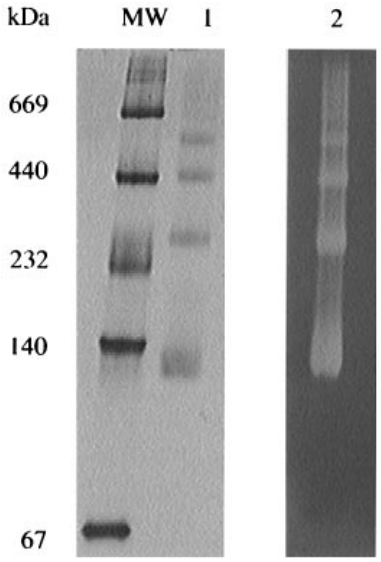

Figure 6 Non-denaturing gel electrophoresis of EC SOD

A gel with molecular-mass standards (MW) and 25 μg of EC SOD (lane 1) stained with Coomassie Blue (left panel) and a gel loaded with 2 μg of EC SOD, subjected to non-denaturing PAGE and stained to show EC SOD activity (lane 2, right panel) are shown.

to the heparin-binding domain is involved in the interchain disulphide bond of EC SOD.

Demonstration of EC SOD multimer formation

Analysis of EC SOD in non-denaturing PAGE revealed that the majority of native EC SOD migrated as a tetramer with a molecular mass of 125 kDa (Figure 6, left panel). However, many bands with higher molecular mass were also present. Staining for SOD activity revealed that all bands identified with Coomassie Blue also contained SOD activity (Figure 6, right panel), indicating that EC SOD exists as tetramers and larger multimers. EC SOD was further analysed using transverse urea-gradient PAGE. Tetrameric EC SOD unfolded rapidly with increasing concentrations of urea. However, the larger multimers of EC SOD were found to resist denaturation (results not shown).

DISCUSSION

A three-step purification protocol for EC SOD is presented which resulted in a high yield recovery of pure EC SOD from human aorta. Previous methods reported for the purification of EC SOD [9,33,35] used five or more steps including heparin–Sepharose chromatography late in the purification process and resulted in poor yields (2% to 25%) of pure protein. Aorta was used as a starting material in the present protocol, since recently arteries have been shown to contain very high concentrations of EC SOD [6,24]. In addition, heparin–Sepharose chromatography was used as a first step to take advantage of the high affinity of EC SOD for heparin. Using the heparin–Sepharose column first resulted in a highly enriched pool of EC SOD, which was then easily purified using a combination of cation- and anion-exchange chromatography.

The largest loss of EC SOD activity occurred during Mono S chromatography, which was performed at pH 4.8. EC SOD has been reported to be unstable below pH 5 [27]. However, the EC SOD was at pH 4.8 for only 24 h for this step in the purification procedure and we have noted no loss of EC SOD activity in samples stored at pH 4.8 in excess of 72 h.

Analysis of EC SOD in non-denaturing polyacrylamide gels revealed that native EC SOD exists as a tetramer and as larger polymers (Figure 6). The formation of EC SOD into multimers has been noted previously with recombinant human EC SOD [11]. Conformation into multimers resulted in increased stability of EC SOD to denaturation, which was demonstrated by transverse urea-gradient PAGE (results not shown). Because of the different sized multimers, gel filtration should not be used to purify EC SOD.

Analysis of EC SOD by non-reducing SDS/PAGE demonstrated that the protein subunits were arranged as disulphide-linked dimers. Treatment of EC SOD with trypsin or endoproteinase Lys-C dissociated the dimers and resulted in a loss of all of the higher-molecular-mass monomers, shown by examination of SDS/PAGE gels (Figure 4). N-terminal sequence analysis of the lower-molecular-mass subunit following treatment with trypsin revealed that the N-terminus of EC SOD had not been cleaved. This suggests that the loss of mass of the higher-molecular-mass subunit was because of C-terminal cleavage by trypsin and endoproteinase Lys-C. This is consistent with a previous report which demonstrated that both trypsin and endoproteinase Lys-C cleaved EC SOD only in the heparin-binding domain [34], which is near the C-terminus of the protein. Notably, treatment with high concentrations of trypsin resulted in a small amount of N-terminal cleavage between arginine-34 and arginine-35 of EC SOD (Figure 5). However, there are no cysteine residues N-terminal to this cleavage site and therefore cleavage at this site cannot cause dissociation of the EC SOD dimer.

There is only one cysteine residue (cysteine-219) C-terminal to the heparin-binding domain of EC SOD. This cysteine residue is, therefore, likely to be involved in the interchain disulphide bond forming dimeric EC SOD and may be linked either to the same cysteine residue on the other subunit of EC SOD or to another cysteine residue within the EC SOD protein. If the latter were true, N-terminal sequence analysis of the upper band of EC SOD in non-reducing SDS/PAGE should have revealed the sequence of the C-terminal peptide released from the proteolysed subunit. However, this was not observed. Therefore the interchain disulphide bond appears to be between cysteine-219 on one subunit and cysteine-219 on another subunit. This interchain disulphide bond would consequently link the heparin-binding domains of two EC SOD subunits in close proximity (Figure 5). Notably,

this cysteine residue in the C-terminal region of EC SOD is conserved in all species so far examined [35–37].

The linkage of two heparin-binding domains by this disulphide bond may be of structural importance in maximizing the affinity of EC SOD for heparan sulphate in the extracellular matrix and, fortuitously, in maximizing the power of heparin–Sepharose chromatography in the purification of this protein. However, a previous report indicated that in a mutation where alanine was substituted for cysteine-219, no change in the affinity for heparin resulted [14], which suggests that the disulphide bonds may not play a role in the interaction of the enzyme with heparin, but possibly may have some other structural role.

Reduction of EC SOD also resulted in a shift in migration of the monomeric subunits. This indicates the presence of intra-subunit disulphide bonds which were highly resistant to reduction (Figure 2). The active site of EC SOD is similar to that of the dimeric, intracellular CuZn-SOD [36] which, although a cytoplasmic protein, contains a disulphide bond. The cysteine residues forming this bond are conserved in EC SOD and are likely to be responsible for the intra-subunit disulphide bond in EC SOD. The presence of this disulphide bond is consistent with its resistance to reduction, which would be necessary for a cytoplasmic disulphide bond.

In conclusion, by using the high affinity of EC SOD for heparin, a simple, high-yield purification of EC SOD from human aorta has been demonstrated. Preliminary studies suggest that this protocol can be adapted easily for the purification of EC SOD from other species, thus simplifying further study of this protein. In addition, larger-scale preparation of EC SOD should be feasible, allowing large quantities to be purified for pharmacological applications. Finally, EC SOD has been shown to exist predominantly as a tetramer composed of two disulphide-linked dimers. The interchain disulphide bond appears to involve cysteine-219 near the C-terminal region of the protein and links the heparin-binding domains of two subunits (Figure 5).

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