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Extracellular superoxide dismutase exists as an octamer

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Abstract

Human extracellular superoxide dismutase (EC-SOD) is involved in the defence against oxidative stress induced by the superoxide radical. The protein is a homotetramer stabilised by hydrophobic interactions within the N-terminal region. During the purification of EC-SOD from human aorta, we noticed that material with high affinity for heparin–Sepharose formed not only a tetramer but also an octamer. Analysis of the thermodynamic stability of the octamer suggested that the C-terminal region is involved in formation of the quaternary structure. In addition, we show that the octamer is composed of both aEC-SOD and iEC-SOD folding variants. The presence of the EC-SOD octamer with high affinity may represent a way to influence the local concentration of EC-SOD to protect tissues specifically sensitive to oxidative damage.

Keywords

Superoxide dismutase; Quaternary structure; Tissue distribution; Oxidative stress

1. Introduction

The superoxide dismutases (SODs; EC 1.15.1.1) catalyze the rapid dismutation of two superoxide anions to hydrogen peroxide and molecular oxygen [1]. Extracellular SOD (EC-SOD; SOD3) is a tetramer and present in the extracellular space and to a lesser extent in the extracellular fluids [2]. In addition, EC-SOD was recently observed in the nucleus [3]. The N-terminal region of EC-SOD is likely to form an amphipathic α -helix which is responsible for the formation of tetramers [4,5]. The importance of hydrophobic interactions in tetramer formation is illustrated by rat EC-SOD, which exists as a dimer due to the presence of an Asp residue within this region [6,7]. We have recently shown that the central part of human EC-SOD can exist in two different variants producing subunits with SOD activity (aEC-SOD) or without (iEC-SOD) [8]. These two variants are maintained by distinct connectivity of cysteine residues. Notably, iEC-SOD presents a disulfide bridge between two adjacent cysteine residues [8]. This type of disulfide bridge is very rare, and likely to have a pronounced impact on the tertiary structure. It is not clear whether these two variants have any impact on the quaternary structure of EC-SOD. The C-terminal region of EC-SOD mediates the binding to ligands in the extracellular matrix (ECM) including heparan sulfate proteoglycans and type I collagen

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[9–11]. This region is thus responsible for the immobilization of EC-SOD and is hence referred to as the ECM-binding region [11]. Prior to secretion of EC-SOD, the ECM-binding region can be proteolytically removed by a two-step event [12–14]. Consequently, both intact and cleaved EC-SOD subunits are produced. The mature tetramer can be composed of cleaved subunits only (type A), both intact and cleaved subunits (type B), or of intact subunits only (type C) producing EC-SOD with no, intermediate, or high affinity for the ECM, respectively [15,16]. The ratio between intact and cleaved subunits is tissue specific, indicating that the proteolytic events are regulated [12]. In addition, it has been shown that the ratio can be modulated by oxidative stress [17–19].

In this study, we show that EC-SOD purified from human aorta exists as a major population of tetrameric EC-SOD and a minor population of octameric EC-SOD estimated to represent approximately 1% of the purified material. The EC-SOD octamer is composed of intact subunits only and presents an increased affinity for heparin–Sepharose. The stability of octameric EC-SOD was increased relative to the tetramer, indicating that the octamer forms a very stable and compact structure. The presence of an EC-SOD octamer adds to the flexibility of EC-SOD, and supports the fact that the physiological function of EC-SOD is intricately regulated by the use of several structural modulations.

2. Materials and methods

2.1. Purification of octameric EC-SOD

EC-SOD was purified from human aorta (~130 g) as previously described using heparin affinity chromatography and omitting cation exchange chromatography [20]. Briefly, a 5 ml HiTrap heparin–Sepharose column (Amersham Biosciences) was operated at a flow rate of 2 ml/min using an FPLC system (Amersham Bioscience). The aorta homogenate was applied in 20 mM Tris–HCl, 50 mM NaCl, and 5 mM MgCl₂, pH 7.4. The column was subsequently washed in 20 mM Tris–HCl, 50 mM NaCl, pH 7.4, and bound proteins eluted by using a linear gradient from 50 mM to 1M NaCl in 20 mM Tris–HCl, pH 7.4. Fractions of 2 ml were collected and analysed for protein by SDS–PAGE, for SOD activity using the xanthine oxidase/cytochrome *c* assay [21], and for the presence of high molecular weight EC-SOD by non-denaturing PAGE and Western blotting. Fractions containing high molecular weight EC-SOD were pooled and dialysed against 20 mM Tris–HCl, pH 7.4. Further fractionation was performed by anion exchange chromatography using a 1 ml HiTrap Q–Sepharose column (Amersham Biosciences). The column was equilibrated in 20 mM Tris–HCl, pH 7.4, and the dialyzed sample applied at 1 ml/min flow rate. The column was washed and proteins eluted using a linear gradient from 0 to 500 mM NaCl in 20 mM Tris–HCl, pH 7.4. Fractions of 1 ml were collected and analysed as described above. The fractions containing high molecular weight EC-SOD were pooled and stored at –20 °C until further use.

2.2. Polyacrylamide gel electrophoresis

SDS–PAGE and non-denaturing PAGE was performed as previously described [20]. Transverse urea-gradient PAGE (TUG–PAGE) was performed by using 7% acrylamide gels with a horizontal and linear gradient from 0 to 8M urea [22]. Electrophoresis was carried out using the same buffer system as for non-denaturing PAGE and the samples were prepared accordingly. The activity of the separated material was assessed by activity staining [20] or Western blotting by using a polyclonal rabbit anti-human EC-SOD antiserum and HRP-conjugated goat anti-rabbit Ig [8].

2.3. Analytical size exclusion chromatography

EC-SOD was subjected to analytical size exclusion chromatography using a Superose 6 PC 3.2/30 column (Amersham Biosciences) attached to a SMART system (Pharmacia). The

column was equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, and operated at 40 μ l/min. Approximately 10 μ g protein was applied in a volume of 20 μ l. The protein elution was monitored by absorbance at 280 nm. Fractions were collected manually and analysed by non-denaturing PAGE and Western blotting. The column was calibrated by assessing the elution volume (V_e) of the following globular protein standards: thyroglobin (669 kDa), ferritin (440 kDa), adolase (158 kDa), bovine serum albumin (67 kDa), ribonuclease A (13.7 kDa) (HMW and LMW gel filtration calibration kit, Amersham Biosciences). The void volume of the column (V_o) was determined as the elution volume of blue dextran (~2000 kDa). The coefficient K_{av} was calculated according to the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_t is the total column volume (2.4 ml). A plot of K_{av} versus Log (M_w) was used to estimate the size of EC-SOD.

2.4. Mass spectrometric identification of aEC-SOD and iEC-SOD

The collected fraction from size exclusion chromatography containing octameric EC-SOD was lyophilized and resuspended in 100 μ l of 25 mM Tris-HCl, pH 7.4, containing 2.5M guanidinium hydrochloride, 20 mM 8-hydroxyquinoline, and 25 mM iodoacetamide. The sample was left for 2 h at 23 $^{\circ}$ C to allow for the alkylation of free cysteine residues. The buffer was subsequently exchanged using a Fast Desalting PC 3.2/10 column (Amersham Biosciences) connected to the SMART system and operated in 50 mM NH_4HCO_3 at a flow of 200 μ l/min. The collected S-carboxymethylated protein was digested over night at 37 $^{\circ}$ C using porcine trypsin at an approximate weight ratio of 1:40. The peptides were reduced by the addition of 20 mM DTT and subsequently absorbed by using a C_{18} reverse-phase ZipTip (Millipore) according to manufacturer's instructions. Bound peptides were eluted and spotted on a sample plate using 1 μ l of a saturated matrix solution (0.4% (w/v) α -cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid). The peptides were analyzed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) using a quadrupole/time-of-flight (QTOF) Ultima Global mass spectrometer (Micromass) as previously described [23].

3. Results

3.1. EC-SOD type C forms two distinct quaternary structures

When EC-SOD extracted from human aorta tissue was subjected to heparin-Sepharose affinity chromatography, the majority eluted between 380 and 550 mM NaCl (Fig. 1A) [20]. A minor fraction of the material (~1% as evaluated by SOD activity) expressed a higher heparin affinity and eluted in subsequent fractions (600–650 mM NaCl) (Fig. 1A). Analysis by reducing SDS-PAGE showed that the major peak contained both intact and cleaved subunits whereas the material with higher heparin affinity consisted of intact subunits only (Fig. 1B). EC-SOD in the major peak corresponded to type B EC-SOD whereas the minor fraction of EC-SOD represented type C. Analysis by non-reducing SDS-PAGE showed that type B EC-SOD is composed of dimeric and monomeric subunits whereas type C consisted of dimers only (Fig. 1C). This finding corroborates previous reports showing that the intact subunit has the capacity to form an inter-subunit disulfide bond [8,20,24,25]. To investigate the quaternary structure of the collected material, we analyzed the fractions by non-denaturing PAGE (Fig. 2). Fractions representing type B EC-SOD migrated as a tetramer, however, the fractions representing type C EC-SOD were found to contain both a tetramer and a higher molecular weight species (Fig. 2). This species was repeatedly observed during separate purifications using distinct starting material. These results show that EC-SOD composed of intact subunits form molecules of two distinct quaternary structures.

3.2. EC-SOD of high molecular weight is an octamer

In order to corroborate the size estimated by non-denaturing PAGE, the high molecular weight material was subjected to analytical size exclusion chromatography (Fig. 3). Although the applied material did not separate into two distinct peaks, two fractions were manually collected and denoted fraction 1 and 2 (Fig. 3). The size of EC-SOD collected in fraction 1 and fraction 2 was estimated to be 270 and 150 kDa, respectively (Fig. 3). As the mass of the intact EC-SOD subunit is ~33 kDa, the material collected in fraction 1 is estimated to be octameric whereas EC-SOD in fraction 2 represents a tetramer. When fraction 1 was analysed by non-denaturing PAGE a single band corresponding to the octameric EC-SOD was detected (Fig. 3, inset; lane 2). Analysis of fraction 2 by non-denaturing PAGE produced two bands including tetrameric EC-SOD and octameric EC-SOD (Fig. 3, inset; lane 3). The presence of octameric EC-SOD is likely due to the inability to achieve sufficient separation. The data supports the result obtained by non-denaturing PAGE and we conclude that EC-SOD type C exists as both a tetramer and an octamer.

3.3. Octamerization of EC-SOD leads to enhanced stability

To further characterize octameric EC-SOD, we investigated the conformational stability by denaturant-induced unfolding using TUG-PAGE analysis. The purified material representing both tetrameric and octameric EC-SOD was subjected to TUG-PAGE using a horizontal gradient from 0 to 8M urea. The migration patterns of the proteins were detected by Western blotting. Low concentrations of urea had no effect on the electrophoretic mobility of tetrameric EC-SOD (Fig. 4). However, at approximately 5 M urea the mobility was reduced. At concentrations of urea above the transition no mobility change was observed (Fig. 4). In contrast, octameric EC-SOD showed a marked increase of stability and was not affected by 8 M urea. This finding suggests that the EC-SOD octamer has gained conformational stability as compared to the tetramer composed of intact subunits only. It is interesting to note that the activity of both the tetramer and the octamer is maintained across the full range of urea concentrations as assessed by activity staining (data not shown) suggesting that the unfolding of the tetramers do not involve the catalytic region.

3.4. The EC-SOD octamer is composed of both aEC-SOD and iEC-SOD subunits

The EC-SOD octamer purified by size exclusion chromatography (Fig. 3, inset; lane 2) was used to investigate if the formation of the octamer depends on the EC-SOD folding variants (aEC-SOD only, iEC-SOD only, or both). The material was incubated with iodoacetamide to alkylate free cysteine residues and the alkylated material was subsequently digested with trypsin. The resulting peptides were reduced with DTT and analyzed by MALDI-MS. Peptides derived from aEC-SOD and iEC-SOD show different mass fingerprints as the position of the single free cysteine residue is different in the two forms (aEC-SOD, Cys195; iEC-SOD, Cys45) [8]. Hence, the identification of *S*-carboxymethylated peptides (+57 Da) at Cys19 or Cys45, reveal the presence of aEC-SOD or iEC-SOD, respectively. The remaining cysteine residues in both aEC-SOD and iEC-SOD are involved in disulfide bonds during alkylation and are not derivatized. Both peptides containing Cys45 and Cys195 were detected with and without a mass increase of 57 Da (data not shown). This finding indicates that the octamer contains both folding variants and that there is no structural constraints of either aEC-SOD or iEC-SOD to participate in the formation of octameric EC-SOD.

4. Discussion

During the purification of EC-SOD from human aorta extracts, we have consistently observed a high molecular weight form with an increased affinity for heparin–Sephacrose. This species has been observed in separate purifications using distinct starting material. Here we show that the high molecular weight EC-SOD is an octamer composed of intact subunits only, indicating

that type C EC-SOD can exist as both a tetramer and an octamer. We have previously published that type B EC-SOD purified from human aorta and mouse lungs form multimers [20]. However, subsequent analyses have shown that these multimers were induced by the low pH (4.8) used during cation exchange chromatography (data not shown). In this report, we have subjected the aortic extract to purification steps at neutral pH only. In addition, it should be stressed that the EC-SOD octamer is observed only in the late fractions collected from the heparin–Sephacel and not in the major peak containing type B EC-SOD (Fig. 2). It does not appear that the octamer and tetramer is in a dynamic equilibrium, as we did not observe any tetramer in the purified octameric material upon storage. Moreover, we did not observe any dissociation of the octamer by varying ionic strength or pH, suggesting that the octameric structure is not stabilized by ionic interactions (results not shown).

The finding that only the intact EC-SOD subunit has the capacity to generate the octamer, suggests that the ECM-binding regions are involved in the stabilization. This notion is supported by thermodynamic stability studies using TUG-PAGE analysis. If tetrameric EC-SOD composed of intact subunits are dissociated by urea the migration of the protein will increase and be represented by a band corresponding to the dimer (~70 kDa). However, the finding that the migration of tetrameric EC-SOD is reduced at concentrations above 5M urea suggests that the N-terminal interactions are maintained. The stability of the tetramer in the presence of strong denaturants such as 5 M guanidinium hydrochloride or 8 M urea has previously been noted [4,26]. The enzymatic activity of EC-SOD is also maintained in TUG-PAGE analysis (0–8M urea) (data not shown). The structure of the central part of the protein is essential for activity, suggesting that the structural perturbation observed at concentrations above 5 M urea only involves unfolding of the ECM-binding regions. However, the migration of octameric EC-SOD is not affected by urea. This indicates that the octamer is relatively more stable than the tetramer and that the increased stability is caused by interactions between the ECM-binding regions in the EC-SOD octamer. The presence of both aEC-SOD and iEC-SOD in the octamer indicates that there are not structural requirements for the presence of one form only.

In the extracellular space, the EC-SOD octamer is likely to bind strongly to type I collagen and heparan sulfate, based on the presence of eight ECM-binding regions. We have previously shown that the intracellular cleavage of the ECM-binding region is tissue specific [12]. Liver and lungs were found to contain equal amounts of the cleaved and intact subunits, supporting the generation of type B EC-SOD. However, organs like brain and heart contain mainly intact subunits [12], supporting the formation of tetrameric and octameric type C EC-SOD. Due to a relatively low affinity for the ECM, we hypothesize that type B EC-SOD diffuse into the tissue from the site of synthesis to confer global protection against damage induced by the superoxide radical. In contrast, type C with a relative high affinity for the ECM, is likely to be localized at the site of synthesis. The formation of octameric EC-SOD support maximum ECM-binding capacity and may thus reflect virtually immobilized EC-SOD. The finding that heart and brain largely contain intact subunits may reflect the physiological importance of superoxide protection in these tissues.

Abbreviations

ECM, extracellular matrix; EC-SOD, extracellular superoxide dismutase; TUG-PAGE, Transverse urea-gradient PAGE.

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Fig. 1. Heparin-Sepharose chromatography

(A) An aorta homogenate was applied to a heparin-Sepharose column and proteins eluted with a linear gradient of NaCl (stippled line). Protein was detected by absorbance at 280 nm. SOD activity measured by the oxidase/cytochrome *c* assay. Fractions were subjected to SDS-PAGE analysis under reducing (B) and non-reducing (C) conditions and EC-SOD subsequently detected by Western blotting. Purified EC-SOD was included as a control (C) in both conditions. The position of intact and cleaved subunits (reducing conditions) and the monomer and dimer (non-reducing conditions) are indicated on the left. The horizontal bars in all panels indicate the fractions pooled for further analysis.

Fig. 2. Analysis of quaternary structure by non-denaturing PAGE

Fractions analyzed by SDS-PAGE (Fig. 1) were also subjected to non-denaturing PAGE to determine the subunit composition. The analysis was performed using approximately the same amount of SOD activity. EC-SOD was subsequently detected by Western blotting. Arrows indicate the positions of tetrameric and high molecular weight (HMW) EC-SOD in the gel. The fractions pooled for further analysis are indicated by the horizontal bar. The position of molecular weight markers are indicated on the left.

Fig. 3. Analytical size exclusion chromatography

The pooled material containing both tetrameric and high molecular weight EC-SOD was applied to a Superose 6 PC 3.2/30 column. Protein elution was detected by absorption at 280 nm. The two fractions collected were analyzed by non-denaturing PAGE/Western blotting as indicated (inset). Lane 1 represents the pooled material applied to the column. The analysis indicates that the EC-SOD octamer has a globular shape.

Fig. 4. Transverse urea-gradient PAGE of purified EC-SOD

The pooled material containing EC-SOD of both quaternary structures was applied across the top of a 7% polyacrylamide TUG gel with a horizontal linear gradient of urea as indicated. EC-SOD was detected by Western blotting. The position of tetrameric and octameric EC-SOD is indicated by arrows. The shapes of the bands suggest that the EC-SOD octamer is more stable than the tetramer.