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The rat extracellular superoxide dismutase dimer is converted to a tetramer by the exchange of a single amino acid

(oxygen radicals/heparin/CuZn-SOD/evolution/tetramerization)

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ABSTRACT Extracellular superoxide dismutase (EC-SOD) is a secreted Cu and Zn-containing glycoprotein. While EC-SOD from most mammals is tetrameric and has a high affinity for heparin and heparan sulfate, rat EC-SOD has a low affinity for heparin, does not bind to heparan sulfate *in vivo*, and is apparently dimeric. To examine the molecular basis of the deviant physical properties of rat EC-SOD, the cDNAs of the rat and mouse EC-SODs were isolated and the deduced amino acid sequences were compared with that of human EC-SOD. Comparison of the sequences offered no obvious explanation of the differences. Analysis of a series of chimeric and point mutated EC-SODs showed that the N-terminal region contributes to the oligomeric state of the EC-SODs, and that a single amino acid, a valine (human amino acid position 24), is essential for the tetramerization. This residue is replaced by an aspartate in the rat. Rat EC-SOD carrying an Asp → Val mutation is tetrameric and has a high heparin affinity, while mouse EC-SOD with a Val → Asp mutation is dimeric and has lost its high heparin affinity. Thus, the rat EC-SOD dimer is converted to a tetramer by the exchange of a single amino acid. Furthermore, the cooperative action of four heparin-binding domains is necessary for high heparin affinity. These results also suggest that tetrameric EC-SODs are not symmetrical tetrahedrons, but composed of two interacting dimers, further supporting an evolutionary relationship with the dimeric cytosolic Cu and Zn-containing SODs.

Extracellular superoxide dismutase (EC-SOD) is a secreted Cu and Zn-containing glycoprotein (1, 2). The central region of the amino acid sequence is highly homologous to that of the dimeric cytosolic CuZn-SODs that defines the active site, suggesting an evolutionary relationship (3, 4). In humans and most other mammals, EC-SOD is homotetrameric and has a high affinity for heparin and heparan sulfate, conferred by a positively charged cluster of amino acids in the carboxyterminal ends of the subunits (5). More than 98% of the EC-SOD in the body exists in tissues (6, 7), bound to heparan sulfate proteoglycans on cell surfaces and in the connective tissue matrix (8, 9). In the vasculature, EC-SOD is in an equilibrium between the heparan sulfate on the endothelial cell surfaces and the plasma phase (6, 10, 11). Plasma also contains EC-SOD fractions with reduced heparin affinity (6, 11), resulting from proteolytic truncations (12, 13) and other modifications (14) of the carboxyterminal ends.

In the rat, the properties and distribution of EC-SOD are different from those of other investigated mammals. Rat EC-SOD is smaller, having an apparent molecular mass of 85 kDa instead of 155 kDa by size exclusion chromatography (6). In the vasculature, there is no binding of EC-SOD to the endothelial cell surfaces as can be revealed by injections of heparin, and there is no EC-SOD with high affinity for heparin, either in tissues or in plasma (6). Finally, the ratio of EC-SOD in the body existing in the tissue interstitium versus the extracellular fluids is much smaller than in other mammals.

To examine the molecular basis of these differences, EC-SOD cDNAs from the rat and mouse were isolated and sequenced. A high degree of similarity between the EC-SOD from these species and from humans was found. However, the deviant properties of rat EC-SOD could be explained by the difference of a single amino acid. Exchange to the amino acid found in human and mouse EC-SODs resulted in the conversion of rat EC-SOD dimer to a tetramer, with high heparin affinity.

MATERIALS AND METHODS

Cloning of the Rat and Mouse EC-SOD Genes and Construction of Expression Plasmids. Primers corresponding to the human EC-SOD cDNA sequence (5′-AAC GAA TTC AGC CG(G/C) GC(G/C) ATC CAC GT(C/G) CA-3′, and 5′-CAC CTC CAT CGG GTT GTA GT-3′) were used to PCR-amplify fragments from rat and mouse genomic DNA. These fragments were used as probes to isolate full-length rat (*Rattus norwegicus*, strain Sprague–Dawley) and mouse EC-SOD (*Mus musculus*, strain 129/SV) cDNA from λgt11 libraries (Clontech) carrying cDNA from uterine tissue. The cDNA fragments were cloned into the expression vector pECE (2). The mouse and rat EC-SOD sequences have the accession numbers X84940 and X94371, respectively, in the EMBL GenBank data base. The four chimeric expression plasmids (HRat, RHuman, MRat, RMouse) were constructed using an internal *Bgl*II-site (see Fig. 1). The four point mutations were generated by PCR, and the mutated DNA fragments were exchanged for the wild-type fragments in the original expression constructs, using the same *Bgl*II-site (Rat S26A, Mouse A26S, Rat D28V, Mouse V28D).

Recombinant Expression of the Different EC-SODs. Chinese hamster ovary (CHO-K1) cells were transfected with the expression plasmids carrying human, mouse, and rat EC-SOD cDNA, the four chimeric EC-SOD cDNAs, and the four cDNAs carrying point mutations using Lipofectamin (GIBCO/Life Technologies, Gaithersburg, MD). The CHO-K1 cells were cultivated in OPTIMEM (GIBCO) without serum, in the presence of 10 mg of pepstatin per liter, 10 mg of chymostatin per liter, and 10 mg of leupeptin per liter to prevent proteolysis of the EC-SODs (12). The conditioned media were harvested 72 h after transfection.

Preparation of EC-SOD from Tissue. Rat and mouse lungs were homogenized in 10 vol of 50 mM potassium phosphate (pH 7.4), with 0.3 M KBr and antiproteolytic agents (0.5 mM

Abbreviation: EC-SOD, extracellular superoxide dismutase.
Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X84940 and X94371).
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phenylmethylsulfonyl fluoride/3 mM diethylenetriamine pentaacetic acid/90 mg of aprotinin per liter/10 mg of pepstatin 10 per liter/10 mg of chymostatin per liter/10 mg of leupeptin per liter) (12). The homogenates were then sonicated, extracted for 30 min at 4°C, and subsequently centrifuged (20,000 × *g* for 15 min).

Separation on Con A-Sepharose. To separate EC-SOD from other SOD isoenzymes, tissue extracts and conditioned media were applied to Con A-Sepharose columns as previously described except that the equilibration buffer used was the same as the one used for tissue extraction (see above) (7). The glycoprotein EC-SOD was eluted with 0.5 M α-methylmannoside.

Heparin-Sepharose Chromatography. After dialysis against 15 mM sodium cacodylate/50 mM NaCl (pH 6.5), fractions from the Con A-Sepharose chromatography were applied to 1 ml heparin-Sepharose columns equilibrated with the same buffer. Bound fractions were eluted with a 0–1 M NaCl gradient.

Size Exclusion Chromatography. After separation on Con A-Sepharose, conditioned cell culture media and lung extracts were concentrated and applied to a Sephacryl S-300 column (1.6 × 90 cm) with 10 mM potassium phosphate, pH 7.4/0.15 M NaCl as eluant. The column was calibrated with IgG (150 kDa), bovine serum albumin (67 kDa), and carbanhydrase (30 kDa).

SOD Activity. SOD activity was determined by the direct spectrophotometric method employing KO_2 (15, 16). One unit corresponds to 8.6 ng of human EC-SOD (1).

RESULTS

Sequence Comparison of Rat, Human, and Mouse EC-SOD. To explore the molecular basis of the deviant physical properties of rat EC-SOD, the cDNAs of the rat and mouse EC-SODs were isolated and the deduced amino acid sequences were compared with human EC-SOD. The overall sequence comparison offers no obvious explanation of the different physical properties and distribution of rat EC-SOD (Fig. 1). The heparin-binding domain of human EC-SOD is located in the carboxyl-terminal end, with a unique cluster of six consecutive positively charged amino acid residues (human EC-SOD amino acid positions 210–215), forming the essential region (5). The carboxyl-terminal ends of rat and mouse EC-SOD are identical and very similar to that of human EC-SOD. The heparin-binding clusters are identical in EC-SOD from all three species. The rat EC-SOD sequence has been presented previously (18, 19), and our sequence analysis confirms the findings presented in ref. 18. According to ref. 19, however, two basic amino acids in the heparin-binding cluster, Lys-217 and Arg-219, are replaced by tryptophans. We have sequenced genomic DNA in this region from six different strains of laboratory rats and also from wild rats, and find that they all carry the sequence presented in Table 1 and ref. 18 (data not shown). By Southern analysis, we have also excluded the presence of a second gene copy (data not shown). We thus conclude that rat EC-SOD is identical to mouse and human EC-SOD in the cluster of six basic amino acids that defines the essential part of the heparin-binding domain. The glycosylation site of human EC-SOD, asparagine-89 (3, 20), is also present in rat and mouse EC-SOD, and the native enzymes are glycosylated (6, 7). According to the deduced amino acid sequences, the EC-SOD subunits should have molecular masses of 24.2–25.4 kDa (Fig. 1). However, upon SDS/PAGE, the subunits display molecular masses of 30–32 kDa (data not shown). Native mouse and human EC-SODs elute from size exclusion chromatography columns at ≈155 kDa. These apparently deviant molecular masses are due to hydrodynamic effects of the carbohydrate moieties, since native mutant nonglycosylated human EC-SOD elutes at 96 kDa as predicted for a tetramer (20). Thus, the apparent molecular masses of 155 kDa for the mouse and human EC-SODs and 85 kDa for the rat EC-SOD (Table 1) suggest that these enzymes are tetrameric and dimeric, respectively. Taken together, this suggests that rat EC-SOD has a low heparin affinity because it is a dimer and not a tetramer.

Analysis of Chimeric EC-SOD Proteins. To search for structural elements that may contribute to this difference in oligomerization, we focused on the extended N terminus of the EC-SODs that have no counterpart in the dimeric cytosolic CuZn-SODs (Fig. 1). The most obvious difference between the EC-SODs is a stretch of amino acids, DRLDVEKI, which is absent from human EC-SOD but exists as one and two repeats in rat and mouse EC-SOD, respectively (Fig. 1). This element is unlikely to cause the differences in subunit organization because it is absent in the human EC-SOD and present in mouse EC-SOD, both of which are tetrameric. To examine the involvement of other amino acids in the N-terminal region, expression plasmids carrying chimeric cDNAs were constructed (Table 1). The proteins were expressed in CHO cells, and the secreted EC-SODs were analyzed by size exclusion and

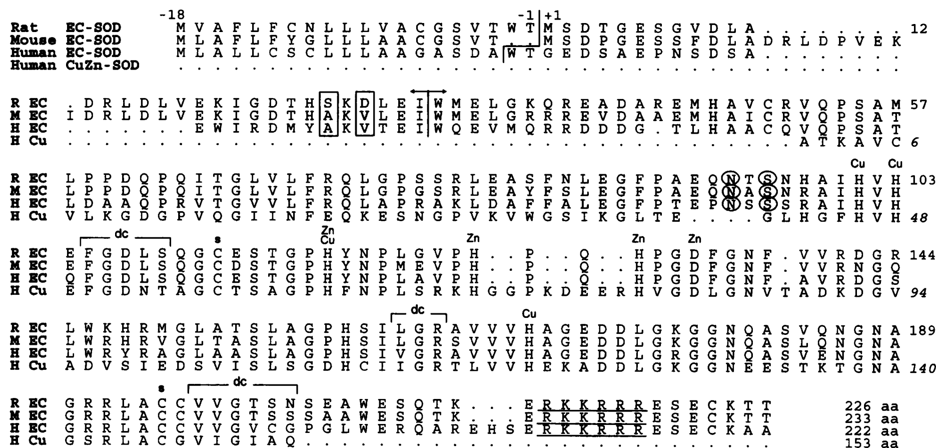

FIG. 1. Alignment of the amino acid sequences of rat, mouse, and human EC-SOD (3), and human cytosolic CuZn-SOD (17). The numbering of the EC-SODs is based on the rat EC-SOD amino acid sequence; numbering in italics, the amino acid positions of the human CuZn-SOD; Cu, Zn, amino acid residues involved in binding of copper and zinc (4); ┌dc┐, residues forming the dimer contact in the cytosolic CuZn-SODs (4); s, residues forming a disulfide bridge (4); ←|→, location of the *Bgl*II site used for the construction of the chimeric EC-SODs; circled amino acids, the glycosylation site of the EC-SODs; underlined amino acids, the heparan sulfate binding cluster in the EC-SODs (5); boxes, amino acids upstream of the *Bgl*II-site that are identical in mouse and human EC-SOD, but different in the rat EC-SOD. The shaded box indicates the site essential for tetramer formation. Because of the high sequence homology, the first N-terminal amino acid in mature mouse EC-SOD was assumed to be the same as in the rat EC-SOD (18).

Table 1. Apparent molecular weights and heparin-affinities of wild-type, chimeric and point-mutated human, rat and mouse EC-SODs

Expressed cDNAs/ EC-SODs	Molecular weight, kDa	Heparin affinity
Human (H) wild type	155	High
Mouse (M) wild type	155	High
Rat (R) wild type	85	Low
H Rat	155	High
R Human	85	Low
M Rat	155	High
R Mouse	85	Low
Rat S26A	85	Low
Mouse A26S	155	High
Rat D28V	155	High
Mouse V28D	85	Low

The apparent molecular weights and heparin-affinities were determined by size exclusion chromatography and heparin-Sepharose chromatography.

heparin-Sepharose chromatography. Chimeric proteins that carry the 31 most N-terminal amino acids from the rat and the remaining sequence from mouse or human EC-SOD (RMouse, RHuman) are dimeric and lack high heparin affinity (Fig. 1 and Table 1). Chimeric proteins carrying the N terminus from mouse or human EC-SOD and the remainder of the rat protein (HRat, MRat) are tetrameric and have a high affinity for heparin (Fig. 1 and Table 1). These results show that structural elements in the extreme N terminus of the protein are involved in tetramer formation in mouse and human EC-SOD.

Analysis of Rat and Mouse EC-SOD Carrying Point Mutations. Among the first 31 amino acids (numbering based on the rat sequence) there are only two positions at which the amino acids are identical in human and mouse EC-SOD but different in rat EC-SOD (Fig. 1). Nucleotide sequence analysis of this region of EC-SOD from other laboratory rat strains and a wild rat showed that these differences are characteristic of all rats, and not only of the highly inbred Sprague–Dawley strain (data not shown). The importance of these two amino acids, Ala-26 and Val-28, in tetramer formation was analyzed by site-directed mutagenesis. In the rat sequence, the amino acids in these positions are serine and aspartate, respectively. Exchanges at position 26 do not change the physical properties of the enzymes (Table 1). In contrast, a valine to aspartate (V28D) exchange in mouse EC-SOD results in a dimeric enzyme which has lost its high heparin affinity (Figs. 2*A* and 3*A*). Strikingly, an aspartate to valine (D28V) exchange in rat EC-SOD converts the rat enzyme into a tetramer with high heparin affinity (Figs. 2*B* and 3*B*). Thus, these results show that a single amino acid substitution can convert the rat EC-SOD dimer into a tetramer with high heparin affinity.

DISCUSSION

This study shows that the failure of rat EC-SOD to form a tetramer can be attributed to the presence of the negatively charged aspartate in position 28 rather than the lipophilic

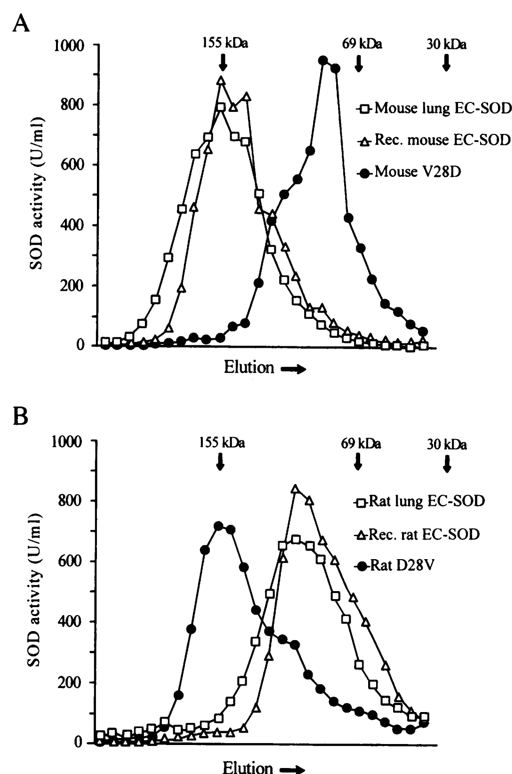

FIG. 2. Size exclusion chromatography displaying native EC-SOD from mouse lung, recombinant wild-type mouse EC-SOD, and the mouse V28D mutant (*A*), and native EC-SOD from rat lung, recombinant wild-type rat EC-SOD, and the rat D28V mutant (*B*).

valine which is found in mouse and human EC-SOD. This does not necessarily mean that the valine is part of the only tetramer-forming contact site, but rather that other interactions alone are not strong enough to ensure tetramer integrity under physiological conditions. With aspartate in position 28, the EC-SOD subunits still combine to form dimers, which must be held together by other interactions (Table 1 and Fig. 2). This suggests that in the tetrameric EC-SODs, the subunits do not form a symmetrical tetrahedron, but rather two interacting dimers.

In all three EC-SODs, there is a central region from His-101 to Gly-199 (numbering based on the rat sequence) that is highly homologous (≈50%) to the active site of the cytosolic dimeric Cu and Zn-containing SODs (3, 4). This and the apparent organization of EC-SOD as two interacting dimers suggest that EC-SOD and the cytosolic dimeric CuZn-SODs have a distant, but common, ancestor, most likely the dimeric bacteriocupreins (21). Indeed, the amino acid residues involved in dimer contact in the CuZn-SODs (4) are identical or very similar to the corresponding residues of EC-SOD (Fig. 1). Fe or Mn-containing SODs also occur as dimers and tetramers (22–25). Similarly, tetrameric Mn-SODs have been shown to be composed of two interacting dimers (26, 27), in which the intrinsic dimer interface is structurally similar to that of the dimeric Mn-SODs.

The physiological significance of the tetramerization of EC-SOD is unknown. However, the present results lend further support to the notion that a cooperative action of the heparin-binding domains of all four subunits is necessary for high heparin affinity. The physiological ligand of EC-SOD is another sulfated glycosaminoglycan, heparan sulfate, which binds EC-SOD with lower affinity than heparin (28). Rat EC-SOD appears to lack significant affinity for heparan sulfate proteoglycans under physiological conditions, resulting in a drastically altered distribution of the enzyme in the body. Thus, there is no heparin-induced increase in plasma EC-SOD

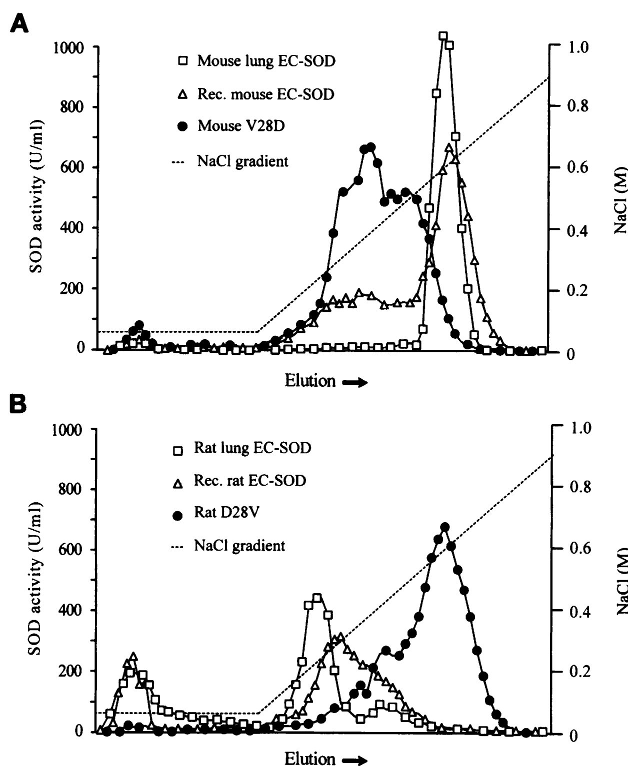

FIG. 3. Heparin-Sepharose chromatography of native EC-SOD from mouse lung, recombinant wild-type mouse EC-SOD, and the mouse V28D mutant (*A*), and native EC-SOD from rat lung, recombinant wild-type rat EC-SOD, and the rat D28V mutant (*B*). Large amounts of protein elute early in the NaCl gradient when the lung extracts are applied. Interference by this bulk of protein may be the cause of the difference in elution profiles between rat EC-SOD from the lung extract and from the conditioned medium which contains much less protein.

in the rat, showing that no enzyme is bound to the glycocalyx of the endothelium (6). The retention of EC-SOD in the connective tissue interstitium is critically dependent on binding to heparan sulfate proteoglycans (8). The lack of such tissue retention explains the low EC-SOD content in rat tissues and the high content in plasma (6, 7). Compared with most other species, the rat tissue interstitium is thus poorly enzymatically protected against superoxide radicals.

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