The Structure of Rabbit Extracellular Superoxide Dismutase Differs from the Human Protein†

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ABSTRACT: The cDNA sequence encoding rabbit, mouse, and rat extracellular superoxide dismutase (EC-SOD) predicts that the protein contains five cysteine residues. Human EC-SOD contains an additional cysteine residue and folds into two forms with distinct disulfide bridge patterns. One form is enzymatically active (aEC-SOD), while the other is inactive (iEC-SOD). Due to the lack of the additional cysteine residue rabbit, mouse, and rat EC-SOD are unable to generate an inactive fold identical to human iEC-SOD. The amino acid sequences predict the formation of aEC-SOD only, but other folding variants cannot be ruled out based on the heterogeneity observed for human EC-SOD. To test this, we purified EC-SOD from rabbit plasma and determined the disulfide bridge pattern. The results revealed that the disulfide bridges are homogeneous and identical to human aEC-SOD. Four cysteine residues are involved in two intra-disulfide bonds while the C-terminal cysteine residue forms an intersubunit disulfide bond. No evidence for other folding variants was detected. These findings show that rabbit EC-SOD exists as an enzymatically active form only. The absence of iEC-SOD in rabbits suggests that the structure and aspects of the physiological function of EC-SOD differs significantly between rabbit and humans. This is an important notion to take when using these animals as model systems for oxidative stress.

The family of superoxide dimutase (SOD; EC 1.15.1.1) enzymes converts the superoxide radical to hydrogen peroxide and oxygen (*1*). In mammals, two isoforms of SOD contain copper and zinc atoms. The copper atom is directly involved in the enzymatic process of superoxide dismutation. Cu/Zn-SOD (SOD1) is located in intracellular compartments including the cytosol, the nucleus, and the intermembrane space of mitochondria (*2*−*4*), whereas extracellular SOD (EC-SOD; SOD3)[1] is predominantly found in the extracellular matrix of tissues (*5*−*8*). Recently, EC-SOD was also found to be located in the nucleus (*9*). SOD1 is a homodimer of 32 kDa with a subunit structure represented by a compact and highly stable β-barrel structure (*10*, *11*), while EC-SOD is a tetramer of 135 kDa (*12*). The primary structure of the central part of human EC-SOD (His96-Gly193) is homologous to SOD1 and contains all the residues essential for the coordination of the Cu(II) and Zn(II) ions (*13*). However, it is not clear whether SOD1 and EC-SOD have similar tertiary structures because no structural data exists for the central part of EC-SOD. The N-terminal region of EC-SOD is important for the formation of tetramers (*14*, *15*). Interestingly, rat EC-SOD was found to form a dimer only (*16*). However, a single amino acid substitution in the N-terminal region (Asp → Val) converts the protein into a tetramer (*17*). The C-terminal region of EC-SOD contains a high amount of basic amino acid residues (*13*). This polybasic region is involved in the binding to heparin/heparan sulfate (*18*, *19*) and type I collagen (*20*, *21*) and is referred to as the extracellular matrix (ECM)-binding region (*21*). We have previously shown that this region can be proteolytically removed immediately before secretion and that this process is a two-step event involving an initial cut by furin or another member of the proprotein convertase family of processing proteinases followed by the action of an unknown Arg/Lys-specific carboxypeptidase (*22*−*24*). This generates both intact and cleaved subunits of EC-SOD. It is thus possible to generate EC-SOD tetramers with no (type A), intermediate (type B), and high (type C) affinity for the ECM depending on the ratio between intact and cleaved subunits (*25*).

Mature human EC-SOD contains six cysteine residues (*13*) (Figure 1). We have recently shown that human EC-SOD exists as two different forms with distinct disulfide bridge patterns (*26*). One form has SOD activity (aEC-SOD) and has a free cysteine residue in position 195 (Figure 1). Based on sequence homology to SOD1, we suggested that Cys107 and Cys189 formed the intrasubunit disulfide bridge essential for SOD activity (*26*). The other form of human EC-SOD has a free cysteine residue at position 45 and is inactive (iEC-SOD). This form contains an intrasubunit disulfide bridge between Cys107 and Cys195. This finding correlates with

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[1] Abbreviations: EC-SOD, extracellular superoxide dismutase; ECM, extracellular matrix; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; SOD1, CuZn-SOD.

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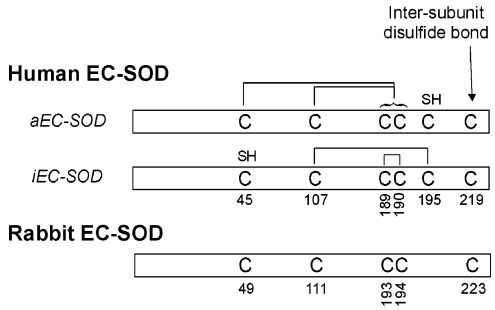

FIGURE 1: Schematic representation of the cysteine residues of human and rabbit EC-SOD. The exact connectivity of human aEC-SOD was not previously established (*26*). The C-terminal Cys219 of human EC-SOD forms an intersubunit disulfide bond as indicated. The primary sequence of rabbit EC-SOD does not contain the cysteine residue homologous to Cys195 of human EC-SOD.

the lack of SOD activity due to the absence of the predicted Cys107/Cys189 disulfide bridge.

The amino acid sequence of rabbit EC-SOD does not contain a cysteine residue homologous to Cys195 in human EC-SOD (*27*) (Figure 1) and rabbits are thus unable to produce iEC-SOD identical to human EC-SOD (*26*). In addition, the specific SOD activity of rabbit EC-SOD was approximately 2-fold higher than that of human EC-SOD. To determine the relationship between these observations, we purified rabbit EC-SOD and determined the disulfide bridge pattern. The analyses showed that the disulfide bridge pattern of rabbit EC-SOD was homogeneous and identical to that of human aEC-SOD with no evidence of other disulfide variants. Apparently, rabbits do not generate an inactive variant of EC-SOD, and the ability to do so may depend on the presence of Cys195. The absence of iEC-SOD in rabbits suggests that aspects of the biological functions and regulation of the EC-SOD activity in humans and rabbits may differ. This may have important implications when using rabbits as model systems for oxidative stress.

MATERIALS AND METHODS

Reagents. Rabbit plasma was from Pel Freeze. Sequence grade porcine trypsin was purchased from Promega. Human EC-SOD was purified from aorta as previously described, except that cation-exchange chromatography was omitted (*28*). Cytochrome *c* and xanthine were obtained from Sigma. Xanthine oxidase was from Roche.

Purification of Rabbit EC-SOD. Approximately 500 mL of rabbit plasma was centrifuged at 13 000*g* for 30 min. To the supernatant, PEG-8000 was added to a final concentration of 8% (w/v), and the sample was left stirring for 1 h at 4 °C. The precipitate was collected by centrifugation at 13 000*g* for 30 min and discarded. The supernatant was brought to 20% (w/v) PEG-8000 and left for 1 h at 4 °C as described above. After a final centrifugation, the pellet was recovered and resuspended in 20 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, pH 7.4. The material was then applied to a 200 mL heparin−Sepharose column (20 mm × 160 mm) preequilibrated in 20 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, pH 7.4. The column was subsequently washed using the same buffer until the absorbance (A_{280}) of the eluate was below 0.01. Heparin-binding proteins were eluted using a linear gradient of NaCl from 50 mM to 1 M in 20 mM Tris-HCl, pH 7.4, at a flow rate of 40 mL/h. Fractions of 4 mL were collected and analyzed by SDS−PAGE and for SOD activity using the xanthine oxidase/cytochrome *c* assay (*29*). Fractions containing rabbit EC-SOD were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4. The material was further fractionated by anion-exchange chromatography using a 5 mL HiTrap Q column connected to a fast protein liquid chromatography (FPLC) system (Amersham Biosciences). The column was preequilibrated in 20 mM Tris-HCl, pH 7.4, and operated at 2 mL/min. After sample application, the column was washed in 20 mM Tris-HCl, pH 7.4, and developed using a linear gradient from 0 to 500 mM NaCl in 20 mM Tris-HCl, pH 7.4. Fractions of 2 mL were collected and analyzed for the presence of rabbit EC-SOD as described above. Selected fractions were pooled and concentrated to ~600 μL using a Centriprep YM-10 (Millipore). The sample was finally subjected to size-exclusion chromatography using a Superose 12 HR 10/30 column (Amersham Biosciences). The column was equilibrated in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.4, and connected to a FPLC system operated at 200 μL/min. Fractions containing EC-SOD were identified as above and pooled accordingly. The material was stored at −20 °C until further use.

Polyacrylamide Gel Electrophoresis. Proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 5−15% gradient gels and the glycine/2-amino-2-methyl-1,3-propanediol-HCl system described by Bury (*30*). Prior to electrophoresis, samples were boiled in the presence of 1% SDS. For reducing conditions, 30 mM dithiotreitol (DTT) was included. Nondenaturing PAGE was performed in the same system omitting boiling of samples prior to analysis and SDS from all buffers used.

Separation of Rabbit EC-SOD by Reverse-Phase High-Performance Liquid Chromatography (HPLC). Rabbit EC-SOD was subjected to reverse-phase HPLC chromatography as previously described (*26*). Approximately 40 μg of purified rabbit EC-SOD in 50 mM HEPES, 150 mM NaCl, pH 7.4, was acidified by the addition of trifluoroacetic acid (TFA) and applied to a 2.1 mm × 220 mm Aquapore RP-300 C_8 reverse-phase HPLC column (Brownlee Labs). Bound proteins were eluted using a two-step linear gradient as described previously (*26*) using solvent A (0.1% TFA) and solvent B (90% acetonitrile, 0.08% TFA). The column was operated at 23 °C at a flow rate of 200 μL/min. Protein was detected at 220 and 280 nm, and fractions were collected manually.

Alkylation and Reverse-Phase HPLC Analysis of Monomeric Rabbit EC-SOD. The collected fraction containing monomeric rabbit EC-SOD was lyophilized and redissolved in 30 mM HEPES, pH 8.3, containing 5 M guanidinium hydrochloride and 25 mM iodoacetamide, as previously described (*26*). The sample was then acidified by the addition of TFA and subjected to reverse-phase HPLC analysis as described above.

Tryptic Digestion of Monomeric Rabbit EC-SOD. The collected *S*-carboxyamidomethylated monomeric rabbit EC-SOD was digested overnight at 37 °C in 0.5 M HEPES, pH 8.3, using porcine trypsin with an approximate weight ratio of 1:20. The sample was subsequently lyophilized and rehydrated in 0.1% TFA. The tryptic peptides were separated

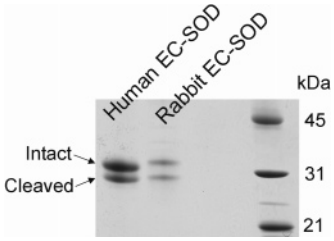

FIGURE 2: The specific activity of rabbit EC-SOD is higher than human EC-SOD. The SOD-acitivity of plasma-derived rabbit EC-SOD and human EC-SOD isolated from aorta tissue was evaluated using the xanthine oxidase/cytochrome *c* assay. EC-SOD from both preparations corresponding to 100 U of SOD activity was subjected to SDS−PAGE analysis. The gel was stained by Coomassie Brilliant Blue. A marker is indicated on the right.

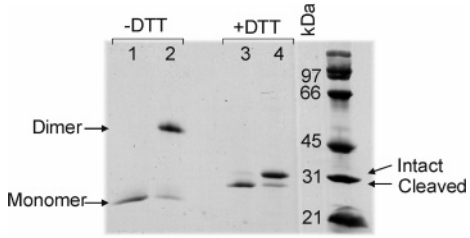

FIGURE 3: Rabbit EC-SOD exists as a disulfide-linked homodimer. Fractions collected from reverse-phase HPLC analysis of rabbit EC-SOD were subjected to SDS−PAGE analysis in nonreducing (−DTT) and reducing (+DTT) conditions. Lanes 1 and 3 represent fraction 1, and lanes 2 and 4 represent fraction 2. Dimeric and monomeric rabbit EC-SOD identified by nonreducing conditions are indicated on the left. The intact and cleaved subunits detected by reducing conditions are indicated on the right. A molecular weight marker is included. The gel was stained with Coomassie Brilliant Blue.

by reverse-phase HPLC and collected manually using the HPLC system described above.

Analyses of the Tryptic EC-SOD Triple Peptide. The fraction obtained by reverse-phase HPLC containing the disulfide-linked triple peptide (i.e., the three cysteine-containing peptides connected by disulfide bridges, see Results) was lyophilized and rehydrated in 200 μL of 0.1% TFA. The peptide was applied to a preactivated ProSorb membrane (Applied Biosystems). The membrane was washed in 0.1% TFA and subjected to three cycles of automated Edman degradation as previously described (*28*). The membrane was subsequently removed from the sequencing cartridge, rinsed with water, and incubated in extraction buffer (150 μL 50 mM Tris-HCl, pH 8.5, containing 40% acetonitrile) for 3 h at 37 °C. The extraction buffer was subsequently removed, and the membrane was lyophilized and rehydrated in 30 μL of 0.1% TFA. The extracted peptides were concentrated using C_{18} ZipTip (Millipore) according to manufacturer's instructions. Bound peptides were eluted using 1 μL of matrix solution (0.4% α-cyano-4-hydroxy-cinnamic acid in 70% (v/v) acetonitrile, 30% (v/v) 0.1% TFA) and spotted onto the target used for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Mass Spectrometric Analysis. Peptides were analyzed by MALDI-MS using a quadropole/time-of-flight (Q-TOF) *Ultima* Global mass spectrometer (Micromass) with α-cyano-4-hydroxycinnamic acid (Sigma) as the matrix. Prior to analysis, the mass spectrometer was calibrated using a mixture of PEG-200, -600, -1000, and -2000 and NaI. Identification of the peptides was performed by using the GPMAW software (http://welcome.to/gpmaw).

RESULTS

Specific Activity of Human and Rabbit EC-SOD. EC-SOD was purified from rabbit plasma because the concentration of EC-SOD in plasma is relatively high in rabbits compared to other mammals (*6*). The specific activity of purified plasma-derived rabbit EC-SOD was compared to human EC-SOD. The activities of the preparations of human EC-SOD and rabbit EC-SOD obtained from size-exclusion chromatography were evaluated by using the xanthine oxidase/cytochrome *c* assay. The amount of EC-SOD corresponding to the same activity (100 U) from both preparations was subjected to SDS−PAGE analysis (Figure 2). The amount of EC-SOD was estimated by Coomassie blue staining. Visual inspection of the bands suggested that a significantly higher amount of human EC-SOD was applied (>2-fold) (Figure 2). It was apparent that the specific activity of rabbit EC-SOD isolated from plasma is higher than that of human EC-SOD isolated from aorta.

Intact Rabbit EC-SOD Is a Disulfide-Linked Dimer. To further investigate the basis of the difference in specific activity, we subjected rabbit EC-SOD to reverse-phase HPLC analysis. We have previously shown that monomeric and dimeric human EC-SOD can be separated using this methodology (*26*). The analysis of rabbit EC-SOD produced two peaks (fractions 1 and 2) eluting at positions corresponding to those of monomeric and dimeric human EC-SOD, respectively (data not shown). When fraction 1 was analyzed by SDS−PAGE under nonreducing conditions, a single band of 26 kDa was detected (Figure 3). Following reduction, the band migrated as a 28 kDa band. This material represents monomeric and cleaved rabbit EC-SOD. Fraction 2 contained a single band of 55 kDa when analyzed in nonreducing conditions (Figure 3). This band corresponds to the size of dimeric rabbit EC-SOD. After reduction, the material in fraction 2 migrated as a major band of 32 kDa and a minor band of 28 kDa, corresponding to intact and cleaved subunits of rabbit EC-SOD, respectively (Figure 3). The presence of trace amounts of the cleaved subunit is likely due to the inability to achieve baseline separation during reverse-phase HPLC chromatography. These analyses show that the intact form of rabbit EC-SOD is a disulfide-linked homodimer, whereas the cleaved subunit exists as a monomer. By homology to human and mouse EC-SOD (*28*, *31*), it is likely that the intersubunit disulfide bond of rabbit EC-SOD is formed by the C-terminal Cys223 (see Figure 1).

Tryptic Digest of Monomeric Rabbit EC-SOD. To analyze the disulfide connectivity of rabbit EC-SOD, we used the material collected in fraction 1. Because this material represents cleaved rabbit EC-SOD, the ECM-binding region containing Cys223 has been removed. Therefore, the cleaved subunit of rabbit EC-SOD contains only four cysteine residues (see Figure 1). The collected material was digested with trypsin, thus producing three cysteine-containing peptides of which one contains the two vicinal cysteines, Cys193 and Cys194. The generated peptides were subsequently separated by reverse-phase HPLC (Figure 4). The collected peptides were analyzed by MALDI-MS and assigned to the sequence of rabbit EC-SOD (*27*). The cysteine-containing peptides were only detected in peak 11 (Figure 4). The

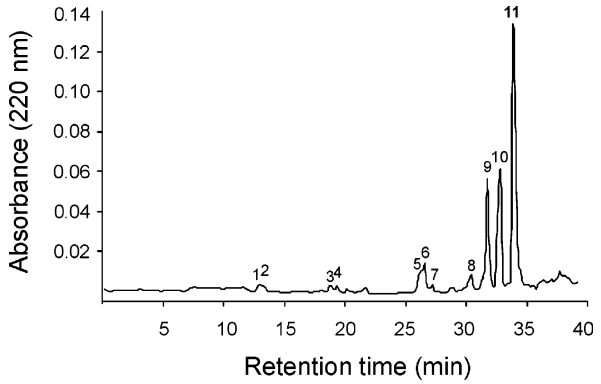

FIGURE 4: Separation of tryptic rabbit EC-SOD peptides by reverse-phase HPLC. Rabbit EC-SOD was digested with trypsin, and the generated peptides were separated by HPLC using a C_8 reverse-phase column. Numbered peaks were analyzed by MALDI-MS. The peak encompassing all cysteine-containing peptides is shown in bold (peak 11).

MALDI-MS analysis of the material in nonreducing conditions presented the combined mass of all three peptides (Table 1). The analysis of the reduced material produced the masses of the individual peptides (Table 1). These analyses indicate that the peptides are connected by disulfide bonds.

The Disulfide Connectivity of Rabbit EC-SOD. The isolated complex containing the three cysteine-containing tryptic peptides of rabbit EC-SOD can be connected in two different ways: Cys49/Cys194 and Cys111/Cys193 (possibility 1) or Cys49/Cys193 and Cys111/Cys194 (possibility 2) (Figure 5). To establish the exact connectivity, we used a combination of Edman degradation and mass spectrometry. The sample was initially applied to a ProSorb membrane and subjected to three cycles of automated Edman degradation. Three N-terminal residues are thus removed from each of the peptides. The third position of the peptide (Leu191−Arg206) containing the vicinal cysteines is occupied by Cys193. This residue is removed by Edman chemistry but remains bound as a phenylthiohydantoin-derivative to the peptide containing Cys111 (possibility 1) or Cys49 (possibility 2) (Figure 5). The remainder of the peptide containing Cys194 is connected to the peptide containing Cys49 (possibility 1) or Cys111 (possibility 2). After the three cycles of Edman degradation, the peptides were eluted from the ProSorb membrane and analyzed by MALDI-MS. The mass of the peptide was 2448.18 Da (Table 2). This corresponds to the combined mass of the peptides containing Cys49 and Cys194. Masses of 1180.58 and 1270.63 Da corresponding to the individual peptides containing Cys49 and Cys194,

Table 1: Assignment of Cysteine-Containing Peptides Detected in Peak 11[a]

oxidation	obsd mass (Da)	assignment	theoretical mass (Da)
unreduced	7432.32 (avg)	Cys49 + Cys111 + Cys193/194	7432.30 (avg)
reduced	1450.72 (mo)	Cys49	1450.72 (mo)
	1557.76 (mo)	Cys193/194	1557.77 (mo)
	4428.20 (avg)	Cys111	4427.83 (avg)

[a] The material in peak 11 in Figure 4 was analyzed by MALDI-MS before and after reduction. The observed masses are given as monoisotopic (mo) or average (avg) masses and assigned to peptides containing the indicated cysteine residues.

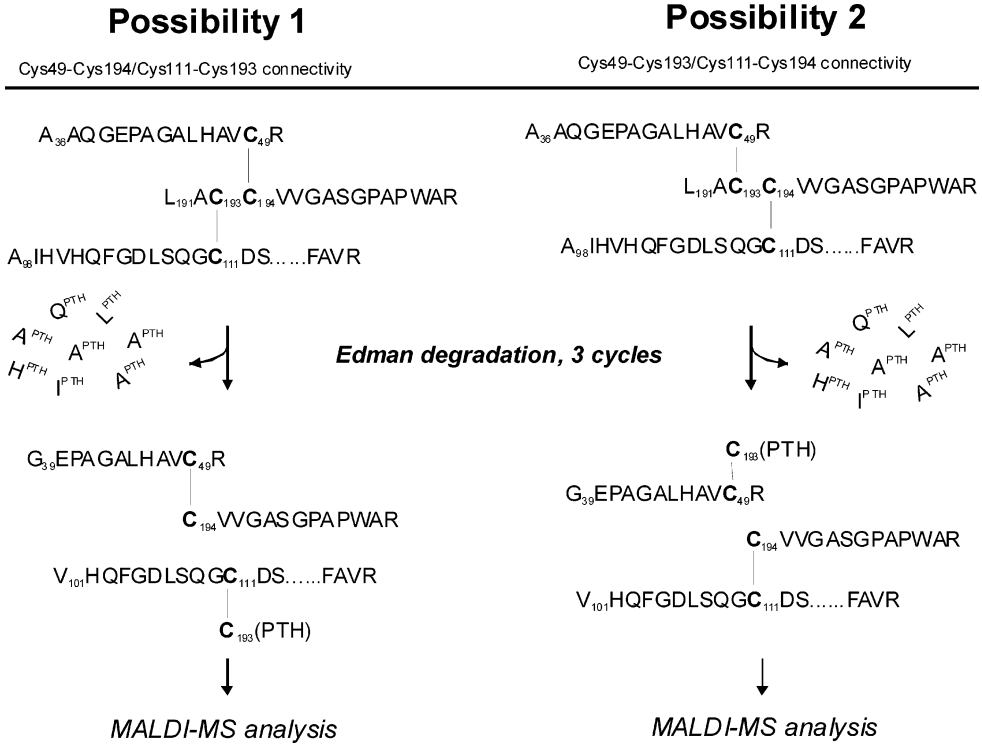

FIGURE 5: Schematic representation for determination of cysteine connectivity. The two possibilities for connecting all three cysteine-containing tryptic peptides of rabbit EC-SOD are depicted. Three N-terminal amino acid residues of each peptide in the complex are removed as phenylhydantoin (PTH) derivatives by three cycles of Edman degradation. Cys193 remains disulfide-bonded as a PTH derivative. This procedure separates the isolated triple peptide into two distinct disulfide-linked double peptides. The material is subsequently analyzed by MALDI-MS. The exact cysteine connectivity can be delineated because each possibility generates a distinct mass pattern (see Table 2).

Table 2: Theoretical and Observed Masses of Peptides Depicted in Figure 4[a]

	masses of cysteine-containing peptides generated				combined mass
	Cys49	Cys111	Cys193(PTH)	Cys194	
possibility 1	1180.59			1270.64	2448.20
		4103.88	356.02		
possibility 2	1180.59		356.02		
		4103.88		1270.64	5371.51
observed	1180.58	*b*	*b*	1270.63	2448.18

[a] The triple peptide of rabbit EC-SOD was subjected to three cycles of Edman degradation and subsequently analyzed by MALDI-MS. The theoretical monoisotopic masses of the individual peptides representing possibilities 1 and 2 are indicated. The combined masses representing the potential disulfide-linked peptides are also indicated. The observed masses are given as monoisotopic. [b] Not determined.

respectively, were also detected. The presences of these masses are due to dissociation of the disulfide bonds during MALDI-MS analysis (*32*). These data show that the cysteines of rabbit EC-SOD are connected as depicted in possibility 1 (Figure 5). Using the same procedure for human aEC-SOD, we established that Cys45/Cys190 and Cys107/Cys189 are connected as previously suggested (data not shown) (*26*). The disulfide bridge pattern of rabbit EC-SOD thus corresponds to that of human aEC-SOD.

DISCUSSION

We have recently shown that the disulfide bridge pattern of human EC-SOD is heterogeneous (*26*). Because EC-SOD from mouse, rat, and rabbit contains one cysteine residue less than human EC-SOD, the disulfide bridge patterns of EC-SOD from these species are likely to be different (Figure 1). To investigate this hypothesis, we purified EC-SOD from rabbit plasma. Rabbit EC-SOD subunits could be separated into monomers and dimers by using reverse-phase HPLC. The monomeric material represents the cleaved subunit lacking the ECM-binding region, while the dimer represents that intact subunit. We have previously suggested that rabbit EC-SOD exists as a monomer only (*33*). However, the present study shows that rabbit EC-SOD forms an intersubunit disulfide bond homologous to human (*28*), mouse (*31*, *34*), and rat (*16*, *17*) EC-SOD. This disulfide bond is likely to be established between the C-terminal cysteine residues, as shown for human EC-SOD (*26*). The analysis of monomeric rabbit EC-SOD by nonreducing SDS−PAGE produced one band only (Figure 3). This is in contrast to analysis of monomeric human EC-SOD, which produces a closely spaced doublet representing aEC-SOD and iEC-SOD (*26*). In addition, monomeric rabbit EC-SOD could not be separated into iEC-SOD and aEC-SOD by reverse-phase HPLC after *S*-carboxyamidomethylation as previously shown for human EC-SOD (*26*) (data not shown). These analyses indicate that only one form of rabbit EC-SOD exists. This hypothesis was subsequently verified using a combination of enzymatic digestion, Edman degradation, and mass spectrometry. It was established that Cys49/Cys194 and Cys111/Cys193 were disulfide-bonded (Figure 6A). We did not find any evidence for a heterogeneous disulfide bridge pattern as is seen with human EC-SOD. Using the same methodology, we established a homologous disulfide bridge pattern for human aEC-SOD (Figure 6B). The presence of the Cys111/Cys193 disulfide of rabbit EC-SOD supports enzymatic activity, as the homologous disulfide bond is known to maintain an active structural fold of SOD1 (*10*). EC-SOD purified from human aorta tissue is composed of approximately equal amounts of iEC-SOD and aEC-SOD (*26*). It is thus likely that the specific SOD activity of human EC-SOD is lower than that of rabbit EC-SOD. Indeed we found that the specific activity of rabbit EC-SOD was approximately 2-fold higher as compared to human EC-SOD (Figure 2).

Plasma-derived rabbit EC-SOD was found to contain approximately equal amounts of intact and cleaved subunits (Figure 2). The analysis of rabbit EC-SOD shows that the native protein is a tetramer homologous to human and mouse EC-SOD (*12*, *31*, *34*) (data not shown). These findings suggest that the purified material is type B EC-SOD composed of both intact and cleaved subunits (*25*). However, the heparin affinity of the type B-variants of human and rabbit EC-SOD does not appear to be different as determined by heparin−Sepharose affinity chromatography (*35*). It is thus interesting to note that the plasma level of rabbit EC-SOD is significantly higher than that of human EC-SOD (*6*). It could be speculated that the absence of iEC-SOD modulates the affinity of rabbit EC-SOD for ECM-binding partners, for example, collagen.

By searching the GenBank for sequences homologous to human EC-SOD, we identified two clones representing pig (accession no. BX922265) and cow (accession no. CB468620) EC-SOD. In addition, by using the Ensembl genome browser (www.ensembl.org), we identified the gene encoding chicken EC-SOD (Ensembl gene annotation ENSGALG00000014393). The primary sequence of EC-SOD from these animals and the published sequences of human, rabbit, mouse, and rat EC-SOD are aligned in Figure 6C. The sequences of EC-SOD from human, pig, cow, and chicken contain six cysteine residues whereas those of rabbit, mouse, and rat EC-SOD contain only five. Based on homology to human and rabbit EC-SOD, respectively, it is hypothesized that pig, cow, and chicken have the capacity to generate both active and inactive EC-SOD, while mouse and rat can generate the active form only. As previously discussed, the presence of two variants is likely to change the biological properties of EC-SOD as compared to the presence of one variant (*26*). In an evolutionary context, the ability to produce aEC-SOD and iEC-SOD could either be acquired in humans, cows, pigs, and chickens or lost in mice, rats, and rabbits. However, due to the distant evolutionary relationship between mammals and birds, it is likely that the ability to generate iEC-SOD has been lost in rabbits, mice, and rats. The biological significance of this is currently unknown.

We have shown that rabbit EC-SOD exists in one folding variant supporting SOD activity. This contrasts with human EC-SOD that exists in two folding variants of which one is enzymatically inactive (*26*). As previously discussed, human EC-SOD may have the capacity to generate tetramers with variable SOD activity (*26*). However, due to the absence of iEC-SOD, rabbits can only generate fully active tetramers. Although the average activity of EC-SOD in tissues from humans and rabbits was found to be similar (*6*), it may be

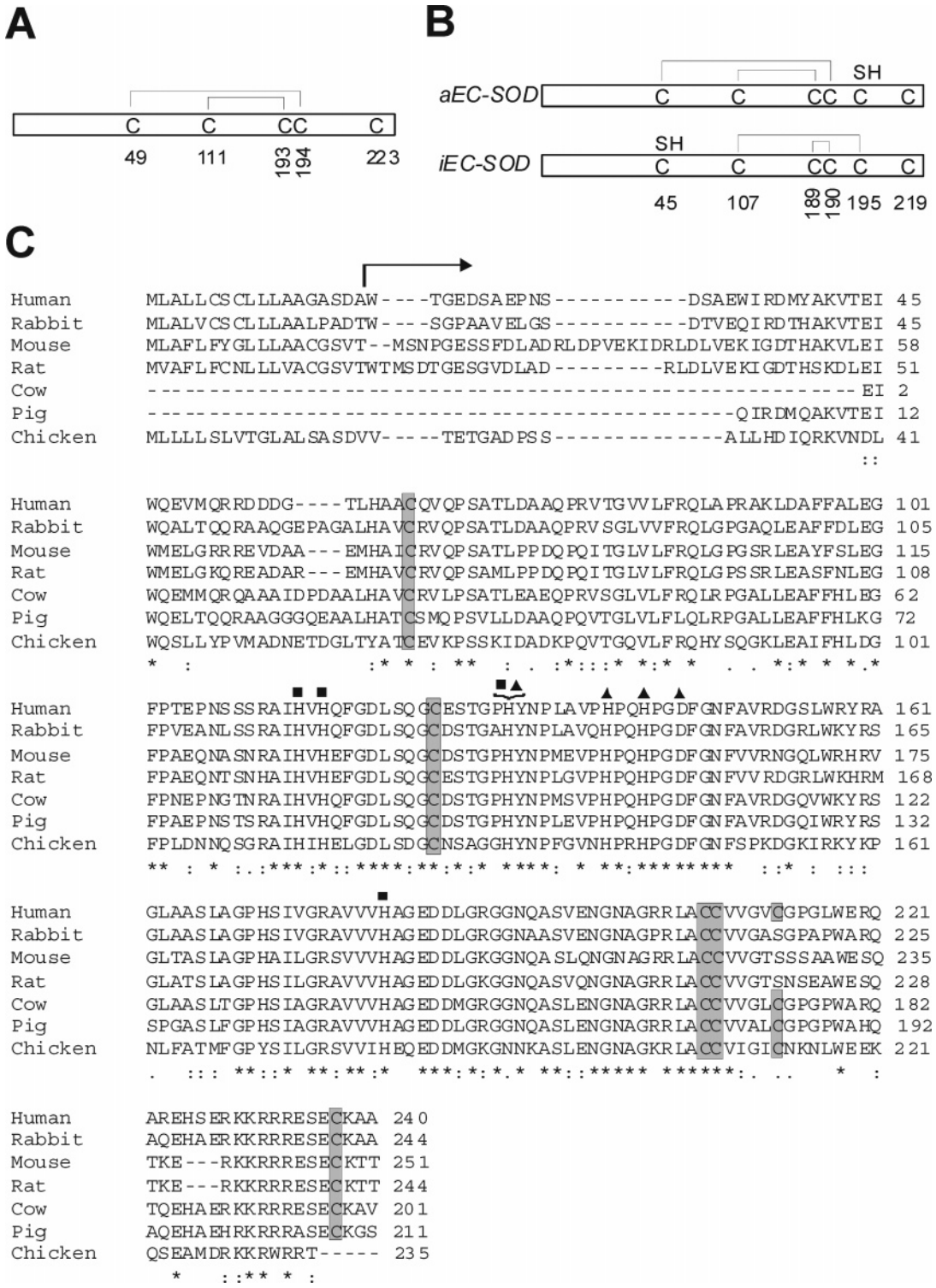

FIGURE 6: Structure of EC-SOD from seven species. Panel A shows a depiction of the disulfide bridge pattern of rabbit EC-SOD. The formation of homodimers is supported by an intersubunit disulfide between Cys223. Panel B shows a depiction of the established disulfide bridge pattern of human aEC-SOD and iEC-SOD. Cys219 is involved in an intersubunit disulfide bond. In panel C, the amino acid sequences (including signal peptide) of human (P08294), rabbit (P41975), mouse (O09164), and rat (Q08420) and the translated nucleotide sequences of pig (GenBank BX922265), cow (GenBank CB468620), and chicken (Ensembl gene annotation ENSGALG00000014393) were aligned using the ClustalW software (*36*). The mature N-terminus of human and rabbit EC-SOD is indicated by an arrow. Cysteine residues are marked by shaded boxes, and residues involved in the coordination of the zink (▲) and copper (■) atoms are indicated.

that humans have the capacity to regulate this activity locally by changing the ratio between aEC-SOD and iEC-SOD. Moreover, because the folding of iEC-SOD is different from aEC-SOD, human EC-SOD may interact differently with extracellular matrix components as compared to rabbit EC-SOD. It is thus important to emphasize that the biological and physiological properties of human EC-SOD, and likely EC-SOD from pig, cow, and possibly chicken (Figure 6C), are significantly different from those of rabbit EC-SOD. This is an important notion to take when analyzing the data obtained from disease models established in, for example, rabbits or mice.

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