The Folding of Human Active and Inactive Extracellular Superoxide Dismutases Is an Intracellular Event*

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Human extracellular superoxide dismutase (EC-SOD) is a tetrameric glycoprotein responsible for the removal of superoxide generated in the extracellular space. Two different folding variants of EC-SOD exist based on the disulfide bridge connectivity, resulting in enzymatically active (aEC-SOD) and inactive (iEC-SOD) subunits. As a consequence of this, the assembly of the EC-SOD tetramers produces molecules with variable activity and may represent a way to regulate the antioxidant level in the extracellular space. To determine whether the formation of these two folding variants is an intra- or extracellular event, we analyzed the biosynthesis in human embryonic kidney 293 cells expressing wild-type EC-SOD. These analyses revealed that both folding variants were present in the intra- and extracellular spaces, suggesting that the formation is an intracellular event. To further analyze the biosynthesis, we constructed mutants with the capacity to generate only aEC-SOD (C195S) or iEC-SOD (C45S). The expression of these suggested that the cellular biosynthetic machinery supported the secretion of aEC-SOD but not iEC-SOD. The coexpression of these two mutants did not affect the expression pattern. This study shows that generation of the EC-SOD folding variants is an intracellular event that depends on a free cysteine residue not involved in disulfide bonding.

Human extracellular superoxide dismutase (EC-SOD2; SOD3) is present in the extracellular matrix of tissues, where it functions as a scavenger of the superoxide radical (1–3). The protein is a highly stable metalloenzyme containing copper and zinc ions, which support enzymatic and structural activities, respectively, and is predominantly found as a tetramer, although an octamer can be detected in EC-SOD purified from human tissue (4–6). The EC-SOD subunit contains three distinct functional regions: the N-terminal region, which is involved in the tetramerization of subunits (7, 8); the central region, which contains amino acid residues essential for the coordination of metals (9); and the C-terminal region, which harbors the affinity for ligands in the extracellular space, including heparan sulfate (10, 11), type I collagen (12), and hyaluronan (13). The C-terminal region can be proteolytically removed intracellularly, resulting in the secretion of an intact subunit (Trp1–Ala222)3 and a cleaved subunit (Trp1–Glu209) (14–16).

The primary sequence of human EC-SOD contains six cysteine residues (9). One cysteine residue (Cys219) is involved in the formation of disulfide-linked homodimers (5), and the remaining five residues are disulfide-bonded in two different ways, generating distinct folding variants (17). One isozyme, aEC-SOD is active, whereas the other isoform, iEC-SOD, is not. Interestingly, the primary sequence of EC-SOD from rabbit (Swiss-Prot accession number P41975), mouse (accession number O09164), and rat (accession number Q08420) does not contain a specific cysteine residue essential for the formation of iEC-SOD, indicating that these species produce only aEC-SOD (18).

Disulfide bonds of secreted proteins are generated in the endoplasmic reticulum by the action of thiol isomerase enzymes, including protein-disulfide isomerase (19). Although the majority of thiol isomerase enzymes are present in the endoplasmic reticulum, increasing evidence suggests that these proteins are also present in the extracellular space (20). The formation of disulfide bonds may serve several functions: (i) to confer stability to the overall protein structure; (ii) to participate directly in protein activity as seen in thioldisulfide oxidoreductases, including thioredoxin; and (iii) to allosterically regulate protein activity by the reduction/oxidation of cysteines not directly participating in protein activity (21). The presence of extracellular thiol isomerases and allosteric disulfide bonds demonstrates that the activity of extracellular proteins can be modulated by changing the redox status of specific cysteine residues. This type of regulation is now becoming evident in a number of proteins, including tissue factor (22) and CD4 (23).

The two EC-SOD folding variants have been identified by analyzing authentic EC-SOD purified from human aorta (24),...
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but it has until now been unclear whether the generation of aEC-SOD and iEC-SOD is an intra- or extracellular event. In this study, we present evidence that both variants are synthesized intracellularly. Moreover, our analyses indicate that the correct assembly of disulfide bridges in iEC-SOD depends on a free cysteine residue not involved in disulfide bonding, further supporting a role for thiol/disulfide exchange reactions in the biosynthesis of EC-SOD.

EXPERIMENTAL PROCEDURES

Materials—Porcine trypsin (sequence grade) was purchased from Promega. A peptide representing Ala94–Cys107 (AIH-VHQFGDLSQLC) of human EC-SOD was coupled to keyhole limpet hemocyanin by m-maleimidobenzoyl-N-hydroxysuccinimidyl ester chemistry, and the keyhole limpet hemocyanin–peptide conjugate was subsequently used as an antigen emulsified in Freund’s adjuvant (Sigma Genosys). The generated rabbit antiserum detects both aEC-SOD and iEC-SOD by Western blotting following separation of nonreduced tryptic digests by electrophoresis using a Tris/Tricine/SDS buffer system (see below).

Proteins—EC-SOD was purified from human aorta or from cell culture supernatants using heparin affinity chromatography and anion exchange chromatography as described previously (5). The intracellular pool of EC-SOD was enriched from cells transiently expressing wild-type EC-SOD. Cells were washed with Hanks’ balanced salt solution; lysed by the addition of 25 mM Tris-HCl, 1 mM NaCl, 0.5% (v/v) Triton X-100, and 5 mM EDTA (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride; and purified by immunoaffinity chromatography using a monoclonal antibody directed against human EC-SOD (16). Protein was eluted using 0.1 M glycine (pH 2.7), and fractions were immediately neutralized by the addition of 1 M Tris-HCl (pH 8.0). Fractions containing EC-SOD (evaluated by SDS-PAGE and Western blotting) were pooled and stored at 4 °C.

Construction of Expression Plasmids—The C45S and C195S EC-SOD mutants were constructed according to the QuikChange method using Herculease enhanced DNA polymerase (Stratagene) and DpnI (New England Biolabs). The template was pCDNA3-cloned wild-type EC-SOD cDNA. PCR was performed with annealing at 60 °C and elongation times of 10 min in 16 cycles. The primers used were as follows (with the mutation underlined): C45S, 5′-CAGGGTCACC-GCCGCCCTCCCAAGCTGACGCGCCGTGG-3′ (forward) and 5′-CCGACGCGTCACTGGAGCCGCGCTGGACGTG-3′ (reverse); and C195S, 5′-CTCGGTGGTGCCGCCTTCCGGGCCTGGGCTCTGGG-3′ (forward) and 5′-CCAGAGCCCGGCCCAGGACACGCCCACCCACCGCAG-3′ (reverse). The sequences of expression constructs generated were verified by sequence analysis of both strands.

Expression of Recombinant Protein—Human embryonic kidney (HEK) 293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated onto 9-cm tissue culture dishes and transfected 18 h later by calcium phosphate co–precipitation (26) using 17 μg of plasmid DNA or, in the case of cotransfections, 8.5 μg of each plasmid DNA encoding EC-SOD variants. Plasmid DNA was prepared using the Quantum Prep midi-prep kit (Bio-Rad). After 48 h, the culture medium was replaced by serum-free medium. The medium was changed three times at intervals of 24 h, pooled, clarified by centrifugation, and subsequently analyzed for expression of EC-SOD by Western blotting. Cells used for the analysis of intracellular protein were harvested after 48 h in serum-free medium.

Trypsin Digestion—Purified EC-SOD was lyophilized, and free cysteine residues were blocked by the addition of 20 mM iodoacetamide in 50 mM HEPES, 6 mM guanidinium hydrochloride, and 20 mM 8-hydroxyquinoline (pH 8.3). The S-carbamidomethylated protein was recovered by reverse-phase chromatography using microcolumns containing Poros reverse-phase R1 resin (27) and eluted using 90% acetonitrile containing 0.1% trifluoroacetic acid. The lyophilized material was resuspended in 50 mM ammonium bicarbonate; trypsin was added at an enzyme/protein ratio of 1:50; and the reaction was allowed to proceed overnight at 37 °C.

Gel Electrophoresis—Proteins were separated by polyacrylamide gel electrophoresis using 5–15% gradient gels and the glycine/2-amino-2-methyl-1,3-propanediol HCl buffer system (28). Samples analyzed under denaturing conditions were boiled in the presence of 0.5% (w/v) SDS. Reducing conditions were obtained by the addition of 30 mM dithiothreitol. Samples analyzed by nondenaturing gel electrophoresis were analyzed without boiling prior to electrophoresis, and SDS was omitted in all buffers used. Peptides were separated by Tris/Tricine/SDS-gel electrophoresis using 16.5% acrylamide gels (29).

Western Blotting—Proteins separated by electrophoresis were electrophoretically transferred to a polyvinylidene difluoride membrane in 10 mM CAPS and 10% methanol (pH 11) (30). Peptides separated by Tris/Tricine/SDS-gel electrophoresis were transferred to a polyvinylidene difluoride membrane in 25 mM Tris-HCl and 192 mM glycine (pH 8.3) (31). The membranes were blocked with 5% (w/v) skimmed milk in 20 mM Tris-HCl and 150 mM NaCl (pH 7.4). EC-SOD protein was subsequently detected using rabbit anti-human EC-SOD antisera and, for EC-SOD peptides, using rabbit anti-EC-SOD antisera directed against Ala94–Cys107. The blots were developed using peroxidase-conjugated goat anti-rabbit Ig (Sigma) and enhanced chemiluminescence.

SOD Activity—Proteins were separated by nondenaturing gel electrophoresis, and the gel was subsequently equilibrated three times for 10 min each with 20 mM Tris-HCl (pH 7.4). The gel was equilibrated with nitro blue tetrazolium, riboflavin, and TEMED as described (5), and SOD activity was detected by exposure to light. The activity was also determined in fluid phase using the xanthine oxidase/cytochrome c assay (32). The protein concentration used to calculate specific activity was estimated using absorption at 280 nm.

RESULTS

EC-SOD Folding Variants Are Generated Intracellularly—We detected previously the presence of aEC-SOD and iEC-SOD in purified EC-SOD by reverse-phase high pressure liquid chromatography of tryptic digests (17). However, the presence of aEC-SOD and iEC-SOD may also be analyzed conveniently by nonreducing Tris/Tricine-gel electrophoresis. The detec-
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FIGURE 1. Schematic illustration showing the disulfide-bonded peptides generated by trypsin cleavage of human EC-SOD folding variants. The N-terminal region of EC-SOD is shaded dark gray; the central region with high sequence identity to SOD1 is shaded light gray; and the extracellular matrix-binding region is unshaded. The five cysteine residues involved in intramolecular disulfide bridges are numbered, and the connectivity representing aEC-SOD and iEC-SOD is shown. The calculated masses of the disulfide-linked peptides are shown on the right.

FIGURE 2. Analysis of authentic EC-SOD by Tris/Tricine-gel electrophoresis. A, human EC-SOD purified from aorta and medium collected from transfected HEK 293 cells was digested by trypsin and analyzed by Tris/Tricine-gel electrophoresis under nonreducing conditions. Intact EC-SOD and trypsin were included as controls. Molecular mass markers are included on the left. Bands were visualized by Coomassie Blue staining. B, EC-SOD isolated from the intracellular compartment of transfected HEK 293 cells was digested with trypsin. The generated disulfide-linked peptides were separated by Tris/Tricine-gel electrophoresis under nonreducing conditions and detected by Western blotting. Purified intact EC-SOD (aorta) and digested material (aorta + trypsin; two concentrations) were included as controls. This analysis showed that both folding variants are present in authentic and recombinant EC-SOD.

FIGURE 3. Schematic representation of generated mutants supporting the formation of aEC-SOD or iEC-SOD. Amino acid substitutions of wild-type (wt) EC-SOD were designed to allow for the expression of either aEC-SOD or iEC-SOD analogs. Pseudo-aEC-SOD was generated by the substitution of Cys45 for Ser, and pseudo-iEC-SOD was generated by the substitution of Cys195 for Ser. Cysteine residues involved in intramolecular disulfide bonding are shown, and serine residues are indicated by –OH.

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In an attempt to analyze the biosynthesis of iEC-SOD or aEC-SOD separately, two EC-SOD mutants were constructed. The substitution of Cys45 for Ser (C45S) prevents the formation of aEC-SOD, as Cys45 is involved in the formation of an intramolecular disulfide bond (Fig. 3). The C45S substitution thus permits the generation of iEC-SOD only. Likewise, the substitution of Cys195 for Ser (C195S) eliminates the synthesis of iEC-SOD, forcing the cells to produce only aEC-SOD (Fig. 3). Expression of EC-SOD mutants was analyzed by SDS-PAGE, followed by Western blotting. The analysis of the medium derived from the C45S-transfected cells showed that it did not contain any detectable iEC-SOD (Fig. 4). The analysis of the C45S cell lysate showed two iEC-SOD bands of 32 and 29 kDa under reducing conditions (Fig. 4). Following N-glycosidase F treatment, only one band was detected, indicating that the difference in migration was caused by carbohydrate heterogeneity (data not shown). Moreover, it was shown previously that the EC-SOD subunit is cleaved immediately before secretion, underscoring the absence of any cleaved subunit in the intracellular compartment (14). Thus, the two bands detected represent the intact subunit in a glycosylated (32 kDa) and non-glycosylated (29 kDa) form (Fig. 4). When the analysis of the C45S cell lysate was performed using nonreduc-
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C195S EC-SOD is an Active Tetramer—To establish whether C195S EC-SOD retains the structural and functional activities of native EC-SOD, we subjected the purified material to native gel electrophoresis. The analysis established that pseudo-aEC-SOD forms a tetramer (Fig. 5) and that the generated material is enzymatically active, as evident from the spectroscopic analysis of activity and by activity staining of protein separated by native gel electrophoresis (Fig. 5). These analyses established that C195S EC-SOD is correctly folded and retains activity.

The triple peptide derived from aEC-SOD (C195S) was detected (Fig. 6). This result demonstrates that the folding of iEC-SOD (C45S) depends on factors other than aEC-SOD (C195S).

DISCUSSION

The two folding variants called aEC-SOD and iEC-SOD were originally identified and characterized using authentic protein purified from human aorta (17). Our present analyses of cells expressing wild-type EC-SOD revealed that the two folding variants are generated intracellularly before secretion, demonstrating that the expression system contains the required biosynthesis machinery for production of both forms. In an attempt to investigate the biosynthesis of the two forms separately, we generated expression constructs encoding EC-SOD proteins in which serine residues were substituted for cysteine residues essential for the folding of the two forms (see Fig. 3). In theory, the C195S substitution will force the cells to produce only aEC-SOD, and the biochemical analyses of the protein produced supported this, as the cells secreted enzymatically active tetrameric EC-SOD. The substitution of Cys\(^{195}\) for Ser corresponds to the amino acid in rabbit, mouse, and rat EC-SOD (18), and it is thus not surprising that this mutant was readily expressed. In contrast, the expression of C45S resulted in intracellular disulfide-bonded aggregation, and iEC-SOD was not detected in the medium. The substitution of serine for cysteine is generally used to analyze the function of cysteine residues because of the structural similarity of the side chains, and it is therefore intriguing that the C45S mutation generated large disulfide-bonded intracellular aggregates. This indicates
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that the inability to secrete iEC-SOD resides in the failure to generate the correct disulfide bridge pattern. This suggests that Cys\textsuperscript{45} (although free in mature iEC-SOD) is involved in the folding process, most likely by forming a transient disulfide bridge. Alternatively, aEC-SOD might promote the proper folding of iEC-SOD. However, coexpression experiments did not support this scenario, as only aEC-SOD was secreted. Together, these analyses have demonstrated that the presence of Cys\textsuperscript{45} is essential for the generation of iEC-SOD, suggesting that this residue participates actively during the folding process.

The folding of EC-SOD is likely to involve metallochaperones during the insertion of copper and zinc and oxidoreductases to establish the correct disulfide bridge patterns. In this respect, it is interesting to note that the insertion of copper into Cu,Zn-SOD (9) involves a transient interprotein disulfide bridge between a metallochaperone and Cu,Zn-SOD (33). Following the insertion of copper, the cysteine residue of Cu,Zn-SOD involved in the interprotein disulfide bridge is oxidized to generate a disulfide bond essential for catalytic activity (34, 35). The insertion of copper into EC-SOD involves the activity of the metallochaperone Atox1 (antioxidant-1; also known as HAHI) (36), which is a cytosolic protein that mediates the transport of copper into the trans-Golgi network via copper transporters in the membrane (37). The transport of copper from Atox1 to EC-SOD appears to be mediated by ATP7A (Menkes protein) (38). Importantly, this copper shuttle involves the chelation of copper by cysteine residues in both Atox1 (39, 40) and ATP7A (41). In analogy to the folding of Cu,Zn-SOD, it may be hypothesized that the folding of EC-SOD involves a number of transient inter- or intramolecular disulfide bridges or that free cysteine residues are important for the acceptance of copper from the ATP7A copper transporter. This hypothesis is substantiated by our studies showing that Cys\textsuperscript{45} is essential for the folding of iEC-SOD, whereas Cys\textsuperscript{195} does not seem to have any effect on the folding of aEC-SOD.

The regulation of protein activity by allosteric disulfide bridges involves the redox status of cysteine residues not directly involved in protein function, as \textit{e.g.} the regulation of CD4 (23). It is not clear if the two folding variants of human EC-SOD can interconvert or whether these two forms are the result of different folding pathways generating stable end products. However, a putative interconversion between the two folding variants of human EC-SOD will require the disruption and regeneration of two disulfide bonds, and it is therefore likely to involve chaperones. Although generation/isomerization of disulfide bridges may be complex, increasing evidence suggests that these covalent bonds are more dynamic than previously thought and can undergo significant rearrangements to modulate functional activity. An example of this is the mitochondrial copper chaperone Cox17, which contains six highly conserved cysteine residues. This protein can exist in three different conformers: (i) fully reduced with the capacity to bind four copper ions, (ii) containing two disulfide bridges with the capacity to bind one copper ion, and (iii) fully oxidized without copper binding capacity (42). The physiological function of this protein has been speculated to reside in the redox status of cysteine residues (43). Interestingly, the interconversion of the fully oxidized form requires the reduction of a disulfide bridge between vicinal cysteine residues and a subsequent isomerization of disulfide bridges (43), a sequence of steps that would also convert iEC-SOD into aEC-SOD.

We have shown that the two folding variants of human EC-SOD are generated intracellularly and that the folding of these two forms is likely to involve transient disulfide bridges. It was shown previously that post-translational modifications of EC-SOD can be modulated tissue-specifically (14), by the availability of metal ions (44), and by the oxidative load in the extracellular matrix (45). At present, it is not clear whether the synthesis of the two folding variants of human EC-SOD can be regulated; however, the presence of extracellular oxidoreductases (20) and redox-sensitive chaperones (46) sets the basis for regulating the antioxidant level in the extracellular space by modulating the folding of human EC-SOD.

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