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Murine Extracellular Superoxide Dismutase Is Converted into the Inactive Fold by the Ser195Cys Mutation

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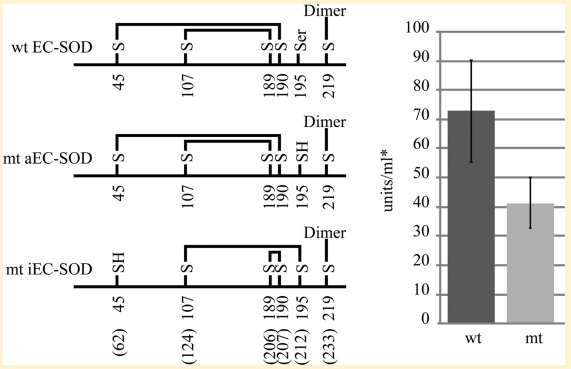
ABSTRACT: We have previously shown that human extracellular superoxide dismutase (EC-SOD) exists as two variants with differences in their disulfide bridge patterns: one form is the active enzyme (aEC-SOD), and the other is inactive (iEC-SOD). The availability of both active and inactive folding variants significantly reduces the specific activity of EC-SOD *in vivo*. Both forms are produced during biosynthesis, but the underlying folding mechanisms remain unclear. To address this issue, we expressed EC-SOD in heterologous systems that do not endogenously express iEC-SOD. Rodents express only aEC-SOD because they lack Cys195 (human EC-SOD sequence numbering), which is essential for the formation of iEC-SOD. However, cultured hamster cells and transgenic mice expressing human EC-SOD were able to produce both human a- and iEC-SOD variants, which led us to hypothesize that the folding was sequence-dependent rather than a property of the expression system. To substantiate this hypothesis, we expressed murine EC-SOD in a human cell line, and as expected, only aEC-SOD was produced. Significantly, when Cys195 was introduced, both murine aEC-SOD and a novel murine iEC-SOD were generated, and the specific activity of the murine EC-SOD was significantly reduced by the mutation. Collectively, these data suggest that Cys195 actuates the formation of iEC-SOD, independent of the expression system or host. In addition, the dual-folding pathway most likely requires biosynthesis factors that are common to both humans and rodents.



It has previously been assumed that disulfide bonds serve as stabilizers of protein structure during folding and protect the protein against degradation in the extracellular space. However, it is now apparent that disulfide bonds may be dynamic structures involved in the allosteric control of enzyme activity, as exemplified by thrombospondin,[1−3] CD4,[4] and tissue factor.[5,6] Extracellular superoxide dismutase (EC-SOD, SOD3) is the only antioxidant enzyme responsible for the dismutation of extracellularly generated superoxide. We have previously shown that human EC-SOD exists in both active (aEC-SOD) and inactive (iEC-SOD) folding variants, which are characterized by two different intrasubunit disulfide bridge patterns (Figure 1).[7] aEC-SOD resembles intracellular copper/zinc superoxide dismutase (Cu/ZnSOD, SOD1) and contains a homologous disulfide bridge (EC-SOD, Cys107−Cys189; Cu/ZnSOD, Cys57−Cys146) that is essential for its enzymatic activity.[8] In contrast, iEC-SOD contains a disulfide bridge between Cys107 and Cys195 and adopts a different tertiary structure.

The existence of a- and iEC-SOD supports the idea that disulfide interchange of EC-SOD might be involved in regulating the activity of the enzyme either during folding or by fold switching in the mature protein. However, for a switch to occur, the two disulfide bridges must be reduced and reoxidized in a different pattern to convert aEC-SOD to iEC-SOD and vice versa. Such a process, if it exists, likely requires the involvement of extracellular chaperones[9] and disulfide isomerases.[10]

The availability of the inactive fold has a significant influence on the activity of EC-SOD. The ratio of aEC-SOD to iEC-SOD is approximately 1:1 in the human aorta;[7] in contrast, mouse, hamster, and rabbit EC-SOD lack Cys195, the residue involved in the formation of the Cys107−Cys195 disulfide bond found in iEC-SOD, and thus express only aEC-SOD. We have previously shown that rabbits secrete only aEC-SOD and that the specific activity of rabbit EC-SOD is 2-fold higher than that of human EC-SOD. The approximately 1:1 ratio of aEC-SOD

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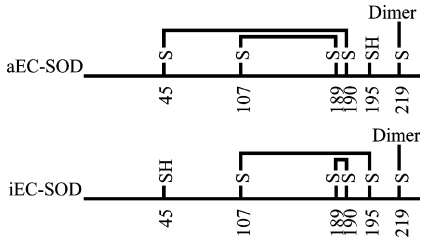

Figure 1. Schematic representation of active and inactive human EC-SOD. The illustration shows the disulfide bond connectivity of the enzymatically active human EC-SOD subunit (aEC-SOD) and the inactive human EC-SOD subunit (iEC-SOD).[7] The Cys residues are indicated by residue number. SH denotes a Cys with a free thiol group, whereas S- indicates Cys residues involved in a disulfide bond. Cys45−Cys190 and Cys107−Cys189 disulfide bonds are formed in aEC-SOD, whereas Cys107−Cys195 and Cys189−Cys190 links are formed in iEC-SOD. The tertiary structure of aEC-SOD is similar to that of SOD1, and the bridge between Cys107 and Cys189 is essential for enzyme activity. This disulfide bridge is broken in iEC-SOD, resulting in an inactive variant. Cys219 is involved in an intersubunit bridge, resulting in dimer formation.

to iEC-SOD in humans accounts for the reduction in enzymatic activity compared to that of the orthologous rabbit protein,[11] indicating that species lacking Cys195 are unable to synthesize iEC-SOD. However, the cDNA sequences of bovine, pig, and chicken EC-SOD all have a Cys residue at position 195, suggesting that, as in humans, these animals are able to generate both folding variants. On the basis of previous studies, it is evident that Cys195 is essential for the folding of iEC-SOD because this fold requires the formation of the Cys107−Cys195 disulfide bridge. However, it is not known whether the two-pronged folding pathway is species specific or a property of the amino acid sequence.

To elucidate the essential factors for a- and iEC-SOD formation, we examined whether human EC-SOD is processed to produce both the a- and iEC-SOD variants when expressed in transgenic mice or CHO cells. Moreover, we examined the expression of murine wild-type EC-SOD and the S195C variant (human EC-SOD sequence numbering; murine numbering is shown in parentheses throughout) in a human cell line. Collectively, the results show that the ability of an organism to generate iEC-SOD, and thereby lower the activity of EC-SOD, is a general phenomenon permitted by the presence of a cysteine at position 195.

EXPERIMENTAL PROCEDURES

Purification of EC-SOD from Human Aorta. EC-SOD was purified from human aorta tissue as described previously,[12] omitting cation exchange chromatography.

Purification of Human EC-SOD from Transgenic Mice. The production of the mice carrying the human EC-SOD transgene has been described previously.[13] Brain tissue from several mice was combined and homogenized in liquid nitrogen, and the proteins were extracted using 50 mM KH_2PO_4 and 300 mM KBr (pH 7.4) containing 0.1 mM *o*-phenanthroline and 0.15 mM phenylmethanesulfonyl fluoride. The homogenate was clarified and dialyzed against 50 mM Tris-HCl and 50 mM NaCl (pH 7.4) overnight at 4 °C. EC-SOD was subsequently enriched by immunoaffinity chromatography using a monoclonal antibody directed against human EC-SOD.[14] The column was washed using 50 mM Tris-HCl and 50 mM NaCl (pH 7.4). The bound protein was eluted using 0.1 M glycine (pH 2.7), and the pH was immediately neutralized by adding 1 M Tris-HCl (pH 7.5). Fractions containing EC-SOD [as analyzed by reducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting] were combined and stored at −20 °C for use in further analyses.

Cell Culture, Expression, and Purification of Human EC-SOD from CHO Cells. CHO cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% bovine calf serum and 1% penicillin/streptomycin. The cells were incubated in a 5% CO_2 atmosphere at 37 °C. Transfection was performed using pcDNA5/FRT (Invitrogen)-cloned human EC-SOD cDNA and the calcium phosphate precipitation method.[15] After approximately 24 h, the culture medium was replaced with serum-free medium, which was collected and replaced five times at 24 h intervals.

The expression medium from transfected CHO cells was dialyzed against 50 mM Tris-HCl and 50 mM NaCl (pH 7.4), and EC-SOD was purified by affinity chromatography using a heparin-Sepharose column (5 mL HiTrap Heparin HP, GE Healthcare), followed by anion exchange chromatography using a HiTrap Q column (5 mL HiTrap Q HP, GE Healthcare), as previously described.[12] The collected fractions were analyzed by Western blotting, and those containing EC-SOD were pooled.

Construction of the S195C (S212C) Murine EC-SOD Expression Plasmid. The S195C (S212C) mutation in murine EC-SOD was generated using the pcDNA5/FRT-cloned wild-type EC-SOD cDNA as the template. The mutation was introduced using the Herculase II polymerase (Stratagene) in an 18-cycle polymerase chain reaction (PCR), with an annealing temperature of 60 °C and a 6.5 min elongation. The following primers were used: 5′-TGCTGCG-TGGTAGGCACCAGCAGCTCCGCCGCCTGG-3′ (forward) and 5′-CCAGGCGGCGGAGCTGCAGGTGCCTAC-CACGCAGCA-3′ (reverse). The PCR product was treated with Fast Digest DnpI (Fermentas) and transformed into competent NovaBlue *Escherichia coli* using the heat shock method. The transformed *E. coli* cells were plated on LB selection plates (LB agar containing 100 μg/mL ampicillin), and the selected colonies were further propagated in LB selection medium (LB medium containing 100 μg/mL ampicillin). The plasmid was purified using a miniprep kit (Fermentas), and the sequence was verified at the Eurofins MWG Operon nucleotide sequencing facility using the T7 (forward) and BGH (reverse) primers. Both the murine wild-type EC-SOD and the S195C (S212C) mutant were transformed into competent NovaBlue *E. coli*, as previously described, and grown in LB ampicillin selection medium for 20 h before the plasmids were purified using a MaxiPrep Kit (Sigma-Aldrich).

Expression of Murine EC-SOD in HEK293 Cells. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% (v/v) calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco) and were transfected at a confluence of 70%. The medium was exchanged 3 h prior to transfection with medium (2 mL) containing 18 μg of plasmid DNA (wild type or S212C mutant) and 180 μg of PEI per plate. At 5 h post-transfection, the medium was changed to growth medium, and after 16 h, the medium was changed to DMEM containing 100 units/mL penicillin and 100 μg/mL streptomycin (expression medium).

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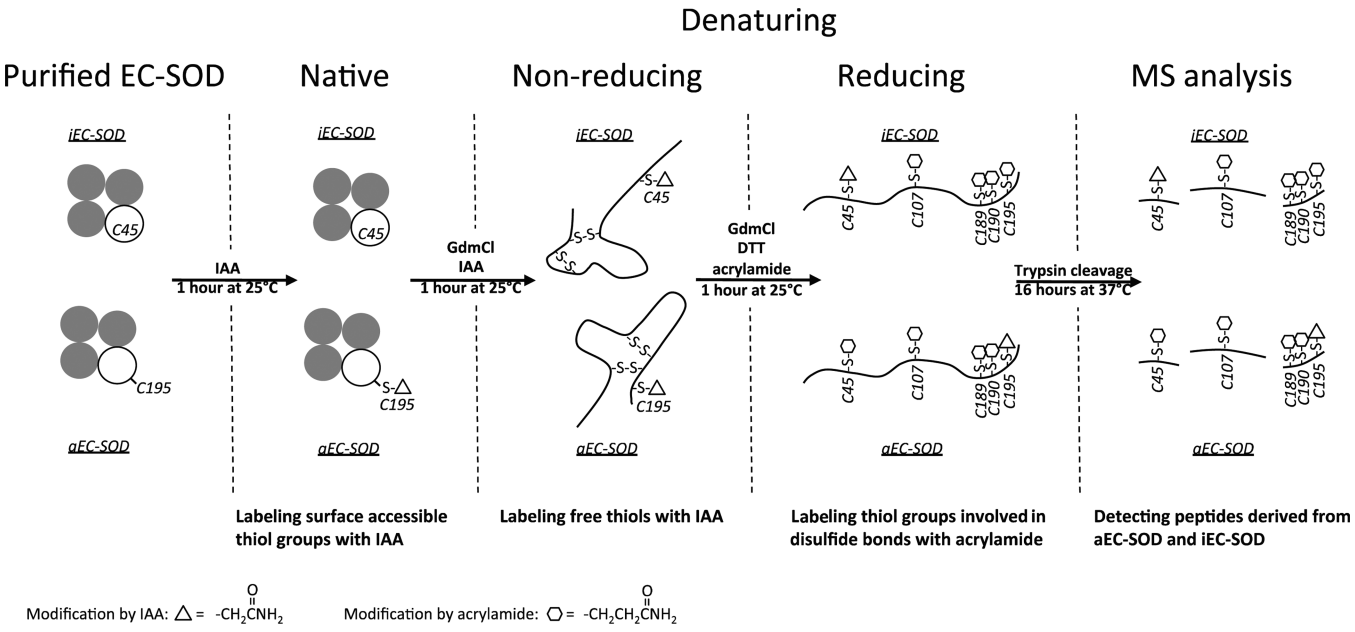

Figure 2. Differential labeling procedures used for human a- and iEC-SOD thiol groups. IAA was added under native conditions to label the surface-accessible thiol groups, which would modify primarily Cys195 of human aEC-SOD. The protein was denatured using GdmCl in the presence of IAA. All the free thiols, Cys45 of iEC-SOD and Cys195 of aEC-SOD, will be labeled with carbamidomethyl (△) using this procedure. Carbamidomethyl-labeled EC-SOD was recovered by micro-reverse-phase purification, and the disulfide bonds were reduced using DTT under denaturing conditions (GdmCl). Subsequent treatment with AA resulted in the propionamide modification (⬡) of the newly formed thiol groups. EC-SOD was cleaved by trypsin, and the resulting peptides derived from a- and iEC-SOD were specifically detected by MS/MS.

The medium was collected after 48 and 96 h, and EC-SOD was purified using a heparin affinity technique, as previously described.

Differential Labeling of aEC-SOD and iEC-SOD. EC-SOD was alkylated in the presence of 15 mM iodoacetamide (IAA) for 1 h at 23 °C, denatured in 6 M guanidinium chloride (GdmCl), and realkylated in 15 mM IAA at 23 °C for 1 h. The carbamidomethyl-labeled EC-SOD was desalted using micro-columns packed with Poros 50 R2 reverse-phase resin.[16] The sample was acidified using 0.1−2% TFA and applied to the microcolumn. The unbound material was removed using 5% acetonitrile in 0.1% TFA, and the bound protein was subsequently eluted using 70% acetonitrile in 0.1% TFA. Acetonitrile and TFA were removed by lyophilization, and the recovered EC-SOD was dissolved in 20 mM Tris-HCl (pH 8.0). EC-SOD was reduced with 5 mM DTT in 6 M GdmCl for 30 min prior to alkylation with 15 mM acrylamide (AA) for 30 min at 23 °C. After an additional microcolumn purification step, the dual-labeled sample was lyophilized and digested with 25 ng/μL trypsin for 16 h at 37 °C. Prior to MS analyses, the tryptic peptides were micropurified using StageTips (Thermo Scientific) according to the manufacturer's instructions and then lyophilized.

Detection of a- and iEC-SOD by Mass Spectrometry Analyses. Nano ESI-tandem mass spectrometry (MS/MS) was performed using an EASY-nLC II system (Thermo Scientific) connected to a TripleTOF 5600 mass spectrometer (AB Sciex) equipped with a NanoSpray III source (AB Sciex) and operated under the control of Analyst TF version 1.5.1. The lyophilized peptides were suspended in 5% formic acid and then injected, trapped, and desalted on a ReproSil-Pur C18-AQ column [5 μm, 2 cm × 100 μm (inside diameter); Thermo Scientific]. The peptides were further separated on an analytical ReproSil-Pur C18-AQ capillary column [3 μm, 10 cm × 75 μm (inside diameter); Thermo Scientific] connected in-line to a mass spectrometer at a flow rate of 250 nL/min using a 50 min gradient from 5 to 35% phase B (0.1% formic acid and 90% acetonitrile). The collected data were processed using Peakview version 1.0 to generate Mascot generic files (MGF files), which were searched using the Mascot search engine against an in-house database containing the EC-SOD sequence. To differentiate between the a-form (aEC-SOD) and the i-form (iEC-SOD), the search was performed using carbamidomethyl and propionamide as variable modifications of Cys residues. The peptide tolerance and MS/MS tolerance were set to 10 ppm and 0.1 Da, respectively. All the reported peptide scores were above the Mascot identity threshold, which indicates a score above which the probability of a random hit is less than 1%.

Activity Measurements of Murine EC-SOD. The relative amount of mutant and wild-type murine EC-SOD in the peak fraction from the heparin-Sepharose column was determined by SDS−PAGE and Western blotting using a polyclonal rabbit murine EC-SOD specific antibody and a Cy5-labeled anti-rabbit secondary antibody (Sigma). An image of the blot was obtained using the Fluorchem Q imaging system (Cell Biosciences), and the relative amount of protein was quantified using the Alpha View software package (Cell Biosciences). The peak fraction was diluted to obtain equal EC-SOD protein concentrations in the mutant and wild-type fractions. The activities of the mutant and wild-type EC-SOD were determined using the previously described cytochrome *c* spectrophotometric assay.[17,18] The activity was calculated as the average of five measurements.

RESULTS

A Mass Spectrometry-Based Assay for the Detection of Disulfide Bridge Variants of EC-SOD. We previously detected human a- and iEC-SOD by separating tryptic digests of the protein by reverse-phase high-performance liquid

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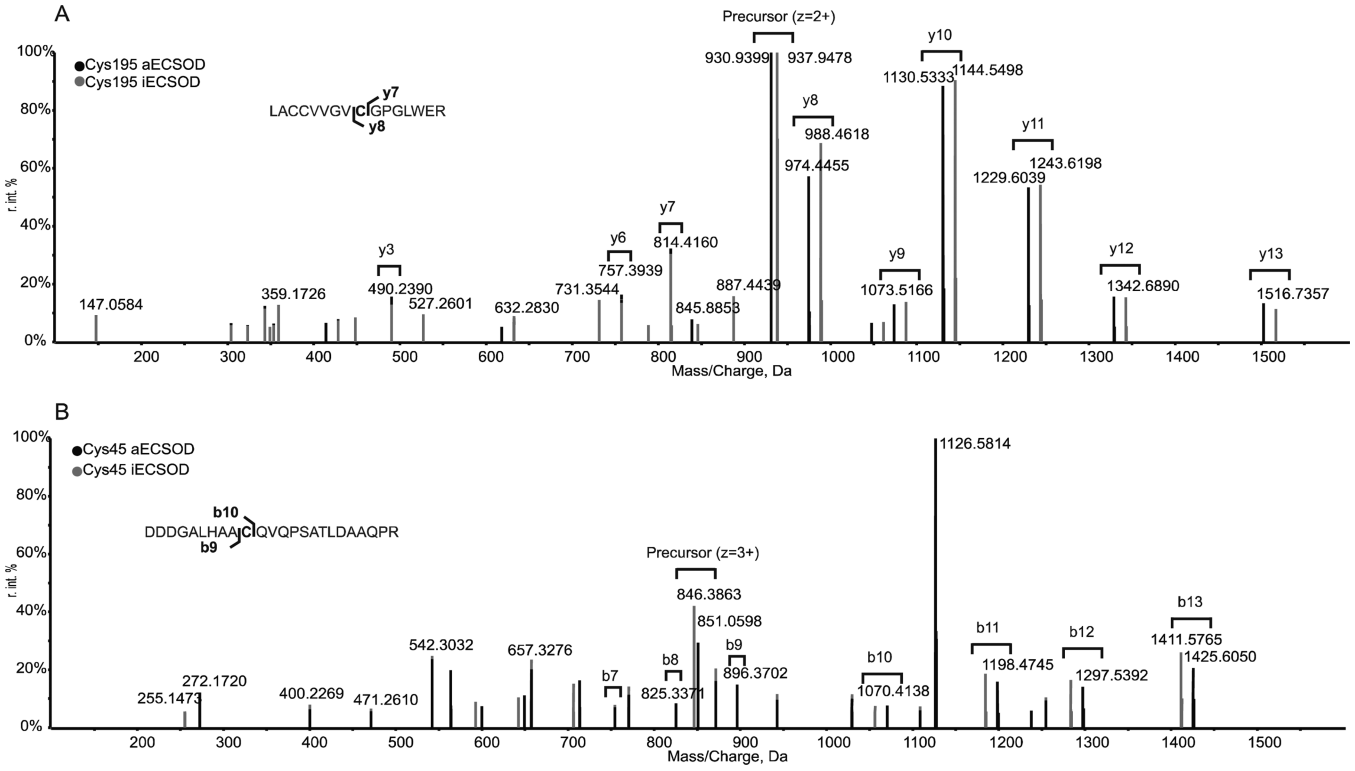

Figure 3. Mass spectrometry data of human EC-SOD from aorta. The figure shows the overlaid MS/MS spectra of the differentially labeled tryptic peptides containing Cys195 (A) and Cys45 (B). The matched y-ions (C-terminal fragments) and b-ions (N-terminal fragments) obtained during MS/MS fragmentation are indicated.[25] Cys195 is modified with carbamidomethyl or propionamide due to the IAA or AA treatment, respectively, giving rise to a 14 Da mass shift from y8. This result indicates the existence of a free thiol group on Cys195 and a disulfide bridge involving this residue, which is consistent with the presence of aEC-SOD and iEC-SOD, respectively. Analyses of the Cys195 peptide also reveal propionamide modification of Cys189 and Cys190, confirming that these residues are involved in disulfide bonds at all times, which is consistent with the structures of a- and iEC-SOD. Similarly, the data show that Cys45 can be modified with either propionamide or carbamidomethyl, resulting in a 14 Da mass shift in the b-ion series from b10. This result suggests that Cys45 may exist within a disulfide bridge or as a free thiol group, consistent with the structure of aEC-SOD or iEC-SOD, respectively.

Table 1. Detection of Cys Modifications of Human EC-SOD by MS/MS[a]

	Cys45 peptide		Cys195 peptide	
expression system	i-form (carbamidomethyl)	a-form (propionamide)	i-form (propionamide)	a-form (carbamidomethyl)
in vivo (aorta)	164	155	99	103
mice	157	171	101	109
CHO cells	183	176	103	95

[a]The numbers indicate the ion score, as evaluated by Mascot (version 2.3.02). The identity threshold varied between 10 and 25.

chromatography (HPLC)[7] or by nonreducing Tris/Tricine SDS–PAGE.[19] However, because of the need for a more sensitive detection method for discriminating the disulfide bridge variants, we developed an MS-based assay in this study (Figure 2). The accuracy of the assay was verified by analyzing authentic human EC-SOD purified from aorta (Figure 3 and Table 1). The free Cys residues were modified with iodoacetamide (IAA) to form S-carboxyamidomethylated free thiol groups (mass increase of 57.02 Da) (Figure 2). The protein was then reduced and treated with acrylamide, producing a propionamide derivatization of the thiol groups previously involved in disulfide bonds (mass increase of 71.04 Da). The protein was then digested with trypsin and analyzed by MS/MS (Table 1). We were able to differentiate between a- and iEC-SOD using this dual-labeling protocol because Cys45 is the only S-carboxyamidomethylated Cys residue in a population composed exclusively of iEC-SOD, and Cys195 is the only S-carboxyamidomethylated Cys residue when only aEC-SOD is present (Figure 2). A mixed population of a- and iEC-SOD produces peptides with a mass difference of 14 Da caused by the dual labeling of both Cys45 and Cys195 (Figure 3). On average, 70 MS/MS spectra were assigned to peptides containing Cys45 or Cys195, and less than 10% of these spectra corresponded to alkylation patterns other than those representing a- or iEC-SOD. These patterns included peptides with only partial alkylation or IAA labeling of disulfide-bound Cys189 and/or Cys190 (data not shown). Although the detection of these aberrant peptides is most likely due to incomplete alkylation in combination with the high sensitivity of the assay, another intriguing explanation might be that these other forms represent intermediates in the folding of EC-SOD. On the basis of these results, we concluded that Cys45 and Cys195 could be used as reporter residues for a- and iEC-SOD in a dual-alkylation protocol.

Heterologous Expression of Human EC-SOD. The a- and iEC-SOD folding variants have been experimentally

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Table 2. Detection of Cys Modifications of Murine EC-SOD by MS/MS[a]

	Cys45 (Cys62) peptide		Cys195 (Cys212) peptide	
murine EC-SOD	i-form (carbamidomethyl)	a-form (propionamide)	i-form (propionamide)	a-form (carbamidomethyl)
wild type	–	69	–	–
S195C (S212C)	61	73	104	98

[a]The numbers indicate the ion score, as evaluated by Mascot (version 2.3.02). The identity threshold varied between 25 and 38.

verified only in humans,[7] and the mechanism underlying the formation of a- and iEC-SOD is not fully understood. To investigate this issue, we tested whether folding depended on the expression system and required specific biosynthesis machinery or was simply a property of the EC-SOD primary structure. Because rodents do not produce endogenous iEC-SOD, we expressed human EC-SOD in both CHO cells and transgenic mice. After the purification of the expressed human EC-SOD, the protein was subjected to the dual-alkylation protocol described in Experimental Procedures, digested with trypsin, and analyzed by MS/MS to identify the disulfide bridge variants (Table 1). The MS/MS analyses detected human EC-SOD peptides containing either Cys45 or Cys195 expressed in the transgenic mice, and these peptides were labeled with both carbamidomethyl and propionamide at the reporter residues. The carbamidomethyl labeling of Cys45 and Cys195 suggested that these residues both existed as free Cys residues in the native protein, whereas the propionamide labeling of Cys45 and Cys195 supported the idea that both of these residues were also involved in disulfide bonds. These alkylation patterns correspond to the patterns observed in authentic human EC-SOD and indicate that mice are able to express both human a- and iEC-SOD. In conclusion, these results show that an organism not usually expressing a- and iEC-SOD is capable of producing both human EC-SOD forms. However, we cannot exclude the possibility that specialized cells in the mice performed the expression of a- and iEC-SOD. To exclude this possibility and further investigate the key components of the dual-folding pathway, we expressed human EC-SOD in CHO cells. When human EC-SOD expressed in CHO cells was analyzed by the dual-alkylation protocol, the peptides containing the reporter residues (Cys45 and Cys195) were labeled with carbamidomethyl and propionamide (Table 1), revealing that both Cys45 and Cys195 were present as free thiol groups and in disulfide bonds. Again, this alkylation pattern revealed that human EC-SOD expressed in CHO cells yields both the a- and i-forms. In conclusion, our data revealed that both mice and cultured CHO cells are able to fold a- and iEC-SOD, suggesting that the folding of a- and iEC-SOD is a property of the human EC-SOD primary structure.

The Murine S195C (S212C) EC-SOD Mutant Is Able To Fold as a- and iEC-SOD. The data described above show that rodents are able to produce human a- and iEC-SOD, which suggests that any biosynthesis machinery required to fold the two forms is also found in mice. Thus, we prepared a murine S195C (S212C) mutant to test whether specific intrinsic properties of the human EC-SOD primary structure played a role in the folding. Wild-type murine EC-SOD contains a Ser residue at position 195 (212) and is therefore not able to form the Cys107−Cys195 (Cys124−Cys212) disulfide bond found in iEC-SOD. Wild-type and S195C (S212C) murine EC-SOD were expressed in HEK293 cells, and the purified proteins were dually alkylated, cleaved by trypsin, and analyzed by MS/MS (Table 2). As expected, we detected only propionamide labeling of Cys residues in wild-type murine EC-SOD, indicating that all the Cys residues were involved in disulfide bonds, consistent with the formation of murine aEC-SOD (Figure 4, left). Significantly, when analyzing the S195C (S212C) mutant, we detected S-carbamidomethyl and propionamide labeling of Cys45 (Cys62) and Cys195 (Cys212), indicating that these residues existed as both free thiol groups and disulfide bonds in the native structure. This alkylation pattern corresponded to the alkylation observed for human EC-SOD (Table 1). These data show that the murine S195C (S212C) EC-SOD mutant is able to fold as both aEC-SOD and iEC-SOD variants, thereby allowing the regulation of enzymatic activity.

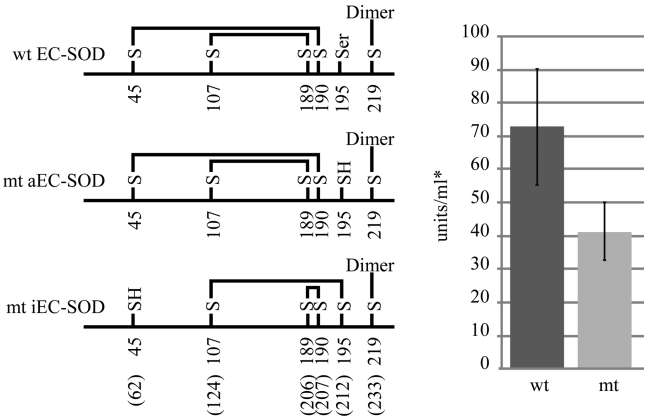

Figure 4. Activity of S195C (S212C) murine EC-SOD that is significantly reduced compared to that of wild-type murine EC-SOD. Schematic representation of the different disulfide bridge patterns found in wild-type and S195C (S212C) murine EC-SOD (left). Position 195 in human EC-SOD corresponds to position 212 in murine EC-SOD. Introducing a Cys at position 195 leads to the generation of both iEC-SOD and aEC-SOD. The numbers in parentheses correspond to the murine numbering system. The activity of wild-type and S195C (S212C) murine EC-SOD was measured using the cytochrome *c* assay (right). The relative concentrations of wild-type and mutant EC-SOD were determined using Western blotting, and the concentration was equalized by dilution. The indicated activity is based on five measurements, with error bars showing the standard deviation. For the *y*-axis, activity was recorded in samples with matched concentrations.

It has previously been shown that the activity of human EC-SOD is approximately 50% lower than that in species that produce only aEC-SOD.[11] If the main determinant of the two-pronged folding path is Cys195, a similar reduction in the S195C (S212C) murine EC-SOD mutant should be observed. Accordingly, we measured the activity of both wild-type and S195C murine EC-SOD to determine the relative amount of iEC-SOD in the murine S195C (S212C) mutant (Figure 4). The S195C (S212C) mutant exhibited a significant (43%) reduction in activity compared to that of the wild type. These data confirm that S195C (S212C) murine EC-SOD is able to fold as iEC-SOD; moreover, the presence of Cys195 (Cys212)

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in murine EC-SOD is sufficient to produce the aEC-SOD:iEC-SOD ratio observed in humans.

In conclusion, the introduction of a single Cys residue resulted in a two-pronged folding pathway that mediated the formation of different disulfide folding variants, suggesting that additional intrinsic properties of the EC-SOD sequence, apart from Cys195, most likely do not play a role in the folding of iEC-SOD.

DISCUSSION

The presence of human EC-SOD disulfide bridge variants has previously been studied by reverse-phase HPLC or Tris/Tricine gel electrophoresis of trypsin digests.[7,19] However, to evaluate the presence of disulfide bridge variants in samples containing only small amounts of EC-SOD, we developed a more sensitive assay based on tandem mass spectrometry. The validity of this method was demonstrated through the detection of a- and iEC-SOD in authentic EC-SOD purified from human aorta (Figure 2 and Table 1).

In this study, we demonstrate that both human a- and iEC-SOD can be expressed in mice. Thus, although mice do not naturally generate the disulfide bridge variants of human EC-SOD, human iEC-SOD will be folded in these animals. Identical results were obtained for human EC-SOD produced in a rodent cell culture (CHO cells). The formation of a- and iEC-SOD in a cell line is consistent with the previously published data, showing that human a- and iEC-SOD are formed intracellularly during biosynthesis and do not require extracellular components to complete their folding.[19] Moreover, our analysis of the S195C (S212C) murine EC-SOD mutant shows that Cys195 is the main factor regulating the ratio between the a- and i-forms and thereby the level of enzymatic activity.

The classic thermodynamic experiments by Anfinsen et al.[20] provided evidence that the oxidative folding of polypeptide chains is a spontaneous process and that the polypeptide sequence itself is sufficient for achieving the native state *in vitro*. Folding is achieved by the random formation of disulfide bonds that rearrange to generate the most thermodynamically favorable form, a process that is accelerated by disulfide isomerases and chaperones *in vivo*.[21]

These results show that the formation of a- and iEC-SOD is likely a general phenomenon, independent of the expression system. Therefore, in a manner consistent with the results of Anfinsen et al.,[20] supported by our results showing that murine S195C (S212C) EC-SOD forms both a- and iEC-SOD, we suggest that the ability of EC-SOD to form different disulfide variants resides within the amino acid sequence of EC-SOD. Although we did not determine whether the iEC-SOD fold is achieved via the aEC-SOD state, some common steps in the folding pathway seem likely; this model is substantiated by studies of a C45S EC-SOD mutant. Hypothetically, the C45S mutant is able to fold as iEC-SOD only because it cannot form the Cys45−Cys190 bridge found in aEC-SOD. However, attempts to express C45S have resulted in misfolding and the intracellular aggregation of both forms, suggesting some interdependence during folding.[19,22] Similarly, our attempts to refold denatured and reduced EC-SOD *in vitro* have been unsuccessful, most likely because of the lack of the highly specialized chaperone needed to load the copper ion into EC-SOD.[23]

Although the folding of iEC-SOD is a chaperone-dependent intracellular event, the ability to switch between the two forms following secretion and thus regulate SOD activity in the extracellular space is an intriguing proposition. However, no such mechanism has been detected to date, though we note that extracellular disulfide isomerases have been characterized previously.[10]

As previously mentioned, several other proteins, such as thrombospondin,[1−3] CD4,[4] and tissue factor,[5,6] exist as different disulfide-bonded isoforms, implying that disulfides may be dynamic structures involved in the regulation of protein function. Thrombospondin, an extracellular protein that is secreted by activated platelets, exists as a mixture of molecules containing different Cys residues,[1] and the pattern of disulfide bond formation among these residues regulates the proteins' cell adhesive properties.[2] The finding that different isoforms exist *in vivo*[3] led to the idea that disulfide interchange is important for controlling the function of thrombospondin. Membrane protein CD4 is another example of a protein with an allosteric disulfide bond. The cleavage of this disulfide bond appears to be regulated by thioredoxin and plays a role in the entry of HIV-1 into susceptible cells.[4] An allosteric disulfide bond has also been identified in tissue factor, and the oxidation of its Cys residues has been proposed to be involved in the activation of the protein.[5,6]

As mentioned above, it can be suggested that the allosteric disulfides in a- and iEC-SOD provide a means of regulating the extracellular antioxidant level. For this process to occur, two disulfide bridges must be reduced and reorganized to function as an allosteric disulfide switch. If such a mechanism of EC-SOD regulation exists, it is likely to require the involvement of extracellular chaperones and protein disulfide isomerases.[9,10] Although the physiological significance of the a- and i-forms is unknown, the tight regulation of the amount and composition of reactive oxygen species is important for both signaling and the protection of the extracellular matrix.[24]

In conclusion, we found that both human a- and iEC-SOD can be folded and expressed in rodents, even though these animals do not endogenously produce iEC-SOD. Moreover, when Cys195 was introduced into the murine EC-SOD sequence, murine S195C (S212C) iEC-SOD was expressed in human cells, suggesting that Cys195 actuates the formation of iEC-SOD. These results show that the ability to fold both a- and iEC-SOD is a universal property and depends on the presence or absence of Cys195. While Cys195 allows the formation of iEC-SOD, the conserved Cys45 is also vital for the correct folding of both a- and iEC-SOD.[19,22] On the basis of these results, we conclude that the introduction of Cys195 is necessary and sufficient for the dual-folding pathway.

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Author Contributions

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■ ABBREVIATIONS

EC-SOD, extracellular superoxide dismutase; aEC-SOD, active extracellular superoxide dismutase; iEC-SOD, inactive extracellular superoxide dismutase; AA, acrylamide; IAA, iodoacetamide; GdmCl, guanidinium chloride.

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