The Intracellular Proteolytic Processing of Extracellular Superoxide Dismutase (EC-SOD) is a Two-step Event*

Received for publication, February 3, 2004, and in revised form, March 10, 2004
Published, JBC Papers in Press, March 24, 2004, DOI 10.1074/jbc.M401180200

Dorte Aa. Olsen‡, Steen V. Petersen‡, Tim D. Oury§, Zuzana Valnickova‡, Ida B. Thøgersen‡, Torsten Kristensen‡, Russell P. Bowler§, James D. Crapo¶, and Jan J. Enghild‡‡

From the ¶Department of Molecular Biology, University of Aarhus, DK-8000 Århus, Denmark, the §Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15261, and the ¶Department of Medicine, National Jewish Medical and Research Center, Denver, Colorado 80206

Extracellular superoxide dismutase (EC-SOD) is a tetramer composed of either intact (Trp1-Ala222) or proteolytically cleaved (Trp1–Glu209) subunits. The latter form is processed intracellularly before secretion and lacks the C-terminal extracellular matrix (ECM)-binding region (210RKKRRRESECKAA222–COOH). We have previously suggested that the C-terminal processing of EC-SOD is either a one-step mechanism accomplished by a single intracellular endoproteolytic event cleaving the Glu209–Arg210 peptide bond or a two-step mechanism involving two proteinases (Enghild, J. J., Thøgersen, I. B., Oury, T. D., Valnickova, Z., Hojrup, P., and Crapo, J. D. (1999) J. Biol. Chem. 274, 14818–14822). In the latter case, an initial endoproteinase cleavage occurs somewhere in the region between Glu209 and Glu216. A carboxypeptidase specific for basic amino acid residues subsequently trims the remaining basic amino acid residues to Glu209. A naturally occurring mutation of EC-SOD substituting Arg213 for Gly enabled us to test these hypotheses. The mutation does not prevent proteolysis of the ECM-binding region but prevents a carboxypeptidase B-like enzyme from trimming residues beyond Gly213. The R213G mutation is located in the ECM-binding region, and individuals carrying this mutation have an increased concentration of EC-SOD in the circulatory system. In this study, we purified the R213G EC-SOD variant from heterozygous or homozygous individuals and determined the C-terminal residue of the processed subunit to be Gly213. This finding supports the two-step processing mechanism and indicates that the R213G mutation does not disturb the initial endoproteinase cleavage event but perturbs the subsequent trimming of the C terminus.

Extracellular superoxide dismutase (EC-SOD)† is the major scavenger of the highly reactive superoxide anion in the extracellular space and an important enzyme in the prevention of radical-mediated tissue destruction (1). EC-SOD is a tetramer held together by both non-covalent and covalent forces (2–4). We have recently shown that the EC-SOD polypeptide folds in two distinct ways, producing an active and an inactive subunit (5). As a consequence of this, it is theoretically possible to produce five distinct tetratomers with 0, 25, 50, 75, and 100% SOD activity. The C-terminal extracellular matrix (ECM)-binding region of EC-SOD encompasses several basic amino acid residues, which mediates the binding to negatively charged heparan sulfate proteoglycans on cell surfaces (Fig. 1) (6–9). Recently, we have shown that this region also binds to type I collagen (10). The C-terminal region is thus responsible for the immobilization of EC-SOD in the ECM. The capacity to bind several ligands may be advantageous and help optimize the distribution of EC-SOD in the ECM. The ECM-binding region can be removed intracellularly before secretion (Fig. 1) (11). Thus, EC-SOD subunits with or without ECM-binding capacity are produced. This supports the formation of EC-SOD tetramers with no (type A), intermediate (type B), or high (type C) affinity for ECM ligands composed of cleaved, both cleaved and intact, and intact subunits, respectively (1, 9, 12). The removal of the ECM-binding region is likely to be mediated by furin or another endoproteinase of the proprotein convertase family followed by a carboxypeptidase trimming of the C terminus to produce the mature Glu209 C terminus of wild-type EC-SOD (Fig. 1) (11, 13).

A naturally occurring mutation in the ECM-binding region of EC-SOD substitutes Arg213 for Gly (Fig. 1) (14–16). Affected individuals (2–6% of the population) have a higher concentration of EC-SOD in plasma than normal individuals (14, 15, 17–20). A reduction of the heparan sulfate affinity of the R213G mutant was suggested to be the cause of this phenotype. It could be hypothesized that the reduced antioxidant level in the arteries will render affected individuals more sensitive to oxidative stress. This has recently been substantiated by the finding that R213G carriers are more susceptible to ischemic heart disease (18, 21) and most likely also have an increased risk of acquiring other vascular diseases such as myocardial infarction and stroke. In addition to the reduced affinity for heparan sulfate, the R213G substitution apparently also interferes with the proteolytic processing of the ECM-binding region. This is evident by the aberrant migration of the processed subunit in reduced SDS-PAGE of EC-SOD from affected individuals (22).

Here we show that purified EC-SOD from individuals heterozygous or homozygous for the R213G mutation contains a subunit that migrates between cleaved and intact wild-type EC-SOD subunits as evident by SDS-PAGE analysis. We have identified the C terminus of this subunit to be Gly213. This...
suggests that the initial cleavage of the ECM-binding region occurs as normal, but the subsequent carboxypeptidase-trimming event is terminated at Gly^{213}. Consequently, the processing of EC-SOD is a two-step mechanism where the initial cleavage event is accomplished by an endopeptidase, most likely furin or another member of the proprotein convertase family of processing proteinases (13), followed by trimming of likely furin or another member of the proprotein convertase family of processing proteinases (13), followed by trimming of the remaining residues by a carboxypeptidase.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The HiTrap Heparin HP column, cyanogen bromide-activated Sepharose 4B, immobilized pH gradient strips, and other two-dimensional gel electrophoresis reagents were purchased from Amersham Biosciences. PNGase F was from Roche Applied Science, and horseradish peroxidase-conjugated goat anti-rabbit IgG was from Sigma. The Gly-specific papaya proteinase IV (PP-IV) was a kind gift from Dr. David J. Buttle, University of Sheffield Medical School, Sheffield, UK. Wild-type EC-SOD was purified from human aorta as described previously, except that the cation exchange chromatography step was omitted (4).

**Generation of Anti-EC-SOD-Sepharose**—Cyanogen bromide-activated Sepharose 4B was rehydrated and washed as recommended by the manufacturer. Monoclonal antibody 4G11G6 anti-human EC-SOD (2.3 mg) in 0.2 M NaHCO_{3}, 0.5 M NaCl, pH 8.3, was mixed with 3.5 ml of activated Sepharose in a final volume of 7 ml. The coupling reaction was carried out for 2 h at 23 °C, and residual binding sites were subsequently blocked by the addition of 0.2 M glycine, pH 8.0, for 2 h at 23 °C. The resin was washed in 0.2 M acetate, 0.5 M NaCl, pH 4, and stored in 20 mM Tris-HCl, pH 7.4.

**Purification of Mutant EC-SOD from Human Plasma**—Mutant EC-SOD was purified from both homo- and heterozygous individuals. Approximately 50 ml of plasma was diluted 5-fold in 20 mM Tris-HCl, 5 mM EDTA, pH 7.4 (buffer A), and applied to a 5-ml HiTrap Heparin HP column connected to an fast protein liquid chromatography system. The column was washed in buffer A, and heparin-binding proteins were eluted by a linear gradient from 0 to 1 M NaCl in buffer A. Fractions of 1 ml were collected and assayed for the presence of EC-SOD by SDS-PAGE and SOD activity analysis using the xanthine oxidase/cytochrome c assay (23). Fractions containing EC-SOD were pooled and dialyzed against 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, and applied to the anti-EC-SOD-Sepharose column. The column was washed with 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, followed by 10 mM Tris-HCl, pH 7.4. Bound proteins were eluted in 2-ml fractions using 0.1 M glycine, pH 2.7, neutralized by the addition of 1 M Tris-HCl, pH 8, and assayed for EC-SOD activity as described above.

**PAGE**—One-dimensional SDS-PAGE was performed in 12.5% uniform or 5–15% gradient gels using the glycin, 2-amino-2-methyl-1,3-propanediol, HCl system described by Bury (24). Prior to analysis, samples were boiled for 5 min in the presence of 1% SDS. For analysis under reducing conditions, 30 μl dithiothreitol was included in the sample buffer. Two-dimensional SDS-PAGE analysis was performed as described previously using a 3–10 non-linear pH gradient strip in the first dimension (25). Second dimension electrophoresis was performed using a 12.5% uniform gel. For subsequent analysis by Western blotting, proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) in 10 ml CAPS, 10% methanol, pH 11, as described (26). The membranes were blocked with 20 mM Tris-HCl, 137 mM NaCl, pH 7.4 (TBS) containing 5% (v/v) skimmed milk. EC-SOD was subsequently detected using a polyclonal rabbit anti-human EC-SOD antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG. All incubations were done in TBS containing 0.1% (v/v) Tween 20. The membrane was developed using an enhanced chemiluminescence detection system (Amersham Biosciences).

**RESULTS**

**Purification of R213G EC-SOD**—Plasma from individuals homozygous or heterozygous for the R213G mutation (as determined by PCR analysis) was applied to a heparin-Sepharose column. EC-SOD eluted from 200 to 500 mM NaCl with a peak at ~400 mM (Fig. 2, A and B; homozygote not shown). Indicated fractions were pooled, and proteins were further separated by affinity chromatography using a monoclonal anti-EC-SOD antibody column (Fig. 2, C and D). The eluted material migrated as two major bands of ~32 and 31 kDa when analyzed by reduced SDS-PAGE. The 31-kDa band was found to migrate between the intact and cleaved wild-type EC-SOD subunits (Fig. 2C). These data indicate that the upper band of 32 kDa represents the intact EC-SOD subunit (Trp^{1–Ala^{225}}), whereas the aberrant 31-kDa band represents a modified or truncated subunit different from both the cleaved and intact wild-type EC-SOD subunits. The obtained material was more than 95% pure as estimated by SDS-PAGE and Coomassie Blue staining.
Deglycosylation of Wild-type and Mutant EC-SOD—The intermediate migration of the 31-kDa EC-SOD subunit identified in the material from heterozygous and R213G homozygous individuals could be caused by post-translational modifications. Since N-linked glycosylation at Asn 89 is the only post-translational modification of wild-type human EC-SOD (13, 27), we treated mutant and wild-type EC-SOD with PNGase F to remove N-linked glycans. Western blot analysis showed that the relative migration of deglycosylated wild-type and mutant EC-SOD was retained after deglycosylation (Fig. 3). The reaction was not complete as traces of glycosylated material were observed in both preparations after deglycosylation (Fig. 3, +PNGase F). However, as only one glycosylation site is present in EC-SOD, the size reduction after PNGase F treatment represents fully deglycosylated proteins. This analysis shows that the difference in estimated size was not due to the modification or lack of N-linked glycosylation.

Two-dimensional PAGE—The ECM-binding region of wild-type EC-SOD (Glu209–Ala222) is highly basic due to the presence of 3 lysine and 4 arginine residues. The pI of the protein is therefore affected by proteolytic modification of this region. The theoretical pI of intact and cleaved wild-type EC-SOD is 6.3 and 5.5, respectively. This difference was evident when wild-type EC-SOD was analyzed by two-dimensional PAGE (Fig. 4A). The analysis of mutant EC-SOD purified from a homozygous individual indicates that the intact and the intermediate forms have similar pI-values (pI 6.2 of intact mutant subunit) (Fig. 4B). However, the intermediate form seems to be slightly more acidic than the intact mutant EC-SOD subunit. Both the wild-type and mutant material separates into several forms in the first dimension. The reason for this is unresolved but could be caused by deamidation of Asn or by carbamylation of Lys or Arg during gel electrophoresis. However, this micro-heterogeneity was similar in all EC-SOD forms, and the analysis supports the conclusion that the intermediate mutant EC-SOD subunit retains some basic residues in the C terminus not present in the cleaved wild-type EC-SOD subunit.

Proteolysis of Mutant EC-SOD with PP-IV—To investigate whether the mutant EC-SOD subunit of 31 kDa could be generated by cleavage of the Gly213–Arg214 bond in the mutant...
**ECM-binding region, we incubated wild-type and mutant EC-SOD with the Gly-specific PP-IV under native conditions. Wild-type EC-SOD was not affected by the proteinase, suggesting that no glycine residues are accessible for cleavage (Fig. 5A). However, the intact form of mutant EC-SOD was cleaved (Fig. 5B), and the product migrated in the same position as the intermediate 31-kDa band. This suggests that the C terminus of the intermediate band is Gly\textsuperscript{213}.

Identification of the C Terminus of Mutant EC-SOD—To identify the C terminus of the 31-kDa intermediate form of mutant EC-SOD, we subjected reduced and alkylated mutant EC-SOD, purified from a homozygous individual, to nondenaturing gel-electrophoresis. Wild-type EC-SOD was analyzed for comparison. The average mass of intact mutant EC-SOD was determined to be 26729.0 Da (Table I). This mass corresponds to Trp\textsuperscript{1}–Ala\textsuperscript{222} with 6 carbamidomethyl cysteines and a complex type bi-antennary carbohydrate structure with end group sialylation and core fucosylation. The mutant EC-SOD contained an Ala at the Thr/Ala-polymorphism at position 40 (28). The mutant EC-SOD presented another molecular species of 25641.0 Da (Table I). This mass corresponds to alkylated and glycosylated Trp\textsuperscript{1}–Gly\textsuperscript{213}. This shows unequivocally that the C terminus of processed mutant EC-SOD is Gly\textsuperscript{213}. The average masses of intact (Trp\textsuperscript{1}–Ala\textsuperscript{222}) and cleaved (Trp\textsuperscript{1}–Glu\textsuperscript{209}) wild-type carbamidomethylated EC-SOD containing carbohydrate at Asn\textsuperscript{89} and a Thr residue at position 40 were 26857.0 and 25199.8 Da, respectively (Table I). These data confirm that the processing of the mutant subunit takes place but is incomplete as compared with wild-type.

**DISCUSSION**

To investigate the mechanism of the intracellular proteolytic processing of the ECM-binding region of EC-SOD, we purified the R213G variant from the plasma of both hetero- and homozygous individuals. As observed previously (22, 29), EC-

**Gly\textsuperscript{213} of Mutant EC-SOD Disrupts C-terminal Processing**

EC-SOD eluted from the heparin-Sepharose with a broad peak at \(\sim 400\) mM NaCl, indicating that the heparin affinities of mutant EC-SOD are similar or slightly lower than those of EC-SOD purified from wild-type individuals (4, 29). Further purification of mutant EC-SOD by affinity chromatography revealed that the material contained an intact subunit and a subunit with a size intermediate of intact and fully cleaved wild-type subunits. It is interesting to note that the material obtained from heterozygous individuals did not contain the cleaved wild-type subunit as analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 2). However, we were able to detect the cleaved form of the wild-type monomer by Western blotting (extended exposure) and mass spectrometry. Analysis by mass spectrometry did not show the presence of the intact wild-type subunit (data not shown). These analyses indicate that only trace amounts of wild-type subunits are present in the material obtained from the plasma of heterozygous individuals. This finding is supported by Adachi et al. (29), who showed that the heparin affinity of EC-SOD from the plasma of heterozygous individuals is similar to that of homozygous individuals and slightly reduced as compared with wild-type EC-SOD. These observations indicate that 1) some regulation of tetramer assembly occurs, making the combination of mutant and wild-type subunits less likely. Thus, tetramers composed of wild-type subunits only will largely be retained in the tissue, and tetramers composed of mutant tetramers diffuse into the circulation due to reduced affinity; or 2) the tetramers containing both wild-type and mutant subunits have sufficient affinity for ECM ligands and are therefore retained in the tissue, whereas those of mutant subunits only diffuse into the circulation.

N-terminal sequencing of the mutant intact and intermediate subunits produced the mature N terminus WTGEN\textsuperscript{5} (data not shown). Therefore, the intermediate electrophoretic mobility of the R213G mutant subunit was the result of either a lack of post-translational modifications (\(-2\)kDa reduction of size) or the incomplete proteolytic processing of the C terminus. EC-SOD carries a complex-type bi-antennary, end-sialylated, and core-fucosylated glycan at Asn\textsuperscript{89} (13, 27). Removal of the N-linked carbohydrate did not resolve the difference in electrophoretic mobility, indicating that the difference in mobility
resides in the C terminus. Two-dimensional gel-electrophoresis and cleavage by a Gly-specific proteinase in concert with mass spectrometry proved that the mature C terminus of the intermediate form is Gly213. The intermediate migration in SDS-PAGE is thus somewhat surprising since the cleaved wild-type subunit (Trp1–Glu209) and the intermediate subunit only differs by 4 residues (210RKKG213), corresponding to 487.6 Da. However, the presence of three consecutive basic residues may reduce migration slightly due to an increased binding of SDS or affect the conformation of the EC-SOD molecule during electrophoresis (30).

We have previously suggested (11) and presented evidence that a two-step mechanism is involved in the removal of the ECM-binding region (13). The data indicated that the mechanism was initiated by an endoproteinase cleavage followed by C-terminal trimming by a carboxypeptidase. Several lines of evidence suggested that furin or another member of the proprotein convertase family of processing proteinases is responsible for the initial cleavage event. The EC-SOD sequence 209ERKKRRRE216 (Fig. 1) contains three potential furin consensus cleavage sites where Arg213, Arg214, or Arg215 may act as potential P1 sites (13, 31). Since (i) the R213G mutation has no effect on the initial cleavage event and (ii) a P1 Gly residue disrupts both of the furin consensus sequences containing Arg213 or Arg214 in P1, it is most likely that the initial cleavage occurs between Arg215 and Glu216 (Fig. 6). However, the R213G mutation disrupts the C-terminal trimming by an unknown carboxypeptidase. The blocking of this reaction by this mutation suggests that the carboxypeptidase is specific for basic residues. This cleavage between Arg215 and Glu216 complies with the consensus sequence for furin and is compatible with cleavage of both wild-type and mutant EC-SOD (Fig. 1). This suggested that an initial cleavage event would generate a subunit with Arg215 as the C terminus, i.e., with a size intermediate of intact and fully cleaved subunits (Fig. 6). It is interesting to note that no band migrating between the intact and the cleaved wild-type subunits was observed when a cell culture supernatant was analyzed by SDS-PAGE following a pulse-chase protocol (11). This finding indicates that no wild-type subunits are secreted immediately after the initial cleavage event. Therefore, it is most likely that the carboxypeptidase responsible for the second proteolytic event is located intracellularly (Fig. 6).

We have confirmed that the intracellular proteolytic processing of EC-SOD is a two-step event and that the mature C terminus of mutant EC-SOD is Gly213. This suggests that the initial cleavage in the ECM-binding region can take place in both wild-type and mutant EC-SOD. This initial cleavage can be performed by furin or another member of the proprotein convertase family of processing proteinases at position Arg215. The second step of processing involves the activity of a carboxypeptidase. The finding that Gly213 blocks the further trimming of the C terminus to Glu209 suggests that this peptidase

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**TABLE I**

| EC-SOD subunit     | Experimental average mass | Theoretical average mass | Sequence               |
|--------------------|---------------------------|--------------------------|------------------------|
| Wild-type, intact  | 26857.0                   | 26857.3                  | Trp1–Ala222 (Thr40)    |
| Wild-type, cleaved | 25199.8                   | 25200.4                  | Trp1–Glu209 (Thr40)    |
| Mutant, intact     | 26729.0                   | 26728.2                  | Trp1–Ala222 (Ala40)    |
| Mutant, intermediate| 25641.0                   | 25640.0                  | Trp1–Gly213 (Ala40)    |

* The theoretical average mass of alkylated intact and cleaved subunits includes a bi-antennary, end-sialylated, and core fucosylated glycan attached to Asn40 and 6 or 5 carbamidomethyl cysteines, respectively.

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**FIG. 6.** Schematic illustrating the proposed two-step mechanism of processing. Both wild-type and mutant EC-SOD subunits are initially cleaved by an endoproteinase, which is likely to be a member of the furin family of processing proteinases. The C-terminal residues are subsequently removed by the action of a carboxypeptidase specific for basic residues. The R213G mutation (shown in **bold**) interferes with the action of this carboxypeptidase and prohibits the generation of the mature wild-type Glu209 C terminus.
has a carboxypeptidase B-type specificity (Arg or Lys residues). Consequently, the processed subunit of mutant EC-SOD contains 4 additional C-terminal amino acids (210RKKG213). Apart from the reduced heparin affinity of mutant EC-SOD, it could be speculated that the additional amino acids could modulate the interaction with other ligands or proteins. Recent data suggest that EC-SOD can be internalized and transported to the nucleus and that this translocation is dependent on the ECM-binding region (32). Further characterization of the EC-SOD processing pathway will provide a better understanding of how the cell controls type-specific EC-SOD production (Types-A, -B, and -C) and identify potential targets that can regulate this process.

Acknowledgment—We thank Susan Fields for contributing to this study.

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