Proteolytic modification of the heparin-binding affinity of extracellular superoxide dismutase

Kurt KARLSSON,* Anders EDLUND,† Jan SANDSTRÖM,† and Stefan L. MARKLUND*§

*Department of Clinical Chemistry, Umeå University Hospital, S-901 85 Umeå, Sweden; †Department of Microbiology, Umeå University, S-901 87 Umeå, Sweden; and §SYMBICOM AB, P.O. Box 1451, S-901 24 Umeå, Sweden

The heparin-binding affinity of the tetrameric extracellular superoxide dismutase (EC-SOD) is a result of the cooperative effect of the heparin-binding domains of the subunits, located in the hydrophilic, strongly positively charged C-terminal ends. EC-SOD C, the high-heparin-affinity type, exposed to immobilized trypsin and plasmin was found to rapidly lose its affinity for heparin, without any loss of enzymic activity or major change in molecular mass as judged by size-exclusion chromatography. Heparin and dextran sulphate 5000 inhibited the proteolysis, suggesting that EC-SOD C sequestered by heparan sulphate proteoglycan *in vivo* is partially protected against proteolysis. The loss of heparin-affinity occurred with the stepwise formation

of intermediates, and the pattern upon chromatography on heparin-Sepharose and subsequent immunoblotting was compatible with the notion that the changes are due to sequential truncations of heparin-binding domains from subunits composing the EC-SOD tetramers. A similar pattern with intermediates and apparent truncations has previously been found with EC-SOD of human plasma. The findings show that the unique design of the heparin-binding domain of EC-SOD allows easy modification of the heparin-affinity by means of limited proteolysis, and suggest that such proteolysis is a major contributor to the heterogeneity in heparin-affinity of EC-SOD in mammalian plasma.

INTRODUCTION

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is a secretory, Cu- and Zn-containing glycoprotein composed of four 30 kDa subunits [1,2]. EC-SOD in plasma in man [3] and other mammals [4] is heterogeneous with regard to heparin-binding affinity, and can be divided into at least three fractions, A which lacks affinity, B which has weak affinity and C which has relatively strong affinity. The physiological ligand of EC-SOD C is the heparin analogue heparan sulphate [5,6] and *in vivo* EC-SOD C occurs mainly anchored to heparan sulphate proteoglycan in the glycocalyx of cell surfaces and in the connective-tissue matrix [6]. EC-SOD A and B seem to exist mainly in the extracellular fluid phase [4]. The structural background to the heterogeneity *in vivo* is unknown. There is no gross difference between the subclasses with regard to apparent molecular mass, amino-acid composition [1], specific activity [1] and antigenic reactivity [7].

The binding to the negatively charged molecules heparin and heparan sulphate is of electrostatic nature [5]. The C-terminal end of the 222 amino-acid-long EC-SOD C subunit, contains six Arg, three Lys and one His among the last 20 amino-acid residues [8]. Based on studies of a series of EC-SOD variants with sequential truncations or amino-acid substitutions in the C-terminal end, it was recently concluded that this region forms the heparin-binding domain, with an unusual cluster of six positively charged amino-acid residues in positions 210–215 constituting the essential part [9]. The C-terminal end is highly hydrophilic and should extend into the solvent and be easily available for post-translational modifications such as proteolytic cleavage. In the present paper we explore the effect of proteolytic enzymes on the heparin-binding affinity of EC-SOD C. We show that by limited proteolysis a series of fractions with reduced heparin-binding affinity can be created, mimicking the heterogeneity *in vivo*.

EXPERIMENTAL

Chemicals

Heparin-Sepharose, CNBr-activated Sepharose, DEAE-Sepharose and Sepharose S-300 were purchased from Pharmacia LKB Biotechnology, Bromma, Sweden. Human recombinant EC-SOD C [2], was obtained from SYMBICOM AB, Umeå, Sweden. Tosylphenylalanylchloromethyl ketone-treated bovine trypsin (Sigma, St. Louis, MO, U.S.A.) and human plasmin were immobilized on CNBr-activated Sepharose at 12 mg/g and 1 mg/g of wet gel respectively according to the manufacturer's suggestions.

Exposure of EC-SOD C to immobilized trypsin and plasmin

To three tubes containing 1 mg/ml EC-SOD C in 10 mM potassium phosphate, pH 7.4, plus 150 mM NaCl, were added trypsin (64 µg/ml) and plasmin (24 µg/ml), both immobilized on Sepharose 4B, or plain Sepharose 4B in a corresponding amount. The tubes were incubated at 23 °C with shaking. After time intervals of 0 min, 5 min, 20 min, 1 h, 3 h, 6 h and 24 h samples were removed, centrifuged for 2 min and aprotinin 7 µg/ml was added to the supernatants to inhibit any protease lost from the Sepharose. The samples were divided into several aliquots and were kept at –80 °C until required for further assay.

Effect of heparin and dextran sulphate 5000 on cleavage of EC-SOD C and casein by trypsin

To two tubes containing EC-SOD C at a concentration of 1 mg/ml in 10 mM potassium phosphate, pH 7.4, plus 150 mM NaCl was added 1 mg/ml heparin and 0.42 mg/ml dextran sulphate 5000 respectively. To remove excess, but still retain a saturating concentration of sulphated polysaccharide [10] the samples were then washed three times with 10 vol. of buffer with

Abbreviation used: EC-SOD, extracellular superoxide dismutase.

§ To whom correspondence should be addressed.

the polysaccharides at a concentration of 3 $\mu\text{g/ml}$ in an Omegacell ultrafiltration system. The samples were then incubated at 25 °C with trypsin (128 $\mu\text{g/ml}$) immobilized on Sepharose 4B. Aliquots of 500 μl were removed at time zero and after 1 h and 14 $\mu\text{g/ml}$ aprotinin was added to the supernatants after centrifugation. Before separation of the samples on heparin-Sepharose for analysis of conversion into type A, the sulphated polysaccharides had to be removed. The samples were diluted 10-fold with 10 mM sodium phosphate, pH 6.5, and NaCl was added to a final concentration of 0.4 M. The sulphated polysaccharides were then absorbed from the EC-SOD by incubation for 20 min with 0.2 vol. of wet DEAE-Sephacel. After centrifugation, the supernatants were analysed for conversion of EC-SOD C into type A. A third control tube was similarly treated except that no sulphated polysaccharide was added.

To test for the possibility that the effects of heparin and dextran sulphate 5000 on EC-SOD C proteolysis were due to inhibitory effects of the polysaccharides on the trypsin, their effects in a standard caseinolytic assay [11] were also examined. The assay was run for 1 h at 23 °C with 5 mg/ml casein in 10 mM potassium phosphate, pH 7.4, plus 150 mM NaCl and with non-immobilized trypsin at a concentration (10 $\mu\text{g/ml}$) that produced about half-maximal release of perchloric acid-soluble u.v.-absorbing material.

Determination of EC-SOD type A with heparin-Sepharose

Protease-exposed EC-SOD was diluted 10-fold with 15 mM sodium cacodylate, pH 6.50, containing 50 mM NaCl and 0.2 % BSA. A sample (0.5 ml) of the solution, containing about 50 μg of EC-SOD, was then applied to a 1 ml heparin-Sepharose column equilibrated with the same buffer. Three 1 ml volumes of buffer were then added and the eluting non-binding EC-SOD A was collected. The bound EC-SOD B and EC-SOD C was eluted with 0.7 M NaCl in the buffer. EC-SOD concentrations were determined in collected fractions using e.l.i.s.a.

Analytical separation of EC-SOD on heparin-Sepharose

Protease-exposed EC-SOD C was diluted 10-fold with 15 mM sodium cacodylate, pH 6.50, containing 50 mM NaCl and 0.2 % BSA. A sample (0.6 ml) of the solution, containing about 55 μg of EC-SOD was then applied to a 1 ml heparin-Sepharose column equilibrated and run at 10 ml/h with the same buffer. After elution of about 30 ml, bound components were eluted with a linear gradient of NaCl in the buffer (0.05–1 mol/l). The effluent was collected in 0.65 ml fractions. In the fractions, EC-SOD concentration was determined using e.l.i.s.a., and the chloride content with a chloride titrator (American Instrument Co. Inc., MO, U.S.A.).

Size-exclusion chromatography

Control and protease-exposed EC-SOD C were applied to a Sephacryl S-300 column (1.6 cm $\times$ 90 cm) operating at 20 ml/h with 10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl. EC-SOD was determined by means of e.l.i.s.a. on collected 1.35 ml fractions. The column was calibrated with IgG (156 kDa), BSA (67 kDa) and carbonic anhydrase (29 kDa).

Electrophoresis and immunoblotting

SDS/PAGE was run in 12.5 % (w/v) gels. They were stained with Coomassie Brilliant Blue. For immunoblotting the SDS/

PAGE gels were electroblotted at a constant current (2 mA/cm²) for 30 min on to an Immobilon polyvinylidene fluoride membrane (Millipore Inc., Bedford, MA, U.S.A.) using a Transblot SD Transfer cell (Bio-Rad Inc., Richmond, CA, U.S.A.). Blots were developed using affinity-purified rabbit antibodies directed against human EC-SOD followed by a biotinylated secondary antibody and thereafter a biotin/avidin-peroxidase complex (biotin/avidin antibodies were obtained from Dakopatts, Denmark). The developing buffer contained 0.05 M Tris, pH 7.2, 1 mg/ml diaminobenzidine and 0.01 % H₂O₂.

SOD analysis

SOD enzymic activity was determined with the direct spectrophotometric method employing KO₂ [12] as modified in [13]. EC-SOD protein was determined with an e.l.i.s.a. [14].

RESULTS

Figure 1 shows the formation of non-binding type A from EC-SOD C exposed to trypsin and plasmin immobilized on Sepharose 4B. Exposure to both proteases was found to result in rapid loss of heparin-binding affinity, which was nearly complete at 24 h. There was, however, no loss of the EC-SOD enzymic activity as a result of the exposure to the proteases. Trypsin had no significant effect on the activity, whereas there was even some increase in activity after exposure to plasmin.

The conversion into type A was, however, not direct, but occurred via fractions with intermediate heparin-binding affinities, as seen in the examples in Figure 2.

There was no major change in apparent molecular mass of the EC-SOD exposed to immobilized trypsin and plasmin as assessed by size-exclusion chromatography. Control and 24-h-incubation samples eluted at positions not with certainty different, near the elution volume of IgG (results not shown).

Figure 3 shows SDS/PAGE analysis of EC-SOD exposed to trypsin. The initial major band of molecular mass about 32 kDa is gradually converted into another major band with a molecular mass apparently 1.5–2 kDa lower. No evidence of a major

Figure 1 Time-course of proteolytic conversion of EC-SOD C into type A and its effect on enzymic activity

EC-SOD C was exposed to trypsin (64 $\mu\text{g/ml}$) and to plasmin (24 $\mu\text{g/ml}$) immobilized on Sepharose 4B. After indicated times, samples were withdrawn and the non-heparin-binding type A EC-SOD determined by means of chromatography on heparin-Sepharose as described in the Experimental section; $\blacktriangle$, trypsin; $\bullet$, plasmin. The SOD enzymic activity was also determined in the samples and is presented as a percentage of initial activity; $\triangle$, trypsin; $\circ$, plasmin; $\times$, Sepharose 4B control.

Figure 2 Separation of EC-SOD on a heparin-Sepharose column after exposure to trypsin

EC-SOD C was exposed to immobilized trypsin (64 µg/ml), and samples were withdrawn and their heparin-binding-affinity patterns analysed as described in the Experimental section. Key to symbols: ▲, before trypsin; □, after 1 h; ×, after 3 h; and △, after 6 h. The broken line shows the gradient of NaCl. I, II, III, IV and V denote fractions in the 1 h chromatogram analysed by immunoblotting, cf Figure 4.

Figure 3 SDS/PAGE analysis of EC-SOD exposed to trypsin

EC-SOD C was incubated with immobilized trypsin (64 µg/ml) and samples were withdrawn after indicated times for analysis by SDS/PAGE as described in the Experimental section. From left to right lanes contain a molecular-mass standard (kDa), samples withdrawn after 5 min, 20 min, 1 h, 3 h, 6 h, 24 h and finally parental EC-SOD C. About 5 µg of protein was applied.

Figure 4 Immunoblot of trypsin-digested EC-SOD separated by heparin-Sepharose

The lanes are, from the left, the EC-SOD truncation variant T209, fractions I, II, III, IV, V from the chromatogram of the 1 h trypsin digest of Figure 2 and finally parental EC-SOD C. To the left are given mobilities of molecular-mass standards in kDa.

degradation of the enzyme is seen. The pattern upon exposure to plasmin was similar (results not shown).

Figure 4 shows an immunoblot of the five fractions marked in the heparin-Sepharose chromatogram (Figure 2) of EC-SOD C

exposed to trypsin for 1 h. Also applied were a 209 amino-acid-long C-terminal EC-SOD C truncation variant, T209 [9], and parental EC-SOD C. Peak V is apparently similar to the parental EC-SOD C, whereas the peaks with lower heparin-binding affinity contain increasing amounts of low-molecular-mass material. A major truncated peak can be discerned, with an apparent molecular mass which cannot with certainty be distinguished from that of the EC-SOD truncation variant T209. There was only a minor formation of further degraded immunoreactive material, with the largest amounts seen in fractions with low heparin-binding affinity.

Heparin and dextran sulphate 5000 bind very strongly to the heparin-binding domain of EC-SOD C [10]. They were found, at a concentration saturating the binding to EC-SOD C, to efficiently inhibit the conversion into type A. Thus EC-SOD C exposed to immobilized trypsin for 1 h under the conditions described in the Experimental section, was only 3.3 % and 1.7 % converted into type A in the presence of heparin and dextran sulphate 5000 respectively, whereas in the control incubation 51 % was transformed into type A. The effects observed were not due to inhibition of the trypsin since the sulphated polysaccharides, tested at concentrations up to 3 mg/ml, had no effect on proteolysis of casein.

DISCUSSION

As expected from the structural data, the heparin-binding domain of EC-SOD C proved very susceptible to proteolysis, whereas despite prolonged exposure to the proteolytic enzymes no major changes in apparent molecular mass or loss of enzymic activity occurred. Ligands for the heparin-binding domain, heparin and dextran sulphate 5000, inhibited the proteolysis efficiently. Both trypsin and plasmin cleave proteins on the carboxy side of positively charged amino-acid residues and the cluster of positively charged amino-acid residues in positions 210–215 is a probable major target of the enzymes. The SDS/PAGE and immunoblot studies reveal the final formation by proteolysis of one major band with an apparent loss of 1.5–2 kDa in molecular mass. Its apparent molecular mass could not be distinguished from that of the EC-SOD variant T209. The finding is compatible with a final cleavage at one or a few sites in the N-terminal part of the cluster.

Studies on recombinant EC-SOD homo- and hetero-tetramers created by co-transfection of plasmids defining EC-SOD subunits possessing and lacking the C-terminal heparin-binding domain, have demonstrated that the heparin-binding affinity is due to the cooperative effects of the subunits [9]. The present proteolytic truncations of the C-terminal end should therefore result in a series of EC-SOD fractions with reduced and finally lost heparin-binding affinities. Such fractions are also formed as shown in Figure 2. The immunoblot study, Figure 4, also reveals larger amounts of low-molecular-mass material at lower heparin-binding affinities.

In a recent detailed study on the heterogeneity of human plasma EC-SOD (J. M. Sandström, T. Edlund, K. Karlsson and S. L. Marklund, unpublished work) a similar pattern with intermediates upon heparin-Sepharose chromatography was found, and an immunoblot study of fractions similarly showed higher amounts of material with apparently reduced molecular mass the lower the heparin-binding affinity. Again a final major band with an apparent molecular mass indistinguishable from that of the variant T209 was found.

All cDNAs so far isolated have encoded EC-SOD of C type [8]. All cell lines studied, that express EC-SOD, secrete enzyme of C class [15], and human tissues that account for about 99 % of

the EC-SOD of the body contain uniformly EC-SOD of C class (J. Sandström, T. Edlund, K. Karlsson and S. L. Marklund, unpublished work). It is thus likely that EC-SOD is primarily synthesized and secreted as C class, and that fractions with reduced heparin-binding affinity are formed secondarily.

These findings together suggest that limited proteolysis is a major cause of the heterogeneity of EC-SOD in mammalian plasma. It should, however, be noted that apparently there is also some contribution by glycation of lysine residues in the heparin-binding domain [16]. The EC-SOD C of the tissues is almost completely sequestered by heparan sulphate proteoglycan. The present results indicate that this binding reduces the susceptibility to proteolysis. The sequestering is also of fundamental importance for the retention of the enzyme in the tissue interstitium. EC-SOD C subcutaneously and intramuscularly injected is locally retained in the tissue with a half-life 10-fold longer than that of EC-SOD A (K. Karlsson, A. Edlund, J. Sandström and S. L. Marklund, unpublished work). Any proteolysis occurring would thus reduce heparan sulphate affinity and lead to facilitated entry of EC-SOD into the plasma via capillaries and lymphatics. Such a mechanism would explain the heterogenous heparin-binding-affinity pattern of EC-SOD in plasma. Finally, it should be noted that enhanced local proteolytic activity in the tissue caused by e.g. inflammation or tissue injury, may lead to a significant loss of EC-SOD from the interstitium.

The reason for the increase in enzymic activity in EC-SOD treated with plasmin for 24 h, see Figure 1, is not clear. It may be related to changes in electrostatic guidance of the superoxide radical towards the active site [17] or to changes in steric hindrance caused by the loss of the hydrophilic, strongly positively charged C-terminal end. It is peculiar that a similar rise in activity was not seen in EC-SOD C treated with trypsin, but the effect may have been missed in the 6 h to 24 h incubation interval, cf. Figure 1. Some proteolytic degradation beyond the loss of the heparin-binding domain does occur, Figure 4, and at 24 h such degradation may have caused a loss of the activity enhancement. It is furthermore possible that such further degradation primarily occurs with trypsin, which has a wider substrate specificity than plasmin [18].

The heparin-binding domain of EC-SOD C is unique in that it does not conform to the suggested consensus sequence for

heparin-binding domains [19], in that the amino-acid residues involved are located closely together in a readily available extreme end of the subunit polypeptide and in that the heparin-binding affinity of the enzyme is due to the cooperative activity of the subunits [9]. This construction allows easy stepwise reduction of the heparin-binding affinity of the enzyme by limited proteolysis. Whether such proteolysis occurs regulated with the intent to reduce the local tissue concentration of EC-SOD or enhance the protection against superoxide radicals of the extracellular fluid phase, or whether the phenomenon should be regarded only as a step in the degradation of the enzyme remains the subject of speculation.

The skilful technical assistance of Ms. Agneta Öberg is gratefully acknowledged. Professor Per Wallén (Department of Physiological Chemistry, Umeå University) is thanked for advice and the gift of the immobilized proteases. The study was supported by the Swedish Natural Sciences Research Council, grant no. 9204.

REFERENCES

- 1 Marklund, S. L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7634–7638
- 2 Tibell, L., Hjalmarsson, K., Edlund, T., Skogman, G., Engström, Å. and Marklund, S. L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6634–6638
- 3 Karlsson, K. and Marklund, S. L. (1987) *Biochem. J.* **242**, 55–59
- 4 Karlsson, K. and Marklund, S. L. (1988) *Biochem. J.* **255**, 223–228
- 5 Karlsson, K., Lindahl, U. and Marklund, S. L. (1988) *Biochem. J.* **256**, 29–33
- 6 Karlsson, K. and Marklund, S. L. (1989) *Lab. Invest.* **60**, 659–666
- 7 Marklund, S. L. (1984) *Biochem. J.* **220**, 269–272
- 8 Hjalmarsson, K., Marklund, S. L., Engström, Å. and Edlund, T. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6340–6344
- 9 Sandström, J., Carlsson, L., Marklund, S. L. and Edlund, T. (1992) *J. Biol. Chem.* **267**, 18205–18209
- 10 Adachi, T. and Marklund, S. L. (1989) *J. Biol. Chem.* **264**, 8537–8541
- 11 Müllerts, S. (1955) *Biochem. J.* **61**, 424–434
- 12 Marklund, S. L. (1976) *J. Biol. Chem.* **251**, 7504–7507
- 13 Marklund, S. L. (1985) in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.), pp. 249–255, CRC Press, Boca Raton, FL
- 14 Karlsson, K. and Marklund, S. L. (1988) *J. Clin. Invest.* **82**, 762–766
- 15 Marklund, S. L. (1990) *Biochem. J.* **266**, 213–219
- 16 Adachi, T., Ohta, H., Hirano, K., Hayashi, K. and Marklund, S. L. (1991) *Biochem. J.* **279**, 263–267
- 17 Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L. and Hallewell, R. A. (1992) *Nature (London)* **358**, 347–351
- 18 Wallén, P. (1968) *Biochim. Biophys. Acta* **154**, 414–417
- 19 Cardin, A. D. and Weintraub, H. J. R. (1989) *Arteriosclerosis* **9**, 21–32