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Binding of human extracellular superoxide dismutase C to sulphated glycosaminoglycans

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The secretory enzyme extracellular superoxide dismutase (EC-SOD) occurs in at least three forms, which differ with regard to heparin affinity: A lacks affinity, B has intermediate affinity, and C has relatively strong affinity. The affinity of EC-SOD C for various sulphated glycosaminoglycans (GAGs) was assessed (*a*) by determining the concentration of NaCl required to release the enzyme from GAG-substituted Sepharose 4B and (*b*) by determining the relative potencies of the GAGs to release EC-SOD C from heparan sulphate–Sepharose 4B. Both methods indicated the same order of affinity. Heparin bound EC-SOD C about 10 times as avidly as the studied heparan sulphate preparation, which in turn was 10 and 150 times as efficient as dermatan sulphate and chondroitin sulphate respectively. Chondroitin sulphate showed weak interaction with EC-SOD C at physiological ionic strength. Heparin subfractions with high or low affinity for antithrombin III were equally efficient. The binding of EC-SOD C to heparin–Sepharose was essentially independent of pH in the range 6.5–9; below pH 6.5 the affinity increased, and beyond pH 9.5 there was a precipitous fall in affinity. The inhibitory effect of NaCl on the binding of EC-SOD C to GAGs indicates that the interaction is of electrostatic nature. EC-SOD C carries a negative net charge at neutral pH, and it is suggested that the binding occurs between the negative charges of the GAG sulphate groups and a structure in the *C*-terminal end of the enzyme that has a cluster of positive charges. These results are compatible with the notion that heparan sulphate proteoglycans on cell surfaces or in the intercellular matrix may serve to bind EC-SOD C in tissues.

INTRODUCTION

The secretory enzyme [1] extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is the major SOD isoenzyme in extracellular fluids such as plasma, lymph [2] and synovial fluid [3], and occurs also in tissues [4,5]. EC-SOD is a tetrameric Cu- and Zn-containing glycoprotein with an apparent subunit molecular mass of 30 kDa [1,6]. Plasma EC-SOD from man [7] and other mammals [8] is heterogeneous with regard to affinity for heparin–Sepharose and can be separated into three fractions: A, which lacks affinity, B, which has weak affinity, and C, which has relatively strong affinity for heparin. Intravenous injection of heparin in man [7] and most other mammals [8] leads to a prompt release of EC-SOD type C to plasma. Human umbilical-cord endothelial cells and many other cultured cell lines bind human EC-SOD C (K. Karlsson & S. L. Marklund, unpublished work). Cell-surface-associated heparan sulphate is apparently the binding substance. The heparin-induced release of EC-SOD C to plasma may thus be due to displacement of the enzyme from heparan sulphate in the glycocalyx of vessel endothelium. This surface association of EC-SOD C is apparently an important feature of the enzyme.

However, most of the EC-SOD in the body is not located to blood and vessel endothelium, but occurs in other locations in tissues [2,4,5]. Tissues contain different classes of sulphated glycosaminoglycans (GAGs) [9,10]. The aim of the present study was to assess the affinity of human EC-SOD C for various types of GAGs in order to gain further insight into the possible mode of binding of the enzyme in the body.

MATERIALS AND METHODS

Materials

Human EC-SOD C was isolated from umbilical cord [1]. Heparin (sodium salt; 2.3 sulphate groups/disaccharide unit) prepared from pig intestinal mucosa was obtained from KabiVitrum AB, Stockholm, Sweden. The specific anticoagulant activity was stated to be 160 i.u./mg. Heparin, fractionated with regard to affinity for antithrombin III (into high-affinity and low-affinity fractions) [11], was kindly donated by Dr. Hans-Peter Ekre, KabiVitrum AB. Their specific anticoagulant activities were 252 and 25 i.u./mg respectively. Two preparations of heparan sulphate, derived from pig intestinal mucosa, were obtained from products of commercial heparin manufacture by fractional precipitation with cetylpyridinium chloride. One of these preparations (designated HS-1; 0.7 residue of sulphate/disaccharide unit) was obtained by precipitation from 0.4 M-NaCl following removal of material precipitable at 0.8 M-NaCl. The resulting product was purified further

Abbreviations used: EC-SOD, extracellular superoxide dismutase; GAG, glycosaminoglycans; e.l.i.s.a., enzyme-linked immunosorbent assay.
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by digestion of galactosaminoglycans with chondroitinase ABC lyase [12]. The other heparan sulphate preparation (HS-2; 1.5 residues of sulphate/disaccharide unit) was obtained in a similar manner, and contained polysaccharide precipitable with cetylpyridinium chloride at 0.4 M but not at 1.2 M concentration of NaCl. The dermatan sulphate preparation (1.3 sulphate residues/disaccharide unit) was kindly donated by Dr. L. Rodén, Birmingham, AL, U.S.A. The purification of this material involved degradation of heparin-related components by treatment with HNO_2 [13]. Chondroitin sulphate, isolated from bovine nasal septa (1.0 sulphate residue/disaccharide unit), was kindly donated by Dr. Å. Wasteson, Linköping, Sweden. The hexuronic acid contents of the various GAG preparations ranged between 23% (low extreme shown by dermatan sulphate owing to its low colour yield in the carbazole reaction [14]) and 35% on a weight basis (not corrected for moisture). Heparin–Sepharose and Sepharose 4B were products of Pharmacia Laboratory Separation Division, Uppsala, Sweden. Carbazole was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. Bovine serum albumin was a product of Sigma Chemical Co., St. Louis, MO, U.S.A. CNBr was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Determination of EC-SOD

EC-SOD enzymic activity was assayed by means of the direct spectrophotometric method employing KO_2 [15] with modifications, as described in ref. [16]. One unit corresponds to 8.8 ng of human EC-SOD. Quantification of EC-SOD C protein was made by double-antibody sandwich e.l.i.s.a. [17].

Labelling of human EC-SOD C with ^{125}I

EC-SOD C was labelled with ^{125}I by using the Iodogen method [18] with some modifications [17]. About 0.04 mol of ^{125}I was incorporated per mol of EC-SOD C subunit. The ^{125}I radioactivity of samples was counted in a 1260 Multigamma counter (LKB Wallac, Stockholm, Sweden). To keep the counting geometry constant, the volume of the samples was always 1 ml.

GAG-substituted Sepharose 4B

GAG-substituted gels were prepared by mixing GAG (4 mg/ml of wet gel), Sepharose 4B and CNBr under the conditions described by Miller-Andersson *et al.* [19]. To block remaining activated sites on the Sepharose, the gels were incubated with 0.1 M-ethanolamine for 2 h at 4 °C. The gels were then washed with distilled water followed by 2 M-NaCl in 10 mM-potassium phosphate buffer, pH 7.4, then 0.1 M-sodium acetate buffer, pH 4.5, and finally 10 mM-potassium phosphate buffer, pH 7.4. To test for non-specific binding of EC-SOD C to activated Sepharose 4B, a blank gel was prepared by mixing Sepharose 4B and CNBr under the conditions described above. The gels were stored in 10 mM-potassium phosphate buffer, pH 7.4, containing 0.2% NaN_3 at 4 °C until used for experiments within 6 months. The yield of coupling of GAG to Sepharose 4B was determined by analysing the GAG content in the supernatant before and after the coupling, by using the carbazole method [14]. The amounts of coupled GAG/ml of wet gel were 0.6, 0.6, 1.6, 0.7 and 0.5 mg for heparin, heparan sulphate HS-1, heparan sulphate HS-2, chondroitin sulphate and dermatan sulphate respectively.

Binding of EC-SOD C to GAG-substituted Sepharose 4B and its displacement by NaCl

About 5 μg (570 units) of EC-SOD C dissolved in 2 ml of 50 mM-NaCl/15 mM-sodium cacodylate buffer, pH 6.5, containing 0.5% (w/v) bovine serum albumin was added to 200 μl of GAG-substituted Sepharose. The mixture was gently agitated at room temperature. To determine the binding of EC-SOD C, the suspension was centrifuged (20000 *g* for 1 min) and the SOD activity of the supernatant was assayed. After 2 h (maximal binding occurred after about 1.5 h) the NaCl concentration was increased in a stepwise fashion, each consecutive ~ 0.1 M concentration increment being preceded by a 30 min equilibration (a new equilibrium in binding was reached after 20–25 min). The SOD activity in the buffer was determined just before the next NaCl addition.

Displacement of human EC-SOD C from heparan sulphate–Sepharose 4B by GAGs

About 5 μg (570 units) of EC-SOD C dissolved in 2 ml of 0.2 M-NaCl/15 mM-sodium cacodylate buffer, pH 6.5, containing 0.5% (w/v) bovine serum albumin was added to 200 μl of Sepharose 4B substituted with heparan sulphate HS-1. The suspension was gently agitated at room temperature. After 2 h the competitory GAGs were added in a stepwise fashion at 30 min intervals, and the release of EC-SOD C was determined as described above.

pH-dependence of binding of EC-SOD C to heparin–Sepharose

^{125}I-EC-SOD (about 1.5×10^6 c.p.m., 0.4 μg) dissolved in 2.3 ml of the appropriate 25 mM buffer containing 0.1 M-NaCl and 0.5% (w/v) bovine serum albumin was added to 0.2 ml of heparin–Sepharose, previously equilibrated overnight with the same buffer. The suspension was gently agitated at room temperature. After 2 h NaCl was added in a stepwise fashion as described above. The radioactivity was determined in the supernatant after centrifugation just before each NaCl addition.

To assess the binding properties of ^{125}I-labelled as compared with unlabelled EC-SOD C, ^{125}I-EC-SOD C (1.5×10^6 c.p.m., 0.4 μg) and EC-SOD C (5 μg, 570 units) in 2.3 ml of 0.1 M-NaCl/25 mM-Hepes/NaOH buffer, pH 7.0, containing 0.5% (w/v) bovine serum albumin were added to 0.2 ml of heparin–Sepharose. NaCl was then added in a stepwise fashion as described above. Supernatants obtained after centrifugation were analysed for ^{125}I radioactivity, EC-SOD C protein (e.l.i.s.a.) and enzymic activity.

To study the effect of pH on EC-SOD C enzymic activity, EC-SOD C (2.5 μg/ml) was incubated, under gentle agitation at room temperature, in various 25 mM buffers containing 0.5% bovine serum albumin and the NaCl concentration giving 50% release (cf. Fig. 3). The SOD activity was determined at intervals during 7 h, the time required for an NaCl displacement experiment.

RESULTS

Binding of EC-SOD C to GAG-substituted Sepharose 4B and displacement by NaCl

Coupling of the available GAGs by standard procedures, with preformed CNBr-activated Sepharose or epoxy-activated Sepharose, was found to be inefficient.

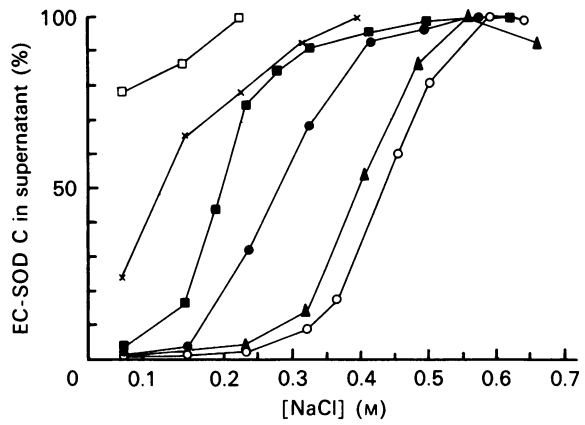

Fig 1. Binding of EC-SOD C to GAG-substituted Sepharose 4B and its displacement by NaCl

EC-SOD C was incubated at room temperature for 2 h with GAG-substituted Sepharose 4B followed by stepwise additions of NaCl, as described in the Materials and methods section. The Figure shows the SOD activity in the supernatant after centrifugation of the suspension, expressed as a percentage of initial activity. □, Blank activated Sepharose 4B; ×, chondroitin sulphate–Sepharose; ■, dermatan sulphate–Sepharose; ●, heparan sulphate–Sepharose (HS-1); ▲, heparan sulphate–Sepharose (HS-2); ○, heparin–Sepharose.

Coupling was, however, achieved by using the procedure of Miller-Andersson *et al.* [19], which involved simultaneous mixing of gel, GAG and CNBr. The GAG contents of the resulting substituted gels ranged between 0.5 and 1.6 mg/ml. Assuming an average molecular mass of 25 kDa for the GAG chains, the substituted gels contained a 400–800-fold molar excess of GAG chains over EC-SOD C subunits in the binding experiments.

EC-SOD C bound to all investigated GAGs (Fig. 1), and could be released by NaCl, indicating that the binding was of electrostatic nature. Heparin showed the strongest interaction, followed by the two heparan sulphate preparations. These differed considerably, the more highly sulphated preparation HS-2 being a more avid ligand than HS-1. The dermatan sulphate bound more strongly than the chondroitin sulphate. At physiological ionic strength (in 0.15 M-NaCl) the enzyme was almost completely retained by the immobilized heparin and heparan sulphates, partially bound by the dermatan sulphate, but poorly bound by chondroitin sulphate.

There was a minimal binding of EC-SOD C to the blank activated Sepharose 4B. At pH 6.5 EC-SOD C has a net negative charge and possibly binds to positive charges in the CNBr + ethanolamine-treated Sepharose. Complete liberation of the enzyme from the gel required about 0.2 M-NaCl.

Displacement of human EC-SOD C from heparan sulphate–Sepharose 4B by GAGs

Fig. 2 shows the release of EC-SOD C bound to heparan sulphate-Sepharose, induced by stepwise additions of GAGs. The competing potencies of the GAGs followed the order expected from the results shown in Fig. 1. Heparin thus was the most potent ligand and released EC-SOD C at a 10-fold lower concentration than the heparan sulphate preparation (HS-1) coupled to the gel. Unfractionated heparin and heparin fractions with high or low affinity for antithrombin III did not differ significantly. The dermatan sulphate was about 10 times less efficient than the heparan sulphate. Chondroitin sulphate was a very weak competitor and about 150 times less efficient than heparan sulphate.

Effect of pH on the affinity of EC-SOD C for heparin–Sepharose

The effect of pH was assessed by determining the releasing effect of stepwise additions of NaCl to ^{125}I-EC-SOD C previously incubated for 2 h with heparin–Sepharose in different buffers (cf. Fig. 1). Fig. 3 shows the NaCl concentration at which 50% of the ^{125}I-EC-SOD C was released into the medium. The heparin affinity did not vary significantly with pH in the range 6.5–9. At lower pH the binding strength increased gradually. Beyond pH 9.5 there was a precipitous decrease in heparin affinity.

At the pH extremes the EC-SOD gradually lost its enzymic activity. After incubation for 7 h at pH 4.5, 10.1 and 10.4 71%, 85% and 70% of the initial activity

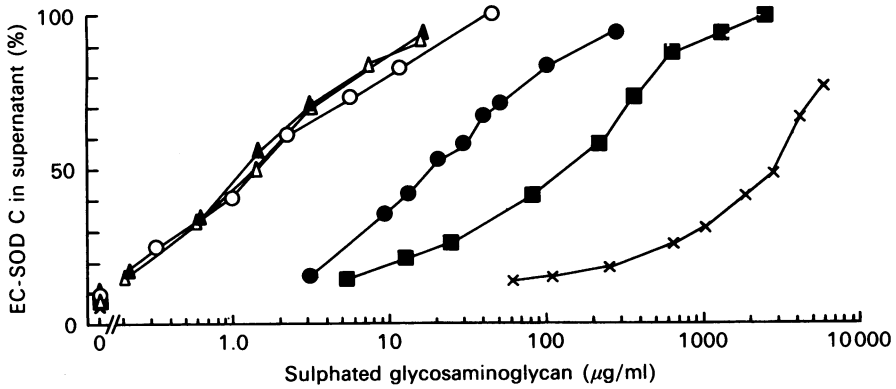

Fig. 2. Displacement of EC-SOD C from heparan sulphate–Sepharose 4B by GAGs

EC-SOD C was incubated for 2 h with heparan sulphate–Sepharose 4B, and GAGs were then added at increasing concentrations, as described in the Materials and methods section. The Figure shows the SOD activity in the supernatant after centrifugation of the suspension, expressed as a percentage of initial activity. ○, Heparan sulphate–Sepharose plus heparin; ▲, plus low-affinity heparin; △, plus high-affinity heparin; ●, plus heparan sulphate (HS-1); ■, plus dermatan sulphate; ×, plus chondroitin sulphate.

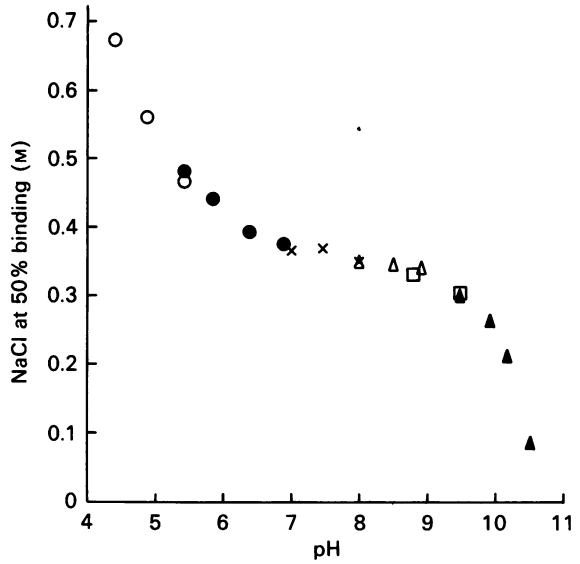

Fig. 3. pH-dependence of binding of EC-SOD C to heparin–Sepharose

^{125}I-labelled EC-SOD C was incubated for 2 h at room temperature with heparin–Sepharose in various 25 mM buffers containing 0.1 M-NaCl followed by stepwise additions of NaCl. The ^{125}I-EC-SOD radioactivity was determined on the supernatants after centrifugation of the suspensions. The Figure shows the NaCl concentration at which 50% release of ^{125}I-EC-SOD C occurred. ○, Sodium acetate buffer; ●, sodium cacodylate buffer; ×, Hepes/NaOH buffer; △, Tris/HCl buffer; □, 2-amino-2-methylpropan-1-ol/HCl buffer; ▲, ethanolamine/HCl buffer.

remained respectively. To avoid confusion induced by EC-SOD C inactivation, ^{125}I-labelled EC-SOD was therefore used for the pH-dependency experiments. The heparin affinity of the ^{125}I-labelled EC-SOD C was found to be a little lower than that of unlabelled enzyme, as tested combined at pH 7.0 50% release occurred at 0.03 M lower NaCl concentration. The results of EC-SOD C determination as enzymic activity and as protein with e.l.i.s.a. agreed in this experiment.

DISCUSSION

EC-SOD is apparently evolutionarily related to the intracellular CuZn-SODs, since the middle portion of the EC-SOD sequence shows strong homology with that part of the CuZn-SOD sequence which defines the active site [20]. EC-SOD is, however, a secretory enzyme [1] and might therefore have some special adaptations for a function in the extracellular space. An interesting feature of EC-SOD is its affinity for heparin [7,8] and, as shown in the present paper, other GAGs. The binding is apparently of electrostatic nature, since the enzyme could be displaced from GAG ligands by NaCl. EC-SOD probably binds to the negatively charged sulphate groups of the GAGs. Since EC-SOD C has a net negative charge at neutral pH (isoelectric point 4.5 [21]), the GAGs presumably interact with one or more clusters of positively charged amino acid residues at specific regions of the enzyme. Such a region occurs at the *C*-terminal end of EC-SOD C, which contains three lysine and six arginine residues among the last 20 amino acid residues [20].

Judged from the experiments with GAG-substituted Sepharose 4B gels (Fig. 1), heparin has the highest affinity for EC-SOD C, followed by the heparan sulphate preparations, the dermatan sulphate and the chondroitin sulphate. The substitution yields could not be controlled and varied between the different GAG preparations. Since this variability might account for part of the differences in binding, we also performed experiments in which GAGs in solution were allowed to compete with a heparan sulphate-substituted gel for enzyme binding (Fig. 2). The order of binding efficiency again agreed with the results illustrated in Fig. 1. Unfractionated heparin and heparin subfractionated with regard to affinity for antithrombin III did not differ in EC-SOD C affinity, which thus is not dependent on the saccharide sequence responsible for antithrombin III binding [22].

By and large, the binding strength would seem to increase with the degree of sulphation of the GAG, as indicated by the relative binding potencies of the three heparin-related polysaccharides. The heparan sulphate preparation HS-2, which has a sulphate density intermediate between those of the fully sulphated heparin and the low-sulphated preparation HS-1, thus shows intermediate affinity for EC-SOD C. However, additional factors, presumably more specifically related to the steric disposition of the negatively charged groups, must also be considered to explain the relative binding potencies of the GAGs. In particular, the heparan sulphate preparation HS-1 clearly has a higher affinity for the enzyme than has the chondroitin sulphate preparation, in spite of a lower sulphate content. The difference in binding strength is not simply accounted for by the presence of L-iduronic acid units in heparan sulphate (which is a co-polymer of L-iduronic acid- and D-glucuronic acid-containing disaccharide units, in contrast with chondroitin sulphate, which is composed of D-glucuronic acid-containing units only; see refs. [9] and [10]), since dermatan sulphate (which also has a co-polymeric structure) had an apparent lower affinity for the enzyme than the less-sulphated heparan sulphate, HS-1. It is noted that the relative affinities of the various GAGs for EC-SOD C conform, on the whole, to those displayed in relation to a different enzyme, lipoprotein lipase [23].

The secretory enzyme EC-SOD occurs both in fluids such as plasma, lymph [2] and synovial fluid [3] and in tissues [4,5]. In man the tissue concentration (per g) is on an average 20 times as high as the plasma concentration (per ml) [4]. The major part of EC-SOD in the body thus occurs in the extravascular space of tissues [4]. Tissue EC-SOD is mainly composed of forms with affinity for heparin (K. Karlsson & S. L. Marklund, unpublished work). It thus seems possible that most tissue EC-SOD is associated with extracellular GAGs. Under normal physiological conditions heparin occurs only intracellularly in mast cells, and should therefore not contribute significantly to the binding of EC-SOD in the tissues. Among the extracellular GAGs, the heparan sulphate preparations showed the strongest enzyme binding. Heparan sulphate occurs in the glycocalyx of most cells, suggesting that a large portion of EC-SOD in tissues is located on cell surfaces. In a recent study (K. Karlsson & S. L. Marklund, unpublished work) encompassing more than 20 different cultured cell lines, EC-SOD C was found to bind to all, and addition of heparin

reversed the binding. A more closely examined cell line, the Chinese-hamster fibroblast V 79, lost its ability to bind EC-SOD C after treatment with heparitinase, whereas chondroitin ABC lyase had no such effect. Dermatan sulphate, which occurs mainly in fibrous connective tissue [9], might to some extent compete with heparan sulphate for the tissue EC-SOD, whereas chondroitin sulphate would be an inefficient competitor. Heparan sulphate is not restricted to cell surfaces but occurs also in the extracellular matrix and in various basement membranes [24]. The distribution of EC-SOD among potential binding sites at cell surfaces and in the extracellular matrix remains to be determined.

The effects of pH on the binding of EC-SOD C to heparin (Fig. 3) should be largely ascribed to changes in the EC-SOD and not in the heparin molecule; sulphate groups do not titrate in the investigated pH range. The binding is largely independent of pH in the range 6.5–9. At lower pH the affinity increases, most probably owing to a decreasing net negative charge as the EC-SOD approaches its isoeletric point. On a somewhat speculative note, the affinity of EC-SOD C for cell surfaces should thus be increased in cases of decreasing tissue pH, such as in ischaemia. Such a stabilization might be of importance in relation to the burst of superoxide radical formation in the extracellular space that has been reported to occur at reperfusion after a period of tissue ischaemia [25]. Above pH 9.5 the affinity of the enzyme for heparin rapidly declines, possibly owing to deprotonation of essential lysine residues (see above). Although the loss of heparin affinity might conceivably also be due to conformational changes at high pH, there was only a slow gradual loss of enzymic activity at the investigated pH extremes. The pH-dependence of the heparin affinity at the alkaline side is similar to that observed with the proteinase inhibitor antithrombin III [26].

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