A non-glycosylated extracellular superoxide dismutase variant

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The secretory tetrameric extracellular superoxide dismutase (EC-SOD) is the only glycosylated SOD isoenzyme. The importance of the carbohydrate moiety for the properties of the enzyme is unknown. An expression vector defining non-glycosylated EC-SOD (ngEC-SOD) was constructed by mutagenesis of the codon for Asn-89 into a codon for Gln. The vector was transfected into Chinese hamster ovary DXB-11 cells and ngEC-SOD was isolated to 70 % purity from the culture media of selected clones. The absence of glycosylation was established by the lack of affinity for various lectins, the absence of staining with the periodic acid–Schiff reagent, the change in mobility and composition of the tryptic peptide containing the mutated glycosylation site, and the reduction in apparent molecular mass upon SDS/PAGE and size-exclusion chromatography. The tetrameric state was retained. The heparin affinity, a fundamental and distinguishing property of EC-SOD, was found to be slightly increased. The enzymic activity was essentially retained. The major difference from native glycosylated enzyme in physical properties was a marked reduction in solubility. Like glycosylated EC-SOD, ngEC-SOD was, after intravenous injection into rabbits, rapidly sequestered by the vessel endothelium, and was promptly released into plasma after injection of heparin. The only difference from glycosylated EC-SOD in this behaviour, was a slightly more rapid elimination of the mutant enzyme from the vasculature. It is concluded that no specific biological role for the EC-SOD carbohydrate moiety could be revealed.

INTRODUCTION

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1) is a secretory tetrameric Cu- and Zn-containing glycoprotein [1,2]. In the mammalian body it is the major (superoxide dismutase) SOD isoenzyme in extracellular fluids such as plasma, lymph and synovial fluid [3–6], although the major part of the enzyme, 90–99 %, exists in the extravascular space of tissues [3–5,7,8]. A prominent feature of EC-SOD is its affinity for sulphated glycosaminoglycans such as heparin and heparan sulphate [1,9]. *In vivo* the major part of EC-SOD appears to be sequestered by heparan sulphate proteoglycans in the glycocalyx of cell surfaces and in the connective-tissue matrix [4,5,10,11].

Unlike other SOD isoenzymes, EC-SOD is glycosylated [1,2]. Judging from amino-acid-sequence data [12] Asn-89 should be a candidate *N*-glycosylation site, and this has been verified by analysis of tryptic peptides [13]. The carbohydrate moiety of recombinant EC-SOD produced by Chinese hamster ovary (CHO) cells appears to be of the complex biantennary type with a core fucose [13]. The carbohydrate moiety of native human EC-SOD is apparently similar [2]. The importance of the glycosylation for the properties of EC-SOD is unknown.

The present paper reports the production and partial isolation of a recombinant non-glycosylated human EC-SOD (ngEC-SOD) variant. To assess the importance of the carbohydrate moiety, both physical properties and the behaviour of the variant *in vivo* were compared with those of recombinant human glycosylated EC-SOD (rEC-SOD).

EXPERIMENTAL

Construction of plasmids

The EC-SOD *EcoRI* cDNA fragment was cloned into phage

M13mp8. The resulting plasmid was used for site-directed mutagenesis using an oligonucleotide-directed mutagenesis system *in vitro* (code RPM.2322) purchased from Amersham International (Aylesbury, U.K.). The oligonucleotide 5'-GACCGAGCCGCAAAGCTCCAGCC-3' was used for the mutagenesis and the mutation was verified by DNA sequencing. Underlined bases were changed compared with the wild-type sequence leading to a codon for Gln (CAA) instead of Asn (AAC) in a position corresponding to residue 89 of the mature enzyme. The mutant *EcoRI* fragment was introduced into plasmid pPS3 [2] by replacement of the wild-type EC-SOD fragment, forming a new plasmid designated pEG1.

The plasmid pSDPdhfr carrying DNA encoding dihydrofolate reductase, lacking the simian virus 40 (SV40) late and early promoter and origin of replication, was derived from pSV2dhfr [14].

Cell culture and transfection

Dihydrofolate reductase-deficient CHO cells, strain DXB-11, were provided by L. Chasin (Columbia University, New York, NY, U.S.A.) and maintained in non-selective medium consisting of α -MEM (Gibco) supplemented with 10 % (v/v) fetal calf serum, 10⁵ units of penicillin/1 and 100 mg of streptomycin/1.

DXB-11 cells were co-transfected with a mixture (20 μ g/0.5 μ g) of linearized pEG1 and pSDPdhfr according to the method of Graham & Van der Eb [15]. Cell clones were selected and propagated in α -MEM without nucleosides (Gibco) supplemented with 10 % (v/v) dialysed fetal calf serum, 10⁵ units of penicillin/1 and 100 mg of streptomycin/1.

Production of ngEC-SOD was made by incubation of cell lines, grown to confluency in roller bottles, with a 1:1 mixture of Opti-MEM without Phenol Red (Gibco) and α -MEM without Phenol Red (Gibco) supplemented with 0.5 % (v/v) newborn

Abbreviations used: SOD, superoxide dismutase; EC-SOD, extracellular SOD; ngEC-SOD, non-glycosylated human EC-SOD; rEC-SOD, recombinant (glycosylated) human EC-SOD; CHO cells, Chinese hamster ovary cells; SV40, simian virus 40; ConA, concanavalin A.

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calf serum, 20 mM-Hepes, 27 mM-NaHCO₃, 2.25 g of glucose/l, 2 μ M-CuSO₄ and 10 mg of gentamicin/l.

Purification of ngEC-SOD

Medium (55 litres) was filtered through three layers of Omegasette filters (Filtron Tech. Corp., Clinton, MA, U.S.A.) and, after adjustment of pH to 7.6, was applied to a 1.7 l Q-Sepharose FF column (Pharmacia-LKB Biotech, Uppsala, Sweden). The column was washed with 26 l of 0.1 M-potassium phosphate, pH 7.6, and bound ngEC-SOD was eluted with 10 l of 300 mM-potassium phosphate, pH 7.6. The pool containing EC-SOD was diluted 5-fold with water and applied to a 1.5 l heparin-Sepharose column (Pharmacia-LKB). The column was washed with 4.5 l of 0.2 M-NaCl in 50 mM-sodium phosphate, pH 7.6, and eluted with a 7.5 l gradient up to 1 M-NaCl in the same buffer. After diluting 2-fold with 5 mM-acetic acid/0.5 M-NaCl/1 mM-CaCl₂/1 mM-MgCl₂/1 mM-MnCl₂, pH 6.9, the pool from the heparin-Sepharose column was applied to a 51 ml concanavalin A (ConA)-Sepharose column (Pharmacia-LKB). The column was washed with the buffer used for dilution and eluted with the same buffer containing 0.5 M- α -methylmannoside. The non-bound material was collected and, to reduce the large volume, was adsorbed a second time to a heparin-Sepharose column (15 ml). The column was eluted similarly to the first heparin-Sepharose column. To increase the solubility, the pool from the column was dialysed against 50 mM-2-amino-2-methyl-1-propanol hydrochloride, pH 9.0, containing 0.5 M-NaCl and was thereafter concentrated using a 30 kDa Omegacell (Filtron) filter. The final 4.7 ml of concentrate contained 76 750 units/ml and had a specific activity of 36 900 (units per ml/*A*₂₈₀).

SOD determination

SOD enzymic activity was determined with the direct spectrophotometric method employing potassium superoxide [16] as modified in [17]. One unit corresponds to 8.6 ng of human EC-SOD [2]. EC-SOD protein was determined with an e.l.i.s.a. [2].

Determination of apparent molecular masses by means of size-exclusion chromatography

The chromatographies were carried out at 4 °C on a calibrated Sephacryl S-300 column (1.6 cm $\times$ 89 cm), equilibrated and eluted with 10 mM-potassium phosphate, pH 7.4, containing 0.15 M-NaCl, as previously detailed [5].

SDS/PAGE

Gradient gels (10–17.5%) were cast according to the discontinuous buffer system of Laemmli [18]. Gels were run in a Pharmacia-LKB Midget Electrophoresis Unit at a constant current of 25 mA/gel.

Immunoblotting

SDS/PAGE gels were electroblotted at constant current (0.8 mA/cm²) for 1 h on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) using a Trans-Blot SD Transfer Cell (Bio-Rad, Richmond, CA, U.S.A.) essentially according to the method of Heegaard & Bjerrum [19]. Blots were developed using highly purified rabbit anti-EC-SOD and alkaline-phosphatase-labelled anti-(rabbit Ig) antibodies (Dakopatts A/S, Copenhagen, Denmark).

Periodic acid-Schiff staining

The SDS/PAGE gel was transferred to Immobilon-P membranes as described above. The membrane was thereafter stained with Schiff's reagent [20].

Lectin affinity

Lectin affinity was analysed using the alkaline-phosphatase-labelled lectins supplied in the LectinLink kit (Genzyme, Boston, MA, U.S.A.). SDS/PAGE gels were run and electroblotted as described above and the blot was developed according to the instructions supplied by the manufacturer.

Peptide mapping

EC-SOD was carboxymethylated, cleaved with trypsin (Boehringer-Mannheim, Germany) and separated on an Ultrapore C8 column as described earlier [13].

Amino-acid analysis

Samples were hydrolysed, derivatized with phenyl isothiocyanate and separated on an Ultrasphere C18 column (150 mm $\times$ 4.6 mm, Beckman Instruments, San Ramon, CA, U.S.A.) as described earlier [13].

Reversed-phase liquid chromatography

A System Gold (Beckman Instruments) with an Ultrapore C8 column (250 mm $\times$ 4.6 mm, Beckman Instruments) was used for reversed-phase h.p.l.c. The separating gradient was 35–45% acetonitrile in 0.1% trifluoroacetic acid in water.

Analytical separation of EC-SOD on heparin-Sepharose

The chromatographies were carried out as previously detailed [5] at room temperature in a 1 ml heparin-Sepharose column with 15 mM-sodium cacodylate containing 50 mM-NaCl, pH 6.5, as equilibration buffer and eluent. Bound material was eluted with a NaCl gradient.

Plasma clearance of EC-SOD in rabbits

About 100 μ g of EC-SOD per kg body wt., recombinant glycosylated [2] or non-glycosylated, dissolved in 50 mM-potassium phosphate, pH 7.4, containing 0.2% BSA, was injected into ear veins of chinchilla ram rabbits, weighing 3–4 kg. Blood samples were tapped at the times indicated in Fig. 4 into tubes containing EDTA as an anti-coagulant. The experiments were terminated at 1, 6 or 24 h (see Fig. 4), by intravenous injection of heparin, 2500 i.u./kg body wt., followed by tapping of blood samples at 5 min intervals thereafter. The plasma volumes of the rabbits were determined by injection of ¹²⁵I-labelled human albumin together with the EC-SOD, and extrapolation of the radioactivity of plasma samples back to time zero. The plasma volumes varied between 34 and 38 ml/kg body wt. The rabbits were used only once. The study was approved by the local ethical committee on animal experiments.

RESULTS

Expression and partial isolation of the ngEC-SOD variant

The expression vector pEG1 was introduced into CHO DXB11 cells by co-transfection with plasmid pSDPdhfr and cell clones were selected as described in the Experimental section. Two clones producing about 0.7 μ g of ngEC-SOD per day and 10⁶ cells were selected for large-scale production in roller bottles.

The isolation procedure for ngEC-SOD was based on previous experience with native and recombinant EC-SOD C [2] and the results are summarized in Table 1. The ConA-Sepharose step was added because a part (about 25%) of the SOD activity was found to bind to ConA. This material may represent endogenous EC-SOD expressed by the CHO cells. The isolation was not driven to complete purity because of handling problems (solubility and stability, see below) with ngEC-SOD. The final specific activity 36 900 (unit per ml/*A*₂₈₀), was lower than that previously

Table 1. Isolation of ngEC-SOD

Results after each step are shown. The SOD activity was determined with the KO₂ method [16,17].

Isolation step	Volume (ml)	10 ⁻³ × Total SOD activity (units)	Recovery (%)
Culture medium	55000	3080	100
Q-Sepharose	11900	1600	52
Heparin-Sepharose	3000	1570	51
ConA-Sepharose	6000	828	27
Heparin-Sepharose	24	473	15.4
Dialysis, concentration	4.7	361	11.7

Fig. 1. Comparison of ngEC-SOD and rEC-SOD on SDS/PAGE

(a) Gel stained for protein with Coomassie Brilliant Blue. Lane 1, 2 μ g ngEC-SOD; lane 2, 3 μ g rEC-SOD; lane 3, 4 μ g ngEC-SOD; lane 4, 10 μ g rEC-SOD. Left, migration of low-molecular-mass standards (Pharmacia-LKB). (b) Immunoblot of ngEC-SOD and rEC-SOD separated by SDS/PAGE. Membrane probed with anti-(human EC-SOD) antibody as described in the Experimental section. Lane 1, 0.5 μ g rEC-SOD; lane 2, 0.5 μ g ngEC-SOD; lane 3, 1 μ g rEC-SOD; lane 4, 1 μ g ngEC-SOD. Left, migration of prestained low-molecular-mass standards (Bio-Rad).

obtained with rEC-SOD 69400 [2]. According to determination of the EC-SOD content of the ngEC-SOD preparation by e.l.i.s.a., one activity unit corresponded to 18.5 ng compared with 8.6 ng for rEC-SOD [2]. The difference in specific activity is partly due to contaminants seen in the analysis by SDS/PAGE (see below). The purity achieved was, however, sufficient for extensive characterization of the enzyme.

Molecular size

The ngEC-SOD eluted from the Sephacryl S-300 size-exclusion chromatography column at a position corresponding to a molecular mass of 96 kDa (results not shown). For comparison rEC-SOD was run in parallel and displayed an apparent molecular mass of 150 kDa.

Upon separation with SDS/PAGE after reduction (Fig. 1a)

Fig. 2. Peptide map obtained by reversed-phase h.p.l.c. separation of the tryptic peptides of (a) rEC-SOD, and (b) ngEC-SOD

Arrows indicate in (a) the glycosylated fragment and in (b) the corresponding non-glycosylated mutated fragment.

and subsequent immunoblotting with anti-(human EC-SOD) antibody (Fig. 1b), ngEC-SOD and rEC-SOD show main bands with apparent molecular masses of 28 kDa and 30–32 kDa respectively. Bands at 55 kDa and 65 kDa are also seen which probably represent dimers. The ngEC-SOD preparation was, however, not pure since a number of bands not staining in the immunoblot could be discerned in the SDS/PAGE. The total amount of contaminants is estimated to represent about 30 % of the protein in the gel. The amino acids in the sequence of EC-SOD C deduced from the cDNA [12] correspond to a molecular mass of 24.2 kDa. The experimental findings are compatible with the loss of the carbohydrate moiety in ngEC-SOD, and with a tetrameric quarternary structure.

Reactivity with carbohydrate-reacting reagents

Unlike rEC-SOD, ngEC-SOD did not stain with Schiff's reagent after SDS/PAGE and subsequent electroblotting to Immobilon membranes or with the labelled lectins ConA, wheat germ lectin, *Ricinus communis* agglutinin, *Phaseolus vulgaris* erythrolectin, and *Datura stramonium* agglutinin (results not shown).

Analysis of tryptic peptides

Figs. 2(a) and 2(b) show chromatograms of tryptic digests of ngEC-SOD and rEC-SOD. The peptide containing the oligosaccharide of rEC-SOD has previously been identified [13] and is indicated with an arrow (Fig. 2a). This is absent in ngEC-SOD and instead a novel peak with a longer retention time is seen (Fig. 2b). The retention time of the non-glycosylated peptide was close to that seen for this peptide when isolated from rEC-SOD and thereafter subjected to enzymic deglycosylation [13].

Table 2. Amino-acid composition of the tryptic fragment (Leu-75 to Arg-93) containing the mutation

The fragment was collected from the reversed-phase chromatography in Fig. 3(b). All calculations are relative to arginine, which was set to 1.0. Results for rEC-SOD are from ref. [13].

Amino acid	Isolated fragment		Predicted value from cDNA sequence	
	ngEC-SOD	rEC-SOD	ngEC-SOD	rEC-SOD
Asx	1.1	2.0	1	2
Glx	2.7	2.2	3	2
Ser	2.9	3.5	3	3
Gly	1.4	1.5	1	1
Arg	1.0	1.0	1	1
Thr	1.0	0.9	1	1
Ala	2.1	2.2	2	2
Pro	1.8	2.3	2	2
Leu	2.6	2.1	2	2
Phe	2.8	2.6	3	3
Others not listed	< 0.3	< 0.3	0	0

Fig. 3. Analytical separation of EC-SOD on a heparin-Sepharose column

ngEC-SOD (●) and rEC-SOD (○), about 11 000 units of each, were applied to and separated on a heparin-Sepharose column as described in the Experimental section. (---), NaCl gradient.

Amino-acid analysis verified the identity of the peptide as well as the exchange of Asn for Gln (see Table 2). ngEC-SOD was not as pure as rEC-SOD before cleavage with trypsin, resulting in more peaks which did not originate from EC-SOD. The main peaks were, however, present in both maps.

Reversed-phase h.p.l.c.

Upon separation by reversed-phase h.p.l.c., ngEC-SOD eluted later than rEC-SOD in the acetonitrile gradient, at 44.1% as compared with 43.4% (chromatograms not shown). The results indicate that the loss of carbohydrate results in increased hydrophobicity.

Affinity for heparin

Analysis on an analytical heparin-Sepharose column showed that ngEC-SOD has a slightly higher affinity for heparin than rEC-SOD. ngEC-SOD eluted at 0.62 M-NaCl as compared with 0.55 M for rEC-SOD (Fig. 3).

Solubility

During development of the isolation procedure it soon became apparent that extensive loss of enzymic activity occurred upon concentration of pooled fractions after the chromatography steps. Concentration of the enzyme was therefore avoided except

Fig. 4. Plasma clearance of EC-SOD in rabbits

The figure shows the time course in plasma of ngEC-SOD (■, ▲, ●) or rEC-SOD (□, ○, △, +) injected intravenously into rabbits, as described in the Experimental section. The expected plasma concentration was calculated from the amount injected and the plasma volume as determined with ¹²⁵I-albumin. The experiments were terminated after 1 h (■, ▲, ●), 5 h (□, ○, △, +) by intravenous injection of a large dose of heparin followed by collection of a blood sample after 5 min.

after the final step in the final sequence (see Table 1). When the enzyme concentration approached about 1 mg/ml a distinct precipitate was formed. SDS/PAGE analysis of the precipitate showed that ngEC-SOD was the main component. The precipitate could be solubilized at high pH and high ionic strength, and this is why the ngEC-SOD pool was dialysed against 50 mM-2-amino-2-methyl-1-propanol hydrochloride, pH 9.0, containing 0.5 M-NaCl before the final concentration step, see the Experimental section. In comparison rEC-SOD has been concentrated to 70 mg/ml without problems.

Plasma clearance in rabbits

After intravenous injection, both rEC-SOD and ngEC-SOD were rapidly sequestered to 97–98% from the blood plasma (Fig. 4). This sequestering is apparently caused by binding to heparan sulphate proteoglycan in the glycocalyx of endothelial cell surfaces [4,5,9–11]. The decline thereafter was slow, although a little more rapid for ngEC-SOD than for rEC-SOD. Injection of large doses of heparin at 1, 5 and 24 h led to prompt release of the enzymes into the plasma. There was slightly less release of ngEC-SOD than of rEC-SOD at 5 h and 24 h.

DISCUSSION

The results document the successful preparation of an ngEC-SOD variant. Thus, the apparent sizes upon gel-exclusion chromatography and SDS/PAGE were predictably reduced, the exchange Asn-89→Gln and lack of glycosylation in the relevant tryptic fragment could be demonstrated, the enzyme did not stain for carbohydrate and the affinity for various lectins was abolished.

The central part of EC-SOD, residues 96–193, shows high sequence similarity to that part of the CuZn SOD's that defines the active site [12]. We have therefore used the crystallographic structure of bovine CuZn SOD [21] to simulate the position of the glycosylation site, Asn-89, in the three-dimensional structure. The atomic co-ordinates were obtained from the Brookhaven Protein Data Bank [22,23] and they were graphically analysed

with the program FRODO [24]. Asn-89 appears to be positioned on the edge of the β -barrel structure in the loop between β -strands 3e and 6d, using the terminology employed for the bovine CuZn SOD structure [21]. This position is on the surface of the subunit, more than 1.5 nm (15 Å) from the active-site region, and opposite to the opening of the active-site cleft. Therefore, direct interaction with the active-site region is unlikely. However, effects of the oligosaccharide on the gross three-dimensional structure may exist. The modelling analysis is compatible with the fact that the SOD activity per Cu atom of EC-SOD is at least as high as that of bovine and human CuZn SOD [1], and suggests that a loss of the oligosaccharide should have no major effect on the enzymic activity of the enzyme. It is difficult to define exactly the specific activity of the ngEC-SOD, since we lack a precise knowledge of the amount of contaminating proteins and their contribution to the absorbance at 280 nm. Further, the reactivity of the ngEC-SOD in the e.l.i.s.a. could be altered as compared with rEC-SOD. In addition, the amount of denaturated enzyme in the preparation is unknown. It can, however, be concluded that the major part of the SOD activity is retained, in accord with the modelling analysis.

The major property change induced by the mutation was a marked reduction in solubility, to a point rendering the isolation of the variant much more difficult than that of the glycosylated rEC-SOD. Precipitated ngEC-SOD could be made soluble and active at high pH (9.0) and high ionic strength (0.5 M-NaCl), which might reflect an involvement of charges. The non-glycosylated CuZn SODs (e.g. bovine CuZn SOD) are highly soluble, and so is glycosylated rEC-SOD. From the modelling analysis no essential differences in surface charge or hydrophobicity between bovine CuZn SOD and the corresponding positions in EC-SOD could be observed. However, nothing is known about the surface charge and hydrophobicity of the N- and C-terminal parts of EC-SOD which have no counterparts in the CuZn SODs. The loss of the oligosaccharide means a loss of hydrophilic carbohydrate and of at least two negatively charged sialic acid residues (complex type of oligosaccharide) and may also have effects on the gross three-dimensional structure. The observed result is an increased hydrophobic behaviour of ngEC-SOD as compared with rEC-SOD, as manifested both in the reversed-phase h.p.l.c. study and in the decreased solubility.

The heparin affinity of EC-SOD is the most distinguishing property of this isoenzyme, and is responsible for its localization *in vivo*. By way of analysis of a series of mutated EC-SOD variants, the heparin-binding domains of EC-SOD have been demonstrated to exist in the strongly positively charged C-terminal ends of the subunits (J. M. Sandström, L. Carlsson, S. L. Marklund & T. Edlund, unpublished work). In accord with this view, the non-glycosylated variant retained the heparin affinity and it was even somewhat increased (Fig. 3). The slightly lower affinity of the glycosylated enzyme may be due to a slight repellent effect between the negatively charged carbohydrate and the negatively charged heparin.

To evaluate the behaviour *in vivo* of ngEC-SOD, the enzyme was injected intravenously into rabbits (Fig. 4). A rapid sequestering of 97–98 % of the enzyme from plasma occurred, as did a prompt return to plasma upon the injection of a large dose of heparin. These phenomena have been extensively investigated with native and recombinant glycosylated EC-SOD [4,5,10,11] and are apparently caused by rapid binding to heparan sulphate proteoglycan in the glycocalyx of the endothelial cell surfaces, which forms an equilibrium with the plasma phase. The enzyme can then be released into plasma by injection of the more strongly bound heparin. The lack of glycosylation had only a minor effect on the behaviour. The extent of initial sequestering was undistinguishable from that of glycosylated enzyme, whereas

the release by heparin at 5 h and 24 h was slightly less extensive, indicating a slightly more rapid turnover of the non-glycosylated enzyme. For several plasma glycoproteins with long plasma survival times, partial degradation of the carbohydrate leads to rapid elimination via cell-surface-bound lectins [25] and this may be an important normal catabolic route for the proteins. In the case of ferritin, glycosylation leads to a slow plasma turnover, whereas the normal non-glycosylated form is very rapidly eliminated [26,27]. For other glycoproteins, the carbohydrate moiety confers resistance towards proteolysis and prolonged survival [28]. The slightly faster elimination of ngEC-SOD may be caused by partial denaturation during purification or by the lower molecular mass allowing a more rapid passage through pores in the vessel endothelium [29]. The findings indicate that the carbohydrate moiety of EC-SOD has no major effect on the turnover and stability of the enzyme in the extracellular space.

To conclude, the present investigation of EC-SOD has, as for so many other secreted glycoproteins, failed to demonstrate any specific physiological properties endowed to the protein by the carbohydrate moiety.

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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 & Marklund, S. L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6634–6638
3. Marklund, S. L., Holme, E. & Hellner, L. (1982) *Clin. Chim. Acta* **126**, 41–51
4. Karlsson, K. & Marklund, S. L. (1987) *Biochem. J.* **242**, 55–59
5. Karlsson, K. & Marklund, S. L. (1988) *Biochem. J.* **255**, 223–228
6. Marklund, S. L., Bjelle, A. & Elmqvist, L.-G. (1986) *Ann. Rheum. Dis.* **45**, 847–851
7. Marklund, S. L. (1984) *J. Clin. Invest.* **74**, 1398–1403
8. Marklund, S. L. (1984) *Biochem. J.* **222**, 649–655
9. Karlsson, K., Lindahl, U. & Marklund, S. L. (1988) *Biochem. J.* **256**, 29–33
10. Karlsson, K. & Marklund, S. L. (1988) *J. Clin. Invest.* **82**, 762–766
11. Karlsson, K. & Marklund, S. L. (1989) *Lab. Invest.* **60**, 659–666
12. Hjalmarsson, K., Marklund, S. L., Engström, Å. & Edlund, T. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6340–6344
13. Strömqvist, M., Holgersson, J. & Samuelsson, B. (1991) *J. Chromatogr.* **548**, 293–301
14. Subramani, S., Mulligan, R. & Berg, P. (1981) *Mol. Cell. Biol.* **1**, 854–864
15. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52**, 456–467
16. Marklund, S. L. (1976) *J. Biol. Chem.* **251**, 7504–7507
17. Marklund, S. L. (1985) in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.), pp. 249–255, CRC Press, Boca Raton, FL, U.S.A.
18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
19. Heegaard, N. H. H. & Bjerrum, O. H. (eds.) (1986) in *Handbook of Immunoblotting of Proteins*, pp. 1–26, CRC Press, Boca Raton, FL, U.S.A.
20. Strömqvist, M. & Gruffman, H. (1992) *Biotechniques*, in the press
21. Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S. & Richardson, D. C. (1982) *J. Mol. Biol.* **160**, 181–217
22. Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, Jr., E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. & Tasumi, M. (1977) *J. Mol. Biol.* **112**, 535–542
23. Abola, E. E., Bernstein, F. C., Bryant, S. H., Koetzle, T. F. & Weng, J. (1987) in *Crystallographic Databases—Information Content, Software Systems, Scientific Applications* (Allen, F. H., Bergerhoff, G. & Sievers, R., eds.), pp. 107–132, Data Commission of the Int. Union of Crystallography, Bonn, Cambridge, Chester
24. Jones, T. A. (1982) in *Computational Crystallography* (Sayre, D., ed.), pp. 303–317, Clarendon Press, Oxford
25. Ashwell, G. & Harford, J. (1982) *Annu. Rev. Biochem.* **51**, 531–534

26. Cragg, S. J., Covell, A. M., Burch, A., Owen, G. M., Jacobs, A. & Worwood, M. (1983) *Br. J. Haematol.* **55**, 83–92
 27. Worwood, M., Cragg, S. J., Williams, A. M., Wagstaff, M. & Jacobs, A. (1982) *Blood* **60**, 827–837
 28. Bernard, B. A., Yamada, K. M. & Olden, K. (1982) *J. Biol. Chem.* **257**, 8549–8554
 29. Arturson, G., Groth, T. & Grotte, G. (1972) *Acta Physiol. Scand. (Suppl.)* **374**, 1–30
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