Purification and Sequence of Rat Extracellular Superoxide Dismutase B Secreted by C6 Glioma*

(Received for publication, May 24, 1993, and in revised form, June 23, 1993)

Jean Willemen†, An Zwijnen‡, Herman Slegers§, Stefan Nicolaš, Jayaram Bettadapura**,
Jos Raymackers***, and Thierry Scarcez**

From the †Interdisciplinary Research Center, KULAK, B-8500 Kortrijk, Belgium, the ‡Department of Biochemistry, University of Antwerp, B-2610 Wilrijk-Antwerpen, Belgium, and ***Innogenetics N. V., B-9050 Zwijnaarde, Belgium

An enzyme which converts radical oxygen, produced by phorbol 12-myristate 13-acetate activated neutrophils, into nonluminescent products is secreted by rat C6 glioma. The enzyme was purified from chemically defined conditioned media and identified as an extracellular superoxide dismutase (EC-SOD).

The purified enzyme is distinct from human EC-SOD C (Hjalmarsson, K., Marklund, S. L., Engström, A., and Edlund, T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6340-6344) by its elution from heparin-Sepharose at 300-400 mM NaCl, in pH 6.1-7.2, and of its native Mr of 85,000 ± 20,000. The rat EC-SOD is a dimer with a subunit Mr of 34,000-36,000 and is extensively modified by post-translational processing.

Although rat EC-SOD has a high sequence homology with the catalytic center and the polybasic heparin-binding site near the COOH terminus of human EC-SOD C, its NH2-terminal sequence and the sequences flanking the heparin-binding site differ substantially. The sequence of the isolated rat EC-SOD cDNA fully confirms the data obtained from amino acid sequence analysis. The amino acid sequence of the enzyme and its biochemical properties support its identification as the rat EC-SOD B.

Superoxide dismutase (SOD, EC 1.15.1.1), which represents one of the main defense enzymes against toxic oxygen species, was originally described by McCord and Fridovich in 1969 (1) and occurs in several forms. Inside eukaryotic cells a CuZn-enzyme is found in the cytosol and a Mn-enzyme is located in mitochondria (for a review, see Ref. 2). Both enzymes are conserved throughout evolution. More recently Marklund and co-workers (3-5) have shown that several extracellular forms of the enzyme exist in the plasma of mammalian species. These extracellular forms can be divided into three classes namely A, B, and C according to their increasing affinity for heparin (6-8). Until now only the human extracellular SOD C has been purified, sequenced, cloned, and expressed.

EC-SOD C avidly binds to anchorage-dependent cell lines but has almost no affinity for blood cells, including neutrophils (9). The enzyme mostly binds to heparan sulfate proteoglycan in the glyocalyx of cell surfaces and in the connective tissue matrix. The EC-SOD A and B subtypes mainly exist in the extracellular fluid (8, 10). The binding of EC-SOD C to sulfated proteoglycan and heparin is due to a hydrophilic COOH-terminal sequence containing 6 arginine and 3 lysine residues in the 21 carboxyl-terminal amino acids (4, 11). The C form is absent in rat and therefore, in this species the B form is the only EC-SOD that is able to bind to heparin (8).

It has been suggested that the A and B forms are derived from the C form by post-translational proteolytic cleavage at the carboxyl terminus (11-13).

Whereas in other mammals EC-SODs have been characterized as tetrameric CuZn-binding glycoproteins with a subunit Mr of 30,000-35,000 and a native Mr of 150,000, rat EC-SOD has a native Mr of 97,000 and seems to be a dimer (8).

EC-SOD is secreted by fibroblast and glial but not by epithelial and endothelial cells (14).

During our studies on rat C6 glioma, we observed that conditioned media of the latter cells contain a protein that strongly reduces the amount of superoxide released by activated neutrophils. In this article we describe the purification, the amino acid and nucleotide sequence determination, and the biochemical properties of the EC-SOD enzyme and identify it as the rat EC-SOD B form with properties distinct from the human EC-SOD C.

**EXPERIMENTAL PROCEDURES

Materials

The rat C6 glioma cell line (ATCC CCL 107) was obtained from Flow Laboratories (Irvine, Scotland).

N-Glycanase (EC 3.5.1.52) was purchased from Genzyme (Cambridge, MA). Bovine and human erythrocyte CuZn-SODs were from Sigma (Brussels, Belgium). Oligo(dT)-cellulose type 3 was from Collaborative Research (Boston, MA). The multiprime labeling kit was purchased from Amersham (Buckinghamshire, United Kingdom) and the afg11 rat glioma cDNA library from Clonetech (San Francisco, CA).

Secondary antibodies were from Dakopatts (Glostrup, Denmark).

Methods

Cell Culture and Conditioned Medium

Rat C6 glioma cells were cultivated in Ham's F-10 supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1% (v/v) MEM vita-
Extracellular Superoxide Dismutase B Secreted by Rat C6 Glioma

mins 100 × 1.5% (v/v) MEM nonessential amino acids 100 × 100 units/ml penicillin, and 100 μg/ml streptomycin. Incubation was at 37 °C in a 5% CO2 humidified atmosphere. At confluence the cells were washed twice with PBS and harvested using 0.025% (w/v) trypsin and 0.01% (w/v) EDTA in PBS. The cells were seeded in serum-free medium (Ham's F-10/MEM 1:1), additives, and 30 nm sodium selenite at a concentration of approximately 5.4 × 104 cells/cm² in double tray cell plates (1,200 cm²). The medium was changed after 72 h and the CM discarded. The CM was harvested for the first time 96 h after the medium change, new medium was added, and a second harvest of CM was made after a further incubation for 72 h. Residual cells were removed from the CM by centrifugation (1,200 × g, 15 min, 4 °C).

Purification of EC-SOD

All chromatographies were performed at room temperature.

Hydroxyapatite Chromatography—Six harvests of 2 liters of CM (400 ml/doubletray) were diluted (2:1, v/v) with 50 mM Tris/HCl (pH 7.5) and the proteins concentrated in batch by binding onto Bio-Gel-HTP hydroxyapatite (4 °C). Hydroxyapatite was equilibrated with 10 mM Tris/HCl (pH 7.5), 10 μM CaCl2 (buffer A), and 10 g of resin added per 1.8 liter of diluted CM. After extensive washing with buffer A the bound material was discontinuously eluted with 500 mM sodium phosphate (pH 7.5).

Heparin-Sepharose Chromatography—The hydroxyapatite eluted material was dialyzed (molecular weight cutoff 3,500) against 50 mM Tris/HCl (pH 7.1), divided into six equal parts, and chromatographed separately onto a 210 × 10-mm column of heparin-Sepharose CL-6B, equilibrated with 50 mM Tris/HCl (pH 7.1). The bound material was eluted with an 80-min linear gradient of NaCl(0-2 M) in equilibration buffer.

Hydrophobic Interaction Chromatography—The active fractions eluted from the heparin-Sepharose CL-6B columns were pooled, dialyzed (m.c.o. 3, 500) against 500 mM (NH4)2SO4, 50 mM sodium phosphate buffer (pH 7.0), and applied onto a Bio-Rad phenyl-HPLC column (75 × 7.5 mm) on-line with a phenyl-Sepharose column (65 × 10 mm) equilibrated with the binding buffer. The bioactivity was not retained on the first phenyl-HPLC column, but was bound on the phenyl-Sepharose column. Bioactive material was eluted from the latter column with a 30-min linear gradient from binding buffer to 50 mM sodium phosphate (pH 7.0).

Reverse Phase Chromatography—The active fractions eluted from

**TABLE I**

Extracellular superoxide dismutase activity as a function of the cultivation time of rat C6 glioma

| Time (h) | Cells/cm² | Units/ml CM | Units/10⁶ cells |
|---------|-----------|-------------|-----------------|
| 72      | 3.2 × 10⁴ | 9.9         | 0.61            |
| 144     | 8.0       | 15.8        | 0.37            |
| 216     | 25.8      | 33.0        | 0.21            |
| 288     | 35.0      | 19.8        | 0.11            |
| 360     | 32.6      | 17.6        | 0.11            |

**Protein Electrophoresis and Electroblotting**

One-dimensional SDS- and two-dimensional PAGE were carried out as described (16–18). Proteins prepared for amino acid sequencing were carboxymethylated (19). Electrophoretic separation was performed on ProBlott PVDF membranes in the presence of 10 mM CAPS (20). Electrophoresis, blotting, and staining were performed in the presence of 1 mM thioglycolic acid.

**Amino Acid Sequence Determination of EC-SOD B**

Purified peptides were sequenced using a pulse-liquid Model 477A sequenator equipped with an on-line 120 phenylthiohydantoin analyzer from Applied Biosystems (Foster City, CA). In the case of NH₂-terminals, the phenyl-Sepharose column were pooled and dialyzed (molecular weight cutoff 10,000) against 20 mM Tris/HCl (pH 7.0). Trifluoroacetic acid was added to a final concentration of 0.1% (v/v) before application on a Bakerbond C₄ column (250 × 4.6 mm). The bound proteins were eluted with an 85-min linear gradient from 25 to 60% acetonitrile in the presence of 0.1% trifluoroacetic acid.

**Luminescence Assay for Inhibition of the Respiratory Burst**

Neutrophils were purified (>97%) from blood taken from healthy human volunteers (15).

Neutrophils (3 × 10⁵ in 150 μl of RPMI) were incubated together with 150 μl of RPMI or test sample diluted in RPMI for 90 min at 37 °C. Subsequently 60 μl of a lucigenin solution (10⁻⁷ M) was added and the tubes placed in a Berthold 6-channel Biliomat LB 9505 apparatus (37 °C) to record the background luminescence. Finally 60 μl of phosphorol 12-myristate 13-acetate (1 μg/ml) in RPMI was added and the luminescence recorded. The peak values were corrected for background and the inhibition calculated in relation to the value of the reference tubes without inhibitor. One unit of SOD is defined as the dilution which gives a 50% reduction of the luminescence of phosphorol 12-myristate 13-acetate-activated neutrophils. One unit in our assay is equivalent to the activity of approximately 50 ng of intracellular SOD and 5.8 ng of the purified rat EC-SOD B (Table II).

**Radioimmunoassay for EC-SOD**

For the preparation of polyclonal anti-EC-SOD B, rabbits were primed and boosted twice with 15–20 μg of purified EC-SOD B together with complete Freund's adjuvant. An IgG fraction was obtained by Na₂SO₄ precipitation (39.1 mg/ml).

The radioimmunoassay for EC-SOD B was performed as follows: cups of a microtiter plate were coated with 50 μl of SOD-containing fractions, postcoated with bovine serum albumin (2 mg/ml in PBS), incubated with 50 μl of the rabbit anti-EC-SOD B (1:250 in PBS), washed three times (PBS containing 0.2% Tween 80), incubated with 125I-labeled sheep anti-rabbit IgG antibodies, washed, and counted in a Packard gamma counter.

Under these conditions less than 1 ng of rat C₆ EC-SOD can be detected. Cross-reactivity of our antiserum with commercial bovine and human erythrocyte CuZn-SOD is about 1.5%.

**TABLE II**

Purification of rat C₆ EC-SOD B

| Volume | Protein concentration | Units | Units | Specific activity | SOD* | Nanograms SOD/units |
|--------|-----------------------|-------|-------|-------------------|------|-------------------|
| ml     | mg                    | units/ml | total | units/mg | mg    |
| 1. C₆ CM | 11,600           | 86.6    | 25    | 290,000 | 3,350 | ND*   |
| 2. Hydroxyapatite | 1,100         | 60.9    | 350   | 385,000 | 6,900 | 2.33  |
| 3. Heparin-Sepharose | 40.0       | 6.1     | 8,000 | 392,000 | 64,300 | 2.30  |
| 4. Phenyl-Sepharose | 32.5       | 0.42    | 1,210 | 62,000  | 147,800 | 0.36  |
| 5. C₆ reverse phase | 4.0        | 0.05    | 75    | 300     | 6,000  | 0.05  |

* The amount of SOD was estimated from radioimmunoassays. Purified rat EC-SOD was used to calculate the absolute amount of the enzyme after each purification step.

ND, not determined.
terminal sequencing of PVDF bound proteins, the excised bands or two-dimensional spots were sliced (2 × 2 mm) and placed in the reaction cartridge on top of a glass fiber filter to achieve an optimal flow of reagents.

Limited Acid Hydrolysis and Peptide Mapping—Partial acid hydrolysis of proteins immobilized in polyacrylamide gels was as described (21). The peptides were separated on a narrow-bore PTC C18 column (2.1 × 220 mm) by a gradient from 0.1% (v/v) trifluoroacetic acid in water (A) to 0.09% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile (B) (6 min to 15% B, 6-55 min to 60% B).

Cyanogen Bromide Cleavage—EC-SOD was deglycosylated with N-glycanase (22). CNBr-cleaved peptides were generated by a modification of the method described by Gross (23). The reaction was performed in the dark in 70% (v/v) formic acid in water with a 50- or 200-fold molar excess of CNBr/methionine residues. Incubation was at 30°C for 24 h. The reaction was stopped by dilution of the reaction sample with 15 volumes of water and subsequent freeze-drying.

Tryptic Digestion—Tryptic digestion of blotted rat EC-SOD was as described (24). The 80 μl reaction mixture contained 50 ng of trypsin, 100 mM Tris/HCl (pH 8.56). Hydrolysis was at 37°C for 18 h. Tryptic peptides were vacuum-dried and resolubilized in 10% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, and separated on a C4 Vydac column by a gradient from 0.1% (v/v) trifluoroacetic acid to 0.08% (v/v) trifluoroacetic acid in 70% acetonitrile (B) (6 min 10% B, 8-60 min to 70% B).

Hydroxylamine Cleavage—EC-SOD was reduced and carboxymethylated with iodoacetamide prior to hydroxylamine hydrolysis (13, 20). The reaction mixture contained 1.5 M hydroxylamine/HCl, 0.15 M K2CO3, and 4.6 M guanidium HCl.

Cloning and Sequencing of EC-SOD

Preparation of mRNA from Rat C6 Glioma—Total RNA was isolated by the acid-guanidinium-thiocyanate phenol method as described by Chomczynski and Sacchi (25). Poly(A)+ RNA was purified concentrated fractions on a heparin-Sepharose column was as described. Activity was eluted using a NaCl gradient (-). Fractions of 3 ml were collected and assayed for SOD activity (block diagram). Optical density (—). Fractions (horizontal bar) were pooled and subjected to hydrophobic interaction chromatography on phenyl-Sepharose 4B. The SOD activity was eluted from the column with a descending gradient of (NH4)2SO4 (-). Fractions of 3 ml were collected and tested for SOD activity (block diagram). Optical density (—). The active pool is indicated by the horizontal bar. C, reverse phase chromatography on C4. The bound material was eluted with a gradient of acetonitrile (-). Fractions of 1 ml were collected, 10 μl of the fractions were freeze-dried and tested for SOD activity (block diagram). Optical density (—). The indicated pool is used for amino acid sequence determination.
The peptides generated by formic acid, tryptic and hydroxylamine cleavage were separated by reverse phase chromatography. The peptide mixture obtained after CNBr cleavage was separated by one-dimensional PAGE and blotted onto a PVDF membrane (Fig. 3). The sequenced polypeptides were generated by the indicated hydrolysis method carboxyl-terminal of the amino acid between parentheses. The latter were deduced from the complete sequence of the rat EC-SOD B. The sequences were aligned with human EC-SOD C (4) using the FASTA program (Fig. 3A). X: unidentifed amino acid residues.

| N-terminal sequence | Corresponding human EC-SOD C residues |
|---------------------|-------------------------------------|
| 2D blot             |                                     |
| 1                   | [MSDTGESGVLDAXMKV]                  |
| 2                   | [MSDTGESVGLDRLVKEK]                 |
| 3                   | [MSDTGESVGLDRLVKEK]                 |
| 4                   | [MSDTGESVGLDRLVKEK]                 |
| CNBr fragments      |                                     |
| 20,800 (M)          |                                     |
| 13,000 (M)          |                                     |
| 10,000 (M)          |                                     |
| Tryptic digest      |                                     |
| T8                  | [LSXVWGTNSSEAXS]                    |
| Acidic hydrolysis   |                                     |
| AH23 (D)            | [FGNFVIR]                           |
| AH16 (D)            | [LGXGQSTGPYYLQVPMVPYQXPQPG]         |
| AH22 (D)            | [LGXGQSTGPYYLQVPMVPYQXPQPG]         |
| Hydroxylamine cleavage | [SEAEWSQTKERKKRREECT][29]         |
| H1 (N)              | [GAGN]                              |
| H2 (N)              | [GAGN]                              |

by chromatography on oligo(dT)-cellulose type 3 (36).

cDNA Synthesis and Cloning—The first cDNA strand was synthesized as described (27) using 50–200 ng/ml poly(A)* RNA and 5,000–10,000 units/ml superscript reverse transcriptase.

For the screening of the agt11 rat glioma cDNA library two degenerated primers, 5'-'CIGGAYCARCCICARATHAC-3' and 5'-'IACRAARTTICRARCT-3', based on the amino acid sequences of peptides 'PDQPIQ' and 'DFGNFV', respectively (Table III), were synthesized by the phosphoramidite method using an Applied Biosystems DNA synthesizer.

Polymerase chain reaction was carried out in a final volume of 50 µl containing 1 µl of cDNA, 10–50 pmol of deoxynucleotide primers, 200 µM dNTPs, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl2, 0.001% gelatin and 1.2 units of Taq polymerase. Routinely 30 cycles were performed using cycles of 2 min at annealing temperatures between 37 and 50 °C, 2 min at 72 °C and 1 min at 94 °C. A 230-bp amplicon was purified, subcloned into EcoRV-cut pBluescript plasmid, and sequenced. Based on the obtained sequence data, a 58-base antisense oligonucleotide probe (5'-GTCTGAGCTCGGCCCCAGCTCGCGGAAGAGGACCAAGCCTGTGATCTGCGG-3' (nucleotides 380-400, fig. 4B) and a primer corresponding to the anchor oligo 5'-GGCGCCGCTTATTAACCTTACTAA-A (39). A 440-bp fragment was obtained and purified. Polymerase chain reaction with a nested antisense primer 5'-ATAAGCTTGGTACCTGCTGCGGCGTAC--3' (nucleotides 380–400, fig. 4B) and a primer corresponding to the anchor linker resulted in the purification of a 400-bp fragment which was cloned into pBluescript(SK*) plasmid and sequenced.

Miscellaneous

The native M, of EC-SOD was measured by gel filtration on a Superose 12 column calibrated with ferritin (440,000), aldolase (128,000), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen A (25,000).

Protein concentrations were determined using the microprotein assay (31).

RESULTS

Secretion of EC-SOD by Rat CG Glioma—CM obtained from rat CG glioma cells grown in chemically defined medium contained an enzyme which reduces the phorbo1 12-myristate 13-acetate-induced superoxide production measured by the
FIG. 4. Amino acid and nucleotide sequence alignments of CuZn-SODs. A, amino acid alignment of mature rat and human SODs. Residues are numbered according to rat mature EC-SOD. Amino acids determined by protein sequencing (summarized in Table I) are indicated (----). The other residues are deduced from the nucleotide sequence of EC-SOD B presented in B. Residues identical to rat EC-
E x t r a c t i o n a r y  S u p e r o x i d e  D i s m u t a s e  B  S e c r e t e d  b y  R a t  C₆  G l i o m a

SOD B are boxed. The NH₂-terminal amino acid of the mature human EC-SOD is indicated by an arrow. The invariant amino acids of Table III are printed in bold and shaded. The glycosylation site is marked by an asterisk. The SOD sequences were taken from Refs. 43 and 4. Based on the comparison of the rat SOD sequences, an NH₂-terminal alignment of the extracellular with the intracellular SODs is proposed, which is slightly different from the one reported in Ref. 2. B, nucleotide sequence alignment of human and rat EC-SOD. The human nucleotide sequence is taken from Ref. 4.
TABLE IV

Location of the invariant amino acids in rat EC-SOD

The invariant amino acids (2, 42) have been deduced from the alignment of rat and human EC-SOD with the intracellular SODs. The endogenous SOD amino acids Gly16 and Gly17 (numbering of Getzoff et al. (42)) are the only invariant residues which are substituted by Pro16 and Cys17 in the extracellular SODs (Fig. 4).

Active site residues:
- Metal liganding residues: His101, His103, His118, His126, His129, His140, Asp142
- Positioning of Cu2+ - and Zn2+ -ligand residues: Asp172, Gly173
- Structure determinants of the active site: Gly46, Gly146, Pro171
- β-Bulge glycines at opposite ends of the active site channel: Gly46, Gly146

Superoxide binding pocket: Arg191
Dimer contacts: Gly146, Gly172
Disulfide bridge: Cys112, Cys174
Greek key β barrel fold: Phe66, Leu74

Fig. 5. Native M, of rat EC-SOD B. EC-SOD B, partially purified by chromatography on heparin-Sepharose and phenyl-Sepharose, was concentrated by lyophilization, dialyzed against 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.06% (w/v) CHAPS, and applied to a Superoxide 12 column. Fractions were assayed for SOD activity (block diagram). Optical density (—). The positions of the M, markers are indicated by the arrow: ferritin (1), aldolase (2), bovine serum albumin (3), ovalbumin (4), and chymotrypsinogen A (5).

and the consensus sequence for glycosylation at Asn194 are also conserved in the rat homologue (22). The main charge is in the primary structure of the human and rat EC-SOD are observed in the NH2-terminal residues Met1-Ile6 and in regions flanking the heparin-binding site, comprising residues Thr196-Lys210 and Thr213-Thr214 (Fig. 4A). The sequence located NH2-terminally of the heparin-binding site lacks the Gly-Pro-Gly sequence only aligns with human EC-SOD if residues carboxyl-terminal of Leu23 are included in the alignment search.

Biochemical Characteristics of Rat EC-SOD B—Although very homologous to the human EC-SOD C, the purified rat enzyme has properties distinct from its human counterpart. The rat enzyme has a slightly lower affinity for heparin-Sepharose and is eluted from the column at 300–400 mM NaCl as proposed for the B-form of human EC-SOD (6–8). In comparison, the human EC-SOD C eluted from the latter column above 500 mM NaCl.

Contrary to other SODs which are acid stable, the purified rat enzyme is acid labile. Its lability was demonstrated by incubation at pH 6.0, 4.0, and 2.0 at 4 °C and for 16 h. After readjustment to pH 7.4, the remaining bioactivity was deter-
mained. The latter decreased to 55, 13, and 8%, respectively.

The pl of 6.1–7.2 measured for the rat enzyme (Fig. 2) is also significantly different from the pl of other SODs. Intracellular SODs have a pl close to 4.7 (38) and the human EC-SOD C has a pl of 4.5 (7).

The native M, for the rat EC-SOD measured by gel filtration on Superose 12 was approximately 85,000 ± 20,600 (Fig. 5) and is significantly less than the M, of 140,000 reported for the human EC-SOD C (3). The M, was confirmed by gel filtration on Toyopearl HW55 (data not shown). The estimated M, is indicative of a dimeric structure rather than a tetrameric structure as reported for the human enzyme.

DISCUSSION

Rat C6, a glioma cell line with oligodendrocytic and astrocytic properties, secretes high amounts of EC-SOD in the logarithmic growth phase. An amount of 100–150 ng/ml is secreted in 72 h by C6 which is approximately 20–30-fold higher than the maximal amount of EC-SOD measured for several other cell lines by Marklund (14). Of the more than 15 rat and human cell lines tested only rat B50 neuroblastoma secretes approximately the same amount of SOD. The secretion of SOD, which converts the superoxide produced by activated neutrophils, may be one of the mechanisms by which C6 survives in rodents and may be able to form solid tumors (37, 38).

Purification, amino acid sequence determination, cDNA cloning, and determination of the deduced amino acid sequence shows that the enzyme is 68% homologous to human EC-SOD C, the only member of the family of extracellular SODs from which the complete amino acid sequence has been described (4). EC-SODs are ubiquitous CuZn-containing enzymes, present in the extracellular fluids of all mammals so far tested (6). The latter enzymes are heterogenous in their affinity for heparin-Sepharose and according to Marklund (14) definition, three subtypes A, B, and C can be distinguished having no, moderate, or high affinity for heparin, respectively (3). Under appropriate conditions, all three isozymes can be detected in the extracellular fluids of humans and other mammals (8). Rat seems to differ from the other mammals by the absence of the C-isoform (8).

Recently, research on EC-SOD has been focused on the differences in heparin binding. It was shown that human EC-SOD C has a carboxyl-terminal sequence containing a cluster of 6 arginine and 3 lysine residues in the last 21 amino acids which are involved in the binding to heparan sulfate proteoglycans and related molecules (10). To account for the observed decrease in heparin binding it has been proposed that the A and B form could be derived from the C form by modifications in the COOH-terminal sequence.

Two explanations, supported by experimental evidence, have been proposed. First, it was shown that EC-SOD A and especially EC-SOD B could be glycosylated in vitro and in vivo, and that nonenzymatic glycation of some of the lysine residues at the COOH terminus could be responsible for the decrease in affinity for heparin (12). Second, the affinity for heparin is not influenced by truncations induced in the human EC-SOD C downstream of Glu216. However, removal of the latter or its substitution by basic residues abolishes the heparin-binding properties. From these data Sandström et al. (11) concluded that the human B-form could be a heterotetramer composed of heparin binding C forms and truncated nonheparin binding A forms.

A. Zwijsen, J. Willems, and H. Slegers, unpublished data.
Although each of the former explanations seems plausible, the amino acid sequence of rat EC-SOD indicates that the B form may exist as a distinct entity which is not derived from the C form.

Although important domains such as the catalytic site, N-glycosylation site, and heparin-binding domain are rather homologous, sequence comparison between human EC-SOD C and rat EC-SOD B indicates an overall sequence divergence of 32%. The observed sequence difference is larger than what is seen between rat and human intracellular CuZn-SOD and mitochondrial Mn-SOD, with 17 and 12% sequence divergence, respectively. Furthermore, interspecies divergence is manifested by sequence variations which are more or less evenly distributed along the molecule. In contrast, the divergence between rat EC-SOD B and human EC-SOD C is less random.

The alignment shows that the rat EC-SOD B has a NH2-terminal sequence in which the nonidentity amounts to over the first 70 residues. The COOH-terminal sequence, which lacks 3 amino acids, contains another stretch of nonidentical amino acids Thr142-Lys143. An important difference between rat and human EC-SOD is the presence of a Gly201-Pro-Gly202 motif in the human enzyme. The latter sequence has a preference to form turns in protein structures (39) and probably favors the exposure of the heparin-binding site in EC-SOD C. The lack of a Gly-Pro-Gly motif in the rat enzyme may be responsible for its reduced heparin affinity. Based on these observations we conclude to the existence of an EC-SOD B isoform distinct from the human EC-SOD C.

SOD has been used in therapeutic approaches to reduce the noxious effects of reactive oxygen in pathophysiological conditions. Its main application is protection against postischemic reperfusion damage in various organs (40). Intracellular CuZn-SODs, however, are rapidly cleared from the circulation and consequently they confer only limited protection. Therefore, in recent experiments human rEC-SOD was used (41), especially since it seems to bind in vivo to the proteoglycans of the endothelial cell surface. This could be due to the Gly-Pro-Gly sequence flanking the heparin-binding site which may give the enzyme more flexibility to interact with the proteoglycans.

Also, chimeric constructs of intracellular SOD and the heparin-binding site of a serpin protein C inhibitor coupled via the Gly-Pro-Gly motif have been used to improve the therapeutic effectiveness of SOD (39). Consequently, the lack of this Gly-Pro-Gly motif in the isolated rat EC-SOD B probably influences its cell binding and cell specificity. In conclusion, EC-SOD B which has biochemical properties distinct from EC-SOD C, is a new SOD to be tested in a number of therapeutic applications related to all types of tissue damage by superoxide.

Acknowledgements—We thank Prof. M. Joniau (IRC, KULAK, Kortrijk) for the preparation of EC-SOD antibody, Dr. J. Heath (University of Oxford, United Kingdom) and Dr. A. Van de Voorde (Innogenetics, Belgium) for helpful suggestions and discussions, and D. Debonnaire for excellent technical assistance. We acknowledge the many volunteers who agreed to provide blood samples for the preparation of neutrophils.

Note Added in Proof—When this article was in press, a rat cDNA for a secreted superoxide dismutase was published by Perry, A. C. F., Jones, R., and Hall, L. (1993) Biochem. J. 293, 21–25. The only difference with the rat C6 glioma sequence is located in the heparin-binding site at positions 215 and 217 at which arginines are mutated into tryptophans. The major site of expression of this EC-SOD is the epithylin.

REFERENCES

1. McCord, J. M. & Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
2. Bannister, J. V., Bannister, W. H. & Rottino, G. (1987) CRC Crit. Rev. Biochem. 22, 111–180
3. Marklund, S. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7634–7638
4. Hjalmarsson, K., Marklund, S. L., Engström, Å. & Edlund, T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6340–6344
5. Tabet, L., Hjalmarsson, K., Edlund, T., Skogman, G., Engström, Å. & Marklund, S. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6634–6638
6. Marklund, S. L. (1984) Biochem. J. 222, 649–655
7. Karlsson, K. & Marklund, S. L. (1987) Biochem. J. 242, 55–59
8. Karlsson, K. & Marklund, S. L. (1985) Biochem. J. 225, 223–228
9. Karlsson, K. & Marklund, S. L. (1989) Lab. Invest. 60, 638–646
10. Adachi, T. & Marklund, S. L. (1989) J. Biol. Chem. 264, 8575–8581
11. Sandström, J., Carlsson, L., Marklund, S. L. & Edlund, T. (1993) J. Biol. Chem. 268, 2530–2535
12. Adachi, T., Otta, H., Hirano, K., Hayashi, K. & Marklund, S. L. (1991) Biochem. J. 279, 265–267
13. Adachi, T., Koder, T., Otta, H., Hayashi, K. & Hirano, K. (1992) Arch. Biochem. Biophys. 297, 155–161
14. Marklund, S. L. (1990) Biochem. J. 266, 212–219
15. Willems, J., Joniau, M., Cinque, S. & Van Damme, J. (1989) Immunology 67, 540–542
16. Laszlo, U. K. (1970) Nature 227, 686–685
17. Schägger, H. & von Jagow, G. (1987) Anal. Biochem. 166, 368–379
18. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 6007–6021
19. Hirs, C. H. W. (1967) Methods Enzymol. 11, 199–203
20. Matsudaïre, P. (1987) J. Biol. Chem. 262, 10055–10058
21. Vanfleteren, J. R., Raymackers, J., Van Buren, S. M. & Meheus, L. A. (1992) Biotechniques 12, 550–557
22. Strömqvist, M., Holgersson, J. & Samuelsson, B. (1991) J. Chromatogr. 548, 285–300
23. Gross, E. (1967) Methods Enzymol. 11, 238–255
24. Ben-Aroya, G., Van Damme, J., Stockbrooke, J., Geasser, B., Ratz, G. P., Lauridsen, J. B. & Cels, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 7301–7305
25. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. 162, 156–159
26. Aviv, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408–1412
27. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)
28. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)
29. Sandstrom, J., Carlsson, L., Marklund, S. L. & Edlund, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5465–5467
30. Troutt, A. B., McFezer, M. P., Pulendran, B. & Nossal, G. J. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 28–31
31. Taggart, J., Blassey, B. K., Johnson, T. R., Rudin, S. D., Tykocinski, M., Itoh, J. & Iman, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4874–4875
32. Boas, J., Kuhn, L. A., Lee, F., Fletcher, C. L., Wang, Y., Halbleib, R. A. & Tainer, J. A. (1993) Biochem. Biophys. Res. Commun. 190, 259–265
33. Ferrari, R., Celoni, C., Curelo, S., Ghielmi, S. & Albertini, A. (1989) Pharmacol. Res. 21 Suppl, 57–66
34. Sjöström, P. O. & Marklund, S. L. (1992) Cardiovasc. Res. 26, 347–350
35. Getzoff, E. D., Tainer, J. A., Stempien, M. M., Bell, G. I. & Hallwell, R. A. (1989) Proteins, Struct. Funct. Genet. 5, 322–336