Journal of Chromatography, 621 (1993) 139–148
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7109

Characterization of recombinant human extracellular superoxide dismutase

Mats Strömqvist

Department of Protein Chemistry, Symbicom AB, P.O. Box 1451, S-901 24 Umeå (Sweden)

(First received March 16th, 1993; revised manuscript received August 23rd, 1993)

ABSTRACT

Recombinant human extracellular superoxide dismutase produced in chinese hamster ovary cells has been characterized using several chromatographic methods. Peptide mapping confirmed the expected primary structure. The 15 amino acids at the N-terminal end were sequenced and were in accordance with expectations in all positions. The C-terminal amino acids have been confirmed both by amino acid composition studies of a peptide of 42 amino acids and by specific sequential cleavage of the last three C-terminal amino acids with carboxypeptidase A. Both methods demonstrated a full length C-terminus. At physiological ionic strength, the dismutase exists for *ca.* 25% as octamers or larger polymers, and the amount of polymers increases at lower ionic strength.

INTRODUCTION

Oxygen is crucial for the aerobic life on Earth, but it is also a potentially very toxic agent because of its ability to form oxy radicals. Superoxide radicals and other oxy-radical intermediates are easily formed *e.g.* by autooxidation. Living organisms have evolved protecting systems to prevent damage from these toxic agents. One example of these protecting factors is the enzyme superoxide dismutase (SOD), which removes the superoxide anion, O_2^-, and converts it into molecular oxygen and hydrogen peroxide. Hydrogen peroxide can thereafter be reduced to water, or to water and molecular oxygen, by the action of other enzyme systems.

Three different SOD isoenzymes in mammalian species are known. The first to be described was CuZn-SOD [1], which is a dimeric protein of two identical subunits each containing one Cu and one Zn atom. It is an intracellular protein of low molecular mass (33 000). Mn–SOD was described a year later; it is a tetrameric protein with a molecular mass of *ca.* 85 000 [2,3] and is located in the mitochondrial matrix [4], and in primates also in the cytosol [5].

Extracellular SOD (EC-SOD) is the major isoenzyme in extracellular fluids [6,7] but is also present in tissues [8]. It is a protein composed of four equal subunits with a molecular mass of 135 000 as assayed by size-exclusion chromatography (SEC) [9], and contains one Cu and one Zn atom per subunit. The protein is glycosylated and has affinity for several lectins [9,10]. The glycosylation was recently identified to be restricted to the only possible N-glycosylation site, asparagine-89 [10].

The isolation of the cDNA clone provided much information about the primary structure of the protein and opened the possibility of overproducing the protein in cell culture. The protein thus became available for more extensive studies than had seemed possible with enzyme prepared from human material.

This paper describes a study of recombinant human EC-SOD produced in chinese hamster

0378-4347/93/$06.00

ovary cells. The purity of the protein was assayed, using several different techniques to explore the types of impurities present. The primary structure of the protein was confirmed by peptide mapping, and the C- and N-terminus were confirmed by sequencing as well as by analysis of terminal fragments. The subunit structure and the oligomerization of the protein at different ionic strengths was studied using SEC.

EXPERIMENTAL

Proteins

Recombinant EC-SOD produced in chinese hamster ovary cells and purified by ion-exchange chromatography, hydrophobic interaction chromatography and heparin affinity chromatography [11], and native EC-SOD purified from human umbilical vein cord, were from Symbicom AB (Umeå, Sweden). Antibodies against EC-SOD, raised in rabbit and in goat using both recombinant and native EC-SOD, were purified by affinity chromatography on EC-SOD Sepharose, prepared by immobilization of EC-SOD onto CNBr-activated Sepharose by the method described by the manufacturer (Pharmacia-LKB Biotechnology, Uppsala, Sweden).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gradient gels (10–17%) were cast and run according to the discontinuous buffer system of Laemmli [12]. Gels were run either in a Midget Electrophoresis unit (Pharmacia-LKB) or in a Protean unit (Bio-Rad, Richmond, CA, USA). Gels were stained with Coomassie Brilliant Blue R-250.

Protein blotting

SDS-PAGE gels were electroblotted at a constant current (0.8 mA/cm^2) for 1 h onto Immobilon-P membranes (Millipore, Bedford, MA, USA) using a Trans-Blot SD Transfer Cell (Bio-Rad). The transfer buffer contained 48 m*M* Tris, 39 m*M* glycine, 1.3 m*M* SDS and 20% methanol. Prior to transfer, the filter was soaked in methanol and then in transfer buffer. Membranes were blocked in 5% defatted milk in 10 m*M* Tris-HCl, 0.5 *M* NaCl (pH 8.2). Rabbit anti EC-SOD (0.3 μg/ml) was used as detecting antibody, alkaline phosphatase conjugated anti rabbit Ig (Dakopatts A/S, Copenhagen, Denmark) was used for enzyme labelling, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrate.

HPLC system

A System Gold software together with a Model 126 pump and a Model 166 variable-wavelength detector (Beckman, San Ramon, CA, USA) was used in all HPLC runs. Column temperature was controlled using a TCM temperature control module (Millipore-Waters).

Size-exclusion chromatography

For purity and size determinations, a 3000SW Spherogel TSK (300 × 7.5 mm I.D., Beckman) column with a guard column (75 × 7.5 mm I.D.) (Varian Micropak TSKgel 3000SW, Varian Assoc., Sunnyvale, CA, USA) was used with several different buffer systems. For determination of molecular masses, the column was calibrated with ferritin (440 000), aldolase (158 000), bovine serum albumin (67 000), ovalbumin (44 000), myoglobin (17 600) and insulin (6000).

Reversed-phase HPLC

For the analysis of intact and trypsin-cleaved EC-SOD, a C_8 Ultrapore column (250 × 4.6 mm I.D., Beckman) was used. The elution system was 0.1% trifluoroacetic acid (TFA) in water (mobile phase A) and 0.1% TFA in 60% acetonitrile (mobile phase B). All separations were made at 38°C.

Carboxymethylation of EC-SOD

Lyophilized EC-SOD (5 mg) was dissolved in 1.5 ml of 0.5 m*M* ammonium acetate with 6 *M* guanidine-HCl (pH 8.0), and the solution was flushed with nitrogen. A 15-μl volume of 1 *M* dithiothreitol was added, the sample was flushed again with nitrogen and then incubated for 1 h at room temperature. A 30-μl volume of iodoacetic acid was added, and the sample was flushed again

with nitrogen and incubated in the dark for 2 h. The reaction was terminated by the addition of 30 μl of 2-mercaptoethanol and acidification with 525 μl of glacial acetic acid.

Trypsin cleavage

Carboxymethylated EC-SOD was dialyzed against 0.1 *M* NH_4HCO_3 (pH 7.8) and mixed with trypsin at a mass ratio of 50:1. The mixture was incubated overnight (16–20 h) at room temperature. The reaction was terminated by adding 1 m*M* phenylmethanesulphonyl fluoride (PMSF).

Chemical cleavage with hydroxylamine

EC-SOD was reduced by adding 10 m*M* 2-mercaptoethanol and incubating at 45°C for 1 h. Thereafter the reduced protein was mixed with 2 *M* hydroxylamine, 4 *M* guanidine–HCl, 0.2 *M* K_2CO_3 (pH 9.0) (cleavage buffer), so that the cleavage buffer always constituted at least 80% of the total volume, and incubated for 4 h at 45°C. The reaction was terminated by immediate chromatographic separation or by freezing at −80°C.

Total amino acid analysis

For the amino acid analysis, the Pico-Tag system (Millipore-Waters) was used, with a few exceptions. Instead of the supplied column, the PTC-amino acids were separated on a Beckman Ultrasphere ODS (150 × 4.6 mm I.D.) at 38°C with an elution programme as described previously [10]. Samples were dehydrated and hydrolyzed at 105°C for 16 h in 6 *M* HCl with 1% phenol, using a Pico-Tag Work Station (Millipore-Waters). Samples were then derivatized with phenyl isothiocyanate (PITC) and prepared for analysis according to the instructions supplied by the manufacturer (Millipore-Waters).

N-Terminal amino acid analysis

Sequence analysis was carried out on an Applied Biosystems (Foster City, CA, USA) 477A Pulsed Liquid Phase sequencer with an online PTH 120A Analyzer. Sequencing was performed with regular cycle programmes and chemicals from the manufacturer.

C-Terminal amino acid analysis

Carboxymethylated EC-SOD was dialyzed against 0.1 *M* N-ethylmorpholine acetate (pH 9.2). Carboxypeptidase A was added at a concentration of 10 U per 1.5 mg of EC-SOD. Samples (30 μl) were withdrawn at specific times and immediately added to 60 μl of 8.7 *M* acetic acid. Free amino acids were determined by lyophilization, derivatization with PITC, and amino acid analysis of the withdrawn samples, as described above.

Fast atom bombardment–mass spectrometry (FAB–MS)

FAB–MS was carried out using a ZAB HF 2F mass spectrometer, as described previously [10].

RESULTS

EC-SOD purified from conditioned media was *ca.* 98% pure according to SDS-PAGE. EC-SOD was distributed in a number of bands ranging from 27 000 to 32 000 (Fig. 1a), and all were immunoreactive against specific EC-SOD antibodies according to protein blotting (Fig. 1b). Two bands at 60 000–65 000 also showed immunoreactivity against EC-SOD antibodies and were found to have the N-terminus expected for EC-SOD. The amounts of these bands, which can be assumed to be dimers of EC-SOD, were independent of the time and temperature at which the samples were treated with the sample cocktail containing SDS and 2-mercaptoethanol. SDS-PAGE in the absence of 2-mercaptoethanol gave the same results as in its presence, indicating that the subunits in EC-SOD are not held together by sulphydryl bonds. Of the protein contaminants present in the preparation, bovine serum albumin (BSA) from calf serum, comigrating with BSA in the molecular mass marker, was the predominant, constituting 0.9–1.1% of the total protein. The identity of this contaminant was also assayed with specific antibodies (not shown). Impurities with subunit molecular masses of *ca.* 15 000 (< 0.5%) and *ca.* 100 000 (< 0.5%) could also be detected when the protein load was high.

The protein contaminants remaining after pu-

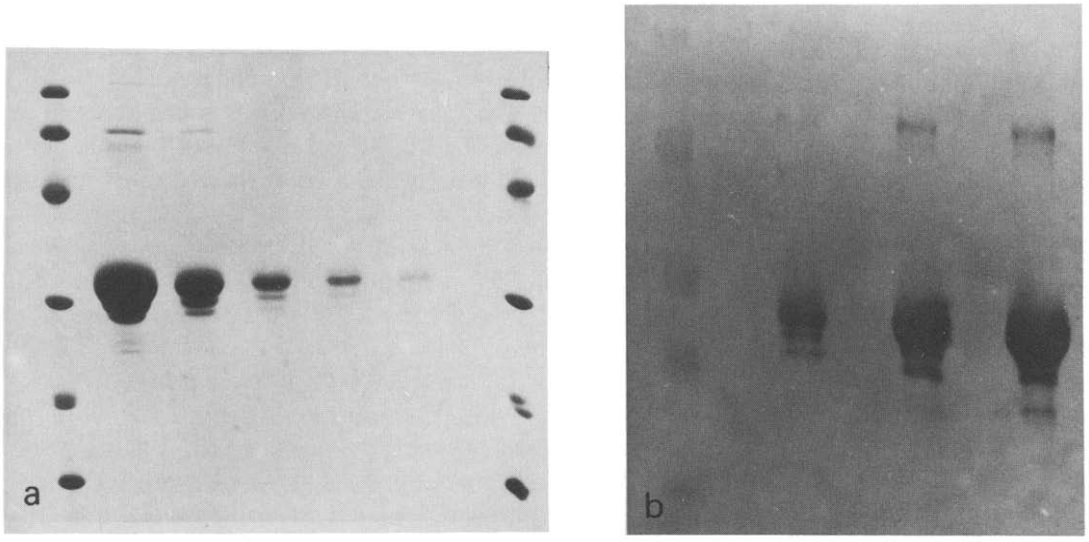

Fig. 1. (a) SDS-PAGE of recombinant EC-SOD. From the left, lanes contain: molecular mass marker (LMW, Pharmacia-LKB Biotechn., 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400), 100, 30, 10, 5, 3, and 1 μg of EC-SOD, and an additional Pharmacia LMW marker. (b) Protein blot of recombinant EC-SOD. From the left, the lanes contain: prestained molecular mass marker (Bio-Rad, 80 000, 49 500, 32 500, 27 500 and 18 000), 0.3, 1 and 3 μg of EC-SOD.

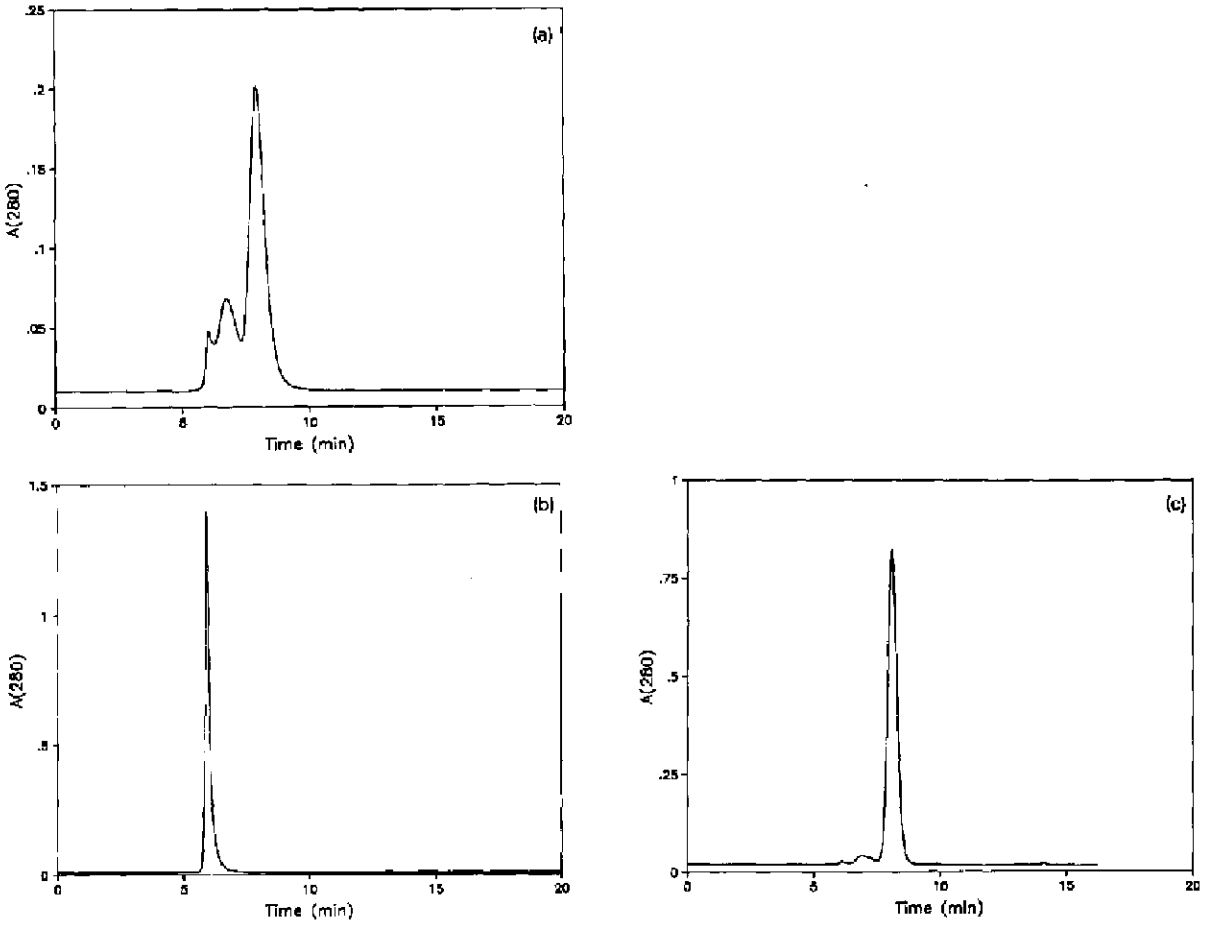

Fig. 2. Size-exclusion chromatography of EC-SOD on 3000 SW Spherogel TSK: (a) recombinant EC-SOD in phosphate-buffered saline (PBS), (b) recombinant EC-SOD in 2 m*M* Tris–HCl (pH 7.5); (c) native EC-SOD in PBS.

rification could not be removed by any standard chromatography, although even affinity chromatography on a monoclonal antibody directed against EC-SOD immobilized on CNBr-activated Sepharose was tested. As BSA was the main contaminant, it should be removable by SEC, but at the elution volume where BSA normally elutes on SEC, no contaminating BSA was detected.

The purified protein consisted partly of higher oligomers than the natural tetramer. Under physiological salt conditions, *ca.* 20% was present as octamers and 7% as larger complexes, according to SEC (Fig. 2a). Decreasing the ionic strength led to more aggregated protein and, in 2 m*M* Tris-HCl (pH 7.5), all eluted as higher mers (Fig. 2b). Native EC-SOD eluted to 94% as tetramers at physiological ionic strength (Fig. 2c) and completely as higher mers in 2 m*M* Tris–HCl (pH 7.5) (not shown). The elution volume on the column used indicated molecular masses of 209 000 for recombinant EC-SOD and 186 000 for native EC-SOD; these values are somewhat higher than those reported earlier [9]. Using a different column (Superdex 200 HR 10/30, Pharmacia-LKB Biotech.), the obtained molecular masses were 191 000 for the recombinant and 182 000 for the native protein. The specific activity of EC-SOD eluting as higher mers was similar to that of EC-SOD eluting as tetramers (100–120 U/μg).

Reversed-phase chromatography of the protein resulted in the appearance of two peaks (Fig. 3). Both peaks contained EC-SOD according to SDS-PAGE and immunoassays.

However, the smaller of the two was enriched in aggregated EC-SOD, and the larger in free tetramer. Some impurities could also be seen. BSA, for example, could now be seen as a small peak eluting after the two peaks containing EC-SOD (Fig. 3), possibly released from EC-SOD by the hydrophobicity of the eluent.

The resistance of EC-SOD to proteolysis was elucidated when the protein was subjected to trypsin cleavage. The native protein was not cleaved, and only small amounts of minor fragments were produced. Therefore the protein was carboxymethylated prior to cleavage. EC-SOD should theoretically give 26 fragments (T1–T26,

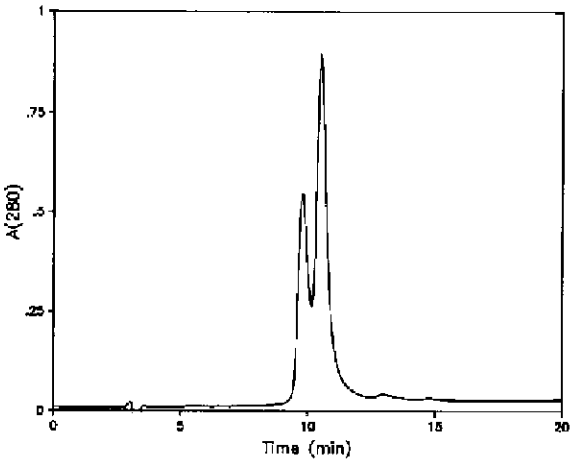

Fig. 3. Reversed-phase LC of recombinant EC-SOD on C_8 Ultrapore (250 × 4.6 mm I.D.). The gradient starts at 25% acetonitrile and 0.1% trifluoroacetic acid, increases from 25% to 32% acetonitrile between 5 and 15 min, and thereafter rises to 60% acetonitrile in 2 min. The small peak eluting at *ca.* 13 min contained BSA.

Table 1) upon cleavage but many of the fragments are too small to detect. The C-terminal portion of the protein has six cleavage sites within six amino acids, resulting probably in very random cleavage of this portion. Reversed-phase HPLC of the digest also confirmed this. The pep-

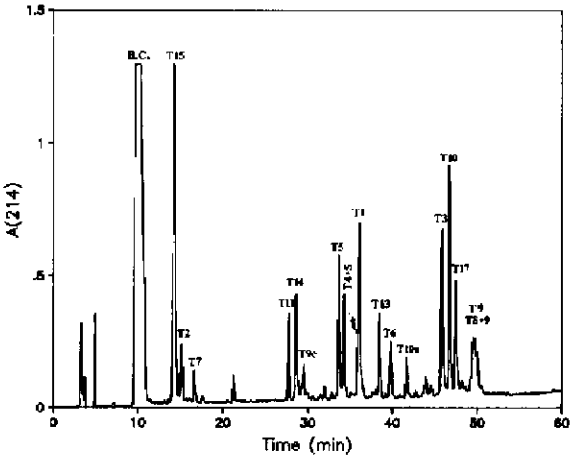

Fig. 4. Tryptic map of recombinant EC-SOD obtained on C_8 Ultrapore (250 × 4.6 mm I.D.). The gradient was 0–25% acetonitrile from 0 to 20 min, 22–40% acetonitrile from 20 to 50 min and 40–60% from 50 to 55 min. The fragments are labelled according to Table I. The letters n and c after the fragment numbers refer to N- and C-terminal parts of the unspecifically cleaved fragment. B.C. refers to a buffer component.

TABLE I

EC-SOD CLEAVAGE

Theoretically obtained fragments upon cleavage of EC-SOD with trypsin (T1–T26) and the C-terminal fragment obtained upon cleavage with hydroxylamine (H2). The fragments are numbered according to their position, starting at the N-terminus.

Fragment	Amino acid sequence
T1	WTGEDSAEPNSDSAEWIR
T2	DMYAK
T3	VTEIWQEVMQR
T4	R
T5	DDDGTLHAACQVQPSATLDAAQPR
T6	VTGVVLFR
T7	QLAPR
T8	AK
T9	LDAFFALEGFPTEPNSSSR
T10	AIHVHQFGDLSQGCESTGPHYN PLAVPHPQHPGDFGNFAVR
T11	DGSLWR
T12	YR
T13	AGLAASLAGPHSIVGR
T14	AVVVHAGEDDLGR
T15	GGNQASVENGNAGR
T16	R
T17	LACCVVGVCGPGLWER
T18	QAR
T19	EHSER
T20	K
T21	K
T22	R
T23	R
T24	R
T25	ESGCK
T26	AA
H2	GNAGRRLACCVVGVCGPGLWERQA REHSERKKRRRESGCKAA

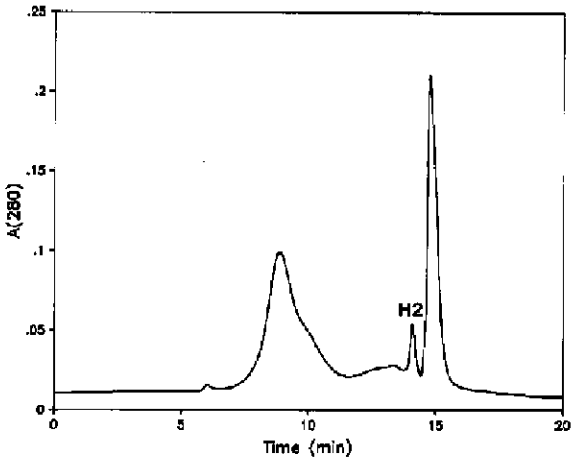

Fig. 5. Size-exclusion chromatography of hydroxylamine-cleaved recombinant EC-SOD on 3000 SW Spherogel TSK. The position of the C-terminal fragment of 42 amino acids (H2) is indicated.

tides at the C-terminal could not be found within the chromatogram. Assuming that not all sites were cleaved, and that the cleavage is not identical for all separate protein subunits in the sample, leads to a huge number of possible fragments. Many of the fragments are basic and highly charged, so they do not bind to the reversed-phase column. Amino acid analysis of the unretarded fraction also indicated that the predominant amino acid in this fraction was arginine. Except for this part of the protein, all fragments containing three or more amino acids could be separated and identified in the tryptic map (Fig. 4). The identification was made by total amino acid analysis of each peak and in some cases also by FAB–MS (T2, T6, T8 + 9, T9, T14).

The problems in confirming the primary structure of the C-terminal portion of the protein led to alternative cleavage methods. Cleaving EC-SOD with hydroxylamine gave a C-terminal peptide consisting of 42 amino acids (H2, Table I), which was easily separated from the rest of the protein by SEC because of the large difference in size compared with the rest of the protein (Fig. 5). This peptide was then analyzed for its amino acid content, and it was found to have the composition of an intact full-length C-terminal fragment (Table II).

The 15 amino acids at the N-terminal end of the protein showed no differences from what was expected from the cDNA sequence. The amino acid analysis of peptide T1 also confirmed the results for the 18 N-terminal amino acids (Table II).

The C-terminal had in part been confirmed by the amino acid analysis of the C-terminal fragment obtained on chemical cleavage with hydroxylamine (Table II). In addition, attempts were made to cleave amino acids sequentially

TABLE II

AMINO ACID COMPOSITION OF C- AND N-TERMINAL FRAGMENTS OF EC-SOD

The tryptic fragment at the N-terminus (T1) was isolated by reversed-phase HPLC and the isolated hydroxylamine fragment at the C-terminus (H2) by SEC.

Amino acid	N-Terminal fragment (T1)		C-Terminal fragment (H2)	
	Theoretical	Experimental	Theoretical	Experimental
Asx	3	2.9	1	1.2
Glx	3	2.7	5	5.2
Ser	3	2.3	2	2.1
Gly	1	1.2	–	
Arg	1	1.1	8	7.9
Thr	1	0.9	–	–
Ala	2	2.0	5	5.1
Pro	1	1.3	1	1.2
Val	–	–	3	2.4
Cys	–	–	4	N.D.
Ile	1	0.7	–	–
Leu	–	–	2	2.2
Lys	–		3	2.8
Trp	2	N.D.[a]	1	N.D.

[a] N.D. = not determined.

from the C-terminus by using carboxypeptidase (Cp). The known sequence, based on the results of cDNA sequence [9], was of help in the choice of enzyme and the conditions for cleavage. The first attempts were made with CpY because of its non-discriminating specificity. The result, though, was that a large number of amino acids were released at about the same rate. Considering the types of amino acid released, and the results of hydroxylamine cleavage, these results indicated that the enzyme acted not only as an exopeptidase but also as an endopeptidase. Similar results were obtained with a mixture of CpA and CpB, where arginine was very predominant and a number of amino acids not present at the end of the protein also occurred. CpA at neutral pH, though, released alanine rapidly but all other amino acids slowly. This result was in agreement with what was expected: at neutral pH only the two alanines at the end should be released. At pH 9.2, CpA was also capable of releasing the third amino acid from the end, lysine (Fig. 6). The rate of lysine release was much lower than that of alanine release, and at equilibrium the amount of lysine released was close to half the amount of alanine (Fig. 6). Neither of the carboxypeptidases should be able to release cysteine, therefore it is not possible to reach further into the sequence by the use of carboxypeptidases.

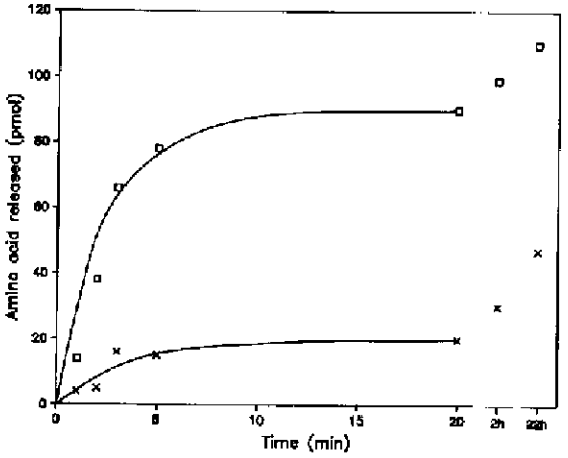

Fig. 6. C-terminal sequencing of recombinant EC-SOD with carboxypeptidase A. The figure shows the amino acid composition of samples withdrawn at specific times after the addition of carboxypeptidase A: (□) alanine; (×) lysine. For details see Experimental.

DISCUSSION

Products from naturally occurring genes expressed in foreign hosts may deviate from their natural counterparts. The deviations can occur for genetic, post-translational, or purification reasons. In many cases, the naturally occurring molecule is difficult to prepare because it is present in very low concentration in its natural source. It is therefore difficult to characterize fully such a protein in its native naturally occurring form, and much more information can be gained when large amounts of recombinant protein are available. The concentration of EC-SOD in human extracellular fluids is very low, and large amounts of native protein are therefore not easily available. Despite that, the protein has been characterized to a large extent. The molecular mass, subunit composition, enzymic characteristics, and metal atom binding are known from studies on human material. The isolation of the cDNA clone encoding human EC-SOD [9] led, in addition, to insight into the primary structure of the protein. A possible glycosylation site was identified. The C-terminal part of the protein contained a cluster of positively charged amino acids possibly responsible for the heparin affinity.

Determination of the molecular mass of the recombinant protein produced by chinese hamster ovary cells indicated that the protein has a tendency to form aggregates at physiological ionic strength. At low ionic strength this property was even more pronounced, and in 2 m*M* Tris–HCl all the protein appeared in the void volume of the column, which corresponded to a molecular mass of over 400 000 (Fig. 2b). However, if the ionic strength was increased (to 0.6 *M*), the ratio of the amount of tetramers to that of higher mers was equal to that at physiological ionic strength (not shown). When the pool containing higher mers at physiological ionic strength was collected and rechromatographed after standing at 4°C for one week, it still contained only higher mers. When the fraction containing tetramers was collected and rechromatographed after one week, it only contained tetramers. The material forced to form higher mers by incubation at low ionic strength could, however, be recovered in the original ratio upon dialysis back to physiological ionic strength. These results imply that the formation of higher mers is facilitated at low ionic strength, both for native and recombinant protein. There is a simple equilibrium between the different mers only for a part of the protein, but for another fraction of the protein the higher mers are irreversibly in that state. The formation of higher mers does not seem to affect the activity to any large extent, however, as both the higher mer and the tetramer fractions showed comparable specific activities. Compared with the native protein, the amount of aggregated EC-SOD is higher. Native EC-SOD prepared from umbilical cord consisted of 94% of the tetrameric form and only 6% of aggregates when run on SEC (Fig. 2c). The figures for the molecular masses obtained by SEC differ somewhat from values published earlier. The shape of the chromatogram, however, is the same, and is independent of the type of column used (although the resolution is less on gels of older type, such as Sephacryl S-300). The molecular mass of the recombinant protein recently obtained on Sephacryl S-300 was 155 000 [13].

Reversed-phase chromatography produced two peaks, both containing EC-SOD according to immunoassays. The smaller was enriched in aggregated EC-SOD, whereas the larger was enriched in free tetrameric EC-SOD when analysed by SEC. Probably the two peaks represented aggregated and free EC-SOD, but at the high concentration of acetonitrile, the equilibrium between aggregated and free EC-SOD was effected.

The many protein subunits migrating at *ca.* 30 000 on SDS-PAGE and protein blotting might be a result of heterogeneous glycosylation. Deglycosylated (N-glycanase treated) recombinant EC-SOD, and a mutant lacking glycosylation, show only one band at *ca.* 28 000 [14]. In the peptide map of the protein, the fragment carrying the glycosylation site (T9) was found in low concentration at additional positions, indicating heterogeneity. This theory is also supported by the finding that some EC-SOD is not bound upon lectin chromatography. Also, when T9 is run on lectin chromatography, a heterogeneity in the

bound material can be seen upon analysis by reversed-phase HPLC [10]. Native EC-SOD is also heterogenous in glycosylation and shows two protein bands at *ca.* 30 000. Like recombinant EC-SOD, the native protein also gives protein bands at 60 000–65 000, possibly corresponding to a dimer of the protein subunit (not shown). To confirm the identity of these bands, they were transferred to PVDF membranes and sequenced after SDS-PAGE. The N-terminus was as expected for EC-SOD, and no N-terminal heterogeneity could be seen.

The genetic stability of a protein can be analysed by peptide mapping. Mutations in the DNA sequence, and hence changes in the amino acid composition, usually result in changes in the mobility of peptides. Trypsin is often the best choice among the proteases because of its specificity and its ability to cleave proteins into peptides of suitable sizes. However, for EC-SOD the choice of protease was not obvious. The high density of cleavage sites for trypsin at the C-terminus resulted in difficulties in separating these fragments. On the other hand the high resistance to proteolysis led to problems with specificity with *Staphylococcus V8* protease. Despite the problems with the C-terminus, trypsin was the best choice and led to identification of all fragments except the fragments in the last 18 amino acids. For larger proteins, it is often a problem to cover the complete sequence using one protease. Certain parts of the protein might be very resistant to cleavage, while at the same time other unspecific sites can be cleaved if the amount of protease, the temperature or the cleavage time is increased. In those cases, an extra cleavage method to elucidate structural homogeneity is often a good choice. In the case of EC-SOD, chemical cleavage with hydroxylamine was the best complement to trypsin cleavage. This created only two fragments, of which the C-terminal was small and easily separated from the rest of the protein. This fragment (H2), consisting of 42 amino acids and with a calculated molecular mass of 4640, covered well the part of EC-SOD that was not identified by trypsin cleavage, and was small enough to give information on the amino acid composition.

By this combination of enzymic cleavage with trypsin and chemical cleavage with hydroxylamine, the complete sequence could therefore be verified. No genetic change or post-translational modification, except the N-glycosylation of the protein, could be detected.

As an additional test for C-terminal homogenity, the protein was sequenced from its C-terminal using carboxypeptidases. The protein appears to be sensitive to cleavage at the C-terminal part, especially at the cluster of lysine and arginine residues when other less specific carboxypeptidases are used. The specificity was increased when carboxypeptidase A was used, and the two terminal alanines were quickly released. When the pH was increased, also the lysine at position 3 from the C-terminus was slowly released. At equilibrium, the amount of released alanine was approximately twice that of lysine, thus confirming the C-terminal sequence expected from the gene (Cys–Lys–Ala Ala).

The results obtained on the characterization of recombinant human EC-SOD show that the properties of the recombinant protein appear to be very similar to those of the native protein. The only difference that could be explored in this work was that recombinant EC-SOD is more easily aggregated. As the primary structure of the protein shows no deviation from what is expected from the cDNA, the aggregation may be a consequence of the fact that recombinant EC-SOD is expressed at a much higher concentration than native EC-SOD. Another reason could be differences in glycosylation and different glycan interactions. As the native enzyme has not been as thoroughly studied, some results cannot be compared with the native protein. The enzymic and physiological properties have in parallel been shown to be similar, with respect to both activity [9] and the pharmacokinetics [15].

ACKNOWLEDGEMENTS

Thanks are due to Mr. Jan-Olov Andersson, Mrs. Helen Fält and Mrs. Else-Britt Lundström for their excellent technical assistance, to Prof. Bo Samuelsson for help with mass spectrometry

and to Drs. Anders Bergman, Lennart Hansson, Gunnar Skogman and Lena Tibell for many stimulating discussions during this work.

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