Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7634–7638, December 1982
Biochemistry

Human copper-containing superoxide dismutase of high molecular weight

(lung/superoxide radical/extracellular fluids)

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Communicated by Irwin Fridovich, August 26, 1982

ABSTRACT **A superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1), distinct from previously known superoxide dismutases, has been isolated from human lung tissue. It is probably of the same nature as a previously demonstrated high molecular weight superoxide dismutating factor in human extracellular fluids. The enzyme has a molecular weight around 135,000 and is composed of four equal noncovalently bound subunits. Each molecule appears to have four copper atoms. No iron or manganese was found in the enzyme. Cyanide inhibits the enzyme efficiently. The enzyme brings about a first-order dismutation of the superoxide radical, the rate constant for the catalyzed reaction being about 1×10^9 M^{-1} s^{-1} per copper atom. The enzyme has hydrophobic properties. Affinity for various lectins indicates the presence of carbohydrate. Upon chromatography on heparin-Sepharose it is divided into three fractions, one with no, one with weak, and one with strong affinity for heparin.**

The first superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) to be described was the CuZn enzyme (1) long known as the protein erythrocuprein or hemocuprein (2). This type of SOD has subsequently been demonstrated in virtually all eukaryotic organisms (3). In prokaryotes, SODs containing Mn (4) or Fe (5) have been described. The Mn type of SOD has also been found in the mitochondrial matrix of eukaryotes (6). The Fe- and Mn-containing SODs show sequence homologies with each other but not with the CuZn SODs (7).

The activities of CuZn SOD and Mn SOD in extracts of eukaryotic origin in general are distinguished from each other by using cyanide; CuZn SOD is very sensitive whereas Mn SOD is almost insensitive. Recently it was demonstrated that CuZn SOD as determined with a radioimmunoassay could account for only a minor part of the cyanide-sensitive SOD activity of human extracellular fluids (8, 9). The rest appeared to be the result of a factor with a higher molecular weight than CuZn SOD (around 130,000) as determined with gel chromatography (9). It was not inhibited by antibodies toward human CuZn SOD. The factor could be demonstrated in homogenates of human lung and some other tissues.

This paper reports the purification and partial characterization of this novel SOD from human lung. Because it was first discovered in extracellular fluids, it is tentatively named EC-SOD.

MATERIALS AND METHODS

Chemicals. Ultrogel ACA-34 was obtained from LKB (Stockholm, Sweden). Sephacryl S-300, DEAE-Sephacel, concanavalin A (Con A)-Sepharose, phenyl-Sepharose CL-4B, wheat germ lectin-Sepharose 6 MB, blue-Sepharose CL-6B, and heparin-Sepharose CL-6B were products of Pharmacia (Uppsala, Sweden). KO_2 was an Alfa Europe product.

Assays. Discontinuous polyacrylamide gel electrophoresis was performed in 7.5% gels at pH 8.9. The electrophoresis in the presence of dodecyl sulfate, and the molecular weight determinations were performed in 7% gels with human serum albumin, ovalbumin, chymotrypsinogen, and RNase as references. SOD was determined with the direct KO_2 assay, essentially as described (10) but all determinations were performed at pH 9.50. One unit is defined as the activity that brings about a decay in $O_2^{\cdot -}$ concentration at a rate of 0.1 s^{-1} in 3 ml of buffer. For purposes of comparison, the enzymic activity was also analyzed by the pyrogallol autoxidation assay (11) and the xanthine oxidase-cytochrome *c* procedure (1). Staining for SOD in gels was performed as described by Beauchamp and Fridovich (12).

Metal analysis was performed by atomic absorption spectrometry in a graphite furnace. Amino acid analysis was performed in a LKB 4400 amino acid analyzer. It was calibrated with norleucine and the samples (≈7 μg) were hydrolyzed for 24 hr in constant boiling HCl under reduced pressure at 110°C. No compensations were made for losses during hydrolysis.

Molecular weight estimation by sedimentation equilibrium analysis according to Chervenka (13) was performed in a MSE Centriscan ultracentrifuge run at 10,000 rpm for 34 hr at 4°C. The samples (about 80 μg/ml) were dissolved in 25 mM Tris·HCl at pH 7.5. The partial specific volume was calculated to be 0.715 from the amino acid composition. Molecular weight estimation by gel chromatography was performed by calibrating the ACA 34 column used in separation step 6 (see below). Bovine catalase, rabbit muscle aldolase, and human serum albumin were used as reference standards.

Determination of EC-SOD Content of Samples. The separation of EC-SOD from other SODs is based on the fact that, unlike CuZn SOD and Mn SOD, EC-SOD binds to concanavalin A. The samples (<2 ml) were applied to a Con A-Sepharose column (2.5 × 1 cm) equilibrated with 10 mM Na phosphate, pH 6.5/120 mM NaCl. After 5 min, 3 ml of buffer was added. The effluent from these two additions was collected and it contained all CuZn SOD and Mn SOD. After that the column was washed with 20 ml of the phosphate buffer. The EC-SOD was then eluted with 5 ml of 150 mM α-methyl D-mannoside/50 mM Na phosphate, pH 6.5. The column was regenerated with 5 ml of 0.5 M α-methyl D-mannoside followed by 20 ml phosphate buffer.

Purification Procedure. The results of the purification steps are summarized in Table 1. For steps 1 and 2, the results of 10 separate batches are summarized; step 3 represents 7 batches, step 4 represents 6 batches, and step 5 represents 2 batches. Thereafter all material was handled as a single batch.

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Abbreviations: SOD, superoxide dismutase; Con A, concanavalin A.

Table 1. Purification of human EC-SOD

Step	Total activity, units	Total protein, mg	Specific activity, units/mg protein	Recovery, %
1. Extract human lung (5,800 g)	2,900,000	480,000	6	100
2. DEAE-Sephacel pH 5.5	1,830,000	10,900	169	63
3. DEAE-Sephacel pH 8.4	1,270,000	2,800	453	44
4. Phenyl-Sepharose CL-4B	573,000	160	3,560	20
5. Con A-Sepharose	320,000	33	9,650	11
6. ACA-34 gel chromatography	220,000	9.3	21,600	7.6
7. Wheat germ lectin-Sepharose 6 MB	187,000	5.3	35,200	6.4
8. Blue Sepharose CL-6B	120,000	4.1	29,300	4.1
9. Heparin-Sepharose CL-6B sum	108,000	3.27	33,100	3.7
Peak A	31,400	1.87	16,600	
Peak B	36,600	0.94	38,900	
Peak C	27,400	0.46	59,500	
10. Sephacryl S-300 peak A	20,100	0.284	70,800	
11. Final yield after dialysis and concentration:				
Peak A	12,600	0.197	64,200	
		(0.093)	(135,400)	
Peak B	16,900	0.452	37,400	1.3
Peak C	8,600	0.153	56,400	
		(0.094)	(91,100)	

Protein content was estimated from the absorbance at 280 nm, assuming that $A = 1$ corresponds to 1 mg of protein per ml. The values in parentheses are based on protein determination from the amino acid content.

1. Lung extract. Human lungs were obtained, within 24 hr after death, at autopsy from nine patients without apparent lung disease. The lungs were cut into pieces and excess blood was washed away in 0.15 M NaCl. The pieces were homogenized with a Waring blender in 5 vol of ice-cold 50 mM Na acetate at pH 5.50. The homogenate was then sonicated, allowed to extract for 30 min at 4°C, and finally centrifuged (6,000 × *g*) for 20 min.

2. Batch adsorption on DEAE-Sephacel at pH 5.5. The supernatant was batch-adsorbed on DEAE-Sephacel (1 vol per 25 vol of homogenate) equilibrated with 50 mM Na acetate at pH 5.50. The ion exchanger was then washed with the buffer, packed in a column, and eluted with a gradient of 0–200 mM NaCl in the acetate buffer. The gradient volume was 10 times the column volume.

3. Batch adsorption on DEAE-Sephacel at pH 8.4. The active fractions from the previous step were pooled, diluted with 1.5 vol of distilled water, and titrated to pH 8.4 with 1 M NaOH. The pool was again batch adsorbed to DEAE-Sephacel equilibrated with 175 mM Tris·HCl at pH 8.4 (≈1 vol of ion exchanger per 10 vol of pool). The DEAE-Sephacel was subsequently washed with the buffer, packed in a column and, eluted with 10 column volumes of a 0–200 mM NaCl gradient in the Tris buffer.

4. Phenyl-Sepharose. The pooled fractions from the previous step were concentrated and dialyzed against 150 mM Na phosphate at pH 6.5. The sample was applied to a column (about 1 ml of gel per 15 mg of protein in the sample) with phenyl-Sepharose equilibrated against the same buffer. The activity was eluted with 20 column volumes of a 0–0.5 M KBr gradient in 50 mM Na phosphate at pH 6.5

5. Con A-Sepharose. Active fractions from phenyl-Sepharose were pooled, concentrated, and dialyzed against 0.15 M Na phosphate at pH 6.5. The sample was applied to a column of Con A-Sepharose (1 mg of gel per 2 mg of protein in the sample), equilibrated against the phosphate buffer, and then pulse-eluted with 50 mM α-methyl D-mannoside in the phosphate buffer.

6. Gel chromatography on Ultrogel ACA 34. Active fractions from the Con A-Sepharose were pooled, concentrated, applied to an ACA 34 column (2.5 × 83 cm), and eluted in 50 mM Na phosphate at pH 6.5. The elution rate was 5 $ml \cdot h^{-1} \cdot cm^{-2}$.

7. Wheat germ lectin-Sepharose 6 MB. Active fractions from ACA 34 were pooled, concentrated, and applied to a wheat germ lectin-Sepharose column (10 ml) equilibrated with 0.15 M Na phosphate at pH 6.5. The enzyme was pulse-eluted with 0.45 M *N*-acetyl-D-glucosamine in the phosphate buffer.

8. Blue Sepharose CL-6B. Active fractions from the above step were pooled, concentrated, dialyzed against 0.15 M Na phosphate at pH 6.5, and applied to a blue Sepharose (Cibacron Blue F3G-A) column (bed volume, 6 ml) equilibrated with the phosphate buffer. After the column was washed with buffer, a pulse of 10 mM NAD and 10 mM NADP in the buffer was introduced. After the pyridine nucleotides were washed out with buffer, the enzyme was pulse-eluted with 0.9 M KBr/50 mM Na phosphate, pH 6.5

9. Heparin-Sepharose CL-6B. The active fractions from the blue Sepharose column were pooled, dialyzed against 25 mM Na phosphate at pH 6.5, and applied to a heparin-Sepharose column (bed volume, 10 ml) equilibrated with the same buffer. The column was then eluted with 140 ml of a 0–1 M NaCl gradient in the phosphate buffer. The activity eluted in three distinct peaks: A did not bind to the heparin, B desorbed early in the gradient, and C desorbed late (Fig. 1).

10. Sephacryl S-300. Peak A contained UV-absorbing material which probably had leaked from the heparin-Sepharose column and was further purified on a Sephacryl S-300 column (1.6 × 90 cm). The sample was eluted in 25 mM Tris·HCl at pH 7.5.

11. Concentration, dialysis, and protein determination. Fractions A, B, and C were dialyzed against 25 mM Tris·HCl at pH 7.5 and then concentrated to about 1 ml on Amicon UM-10 ultrafiltration membranes. These concentrated fractions were used for all investigations reported here. The protein contents of the fractions were calculated from their amino acid contents. Because neither carbohydrate nor tryptophan was measured the estimates of protein content will be somewhat low.

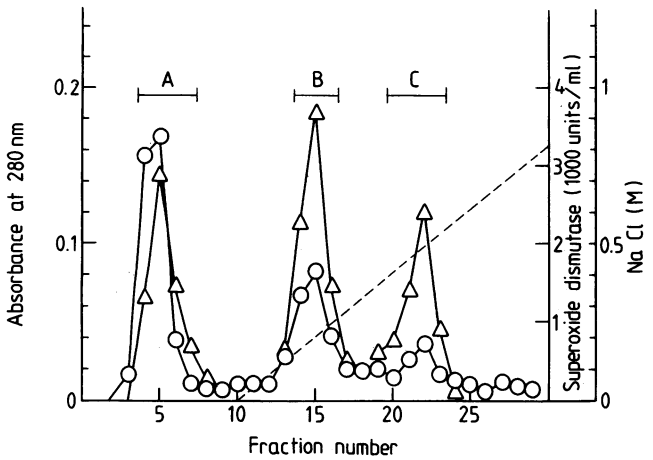

FIG. 1. Chromatography on heparin-Sepharose (step 9 of Table 1). At fraction 10, a gradient in NaCl (broken line) was applied. The active fractions were pooled as shown. ○, Absorbance at 280 nm; △, SOD activity.

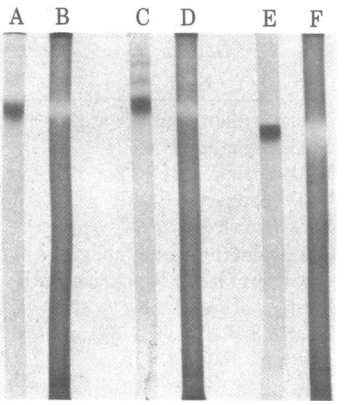

FIG. 2. Electrophoresis of EC-SOD in polyacrylamide gel. Lanes: A, fraction A (10 μg) stained for protein (Coomassie brilliant blue); B, fraction A (2 μg) stained for SOD activity; C, fraction C (10 μg) stained for protein; D, fraction C (2 μg) stained for SOD activity; E, human CuZn SOD (10 μg) stained for protein; F, human CuZn SOD (2 μg) stained for SOD activity.

RESULTS

Enzyme Purification. The enzymic activities presented in Table 1 represent only EC-SOD. The total SOD activity in the lung homogenates was about 40×10^6 units, and thus EC-SOD comprised about 7% of the total activity. CuZn SOD and Mn SOD did not bind to the DEAE-Sephacel in step 2 and were therefore eliminated early. Affinity for phenyl-Sepharose under the conditions used indicates a hydrophobic character of the enzyme. The binding to Con A (step 4) indicates the presence of neutral carbohydrates in the enzyme, and the affinity for wheat germ lectin (step 7) points to the presence of *N*-acetyl-D-glucosaminyl residues. The binding to blue Sepharose (step 8) was probably of hydrophobic nature because desorption was possible only with a strongly chaotropic reagent. The enzyme could not be desorbed by NAD^+ and $NADP^+$, which indicates that the binding to blue Sepharose is hardly because of specific affinity for these cofactors. After steps 7, 8, and 9 the amount of pooled protein, as determined from the absorbance at 280 nm, was somewhat uncertain because the gels leaked UV-absorbing material. In the heparin-Sepharose step the enzyme was divided into three nearly equally large fractions (Fig. 1) with different heparin affinities.

Analysis by Electrophoresis. The results of electrophoresis in polyacrylamide gel at pH 8.9 are shown in Fig. 2. Fraction A revealed one strong homogeneous band and one faint band of lower mobility. When the gels were stained for SOD, the activity coincided with the strong protein band. The faint band cannot be visualized with certainty in the activity staining and may represent an impurity. Fraction C produced one strong protein band and three faint bands with lower mobility. In this case all four bands can be visualized in the activity staining and no impurity can be demonstrated. For comparison, the result with human CuZn SOD is also shown; it has a higher mobility than EC-SOD in this system, due to steric hindrance of the larger EC-SOD in the gel. Upon electrophoresis at alkaline pH in agarose gel, which has a more open structure, EC-SOD has a higher mobility than CuZn SOD (not shown).

The results of electrophoresis in the presence of Na dodecyl sulfate are shown in Fig. 3. The samples shown were boiled in 1% Na dodecyl sulfate and 1% dithioerythritol for 5 min. However, even in the absence of dithioerythritol, most material was at the position seen in the figure, but there was also a band with a lower mobility. The position in the gel indicates molecular weights of 29,000 for peak A, 30,000 for peak B, and 29,000 for peak C. The results cannot be judged to be significantly different. When human CuZn SOD was run in parallel with the EC-SOD samples (not shown), no protein could be seen at the position of EC-SOD, the EC-SOD gels did not show any trace of protein at the position of CuZn SOD. Fraction B was contaminated with several proteins of lower mobility and was obtained in approximately 60% purity. Fractions A and C, on the other hand, were almost completely pure. Two scanty extra bands of low mobility could be seen. Together they comprised approximately 3% of the applied protein, as judged by comparison with gels calibrated with various amounts of protein.

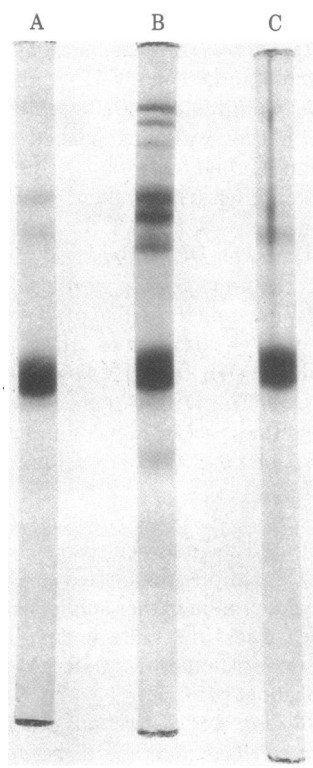

FIG. 3. Electrophoresis of EC-SOD in polyacrylamide gel in the presence of Na dodecyl sulfate. Lanes: A, fraction A, 15 μg; B, fraction B, 15 μg; C, fraction C, 15 μg. The gels were stained for protein with Coomassie brilliant blue. The streak in lane C is caused by a defect in the gel.

The lower band appears to have the same mobility as the not fully dissociated band in the absence of dithioerythritol and does not necessarily represent an impurity. The small bands will not significantly affect the characterization of the enzyme.

Metal Analysis. The results of metal analysis are presented in Table 2. Fractions A and C contained copper, corresponding to a protein molecular weight of 24,700 and 26,300 per copper atom respectively. The fractions contained slightly more zinc than copper. The concentration of EC-SOD in the fractions was very small and, given the well-known problems with zinc contamination, these data should be regarded as preliminary. No manganese or iron could be detected.

Amino Acid Analysis. The amino acid composition of fractions A and C are shown in Table 3. For comparison, the amino acid compositions of human CuZn SOD and human Mn SOD are also shown. There are relatively small differences between fraction A and fraction C. However, the amino acid compositions of the EC-SOD fractions are clearly different from those of human CuZn SOD and human Mn SOD.

Molecular Weight Determination by Gel Chromatography. The EC-SOD eluted from the ACA 34 (step 6) at a position corresponding to a molecular weight of 140,000. When human serum was chromatographed, the EC-SOD peak coincided exactly with the blue peak of ceruloplasmin which has a molecular weight of 134,000 (16).

Ultracentrifugation. The sedimentation equilibrium analysis indicated a molecular weight of 135,000 for fraction C. The material appeared to be homogeneous except for indications for some high molecular weight material. This may be related to the faint bands of enzymic activity with low mobility seen in the electrophoresis experiments (Fig. 2) and probably represents aggregated enzyme. No definite molecular weight could be obtained for fraction A because it appeared to increase with increasing protein concentration in the cell. The molecular weight appeared to be distributed between 40,000 and 100,000, indicating a dynamic equilibrium between various polymerization states of the subunits.

Enzyme Activity. The specific activity, in terms of protein, for the EC-SOD fractions in the various assays was about as high as that of human CuZn SOD (Table 4). The reactivity of copper with $O_2\cdot^-$ was larger in the two fractions than in CuZn SOD. The rate constant for the reaction between the complete enzyme molecule (assuming a molecular weight of 135,000) and $O_2\cdot^-$ is larger than that of CuZn SOD. The ratio between the specific activity of the enzyme in the direct KO_2 assay at pH 9.50 and the xanthine oxidase/cytochrome *c* assay at pH 7.80 is similar to the ratio for human CuZn SOD. The activity of the latter enzyme has a flat pH dependence in this pH range with a rate constant for the catalyzed reaction of about $1.25 \times 10^9\ M^{-1}\cdot s^{-1}$. The similar assay ratios for the two enzymes indicates a similar flat pH dependence for EC-SOD in that pH range. Like human CuZn SOD, EC-SOD was found to catalyze a first-order dismutation of the superoxide radical up to the highest $O_2\cdot^-$ concentration (about 30 μM) obtainable with the KO_2 assay, and no saturation behavior was observed. EC-SOD was at least as sensitive to cyanide as CuZn SOD; it was inhibited to >99.8% in 3 mM cyanide in the KO_2 assay.

Table 2. Metal content of EC-SOD

	Metal content, μM				Protein,
	Cu	Zn	Mn	Fe	mg/liter
Fraction A	3.1	3.4	<0.04	<0.14	78
Fraction C	3.4	4.2	<0.04	<0.14	91
Dialysis buffer	<0.08	0.3	<0.04	<0.14	—

The detection limits under the conditions used were: for Cu, 0.08 μM; Zn, 0.05 μM; Mn, 0.04 μM; and Fe, 0.14 μM.

Table 3. Amino composition of EC-SOD

	Residues per molecule			
	EC-SOD		Human	Human
Amino acid	Fraction A	Fraction C	CuZn SOD (14)	Mn SOD (15)
Aspartic acid	123.4	127.0	37.0	90.0
Threonine	44.4	45.5	16.2	21.4
Serine	88.1	85.5	19.0	25.8
Glutamic acid	137.7	154.5	27.9	88.5
Proline	87.8	73.9	11.7	40.0
Glycine	147.9	135.9	52.0	75.7
Alanine	154.6	160.6	21.1	67.2
Cysteine	35.1	32.6	5.8	10.0
Valine	109.8	104.5	28.1	45.8
Methionine	9.0	10.9	0	10.0
Isoleucine	25.2	23.3	16.0	34.3
Leucine	76.7	73.1	19.3	64.3
Tyrosine	17.3	16.3	0	27.2
Phenylalanine	43.4	40.4	8.4	20.1
Lysine	24.7	20.9	24.8	60.0
Arginine	98.2	105	9.0	20.5

A molecular weight of 135,000 was assumed for EC-SOD. The amount of carbohydrate was not determined and tryptophan was not measured, so the values presented are somewhat too large.

Behavior of EC-SOD During Tsuchihashi Treatment. The initial step in CuZn SOD purification is in general a Tsuchihashi precipitation followed by salting into two phases with K_2HPO_4 and a final precipitation of the enzyme in the upper phase with acetone. To test for similarities between EC-SOD and CuZn SOD, EC-SOD purified to step 4 was subjected to such treatment as described by McCord and Fridovich (1). In parallel, a human hemolysate was run. The EC-SOD was recovered (about 25% recovery) in the acetone precipitate. All recovered activity retained its ability to bind to Con A. No activity from the hemolysate bound to Con A.

Table 4. Specific enzyme activity of EC-SOD

	EC-SOD		
	Fraction A	Fraction C	Human CuZn SOD
Xanthine oxidase/cytochrome *c* assay (1), units/mg protein	3,190	2,970	3,200*
Pyrogallol assay (11), units/mg protein	1,520	1,290	1,200*
KO_2 assay (10), units/mg protein	135,400	91,100	123,000†
Rate constant for reaction with $O_2\cdot^-$	1.0×10^9	0.72×10^9	0.62×10^{9}†

The pyrogallol assay was performed in 50 mM Tris·HCl buffer at pH 8.2 instead of in 50 mM Tris cacodylic acid buffer at pH 8.2 (11), which makes the assay about 30% less sensitive. The rate constant for the reaction with $O_2\cdot^-$ was determined with the KO_2 assay at pH 9.5 and is shown as $M^{-1}\cdot s^{-1}$ based on copper content.

* From ref. 14.

† From ref. 10.

DISCUSSION

The results demonstrate the existence of a SOD distinct from previously described SODs. It has a molecular weight of about 135,000 and is composed of four equal noncovalently bound subunits. The minimal protein molecular weight per copper atom based on the amino acid analyses is a bit lower than the estimated subunit molecular weight. However, because the enzyme appears to contain an unknown amount of carbohydrate and not all amino acids were analyzed, the most reasonable estimate of copper content appears to be one per subunit and four per enzyme molecule. It also may contain zinc in the same amount as copper. No iron or manganese could be demonstrated, and the enzymic activity is probably given by the copper atoms. The enzymic activity is higher than that of human CuZn SOD. It catalyzes a first-order dismutation of the superoxide anion radical, and the activity is very sensitive to cyanide. The behavior of EC-SOD during Tsuchihashi treatment indicates similarities in physical properties between EC-SOD and CuZn SOD. Apart from the presence of copper, the new enzyme is clearly different from CuZn SOD in molecular and amino acid compositions and behavior during protein separation and electrophoresis. The commonly employed practice of designating cyanide-sensitive SOD activity in homogenates from eukaryotic organisms as erythrocuprein probably should be abandoned.

The enzyme now isolated from human lung tissue is probably of the same nature as the high molecular weight SOD activity previously found in human extracellular fluids (8, 9). That activity accounted for most of the SOD activity in the fluids. The molecular weights appear to be equal, and the activity from the extracellular fluids appears to behave in a manner similar to that of the activity from lung extracts in all tested protein separation steps (steps 2, 3, 4, 5, 6, and 9) including the division into three fractions in the heparin-Sepharose step (data not presented). A similar high molecular weight SOD activity was found in serum from all investigated mammalian species (horse, cow, pig, dog, cat, rabbit, rat, and mouse). The interspecies variability was high (9).

The reason for the division into three separate fractions in the heparin-Sepharose step is obscure. The phenomenon may indicate the existence of isoenzymes, but it also may be caused by partial degradation or denaturation of the enzyme during the unusually long isolation sequence. Because lungs were taken from many persons, the existence of genetic variants is another possibility. There were relatively small differences in amino acid composition between fraction A and fraction C and the behaviors upon electrophoresis were not significantly different. The aberrant behavior of fraction A in the ultracentrifugation experiments may be due to secondary changes.

The main localization of EC-SOD is obscure. It is the principal SOD of the extracellular fluids (9). In lung, which is particularly abundant in EC-SOD compared to most other human tissues, it constitutes only 7% of the total SOD activity. Still, 1 g of lung contains several hundred units of EC-SOD whereas the content of human plasma is only 30 units/ml. The affinity of EC-SOD for binding to Con A and wheat germ lectin indicates that it is a glycoprotein, and in that respect it is similar to the majority of the plasma proteins. The enzyme has hydrophobic properties which may indicate an affinity for cell membranes. Affinity for heparin often indicates an affinity for heparan sulfate (17), which is found on cell surfaces and particularly a vessel endothelium. One might speculate that EC-SOD is intended for extracellular function and is partitioned between cell surfaces and the extracellular fluids.

The skillful technical assistance of Mr. Thord Johansson, Ms. Kjerstin Lindmark, and Ms. Agneta Öberg is gratefully acknowledged. I also wish to thank Dr. L. G. Öfverstedt (Uppsala) for performing the ultracentrifugation analysis and Dr. E. Lundberg (Umeå) for atomic absorption spectrometry. The study was supported by Statens Medicinska Forskningsråd Grant 04761 and The Lions Research Foundation, Department of Oncology, Umeå University Hospital.

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