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Properties of extracellular superoxide dismutase from human lung

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A further characterization of human extracellular superoxide dismutase is reported. The study was especially aimed at the interaction with substances known to interfere with CuZn superoxide dismutase and other superoxide dismutases. Extracellular superoxide dismutase is efficiently inhibited by cyanide and is about 3 times more sensitive than is human CuZn superoxide dismutase. The sensitivity to azide is much lower, but still about 3 times higher than that of human CuZn superoxide dismutase. Extracellular superoxide dismutase is about as rapidly inactivated by hydrogen peroxide as is CuZn superoxide dismutase. The sensitivity to diethyldithiocarbamate is very high and more than an order of magnitude larger than that of CuZn superoxide dismutase. Sodium dodecyl sulphate, under conditions suggested as being suitable for distinguishing between the insensitive CuZn superoxide dismutase and the sensitive Mn superoxide dismutase, efficiently inactivated extracellular superoxide dismutase. No antigenic similarities between extracellular superoxide dismutase and CuZn superoxide dismutase could be demonstrated. Anti-(extracellular superoxide dismutase) did not bind CuZn superoxide dismutase, and anti-(CuZn superoxide dismutase) did not bind extracellular superoxide dismutase.

The first superoxide dismutase (SOD) (EC 1.15.1.1) to be demonstrated in mammalian tissues was CuZn-SOD (McCord & Fridovich, 1969). The protein was previously known as haemocuprein or erythrocuprein. It has M_r about 33000 and is composed of two equal non-covalently bound subunits. It possesses 2Cu and 2Zn atoms/molecule. Later the Mn-SOD type, first discovered in prokaryotes (Keele *et al.*, 1970), was found in chicken (Weisiger & Fridovich, 1973) and also in mammalian (McCord *et al.*, 1977; Marklund, 1978) tissues. Mammalian Mn-SODs have M_r just above 80000 and are composed of four equal non-covalently bound subunits. They contain 2–4Mn atoms/molecule. The CuZn-SODs and the Mn-SODs do not show similarities in protein structure.

More recently a third mammalian SOD, EC-SOD, was isolated from human lungs and partially characterized (Marklund, 1982). EC-SOD is a slightly hydrophobic glycoprotein with affinity for heparin and has M_r about 135000. It is composed of four equal non-covalently bound subunits and has a high reactivity with the superoxide radical. It appears to possess 4 copper atoms and possibly also 4 zinc atoms/molecule. In spite of this similarity in prosthetic metals, no similarities with CuZn-SOD were demonstrated in amino acid composition (Marklund, 1982), antigenic properties and (probably) chromosomal localization (Marklund *et al.*, 1982).

Whereas CuZn-SOD and Mn-SOD are primarily intracellular enzymes, EC-SOD appears to be the dominant SOD in the extracellular space. A large interspecies variability in plasma concentration was found among the mammals investigated (Marklund *et al.*, 1982). However, EC-SOD can also be demonstrated in variable amounts in tissues and in most cases in higher concentration than in plasma (S. L. Marklund, unpublished work).

Many substances have been reported to interfere with the SODs, and several of them have been employed for the distinction between the previously known isoenzymes. The present further characterization of EC-SOD is especially aimed at the interaction of the enzyme with such substances.

Abbreviations used: SOD, superoxide dismutase; EC-SOD, extracellular superoxide dismutase.

Materials and methods

Materials

Protein A–Sepharose and CNBr-activated Sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Chemicals were obtained from standard commercial sources.

Human EC-SOD

Human EC-SOD was purified as described previously (Marklund, 1982). The final step is a chromatography on heparin–Sepharose with an NaCl gradient. The EC-SOD that does not bind to heparin is called fraction A. Fraction B is eluted early in the gradient, and fraction C is eluted late.

Human CuZn-SOD

Human CuZn-SOD was isolated as described previously (Marklund *et al.*, 1976).

Human Mn-SOD

Human Mn-SOD was isolated from human liver as described by McCord *et al.* (1977).

SOD activity

SOD was determined in terms of its ability to catalyse the disproportionation of $O_2^{\cdot -}$ in alkaline aqueous solution. The disproportionation was directly studied in a spectrophotometer, essentially as described previously (Marklund, 1976) except that the assay was performed at pH 9.50. One unit in the assay is defined as the activity that brings about a decay of $O_2^{\cdot -}$ concentration at a rate of $0.1\,s^{-1}$ in 3 ml of buffer. It corresponds to 8.8 ng of human EC-SOD and 8.3 ng of human CuZn-SOD.

Inhibition by cyanide and azide

The inhibition was studied with various amounts of the inhibitors present in the buffer during SOD assay.

Inactivation in H_2O_2

The SODs (about 150 units/ml) were exposed to 1 mM-H_2O_2 in 25 mM-2-amino-2-methylpropan-1-ol hydrochloride with 0.1 mM-diethylenetriaminepenta-acetic acid (pH 9.50) at 25°C. After measured time intervals portions were added to catalase (1 μM final concentration) and then analysed for SOD activity.

Inactivation by diethyldithiocarbamate

The SODs (about 300 units/ml) were added to diethyldithiocarbamate in 100 mM-potassium phosphate buffer, pH 7.4, at 37°C. After measured time intervals portions were analysed for SOD activity.

Inactivation in sodium dodecyl sulphate

The SODs were exposed to 2% (w/v) sodium dodecyl sulphate in 50 mM-potassium phosphate buffer, pH 7.4, at 37°C for 1 h as suggested by Geller & Winge (1983). After cooling, the sodium dodecyl sulphate was precipitated with 0.27 M-KCl and the remaining enzymic activity determined.

Immunoprecipitation with antibodies bound to Sepharose 4B

Rabbits were immunized with subcutaneous injections of 100 μg of human CuZn-SOD or 30 μg of EC-SOD fraction A or 30 μg of EC-SOD fraction C. The first injection of each enzyme was given in complete Freund's adjuvant, and the subsequent booster injections in incomplete adjuvant. The immunoglobulin G fractions of the rabbit antisera were isolated by the use of protein A–Sepharose. The immunoglobulin G fractions were then coupled to CNBr-activated Sepharose 4B in accordance with the manufacturer's suggestions. About 5 mg of immunoglobulin G was coupled per ml of gel. To test the binding capacity of the antibodies, 100 μl of 50% gel suspensions were added to the SOD isoenzymes in 1 ml of 0.1 M-sodium bicarbonate buffer, pH 8.3, containing 0.5 M-NaCl. After incubation under shaking for 12 h at 4°C, the solutions were centrifuged and remaining enzyme was determined in the supernatants. To determine the maximal binding capacity, about 700 units of each SOD/ml were incubated with their corresponding antibody–Sepharose gels. When the binding of EC-SOD to anti-CuZn-SOD antibody and of CuZn-SOD to anti-EC-SOD antibodies was to be investigated, the enzyme concentrations were 10 units/ml.

Results and discussion

Inhibition by cyanide

The EC-SOD fractions were very sensitive to cyanide, and 50% inhibition was recorded at 3 μM-cyanide. Human CuZn-SOD was less sensitive, and was inhibited to 50% by 10 μM-cyanide under the same conditions. At 3 mM-cyanide over 99.96% inhibition of EC-SOD was recorded. This means that EC-SOD will be co-determined with CuZn-SOD when cyanide is used for the distinction between CuZn-SOD and Mn-SOD in crude homogenates.

In indirect assays for SOD small amounts (10–20 μM) of cyanide are often added to inhibit cytochrome oxidase and other interfering haemoproteins in crude homogenates (Crapo *et al.*, 1978). Although these assays are performed at neutral pH (7.4–7.8), where the fraction of cyanide in the form of the free inhibitory anion is very low (Beauchamp & Fridovich, 1973), significant inhi-

bition of the very cyanide-sensitive EC-SOD can be expected.

Inhibition by azide

The human EC-SOD fractions were inhibited to 50% by 6.5mM-azide. They are thus more sensitive than human CuZn-SOD, which was inhibited to 50% by 21mM-azide. The sensitivity of human CuZn-SOD is similar to that reported for bovine CuZn-SOD at a pH close to the present pH (Rigo *et al.*, 1975; Misra & Fridovich, 1978). The sensitivity of EC-SOD appears to be more similar to that of Mn-SOD and lower than that of Fe-SOD (Misra & Fridovich, 1978).

Inactivation by H_2O_2

H_2O_2 (1mM) at pH9.50 brought about a first-order inactivation of the SODs. The EC-SOD fractions were very similar: fractions A, B and C were inactivated with half-times of 6.2, 6.8 and 7.1min respectively. Human CuZn-SOD was a little more resistant and was inactivated with a half-time of 9.3min. The inactivation of CuZn-SOD by H_2O_2 appears to be due to a Fenton-type reaction of H_2O_2 with Cu^+, forming a reactive intermediate that destroys an essential liganding histidine residue (Bray *et al.*, 1974; Hodgson & Fridovich, 1975; Blech & Borders, 1983). The finding of a roughly equally high H_2O_2-sensitivity of EC-SOD indicates similarities between the copper ligands of this enzyme and CuZn-SOD. EC-SOD will be as sensitive as CuZn-SOD, *in vitro* and *in vivo*, in systems where H_2O_2 is produced.

Inactivation by diethyldithiocarbamate

Diethyldithiocarbamate is an efficient inhibitor of CuZn-SOD both *in vitro* and *in vivo* (Heikkila *et al.*, 1976). It acts by liganding and removing the copper ions from the enzyme (Cocco *et al.*, 1981). The EC-SOD fractions A, B and C were found to be very sensitive, and were inactivated in accordance with first-order kinetics with half-times around 12min in 0.1mM-diethyldithiocarbamate (Fig. 1). Under comparable conditions human CuZn-SOD was much more resistant and was inactivated with a half-time of 40min in 0.5mM-diethyldithiocarbamate (Fig. 1). The difference in sensitivity was so large that the inactivations of the enzymes were difficult to study with precision at the same diethyldithiocarbamate concentration. The high sensitivity of EC-SOD does not appear to be caused by very loose binding of the copper ions; EC-SOD activity was not affected by incubation with 10mM-diethylenetriaminepenta-acetic acid under the same conditions for 24h.

The results mean that the EC-SOD should be even more sensitive than is CuZn-SOD to inhibition *in vivo* by diethyldithiocarbamate (Heikkila *et al.*, 1976) and its disulphide disulfiram. Physiological effects of diethyldithiocarbamate and disulfiram ascribed to CuZn-SOD inactivation might hence also be caused by EC-SOD inactivation. The very large difference in sensitivity points to the possibility of induction of a rather selective inactivation of EC-SOD with small doses of the dithiocarbamates.

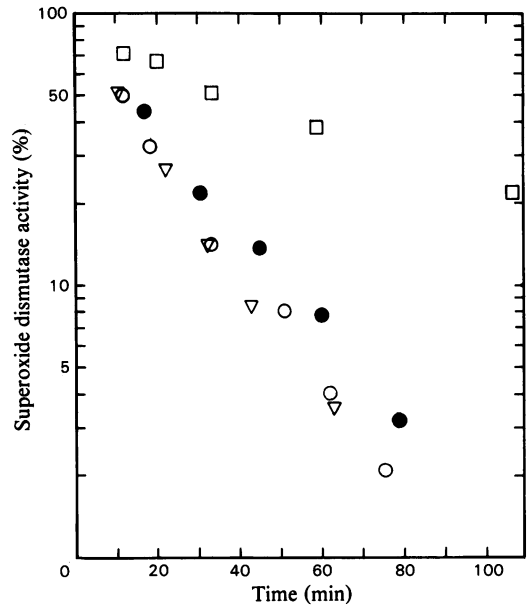

Fig. 1. *Inactivation of SOD enzymes by diethyldithiocarbamate*

Diethyldithiocarbamate was added to the SODs (about 300 units/ml) as described in the Materials and methods section. □, Human CuZn-SOD in 0.5mM-diethyldithiocarbamate; ▽, ● and ○, human EC-SOD, fractions A, B and C respectively, in 0.1mM-diethyldithiocarbamate.

Inactivation in sodium dodecyl sulphate

A new procedure for the distinction between CuZn-SOD and Mn-SOD was described by Geller & Winge (1983). It is based on the resistance and sensitivity of CuZn-SOD and Mn-SOD respectively to inactivation by 2% sodium dodecyl sulphate.

However, under the conditions suggested, human Mn-SOD, pure as well as in a liver homogenate, was in our hands only partially inactivated (55–80%). This indicates that the procedure is not suitable for the assay of human material. The method was developed for rat tissue homogenates, and the discrepancy between the results may be due to the especially low stability of rat Mn-SOD (Salin *et al.*, 1978). EC-SOD, on the other hand, was completely inactivated (>99%) by the sodium

dodecyl sulphate treatment and will be co-determined with Mn-SOD in the suggested procedure.

Immunological comparison between human CuZn-SOD and human EC-SOD

The affinity of anti-(EC-SOD fraction A) and anti-(EC-SOD fraction C) antibodies for CuZn-SOD and of anti-CuZn-SOD antibody for EC-SOD was determined as described in the Materials and methods section. It was found that an amount of anti-CuZn-SOD–Sepharose with a capacity to bind about 500 units of human CuZn-SOD had no detectable affinity for EC-SOD. Under the conditions employed, binding of less than half a unit of EC-SOD would have been detected. Similarly, amounts of anti-(EC-SOD fraction A) and anti-(EC-SOD fraction C) antibodies coupled to Sepharose, with capacities to bind 400 units and 310 units of EC-SOD respectively, showed no appreciable affinity for human CuZn-SOD. There are thus very small or non-existent antigenic similarities between EC-SOD and CuZn-SOD. This is in accord with the fact that the amino acid compositions are clearly different (Marklund, 1982). It appears that, in spite of the similarities in prosthetic metals, the protein parts of EC-SOD and CuZn-SOD are quite different. From what is known at present, there is no justification for grouping EC-SOD and CuZn-SOD together as a separate class of SODs. A final classification must await the availability of sequence data.

Conclusions

EC-SOD and CuZn-SOD are similarly affected by cyanide, H_2O_2 and diethyldithiocarbamate. Procedures employing these substances for presumably specific modification of CuZn-SOD will also modify EC-SOD activity. The sodium dodecyl sulphate procedure for distinction between CuZn-SOD and Mn-SOD (Geller & Winge, 1983) was in our hands erratic for human material. In materials from species where the procedure is applicable, EC-SOD will probably be co-determined with Mn-SOD. No antigenic similarities between EC-SOD and CuZn-SOD were detected, and distinction between the enzymes with immunological methods therefore appears feasible.

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