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Room temperature electron spin resonance of superoxide dismutase-loaded liposomes and erythrocytes. A direct approach to the interaction of O_2^- with cells

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Human erythrocytes were enriched with bovine superoxide dismutase by fusion with liposomes containing the entrapped enzyme. Liquid solution ESR of intact cells at room temperature was used to measure directly the increase in the superoxide dismutase content. From the spectral characteristics (g-value and hyperfine splitting tensor), the structural integrity of the Cu site of the enzyme was found to be unaffected by the liposome preparation procedure or the incubation with cells. Changes in the ESR signal size were used to test directly the interaction of superoxide with the enzyme entrapped in liposomes or delivered to erythrocytes. It was found that the liposome-entrapped enzyme does not react with externally generated O_2^-, but once delivered to red blood cells this reaction can take place. This is the first demonstration of O_2^--scavenging activity by superoxide dismutase delivered into an intact cell structure and is therefore to be considered as strong evidence for activity of this enzyme under in vivo conditions.

Introduction

SOD is considered to be an important enzyme working in the cellular defence against uncontrolled oxidative processes by scavenging O_2^- according to the reaction

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

Cell systems with higher levels of SOD with respect to average values represent an experimental model that gives a better understanding of the biological role of the enzyme and the involvement of oxygen radicals in physiological and pathological processes. Treatment of cells with SOD-containing liposomes has been an obvious approach to this problem. Several papers have dealt with liposomes entrapment of SOD, as well as liposome-mediated augmentation of the SOD content in cells [1–2]. However, no direct spectroscopic studies on liposome-incapsulated protein have been reported in the literature so far; the protein entrapment capacity of the vesicles, or the liposome-mediated increase of cellular SOD, has been quantified either by enzyme activity assays [1], by measurements of radioactively labelled SOD or by liposome fusion kinetics [3].

Being metalloproteins, SODs offer many possibilities for spectroscopic characterization. With the increasing clinical interest in this enzyme, the possibility to analyze SOD inside intact cells with a non-perturbative technique might be an important tool to investigate the formation and the effects of O_2^- in situ. We have used liquid solution room temperature ESR spectroscopy to characterize two kinds of vesicle containing Cu,Zn-SOD. By direct observation of the enzyme-active site, the following parameters were characterized: protein entrapment capacity of the vesicles, influence of the preparation procedure on the SOD-Cu^{2+} site geometry, and quantification of the liposome-mediated increase in human erythrocyte SOD content. Furthermore, from

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Abbreviations: SOD, superoxide dismutase (EC 1.15.1.1); ESR, electron spin resonance; 5-DS, 5-doxylstearate; 16-DS, 16-doxylstearate.

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the decrease of the enzyme ESR signal upon reaction with O_2^-, we have obtained evidence that SOD loaded into erythrocytes is actually functionally active in the intact cells. This appears to be the first direct observation of the activity of SOD in an intact cell system.

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine, stearylamine and cholesterol were obtained from Sigma and used without further purification. The spin labels 5-DS and 16-DS were from Molecular Probes (Junction City, OR). Cu,Zn-SOD were purified from bovine erythrocytes as described by McCord and Fridovich [4].

Liposomes (dipalmitoylphosphatidylcholine/stearylamine/cholesterol 7 : 2 : 1) were prepared by two different procedures, by sonication or by reverse-phase evaporation [5]. Liposome-associated SOD activity was determined by a polarographic method [6] in the presence of Triton X-100 (0.2% final concentration). Phospholipid concentrations were measured by the method of Bartlett [7]. Human erythrocytes were isolated from freshly drawn blood and washed three times in an isotonic NaCl solution, then diluted 1 : 6 with phosphate-buffered saline (7 mM K_2HPO_4 (pH = 7.4)/140 mM NaCl). Aliquots of the suspension were incubated for 2 h at 37 °C with liposomes containing 30–90-fold excess of SOD with respect to the endogenous cell content. At the end of the incubation the blood cells were extensively washed with phosphate-buffered saline. The liposome-mediated increase in the cellular concentration of SOD was determined by the polarographic assay of the enzyme activity of erythrocyte lysate and by ESR measurements on intact cells.

Exposure of the enzyme to controlled fluxes of O_2^- was accomplished by incubating 1 mM xanthine with 1 μM xanthine oxidase at pH 7.4 in the presence of 0.1 μM catalase. The rate of oxygen consumption, as detected by polarographic assays, was 29 $\mu M \cdot min^{-1}$, a rate which assured oxygen availability throughout the time of the ESR measurements. Incubations were carried out under vigorous aeration in large volumes, from which aliquots were rapidly transferred to the ESR flat cell for spectrum recording as a function of time. The sample contained sufficient oxygen to maintain a constant flux of O_2^- during the ESR measurements (approx. 3 min); in control experiments the ESR signal was found to remain stable even after 15 min.

ESR measurements of Cu,Zn-SOD in suspensions of liposomes or red blood cells in standard quartz flat cells were made with a Bruker ESP 300 spectrometer, operating at X-band using a TM_{110}-mode cavity. To obtain maximum sensitivity, spectra were recorded using 200 mW microwave power with a modulation of 16 G at a low frequency, 1.56 kHz, to avoid heating of the sample. A 1000 G scan was completed in 42 s, normally four or ten scans were accumulated. The enzyme concentration in the sample was determined by calibration with standard solutions of the enzyme. Automatic baseline corrections were made before the calculations. For the liposome measurements the absolute concentrations of the enzyme ranged from 20 to 200 μM.

Interaction of the enzyme with the liposome membranes was examined by determining the dynamical properties of the membranes in preparations with or without the protein. 5-DS or 16-DS dissolved in ethanol was deposited as a film at the bottom of a small test-tube by evaporating a few microlitres under a stream of nitrogen. A concentrated suspension of liposomes was added to give a final concentration of the spin label in the membranes of less than 0.4 mol%; after vigorous mixing, the suspension was left to equilibrate for 10 min at room temperature. The sample was drawn into a thin-walled glass capillary, sealed with plasticine and placed inside a 3 mm quartz ESR tube in a standard TE_{102}-mode cavity equipped with a variable-temperature dewar insert. 80 G scans were recorded with 2 C° temperature intervals using 20 mW microwave power and 1.0 G modulation at a frequency of 100 kHz. Due to the drastic changes in the spectral characteristics occurring around the phase-transition temperature, it was not possible to determine either order parameters or rotational correlation times over the entire temperature range studied. Therefore the outer hyperfine splitting tensor, $A_{\parallel}$, was used as an arbitrary parameter of the membrane dynamical properties or 'fluidity'.

Results

Fig. 1 shows the ESR spectra of SOD entrapped in liposomes prepared by sonication or reverse-phase evaporation. The g values and the hyperfine splitting tensors, $A_{\parallel}$, of the three spectra are identical to those of the free enzyme in aqueous solution, indicating that in either preparation procedure the enzyme maintains the integrity of the copper site.

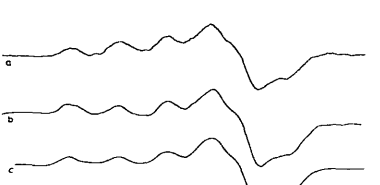

Fig. 1. ESR spectrum of Cu,Zn-SOD in liposomes prepared by sonication (a) or reverse-phase evaporation (b). For comparison a spectrum of free enzyme in solution is included. SOD concentration was 0.1–0.2 mM with the instrument gain setting varied accordingly.

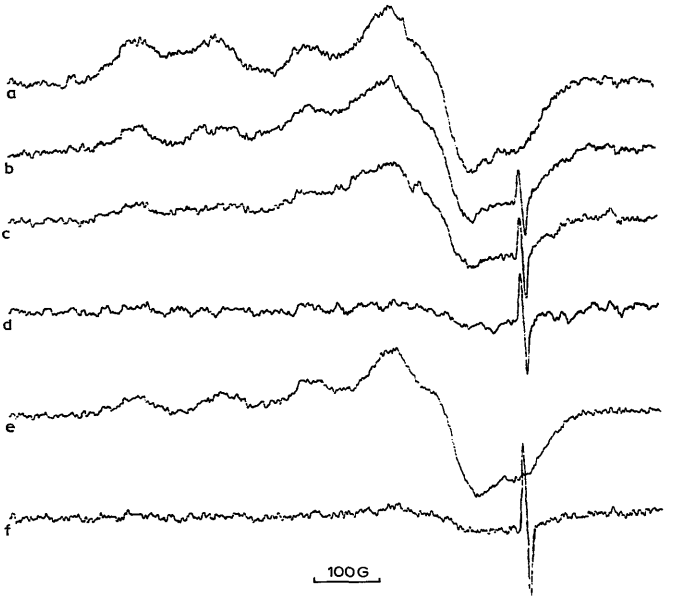

Fig. 2. ESR spectra of Cu,Zn-SOD-containing liposomes before (a) and after addition of 8 mM ascorbate (b–d). Spectrum (b) was recorded immediately after addition (90% signal left), (c) after 2 h of incubation (64% signal left), (d) immediately after (c), in the presence of Triton X-100 (16% signal left), (f) (16% signal left) is free SOD (e) immediately after addition of ascorbate.

The two liposome preparations have protein entrapment capacities of 30–60 μg SOD per μmol phospholipid, as determined by either ESR measurements of intact liposomes or polarographic activity assays after disruption of the liposomes with Triton X-100. On average, the sonication-prepared liposomes seem to be more efficient for protein incapsulation than liposomes prepared by reverse-phase evaporation (data not shown).

To evaluate the protein distribution in the vesicles, a large excess of ascorbate was added to the liposomes and the ESR spectrum was recorded after various time intervals. Immediately after addition of ascorbate only little reduction of the SOD is seen (Fig. 2a, b), in contrast to the results obtained with free SOD (Fig. 2e, f). After 2 h incubation the ESR signal has partially disappeared (Fig. 2c), as would be expected from the time-course of ascorbate penetration into liposomes [8]. Moreover, the remaining signal disappears upon addition of 0.2% Triton X-100, which breaks down the structural integrity of the lipid bilayers, making the protein accessible to the external reductant (Fig. 2d). Similar results were obtained for either type of liposome, demonstrating that liposome-associated SOD is contained inside the vesicles.

That only a small amount of the enzyme is bound to the membrane was shown by the spin-label experiments. Fig. 3 shows that the presence of SOD in the liposomes had only minor effects on the dynamic properties of the membranes. In particular, the sharp phase transition around 46 °C was not blurred, as would have been the case if strong protein–lipid interactive forces existed. The shifts between the two pairs of curves are more probably caused by the inevitable variations in sonication procedure of the lipid suspensions with or without protein. In fact, when probing the membrane close to the surface using the 5-DS label, a smaller difference

was found than for the 16-DS label, although the proximity of the proteins should be expected to have more pronounced effects on the hyperfine splitting parameter of 5-DS label [9].

The SOD-containing liposomes were used to enrich human red blood cells with this enzyme by a simple incubation procedure. In agreement with previous studies [3], both types of liposome were able to load the cells with SOD quite efficiently (up to 25-times the physiological content), without producing any significant extent of hemolysis. The degree of SOD enrichment was dose-dependent and could be regulated by varying the amount of liposome-incapsulated SOD (data not shown).

Fig. 4 shows ESR spectra from intact human erythrocytes after liposome-mediated SOD enrichment. The Cu^{2+} signal characteristic of the native SOD is observed, whereas no signal is seen in cells not pretreated with liposome-SOD. By activity measurements the concentration of SOD in untreated cells was found to be 2 μM, just below the sensitivity limit of our ESR equipment (data not shown). In the enriched cells, concentrations of 10–50 μM could be obtained. In this range, the ESR-determined concentration values were comparable with the values found by activity measurements, showing that no extensive reduction of the enzyme occurred within the cell.

The catalytic dismutation of O_2^- by SOD is a two-step mechanism, where the enzyme is first reduced by one

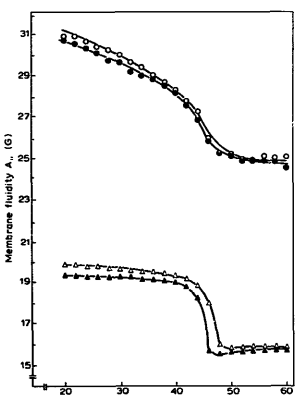

Fig. 3. Temperature-dependence of the outer hyperfine splitting tensor $A_{\parallel}$ of the spin labels 5-DS (○, ●) and 16-DS (△, ▲) was measured in liposomes prepared by sonication with SOD (open symbols) or without (closed symbols).

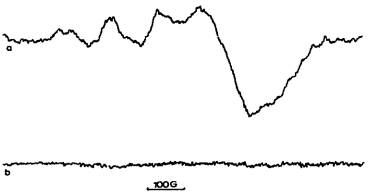

Fig. 4. ESR spectra of intact human erythrocytes after liposome-mediated SOD enrichment (a) and of normal cells (b). The cells were incubated as described in Materials and Methods with reverse-phase evaporation-prepared liposomes.

molecule of O_2^- to give an ESR-invisible Cu^+ intermediate, which is then re-oxidized by another O_2^- back to the Cu^{2+} state. The reaction rates of the two steps are equal under steady-state conditions, the enzyme will thus appear as 50% reduced [10–11]. From the results reported in Table I it appears that in liposomes the enzyme is insensitive to a flux of externally generated O_2^-, which is able to immediately establish and maintain steady-state conditions for the enzyme free in solution. Exposure of enriched red blood cells to externally produced O_2^- resulted in substantial reduction of the SOD ESR signal, although the signal did not reach the 50% reduction level. This may happen either because the passage of the O_2^- across the erythrocyte membrane is too slow, or because a fraction of the liposomes used for enrichment have remained attached to the cells without actually fusing. In any case, the result provides unequivocal evidence that the internal SOD is able to intercept O_2^- of external origin.

Discussion

With the instrumental improvements in the ESR technique obtained within the last couple of years, the sensitivity range has been extended to allow direct observation of the signal from Cu,Zn-SOD inside liposome-enriched red blood cells at room temperature. There are several advantages of using spectroscopic determinations of Cu,Zn-SOD with respect to other types of assay, such as activity or radioactive labelling measurements. From the spectral characteristics it is possible to determine whether the Cu site of the SOD is structurally intact or whether protein denaturation or other damage has occurred during the enrichment procedure, which may result in undesired effects when the protein is administered clinically. Sometimes activity assays may not be fully satisfactory in this respect. First, traditional activity assays cannot be used for studies of SOD localized inside intact cells or liposomes. Secondly, in some cases, metal complexes may interfere

with the standard assays. For instance, although iron-EDTA does not catalyze the dismutation of O_2^- at any significant rate [12–13], it causes unreliable results in assays using cytochrome *c* or nitro-blue tetrazolium reduction because of direct reduction by Fe(II) [14–16]. Here the spectra in Fig. 1 and 4 clearly show that the enzyme has preserved its structural integrity during the preparation of the liposomes used in this study and the interaction with cells. This is not always the case when the enzyme is subjected to manipulations involving organic solvents [17].

Although the use of liposomes as SOD carriers has been extensively treated in previous reports [18–21], it requires thorough knowledge of the properties and behaviour of the liposome system under investigation [22]. The results presented here provide direct evidence for previous suggestions on Cu,Zn-SOD localization in liposomes. The ascorbate reduction experiments (Fig. 2) demonstrate that, in view of the slow diffusion kinetics of ascorbate through liposome membranes at low temperatures [8], the enzyme is actually entrapped inside the vesicles. The spin-label results (Fig. 3) clearly demonstrate that SOD is only loosely associated with the membrane and is not inserted or strongly attached like normal surface-bound proteins [9]. Similar results have been reported for another water-soluble protein, bovine serum albumin, when entrapped in liposomes [23].

A particular advantage of ESR in studies on SOD is the possibility of using the ESR signal of the enzyme as a probe for functional interactions. As shown by the spin-label results (Fig. 3), the liposomes used in this work are in the gel state at physiological temperatures and should thus be virtually O_2^--impermeable [24,25]. This conclusion is confirmed by the experiments in Table I, which demonstrate that external O_2^- does not react with the entrapped enzyme. It is therefore extremely important to know whether the enzyme, once the process of liposome-cell fusion is completed, is still insensitive to O_2^- or not. The results obtained show unambiguously that once the enzyme is delivered to the cells, it is able to react with O_2^-. On the basis of experiments with haemoglobin-free ghosts it has already been established that O_2^- can cross the red blood cell membrane [26,27]. However, direct evidence that SOD integrated within an intact erythrocyte structure is able to scavenge O_2^- has never been obtained and may actually be very difficult to get from activity assay methods. This result is of particular relevance to the possible use of SOD-enriched erythrocytes as physiological scavengers of O_2^- produced in blood.

In conclusion, ESR studies can be used to measure directly the SOD content in intact cells; the type of information obtainable makes the method complementary to the traditional techniques. In the case of cell–superoxide interactions, however, ESR of intracellular SOD may well be a unique tool for further studies.

TABLE I

Decrease of SOD signal amplitude upon reaction between O_2^- and SOD in different environments

Liposomes containing 76 μM SOD, enriched erythrocytes containing 90 μM SOD and a solution of 85 μM SOD in phosphate-buffered saline were exposed to a flux of O_2^- as described in Materials and Methods. Values are expressed as percent of the signal size of the oxidized enzyme.

	Incubation time (min):	Signal size (%) 5	20	30	60
Liposome-entrapped SOD		–	100	100	100
Erythrocyte-delivered SOD		–	83	81	80
SOD free in solution		62	62	52	62

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