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INTERACTION OF SUPEROXIDE DISMUTASE WITH PHOSPHOLIPID LIPOSOMES

AN UPTAKE, SPIN LABEL AND CALORIMETRIC STUDY

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The effect of superoxide dismutases from five species upon phospholipid bilayers has been investigated. The uptake by egg phosphatidylcholine bilayers of the holo and apo forms of bovine superoxide dismutase increases with enzyme concentration and only a fraction of each is removed by treatment with trypsin. These uptake data indicate that both forms of the enzyme associate with and are embedded within lipid bilayers. From the spectrum of the spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl, the binding of superoxide dismutase to egg phosphatidylcholine bilayers can be shown to disorder the lipid packing. The disordering by the bovine holoenzyme is small but increases with increasing enzyme concentration and period of incubation. The disordering effects of the apoenzyme are much larger and are reversible by Cu^{2+}, Zn^{2+} reconstitution of the apoenzyme. The disordering effect of the apoenzyme is further confirmed by differential scanning calorimetry. The gel to liquid crystalline phase transition of egg phosphatidylcholine is lowered 7°C by 25% by weight apo-superoxide dismutase to lipid. Human, dog, swordfish and yeast superoxide dismutases also disorder, and to a greater extent than the bovine enzyme. The greatest perturbation is produced by yeast superoxide dismutase; a 20% decrease in the order parameter by 50% by weight enzyme to lipid.

Introduction

Superoxide dismutase is found in all aerobic cells, in which it has been measured, and plays an important role in the protection of cells from metabolically generated superoxide anions [1–6]. Its radioprotective properties in cells have also been described [7–9] and derive in part from the ability of the cupro-zinc isozyme to penetrate plasma membranes and become associated with the cytosol, mitochondria and nuclei [10]. This ability to penetrate membranes is underscored by the fact that although the cupro-zinc superoxide dismutase is ostensibly synthesized in the cytoplasm, it is nonetheless present in the mitochondrial and nuclear fractions [10]. Thus, it appears that the enzyme is thermodynamically suited to pass through natural membranes, and suggests questions about the nature of the interaction underlying this process.

Recently, it was shown that cupro-zinc superoxide dismutase is internalized in erythrocyte ghost membranes [11], suggesting an interaction with the phospholipid-cholesterol bilayers. It is known that the enzyme binds to egg phosphatidylcholine liposomes and induces a change in the texture of freeze-fracture replicas at different depths of the multilamellar structures [12]. Also, spin label studies have shown that the enzyme affects the order within the hydrocarbon region of the liposomes [12]. The present study

Abbreviation: I(12,3), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl.

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extends these earlier results to a more extensive examination of the interaction of Cu^{2+}, Zn^{2+} superoxide dismutase from several sources with two types of egg phosphatidylcholine and dimyristoylphosphatidylcholine. Bovine erythrocyte apo-superoxide dismutase and γ-radiation-inactivated superoxide dismutase are also studied and differential scanning calorimetry (DSC) is used to investigate the effect of superoxide dismutase upon the phase transition of egg phosphatidylcholine. Also, it is shown that trypsinization of superoxide dismutase-treated phospholipid bilayers does not remove much of the membrane-associated enzyme, thus corroborating the interaction phenomenon observed in the spin label work.

Materials and Methods

Superoxide dismutases. All superoxide dismutases were of the Cu^{2+}, Zn^{2+} form and were obtained from various sources: bovine erythrocyte, human Type I, swordfish and human erythrocyte superoxide dismutases (from Dr. J.V. Bannister, Malta), yeast superoxide dismutase (Pharmacia and Carlsberg), bovine liver superoxide dismutase (Diagnostic Data Inc., Mountain View, CA), and canine erythrocyte superoxide dismutase (Sigma). The superoxide dismutases were stored lyophilized at 4°C until used. Inactivated bovine erythrocyte superoxide dismutase was produced by exposure to 184 krad (1.84 kGy) of Co^{60} γ-radiation. The inactivated superoxide dismutase had a residual activity of less than 1%. Its Cu^{2+}, Zn^{2+} content was 72 and 50%, respectively, of maximal binding as determined by atomic absorption measurements taken on a Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer.

Preparation of the apo form of bovine erythrocyte superoxide dismutase was as follows [13,14]: a 1 mM solution of bovine erythrocyte superoxide dismutase (33 mg/ml) was dialyzed against 1 L of 1 mM EDTA in 0.05 M ammonium acetate buffer, pH 3.6 for two days at 4°C. The dialysate was changed twice and the solution was stirred constantly. The dialysate was then changed to dilute (approx. 5 mM) acetate buffer, pH 4.95, and the solution was changed several times over one day. Ammonium acetate buffer was used since it is volatile and will evaporate to a large extent upon lyophilization of the sample leaving the protein. After extensive washing with dilute buffer, the tubing was removed and covered with Sephadex G-50-150 coarse (M_r exclusion limit 10000) and allowed to stand 6 h at 4°C. Salts, EDTA, and metal chelates are taken up by the beads in such a manner as to concentrate the protein by reducing the volume, while pH and ionic concentration remain constant. The protein was then lyophilized and stored in a vial over phosphorus pentoxide in the dark at 4°C until needed. The Cu^{2+}, Zn^{2+} content of the apoenzyme was 17 and 9.4% of maximum binding, respectively.

Reconstitution of the apoenzyme was done by dialyzing a 1 mM solution against 2 mM $CuSO_4$ or 2 mM $CuSO_4$ followed by 2 mM $ZnSO_4$ in 0.05 M ammonium acetate buffer, pH 5.5, overnight at 4°C in order to, respectively, replace the Cu^{2+} and Cu^{2+}, Zn^{2+}. The dialysate was changed to 0.1 mM EDTA in 0.05 M ammonium acetate buffer, pH 6.0, for 6 h. The reconstituted enzyme was then dialyzed extensively against dilute acetate buffer, pH 6.0, and lyophilized.

Uptake. For the purpose of measuring the uptake by lipid membranes of bovine erythrocyte superoxide dismutase, which was resistant to removal by digestion with trypsin, bilayers of soybean phospholipids (Azolectin®, Associated Concentrates, Woodside, Long Island, NY) were prepared as previously described [15]. Samples of the phospholipid bilayers were incubated for 1 h at room temperature with various concentrations of unlabelled bovine erythrocyte superoxide dismutase (Table I) along with 6.5 μg/ml of ^{125}I-labelled superoxide dismutase. The ^{125}I-labelled bovine erythrocyte enzyme was prepared by the chloramine-T technique as previously described [15]. The treated bilayer membranes were then washed three times in water as previously described, and the final pellets were counted for ^{125}I [12]. The pellets were then dispersed in a trypsin solution (1 mg/ml), incubated for 1 h at room temperature and washed three times in water. The washed pellets were counted for residual ^{125}I activity. The pellets were dispersed in phosphate-buffered saline, pH 7.4, centrifuged and the supernatant was chromatographed on a Bio-Gel P60 column to determine the fractional ^{125}I activity still associated with the enzyme [12]. This fractional activity was related to the total amount of superoxide dismutase (labelled and unlabelled) taken up by the membranes whose

lipid content had previously been determined by dry weight measurement. In a similar fashion, the uptake of the apo form of bovine erythrocyte superoxide dismutase was measured, with the exception that only the ^{125}I-labelled apoenzyme was used.

Spin labelling. L-α-phosphatidylcholines (from egg yolk), Types V-E and VII-E, the sodium salt diphosphatidylglycerol (i.e., cardiolipin from bovine heart) and dimyristoyl-L-α-phosphatidylcholine (approx. 98% pure) were obtained in powdered form from Sigma Chemical Co. and used without further purification. These materials were stored dessicated at −20°C in solution or powdered form.

Purity of the egg phosphatidylcholines was checked by TLC and the Type VII-E was found to contain significantly more impurities than the Type V-E. The Type V-E was found to contain only a very small amount of lysophosphatidylcholine and a barely detectable amount of neutral lipids. The Type VII-E contained lysophosphatidylcholine and small amounts of phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and phosphatidylglycerol.

Some free fatty acids, triacylglycerols and fatty acid methyl esters were detected in the neutral lipid fraction. The concentration of these contaminants was not determined. The Type VII-E egg phosphatidylcholine had a slight yellowish tint and it had 4-times greater absorbance at 232 nm than the Type V-E which was colorless, indicating greater peroxidation of the Type VII-E [15].

The fatty acid spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (I(12,3)) was obtained from Syva. This spin label consists of the oxazolidinyloxyl moiety on the fifth carbon of stearic acid. ESR spectra were recorded with a Varian E-12 EPR spectrometer with a variable temperature accessory.

ESR samples were prepared by drying down 10 μl of a 5 mM or equivalent ethanolic solution of I(12,3) along with a chloroform/methanol solution containing 10 mg lipid under nitrogen, and then drying for an additional 90 min under vacuum at room temperature. In the case of dimyristoylphosphatidylcholine, 10 mg of the dry powdered lipid was added to the spin label dried on the inside of a test tube. For both lipids, the mixture was then vortexed for 5 min with 30–40 μl of phosphate-buffered saline (pH 7.15) to form a 20–25% dispersion of liposomes. The dimyristoylphosphatidylcholine samples were warmed above the phase transition (23°C) and vortexed. The preparation of samples containing superoxide dismutase was made by adding a weighed amount of lyophilized superoxide dismutase to the dry lipid/spin label mixture before addition of the phosphate-buffered saline and vortexing, so that is was present during formation of the bilayers. This procedure contrasts with that used in the superoxide dismutase uptake measurements [12], where the liposomes were prepared first and then incubated with varying amounts of superoxide dismutase for 30 min at room temperature.

The ratio of lipid to spin label was always greater than 200 : 1. Prepared samples were drawn up in thin-walled 50 μl capillaries and the ends flamed shut to prevent loss of water. After standing, the samples separated into two phases; the top phase contained lipid, spin label, some of the enzyme and essentially all the ESR signal while the bottom phase was an emerald-green-blue solution of superoxide dismutase. The samples were incubated at 37°C for stated periods of time.

The order parameter of I(12.3) was calculated using

$$S = \frac{A_{\parallel} - A_{\perp}}{k[A_{zz} - 1/2(A_{xx} + A_{yy})]}$$

where

$$k = \frac{A_{\parallel} + 2A_{\perp}}{A_{xx} + A_{yy} + A_{zz}}$$

is a correction for polarity changes [16]. The values of $A_{\parallel}$ and $A_{\perp}$ are obtained by

$$A_{\parallel} = A_{\max}$$

$$A_{\perp} = A_{\min} + 1.32 + 1.86 \log_{10}(1 - S_{\text{app}})$$

where

$$S_{\text{app}} = \frac{A_{\max} - A_{\min}}{A_{zz} - 1/2(A_{xx} + A_{yy})}$$

and A_{xx}, A_{yy}, A_{zz} are the principal hyperfine coupling constants of 6.3, 5.8 and 33.6 G, respectively [16].

Differential scanning calorimetry. Measurements were made on a Perkin-Elmer DSC-2 with the Intercooler accessory, using either aluminum calorimetric cells with an operational volume of approx. 20 μl for dimyristoylphosphatidylcholine, or large volume stainless steel sample pans (60 μl) for egg phosphatidylcholine. Only heating and cooling rates of 2.5°C/min or 1.25°C/min were used, since at these slow-heating rates little or no hysteresis occurs and results are generally repeatable within ±0.2°C. The enthalpy of the transition was calculated from the area under the peak measured by the cut and weigh method.

DSC samples were prepared similarly to those for spin labelling. For the egg phosphatidylcholine samples, which undergo a phase transition at very low temperatures (i.e., approx. 245–269 K), the phospholipid-superoxide dismutase sample was suspended in a 60% solution of ethylene glycol in phosphate-buffered saline giving a final composition of about 17% lipid, 33% phosphate-buffered saline, and 50% ethylene glycol. The 60% solution of ethylene glycol was found to have a freezing point of about −52°C in the DSC sample pans.

Results

Superoxide dismutase uptake

The uptake by phospholipid bilayers of the ^{125}I-labelled holo and apo forms of bovine erythrocyte superoxide dismutase before and after trypsinization, is documented in Table I. With the holoenzyme, the residual ^{125}I activity after trypsinization varies between 38–42% (Table I, fifth column), while with the apoenzyme it varies from 68–43% at concentrations of 0.15 and 1.55 μg/ml, respectively. Thus, the uptake of the apoenzyme appears more resistant to removal by trypsin, suggesting a greater amount or degree of membrane embedment. In either case, however, the fact that trypsin removes a fraction of the membrane-associated superoxide dismutase is indicative of a surface interaction and is consistent with the increase in surface viscosity of superoxide dismutase-treated liposomes noted previously [12].

TABLE I

UPTAKE OF BOVINE ERYTHROCYTE SUPEROXIDE DISMUTASE BY PHOSPHOLIPID BILAYERS IN 1 h AT ROOM TEMPERATURE AND ITS PARTIAL REMOVAL BY TRYPSINIZATION, ALSO FOR 1 h AT ROOM TEMPERATURE

Concn. of superoxide dismutase		^{125}I-labeled superoxide dismutase uptake					
Unlabeled (μg/ml)	^{125}I-labeled (μg/ml)	Before trypsinization (cpm/ml)	After trypsinization for 1 h at room temperature				
			cpm/ml	% remaining	% bound [a]	Net cpm per mg lipid	Net [b] uptake (μg/mg)
A. With holoenzyme							
0	6.5	$1.87 \cdot 10^5$	$0.79 \cdot 10^5$	42	90	6 192	0.0041
100	6.5	$1.92 \cdot 10^5$	$0.77 \cdot 10^5$	40	88	5 841	0.065
500	6.5	$1.97 \cdot 10^5$	$0.81 \cdot 10^5$	41	85	5 935	0.31
1 000	6.5	$1.82 \cdot 10^5$	$0.69 \cdot 10^5$	38	84	4 996	0.53
B. With apoenzyme							
0	0.15	$1.06 \cdot 10^5$	$0.71 \cdot 10^5$	67	97	4 051	0.0008
0	0.39	$2.07 \cdot 10^5$	$1.27 \cdot 10^5$	61	94	7 022	0.0015
0	0.77	$3.50 \cdot 10^5$	$1.71 \cdot 10^5$	49	95	9 556	0.0020
0	1.55	$4.83 \cdot 10^5$	$2.09 \cdot 10^5$	43	94	11 556	0.0024

[a] Fraction of residual ^{125}I activity after trypsinization which was still bound to superoxide dismutase as determined on a Bio-Gel P-60 column.

[b] Includes both unlabeled and ^{125}I-labeled superoxide dismutase in μg/mg of lipid.

The net uptake of both forms of bovine erythrocyte superoxide dismutase is given in the last column of Table I in μg/mg of lipid and shown to increase with the concentration of the enzyme during incubation. This increase is in agreement with previous results [12]. When normalized to a given concentration of enzyme (e.g., 1.55 μg/ml), the net uptake of the apoenzyme is greater than that of the holoenzyme.

Spin labelling

For the spin label studies, two different preparations of egg phosphatidylcholine were used, both without purification. The first (Type VII-E) was used for the early studies and had a slight yellowish tint and contained far more impurities than the second preparation, Type V-E. The ESR spectra of I(12,3) in these two lipid preparations are shown in Fig. 1. The method of measuring A_{max} and A_{min} is also shown. The egg phosphatidylcholine (Type VII-E) spectrum has more rounded features and an order parameter of 0.562, while Type V-E gives a spectrum with sharper features (the second peak, the low-field A_{min}, is especially sharpened) and has an order parameter of 0.582. Thus, the Type VII-E egg phosphatidylcholine forms a more disordered bilayer. The effect of superoxide dismutase upon the shape of the two different spectra is considerably different, as will be shown below.

The addition of bovine erythrocyte superoxide dismutase to egg phosphatidylcholine liposomes causes a change in the shape of the spectrum. Fig. 2 shows the effect of various concentrations of bovine superoxide dismutase upon the spectrum of I(12,3). Only the low-field region of each spectrum is shown, as the spectral changes are most apparent in this region. The spectra were taken after one day of incubation at 37°C. Enzyme concentrations of 1.44, 5.23 and 11.2 mg/10 mg egg phosphatidylcholine (Type V-E) were used, giving molar ratios of 270, 74 and 35 phospholipid molecules/molecule of superoxide dismutase, respectively. In each case, only part of the superoxide dismutase is bound to the lipid.

The sharp low-field peak corresponding to A_{min} is broadened and shifted outward from the center of

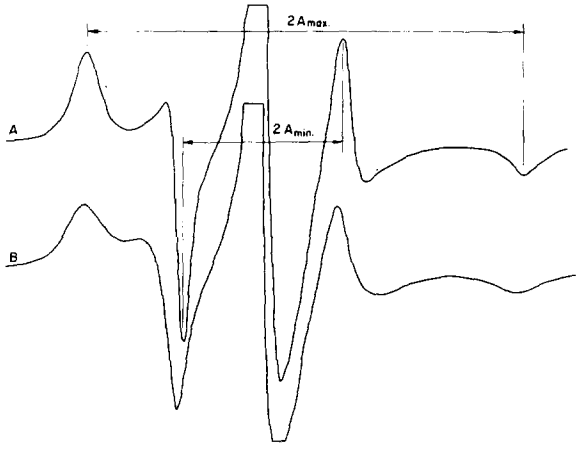

Fig. 1. ESR spectra of I(12,3) in egg phosphatidylcholine liposomes showing the measurement of the A_{max} and A_{min} values. Curve A is a representative spectrum of the egg phosphatidylcholine (Type V-E) used for most of these studies. Curve B is a spectrum of another preparation of egg phosphatidylcholine (Type VII-E) also used. The top and bottom of the mid-field line is clipped-off to show better the low- and high-field regions. This also occurs on many of the following spectra.

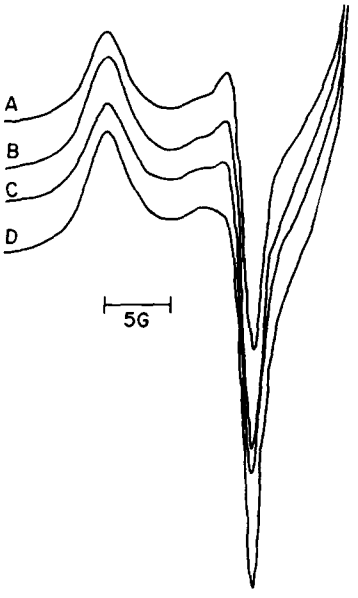

Fig. 2. ESR spectra of the low-field region of I(12,3) in egg phosphatidylcholine (Type V-E) liposomes with bovine erythrocyte superoxide dismutase added. A, no superoxide dismutase added; B, 1.44 mg superoxide dismutase/10 mg lipid; C, 5.23 mg superoxide dismutase/10 mg lipid; and D, 11.2 mg superoxide dismutase/10 mg lipid. The samples were incubated for 24 h at 37°C before spectra were taken.

the spectrum towards the A_{max} peak. The amount of shift and amount of broadening is proportional to the quantity of superoxide dismutase added. The shift appears to be caused by the growth of a new, broader peak about 2 G farther out from the center of the spectrum with the concomitant loss of the sharp A_{min} peak. These spectral shape changes have only a very small effect upon the measured order parameter. The order parameter is slightly decreased, but even for the 11.2 mg superoxide dismutase sample, the change is less than 1%. The order parameters and low-field A_{max} to A_{min} peak separations for the spectra in Fig. 2 are given in Table II. There is a corresponding broadening of the high-field lines, but it is not as apparent as the effect upon the low-field region.

It is difficult to characterize precisely the interaction between superoxide dismutase and the egg phosphatidylcholine liposomes. Only part of the superoxide dismutase added interacts with the bilayers, as there is often a separation of a soluble superoxide dismutase phase and a superoxide dismutase-egg phosphatidylcholine phase with incubation, especially at high enzyme concentrations. This is consistent with partial uptake of superoxide dismutase by egg phosphatidylcholine liposomes [12]. No ESR signal is obtained from the superoxide dismutase phase at room temperature, with the gain used, so the effect of the enzyme must be upon the lipid bilayer. The interaction between superoxide dismutase and the lipid bilayer must be much weaker than that of an integral membrane protein such as cytochrome oxidase, since there is no formation of immobilized boundary lipid [17]. The slight decrease in order parameter, shift of the A_{min} peak towards the A_{max} peak, and broadening of the A_{min} peak may be indicactive of an increase in the disorder of the system. Alternatively, they may be due to a decrease in the rate of rotation of the spin label. Binding of superoxide dismutase to the surface of the bilayer or some penetration of superoxide dismutase into the bilayer cannot be differentiated from these data. The appearance of the new broad peak with increasing superoxide dismutase concentration may be due to the interaction of the enzyme with only a subset of the total lipid at lower concentrations.

The effect of superoxide dismutase increases with time of incubation. Fig. 3 shows a series of spectra of a control egg phosphatidylcholine sample with no superoxide dismutase, and one with 9.91 mg bovine erythrocyte superoxide dismutase/10 mg lipid as a function of time of incubation at 37°C up to 66 h. The separation of the low-field A_{max} and A_{min} peaks and the order parameters are given in Table II. There

TABLE II

PEAK SEPARATIONS (BETWEEN LOW-FIELD A_{max} AND A_{min} PEAKS) AND ORDER PARAMETER (S) OF I(12,3) IN EGG PHOSPHATIDYLCHOLINE (TYPE V-E, 10 mg/SAMPLE) LIPOSOMES WITH BOVINE ERYTHROCYTE SUPEROXIDE DISMUTASE

These measurements were made from the spectra in Figs. 2 and 3.

Sample	Time of incubation (h)	Separation (G)	S
Egg phosphatidylcholine (Fig. 2)	24	9.05	0.565
+1.44 mg superoxide dismutase	24	8.90	0.556
+5.23 mg superoxide dismutase	24	8.75	0.562
+11.2 mg superoxide dismutase	24	7.25	0.562
Egg phosphatidylcholine (Fig. 3)	0	9.10	0.577
+9.91 mg superoxide dismutase	0	9.00	0.577
Egg phosphatidylcholine	17	9.05	0.579
+9.91 mg superoxide dismutase	17	7.95	0.584
Egg phosphatidylcholine	44	9.10	0.574
+9.91 mg superoxide dismutase	44	7.25	0.569
Egg phosphatidylcholine	66	9.10	0.569
+9.91 mg superoxide dismutase	66	7.10	0.568

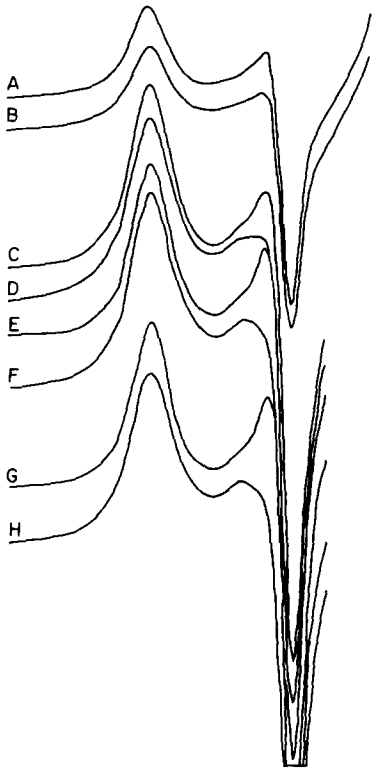

Fig. 3. ESR spectra as a function of time of incubation at 37°C of the low-field region of I(12,3) in egg phosphatidylcholine (Type V-E) liposomes. The spectra labeled A, C, E, and G are of egg phosphatidylcholine with no superoxide dismutase and B, D, F, and H are with 9.91 mg bovine erythrocyte superoxide dismutase/10 mg lipid. A and B are at 0 h; C and D are at 17 h; E and F are at 44 h and G and H are at 66 h.

is no change in the control sample without superoxide dismutase with incubation. Immediately after addition of the superoxide dismutase, there is only a slight broadening of the A_{min} peak and the peak separation decreases from 9.10 to 9.00 G. The broadening increases with time and the peak separation decreases, until after 66 h of incubation, the peak separation is only 7.10 G. There is little change after 60–80 h incubation.

The interaction of superoxide dismutase with a saturated phospholipid, dimyristoylphosphatidylcholine, was also investigated. This lipid has a sharp gel to liquid crystalline phase transition at about 24°C. Spectra taken at 26 and 49.5°C are shown in Fig. 4. The effect of superoxide dismutase on the spectra is similar to its effect on the spectra of egg

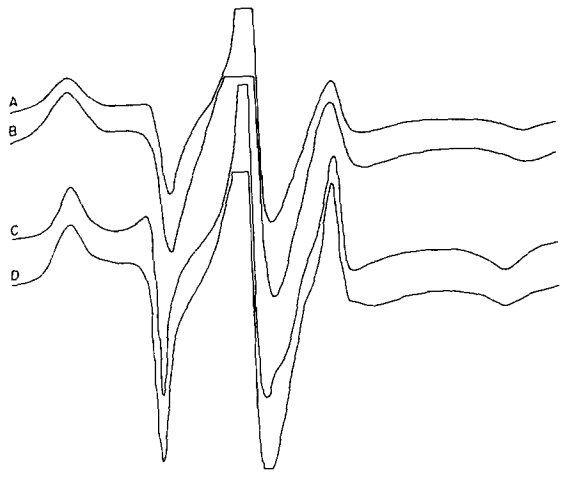

Fig. 4. ESR spectra of I(12,3) in dimyristoylphosphatidylcholine liposomes containing no superoxide dismutase (A, C) or 10 mg bovine erythrocyte superoxide dismutase/10 mg lipid (B, D). The spectra were taken at 26°C (A, B) and 49.5°C (C, D).

phosphatidylcholine. At 26°C, the lines are quite broad and only a slight broadening of the A_{min} peak is apparent. At 49.5°C, the effect is much more apparent and there is also a change in the lower part of the high-field A_{min} peak. There is no change in the measured order parameter at either temperature. Below the phase transition at 24°C, bovine erythrocyte superoxide dismutase has no effect upon the spectrum of I(12,3).

DSC scans of dimyristoylphosphatidylcholine with and without superoxide dismutase were taken to investigate whether or not bovine erythrocyte superoxide dismutase can affect the phase transition in any way, as some integral membrane proteins have been shown to do [18]. No changes in temperature of the transition or in the shape of the transition were found (scans not shown). This indicates that there probably is no binding below the phase transition for a saturated lipid such as dimyristoylphosphatidylcholine where the lipid is in the gel state.

Up to this point, only active bovine erythrocyte superoxide dismutase (holoenzyme) has been used in the spin label studies. The apoenzyme of bovine erythrocyte superoxide dismutase was prepared as described in Materials and Methods to investigate its interaction with egg phosphatidylcholine. Apo-superoxide dismutase, Cu^{2+}-reconstituted apo-superoxide

dismutase, and Cu^{2+}- and Zn^{2+}-reconstituted apo-superoxide dismutase were prepared. The percentages of Cu^{2+} and Zn^{2+} in each preparation are given in Table III. The low-field region of the spectra obtained from I(12,3) in samples made from these preparations and egg phosphatidylcholine (Type V) are shown in Fig. 5. The order parameters are given in Table III.

The effects of the apo-superoxide dismutase are much greater than those of the holoenzyme. The A_{min} peak is similarly broadened but shifted more towards the A_{max} peak than for the holoenzyme, as can be seen in Fig. 5. Cu^{2+} and Cu^{2+}, Zn^{2+} reconstitution tend to sharpen the A_{min} and shift it back towards its original position. This shift for the apoenzyme is large enough to significantly lower the order parameter, the values of which are given in Table III. The holoenzyme lowers S by 1.2%, barely significant, while a smaller amount of apoenzyme lowers it 20.7%. Reconstituting the apoenzyme with Cu^{2+} reduces the size of the effect to 16.2% and a Cu^{2+}, Zn^{2+} reconstitution reduced it further to 2.0% which is close to that for the holoenzyme. Thus, the apoenzyme must differ considerably in structure from the holoenzyme such that it interacts much more strongly with lipid bilayers. The increased interaction could be polar, with the surface of the bilayer. However, the uptake data suggest otherwise, namely that the apoenzyme embeds sufficiently deeply into lipid bilayers that a lesser fraction is accessible to removal by trypsin digestion (Table I). The size of the disordering effect of the apoenzyme is therefore more likely due to extensive penetration into the bilayer interior. This could occur if the structural change exposes more hydrophobic groups on the surface of the apo-molecule. This structural change is reversible, since the Cu^{2+}, Zn^{2+}-reconstituted superoxide dismutase behaves similarly to the holoenzyme.

The radiation-inactivated superoxide dismutase

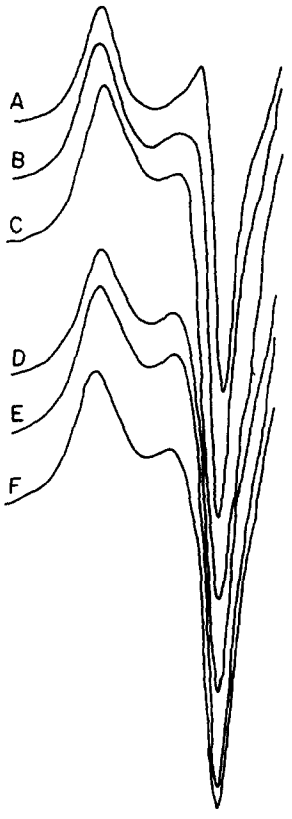

Fig. 5. Low-field region of ESR spectra of I(12,3) in egg phosphatidylcholine (10 mg) liposomes (Type V) containing the following superoxide dismutase preparations: A, no superoxide dismutase; B, 10.4 mg holo-superoxide dismutase (bovine); C, 7.2 mg apo-superoxide dismutase; D, 10 mg Cu^{2+}-reconstituted apo-superoxide dismutase; E, 7.2 mg Cu^{2+}, Zn^{2+}-reconstituted apo-superoxide dismutase and F, 10.6 mg radiation-inactivated superoxide dismutase. The spectra were taken after 46 h of incubation at 37°C.

TABLE III

PERCENTAGES OF Cu^{2+} AND Zn^{2+} IN THE HOLO-SUPEROXIDE DISMUTASE, APO-SUPEROXIDE DISMUTASE AND Cu^{2+}, Zn^{2+}-RECONSTITUTED SUPEROXIDE DISMUTASE AND THE ORDER PARAMETER (S) OF I(12, 3) IN THE SUPEROXIDE DISMUTASE-EGG PHOSPHATIDYLCHOLINE SAMPLES

The percentages given are of the maximum amount of Cu^{2+} and Zn^{2+} that could be bound. Each sample contains 10 mg egg phosphatidylcholine, (Type V). The spectra for these samples are shown in Fig. 5.

Sample	Cu^{2+} (%)	Zn^{2+} (%)	S	ΔS (%)
Egg phosphatidylcholine	–	–	0.598	–
+10.40 mg holo	90	100	0.591	−1.2
+7.2 mg apo	17	9.4	0.474	−20.7
+10 mg Cu^{2+}-reconstituted	–	–	0.501	−16.2
+7.2 mg Cu^{2+}, Zn^{2+}-reconstituted apo	105	89	0.586	−2.0
+10.6 mg radiation-inactivated	72	50	0.502	−16.1

(spectrum in Fig. 5; described in Table III) also had a much larger effect upon the spectrum of I(12,3) than the holoenzyme. The A_{min} peak was extensively broadened and shifted and the order parameter was reduced by 16.1%, almost as great as the reduction caused by the apoenzyme. The inactivated superoxide dismutase had also presumably undergone a structural change causing the loss of activity, which may be related to the structural change undergone by the apoenzyme leading to its increased interaction with egg phosphatidylcholine.

Since the effect of the apoenzyme and inactivated superoxide dismutase is so large compared to that of the holoenzyme, it is possible that most, if not all, of the effect of the holoenzyme is due to an apoenzyme-like or inactivated enzyme-like contaminant. The measured Cu^{2+} content of the holoenzyme is only 90% of the maximum that could bind (Table III) and the Cu^{2+}, Zn^{2+}-reconstituted superoxide dismutase contained only 89% of the maximum amount of Zn^{2+}. Whether these numbers are significantly different from 100% is not clear, but the possibility exists that the holoenzyme contains some apoenzyme or inactivated superoxide dismutase.

To determine whether or not the interaction of bovine erythrocyte superoxide dismutase with lipid bilayers is unique, the effect of superoxide dismutase from other species upon egg phosphatidylcholine liposomes was studied. The sources of the various superoxide dismutases are given in Materials and Methods. The low-field regions of the I(12,3) spectra from the superoxide dismutase-egg phosphatidylcholine liposomes are shown in Fig. 6 and the order parameters measured from these spectra are given in Table IV. All of the superoxide dismutases had a similar effect; the low-field A_{min} peak was broadened and shifted towards the A_{max} peak. The size of the effect varied considerably and the order parameter was significantly reduced for some of the samples. The control egg phosphatidylcholine (Fig. 6A) and bovine erythrocyte superoxide dismutase (Fig. 6B) spectra are similar to those shown previously. The yeast superoxide dismutase (Fig. 6C) produced a large decrease of 17.6% in the order parameter. The decrease in S is also apparent for the human, Type I (Fig. 6D) and the human erythrocyte superoxide dismutase (Fig. 6G). The swordfish (Fig. 6E), the bovine liver (Fig. 6F) and the dog erythrocyte superoxide dismutase (Fig. 6H) had an effect similar to that of the bovine erythrocyte superoxide dismutase. There was little, if any, change in the order parameter but there was a broadening and shifting of the low-field A_{min} peak.

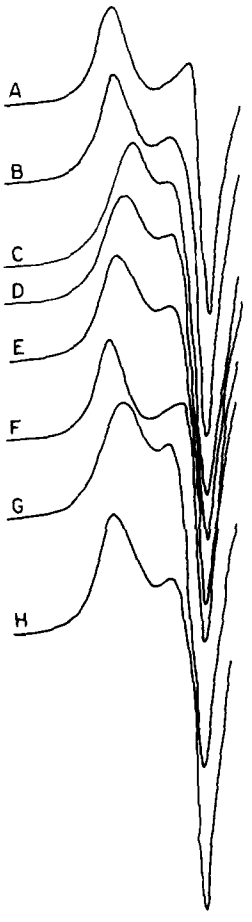

Fig. 6. Low-field region of ESR spectra of I(12,3) in egg phosphatidylcholine (10 mg) liposomes containing superoxide dismutase from the following sources: A, control (none); B, bovine red blood cell (10.3 mg); C, yeast (10.4 mg); D, human Type I (9.57 mg); E, swordfish (18.4 mg); F, bovine liver (10.3 mg); G, human (9.52 mg) and H, dog erythrocyte (10.2 mg). All samples were incubated 46 h at 37°C before the spectra were taken.

Why the various superoxide dismutases interact to such different degrees is not clear. They may differ enough in structure to considerably affect their interaction with egg phosphatidylcholine or, alternatively, they may contain different amounts of inactivated or

TABLE IV

ORDER PARAMETER (S) OF I(12,3) AND PERCENT CHANGES FROM CONTROL FOR VARIOUS TYPES OF SUPEROXIDE DISMUTASE IN EGG PHOSPHATIDYLCHOLINE (10 mg)

These measurements were made from the spectra in Fig. 6.

Sample	S	ΔS (%)
Egg phosphatidylcholine	0.579	–
+10.3 mg bovine erythrocyte	0.577	−0.3
+10.3 mg bovine liver	0.578	−0.2
+10.4 mg yeast (Pharmacia, I)	0.477	−17.6
+10.0 mg yeast (Pharmacia, II)	0.434	−24.9
+10.0 mg yeast (Carlsberg)	0.437	−24.5
+9.57 mg human type I	0.543	−6.2
+9.52 mg human erythrocyte	0.542	−6.4
+18.4 mg swordfish	0.562	−2.9
+10.2 mg dog erythrocyte	0.571	−1.4

apoenzyme which could affect the strength of the interaction.

Measurements were made on two other preparations of yeast superoxide dismutase (spectra not shown), one from Carlsberg and a different one from Pharmacia. Both showed a large decrease in order parameter of 24.5 and 24.9%, respectively, at 10 mg superoxide dismutase/10 mg lipid. This is similar to the decrease in S of the Pharmacia yeast superoxide dismutase, Since the effect of the three yeast superoxide dismutase preparations is similar, yeast superoxide dismutase may differ in structure from the others' in such a way as to increase its perturbing ability.

The effect of superoxide dismutase upon the spectrum of I(12,3) was found to vary, depending upon the preparation of egg phosphatidylcholine used. Fig. 7A and B are of spectra from samples of egg phosphatidylcholine (Type V-E) without and with bovine erythrocyte superoxide dismutase. The broadening of the low-field A_{min} peak with little change in order parameter is again apparent. The order parameters are given in Table V. Fig. 7C and D are of spectra from samples made with egg phosphatidylcholine (Type VII-E). The differences between the two lipid preparations have been previously described but basically the Type VII-E contains more lipid impurities and oxidation products. The addition of superoxide dismutase to the Type VII-E egg phosphatidylcholine

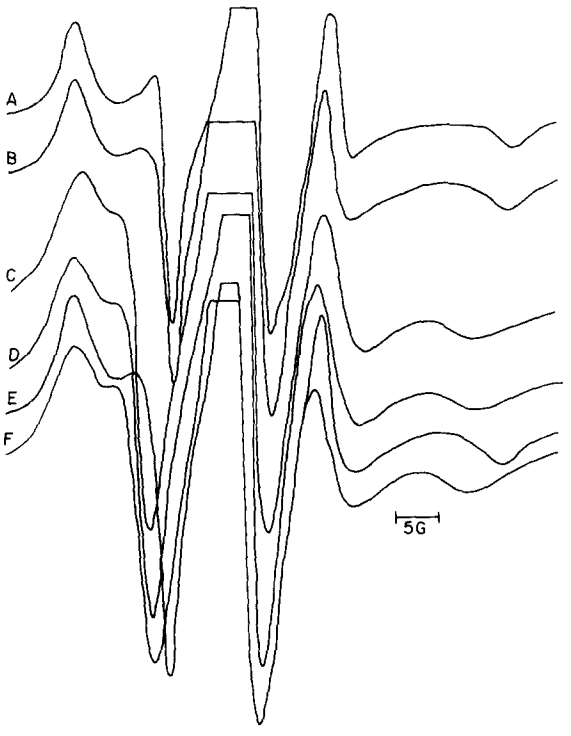

Fig. 7. ESR spectra of I(12,3) in: A, egg phosphatidylcholine (Type V-E); B, same as (A) plus bovine erythrocyte superoxide dismutase (13 mg); C, egg phosphatidylcholine (Type VII-E); D, same as (C) plus bovine erythrocyte superoxide dismutase (7.8 mg); E, egg phosphatidylcholine (Type V-E) and bovine heart cardiolipin (1 : 1) and F, same as (E) plus bovine erythrocyte superoxide dismutase (9.6 mg). All samples contained 10 mg lipid and were incubated at 37°C for 24 h.

causes an increase in the order parameter. There is also a slight sharpening of the low-field A_{min} peak. The increase in S occurred in all cases, but was quite variable and ranged from 2 to 12% for different samples. The reason for this variability was not determined. These results correspond to those previously reported using this same egg phosphatidylcholine preparation [12].

A spectrum similar to that of I(12,3) in egg phosphatidylcholine (Type VII-E) was obtained from a 1 : 1 molar ratio of egg phosphatidylcholine (Type V-E)/bovine heart cardiolipin (Fig. 7E and F). The low-field A_{min} peak was broadened and shifted towards the A_{max} peak. Addition of superoxide dismutase caused a further shift of the A_{min} peak towards the A_{max} peak with little change in the order

TABLE V

ORDER PARAMETER (S) OF I(12,3) AND PERCENT CHANGE FROM CONTROL IN EGG PHOSPHATIDYLCHOLINE TYPES V-E AND VII-E AND EGG PHOSPHATIDYLCHOLINE/BOVINE HEART CARDIOLIPIN (1 : 1) WITH AND WITHOUT BOVINE RED BLOOD CELL SUPEROXIDE DISMUTASE

These measurements were made from the spectra in Fig. 7.

Sample	S	ΔS (%)
Egg phosphatidylcholine (Type V-E)	0.582	–
+ superoxide dismutase (13 mg)	0.581	−0.1
Egg phosphatidylcholine (Type VII-E)	0.562	–
+ superoxide dismutase (7.8 mg)	0.595	+6.0
Egg phosphatidylcholine (Type V-E)/cardiolipin	0.583	–
+ superoxide dismutase (9.6 mg)	0.580	−0.5

parameter, as with the Type V-E egg phosphatidylcholine alone. Thus, the increased order of the Type VII-E lipid is not simply due to the more broadened spectrum, but probably indicates a real increase in order due to superoxide dismutase.

Differential scanning calorimetry

The profile of the gel to liquid crystalline phase transition of the two types of egg phosphatidylcholine and the effect of bovine apo-superoxide dismutase upon this transition for the Type V-E lipid was investigated using DSC. Fig. 8A is a DSC scan of the Type V-E egg phosphatidylcholine. The enthalpy change (area under the curve) is about 6.1 cal/g, the transition (T_c) is centered at 260.3 K and the width at half-maximum is 9.5 K. The samples are run with approx. 50% ethylene glycol to lower the freezing point of the solvent mixture to below that of the lipid. A similar technique has been used to detect low temperature transitions in *Escherichia coli* and has been found to shift the outer membrane transition to a slightly higher temperature, but has no effect on the cytoplasmic membrane transition [19]. The egg phosphatidylcholine transition as detected with spin labels in samples containing no ethylene glycol agrees very well with the DSC result (results not shown). Thus, the ethylene glycol probably has no effect upon the transition of these samples.

Fig. 8B is a scan of egg phosphatidylcholine (Type

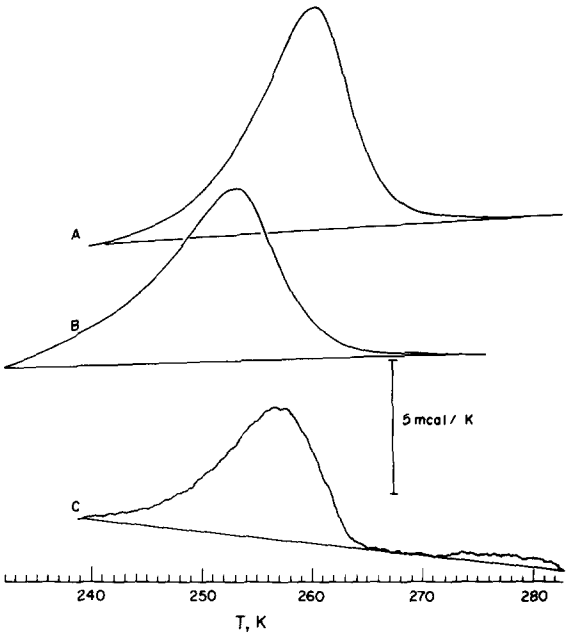

Fig. 8. DSC scans (temperature increasing) of A, egg phosphatidylcholine (Type V-E, 13.9 mg) liposomes; B, egg phosphatidylcholine (Type V-E, 13.2 mg) and bovine apo-superoxide dismutase (4.48 mg) and C, egg phosphatidylcholine (Type VII-E, 11.3 mg). The samples were incubated for 3 h at room temperature before the scans were taken.

V-E, 13.2 mg) and bovine apo-superoxide dismutase (4.48 mg). The ethalpy change is 6.0 cal/g, not significantly different from that for the lipid alone, T_c is centered at 253.2 K, about 7 K lower than that for the lipid alone and the width at half-maximum is 11.6 K, about 2 K greater than for the lipid alone. Scans were also taken with decreasing temperature which differed only in that the transition temperature was about 3 K lower than for the scans with increasing temperature.

The decrease in T_c correlates with the decrease in order due to the apo-superoxide dismutase. The increase in transition width, with no change in the enthalpy, indicates a loss of cooperativity due to superoxide dismutase, which is often associated with a decrease in order. The greater interaction of the apoenzyme with lipid is again illustrated, since the holoenzyme has no effect upon the transition of dimyristoylphosphatidylcholine.

Fig. 8C is a scan of the transition of the Type VII-E egg phosphatidylcholine. The enthalpy change

is 4.6 cal/g, significantly less than that for the Type V-E. The transition is centered at 257.1 K, about 3 K less than that for the Type V-E and the width at half-maximum is 10°C, about the same as for the Type V-E. The major difference between the Type V-E and Type VII-E scans is the appearance of another broad melting peak between 272 and 284 K. This peak probably is due to some of the impurities present in the Type VII-E lipid and may be caused by the free fatty acid impurities, which have a higher melting point than phospholipids with the same fatty acids [20]. These transition profile characteristics further exemplify the differences between the Type V-E and Type VII-E egg phosphatidylcholines and may help explain their different interactions with superoxide dismutase.

Discussion

Both the holo and apo forms of superoxide dismutase bind to egg phosphatidylcholine lipid bilayers. The holo form slightly perturbs and disorders the lipid packing but the apo form has a considerably greater effect. Probably because the apo form is taken up to a greater extent and appears to be embedded deeper into the bilayer, since it is more resistant to trypsin than the holo form in the presence of liposomes. After reconstitution of the apoenzyme with Cu^{2+} and Zn^{2+}, the degree of perturbation is very similar to that of the holoenzyme. The degree of perturbation increases with increasing concentration.

The interaction between superoxide dismutase and egg phosphatidylcholine is greatly dependent upon the condition of the lipid. In pure lipid, superoxide dismutase perturbs, but in egg phosphatidylcholine containing some impurities, mostly neutral lipids which are probably fatty acids and some oxidation products, superoxide dismutase orders the lipid packing. Thus, the final effect of superoxide dismuttase upon membranes is probably quite dependent upon the lipid composition.

The degree of perturbation increases with length of incubation at 37°C to a maximum value at about three days. This may indicate that there is an increase in uptake over this period of time or that once the superoxide dismutase binds it may slowly undergo a confirmational change so that it becomes a more effective perturber of lipid packing.

The disordering effect of superoxide dismutase as shown by spin labelling is confirmed by DSC. Bovine apoenzyme decreases and broadens the phase transition of egg phosphatidylcholine, indicative of a disordering effect. No effect of superoxide dismutase upon the phase transition of dimyristoylphosphatidylcholine was observable by DSC, again in confirmation with the spin labelling which only showed a disordering effect above the phase transition.

The binding of superoxide dismutase to lipid bilayers appears to be hydrophobic in nature, presumably due to the presence of some hydrophobic residues on the protein surface. The binding of the two superoxide dismutase subunits together appears to be primarily hydrophobic [21]. If the subunits separate when bound to a membrane, the exposed non-polar groups would be able to interact with the lipid interior. The increased perturbation and binding of the apoenzyme may be due to a confirmational change, upon removal of the Cu^{2+} and Zn^{2+}, which exposes additional hydrophobic residues on the surface of the protein. Evidence from NMR studies indicate a difference in the three-dimensional structure of the holo and apo forms, although it is not possible to specify the nature and degree of difference [22,23]. In the region of the active site, where the Cu^{2+} and Zn^{2+} are bound, the structural differences appear to be considerable [24].

There are a number of proteins soluble in water which can undergo an interaction with lipids. A spectrin-actin complex binds to and disorders phosphatidylserine [25]. Both oxyhemoglobin and denatured hemoglobin are found associated with erythrocyte membranes with considerably stronger binding of the denatured form [26].

Many membrane proteins order lipids upon binding but others have been shown to disorder. The spectrin-actin complex disorders and the B protein, an intrinsic membrane protein of *fd* phage, broadens and lowers the phase transition of dipalmitoylphosphatidylcholine [27].

The perturbing ability of superoxide dismutase from five different species was measured. The decrease in order parameter varied from 0.2% for bovine liver superoxide dismutase to 24.9% for yeast superoxide dismutase from Pharmacia, preparation II. The varying perturbing ability may be a measure of the difference in structure of superoxide dismutase

from the various species or it may be due to varying amounts of strongly perturbing contaminants, such as inactivated or an apo-like superoxide dismutase. The first possibility is quite likely, since several different preparations from the same species and from different organs of the same species had a similar effect. For example, the decrease in order parameter of the human and bovine superoxide dismutase was about 6 and 0.5%, respectively. The amino acid sequence of human and bovine superoxide dismutase differs in 27 residues out of 153 [28]. Thus, the three-dimensional structures may differ in such a way as to considerably affect the degree of perturbation.

The increased effect of the apoenzyme and radiation-inactivated superoxide dismutase may indicate that the membranes can act as a sink for inactivated forms of superoxide dismutase [29].

The binding of superoxide dismutase to membranes may account, at least in part, for the ability of the active enzyme to protect model lipid membranes against the radiation-sensitizing effect of oxygen [15]. Superoxide dismutase may also be able to protect cellular membranes from superoxide generated by other means, including metabolism.

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