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A Proposed Role of Superoxide Anion as a Biological Nucleophile in the Deesterification of Phospholipids

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Potassium superoxide dissolved in dry dimethyl sulfoxide effects rapid deesterification of ethyl hexadecanoate and of dilauroyl phosphatidyl choline. The reaction with ethyl hexadecanoate is reversible, having an apparent equilibrium constant of 0.4. It is proposed that some of the deleterious effects on biological membranes which have been attributed to oxidation by superoxide may actually be the result of deesterification by superoxide acting as a nucleophile.

INTRODUCTION

Superoxide ($O_2^{\cdot-}$) is a common intermediate in biological oxygen reduction (*1*). The superoxide radical exists in aqueous media at very low steady-state concentration because it readily undergoes a disproportionation to hydrogen peroxide and oxygen. At pH 7.4 the rate constant for this spontaneous dismutation is approximately $2 \times 10^5\ M^{-1}$ sec^{-1} (*1*). In aprotic solvents, however, superoxide is much more stable, having a half-life of several hours in dry dimethyl sulfoxide (DMSO)[1] (*2*) and several minutes in DMSO containing up to 20% water (see Results). Since the reduction of oxygen to superoxide by mammalian cells is intimately related to the function of membrane-bound enzymes (*3*, *4*), the possibility must be considered that appreciable amounts of superoxide exist in the nonaqueous interior of biological membranes. The role of superoxide in oxidative degradation of polyunsaturated fatty acids of biomembranes is consistent with such a distribution (*5*). Recent observations have shown that superoxide anion is also a potent nucleophile toward alkyl halides (*6–9*), and carboxylic esters (*10*, *11*). This communication reports the deesterification of fatty acid esters and of phospholipids by superoxide in dimethyl sulfoxide and proposes on the basis of these data that some of the observed biological effects of superoxide on membrane systems may be due to such a nucleophilic deesterification rather than an oxidative mechanism. Preliminary accounts of this work have been presented.[2]

[1] Abbreviations used: DMSO, dimethyl sulfoxide; 18-crown-6, 1,4,7,10,13,16-hexaoxycyclooctadecane.

[2] E. M. Gregory and W. G. Niehaus, Southeast Regional A.C.S. Meeting, Gatlinburg, Tennessee, October 28, 1976. W. G. Niehaus, 11th Annual Regional Lipid Conference, Winston-Salem, North Carolina, November 5, 1976.

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MATERIALS AND METHODS

Potassium superoxide and lithium hydride were obtained from Ventron, 1,4,7,10,13,16-hexaoxycyclooctadecane (18-crown-6) from Aldrich, and ethyl hexadecanoate, tridecanoic acid, and dilauroyl phosphadityl choline from Sigma. Dimethyl sulfoxide (Aldrich) was distilled from calcium hydride at reduced pressure. Diazomethane was prepared as an ethereal solution from Diazald (Aldrich).

Solutions of potassium superoxide were prepared by suspending finely powdered $KO_2^{\cdot}$ in a dimethyl sulfoxide solution of 18-crown-6. The mixture was stirred at room temperature in the dark for 30–60 min and excess potassium superoxide was removed by centrifugation. The resulting clear light yellow solution was quite stable at room temperature or frozen. Lithium ethoxide solutions were prepared by adding lithium hydride to absolute ethanol, evaporating the ethanol under N_2, and dissolving the residue in dry DMSO. A saturated solution was 14 m*M*, as determined by titration with standard acid.

Superoxide in solution was quantitated polarigraphically by injecting 10–50 μl into 5 ml of deionized water in a closed chamber containing a Clark oxygen electrode (Yellow Springs). One mole of oxygen is produced per 2 mol of superoxide undergoing disproportionation. Oxygen evolution as monitored with a Kiethley digital voltmeter was complete within 2 min. Correction was made for the oxygen dissolved in the DMSO. Superoxide concentrations calculated by this method agreed within 5% with those determined by titration with standard acid after dilution into deionized H_2O.

Deesterification of ethyl hexadecanoate by $KO_2^{\cdot}$ in DMSO was assayed by quenching the reaction mixture with 10 vol of 0.1 *N* HCl and extracting the hexadecanoic acid plus ethyl ester with diethyl ether. The ether solution was dried over $MgSO_4$ and treated with diazomethane to methylate the hexadecanoic acid. Methyl and ethyl hexadecanoate were separated and quantitated by gas–liquid chromatography on an SE-30 column using a Packard–Becker gas chromatograph with hydrogen flame ionization detector. The extent of deesterification was calculated from the ratio of the peak areas.

Deesterification of dilauroyl phosphatidyl choline was assayed in a similar manner, except that an internal standard of tridecanoic acid was introduced prior to ether extraction.

RESULTS

Solubility of $KO_2^{\cdot}$ in DMSO as a function of 18-crown-6. Stable solutions of superoxide in dimethyl sulfoxide were prepared at concentrations up to 75 m*M*, using 18-crown-6 to sequester the potassium counterion. The solubilization appears to be complete within 30 min as evidenced by an unchanging solubility product constant (Table 1).

Stability of superoxide in DMSO–H_2O mixtures. Solutions of superoxide in dry dimethyl sulfoxide are stable at room temperature for several hours, as was reported by Valentine and Curtis (*2*). Addition of deionized water to the solution results in a relatively slow loss of superoxide. The apparent half-life of superoxide is 40 min in DMSO:H_2O (90:10); 20 min in DMSO:H_2O (85:15); 5 min in DMSO:H_2O (80:20); and less than 1 min in DMSO:$H_2O \geq$ (70:30). These data are consistent with

TABLE 1

SOLUBILIZATION OF POTASSIUM SUPEROXIDE

18-crown-6 (mM)	$O_2^{\cdot}$ (mM)	$K = (K^+ \text{ crown})(O_2^{\cdot-})/(\text{crown})$
0	4	
20	20	65
37.5	30	60
75	50	70
150	75	60
		Mean 65

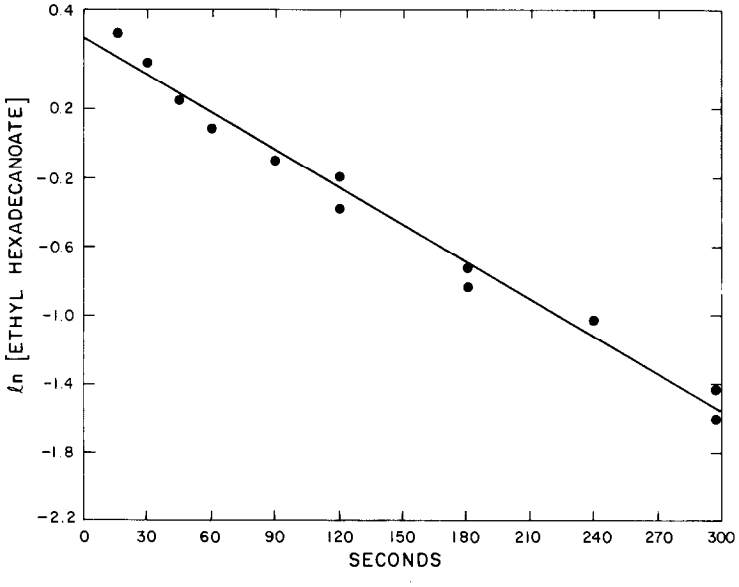

FIG. 1. Kinetics of deesterification of ethyl hexadecanoate by superoxide. Initial concentrations: ethyl hexadecanoate, 1.6×10^{-3} M; superoxide, 3.0×10^{-2} M.

the observation[3] that low concentrations of water have no effect on the rate of deacylation of ethyl hexadecanoate by superoxide.

Kinetics of desterification of ethyl hexadecanoate. The reaction between ethyl hexadecanoate (1.6 mM) and superoxide (30 mM) was followed at 25°C for up to 5 min. Reactions were terminated by addition of 1 ml of 1 N HCl, and the disappearance of ethyl hexadecanoate was assayed as described above. The data do not correlate well with a linear regression straight line plot of ln (ethyl hexadecanoate) vs time (Fig. 1), suggesting that this is not a simple irreversible first-order reaction. The half-time for deesterification of ethyl hexadecanoate under these conditions is about 45 sec.

Reversibility of the deesterification reaction. In order to determine whether the desterification of ethyl hexadecanoate is reversible, reactions were carried out with

[3] W. G. Niehaus, unpublished experiments.

superoxide in the presence of added ethoxide or methanol. Samples of ethyl hexadecanoate (1.1 mM) plus superoxide (25 mM) were allowed to react in the presence or absence of lithium ethoxide or methanol (4.2 mM). Ethoxide significantly slowed the net rate of deesterification (Fig. 2). In the presence of methanol, ethyl hexadecanoate is converted directly to the methyl ester. Since methanol is by many orders of magnitude a weaker acid than is the hydroperoxyl radical ($HO_2^{\cdot}$), these data cannot be explained by reaction of methanol with superoxide to generate methoxide. The apparent

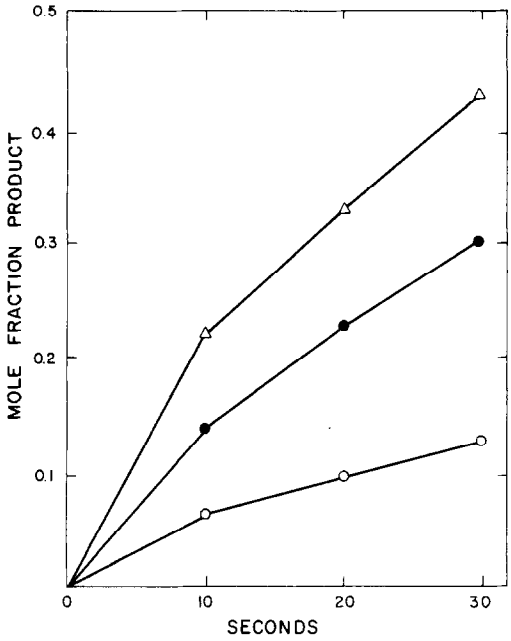

FIG. 2. Deesterification of ethyl hexadecanoate by superoxide (●—●), by superoxide plus ethoxide (○—○), and by superoxide plus methanol (△—△). Initial concentrations: ethyl hexadecanoate, 1.1×10^{-3} M; superoxide, 2.5×10^{-2} M; ethoxide, 4.2×10^{-3} M; methanol, 4.2×10^{-3} M.

half-life of superoxide in the presence of 400 mM methanol is about 1 hr and is much longer at lower methanol concentration. The reaction kinetics in the presence of methanol are complex, suggesting the existence of more than one intermediate species.[3] This reaction is at present undergoing further study.

Equilibrium constant for desterification of ethyl hexadecanoate. Ethyl hexadecanoate (1.6 mM) was incubated with superoxide (14 mM) in the presence of various initial concentrations of ethoxide. Reactions were terminated after 30 min, at which time no further reaction was apparent. Final concentrations of ethyl hexadecanoate and of its deacylation product were determined by gas chromatography after extraction and methylation. Final concentrations of ethoxide and superoxide were calculated assuming the stoichiometry of the reaction to be:

$$\text{Ethyl hexadecanoate} + \text{superoxide} \rightleftharpoons \text{deacylation product} + \text{ethoxide}$$

The apparent equilibrium constant is about 0.4 (Table 2). The precise identity of the

TABLE 2

DETERMINATION OF EQUILIBRIUM CONSTANT FOR THE DEESTERIFICATION OF ETHYL HEXADECANOATE BY SUPEROXIDE

Initial concentration (mM)			Final concentration (mM)		K_{eq}
Et 16:0[a]	$O_2^{\cdot-}$	OEt^-	Me 16:0	Et 16:0	$\frac{[Me\ 16:0][OEt^-]}{[Et\ 16:0][O_2^{\cdot-}]}$
1.6	14	0	1.3	0.3	0.4
1.6	14	2	1.1	0.5	0.5
1.6	14	4	0.8	0.8	0.4
1.6	14	6	0.6	1.0	0.3
					Mean 0.4

[a] Abbreviations used: Et 16:0, ethyl hexadecanoate; Me 16:0, methyl hexadecanoate.

deacylation product is unknown but may well be the peroxyacid radical, as suggested by San Filippo *et al.* (*10*).

Deesterification of phospholipids. In order to project the results of the deesterification experiments to biological systems, phospholipids were used as substrates. Phosphatidyl choline and phosphatidyl ethanolamine containing long-chain fatty acids (palmitoyl, stearoyl, oleoyl) were not sufficiently soluble in dimethyl sulfoxide to be analyzed, and hence the experiments were performed with dilauroyl (dodecanoyl) phosphatidyl choline. Parallel reaction mixtures containing an equimolar concentration of ester (0.9 mM ethyl hexadecanoate or 0.45 mM dilauroyl phosphatidyl choline) were

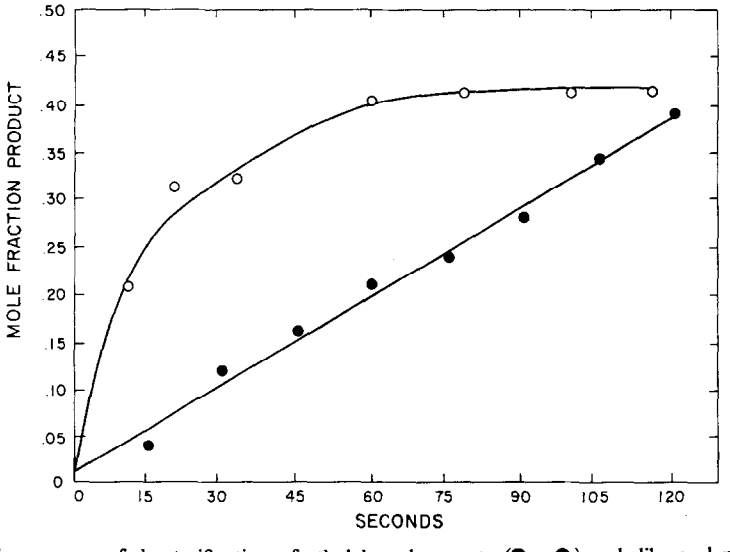

FIG. 3. Time course of deesterification of ethyl hexadecanoate (●—●) and dilauroyl phosphatidyl choline (○—○) by superoxide. Initial concentrations: ethyl hexadecanoate, 9×10^{-4} M; dilauroyl phosphatidyl choline, 4.5×10^{-4} M; superoxide, 3.6×10^{-3} M.

treated with 3.6 m*M* superoxide. The deesterification of phospholipid was appreciably faster than was that of the ethyl hexadecanoate (Fig. 3), which is consistent with the substituted glyceryl anion being a better leaving group than the ethoxide ion. No cleavage of the phosphate ester bonds was observed under these reaction conditions even after 1 hr, although a slow cleavage of triphenyl phosphate by superoxide in benzene has been reported (*10*).

The leveling off of the deesterification of the phospholipid approaching 50% reaction suggests that the diacyl phosphatidyl choline is a much better substrate than is the monoacyl product. This question, and that of positional specificity of the deesterification, are being approached using phospholipids with different fatty acid groups at the 1 and 2 positions.

DISCUSSION

The precise mechanism by which superoxide effects the cleavage of carboxylate esters is uncertain, although, as suggested by San Filippo *et al.* (*10*), a peroxy acid radical is a likely intermediate. In the present study, the identity of the carboxylic acid produced upon quenching of the reaction mixture with aqueous acid has been confirmed by gas chromatography–mass spectrometry.[3] The reaction rates observed in this study are about two orders of magnitude faster than those previously reported (*10*). This difference is presumably due to the use of a homogeneous dimethyl sulfoxide medium, rather than powdered $KO_2^{\cdot}$ in benzene. Recovery of reactant plus product carboxylic esters was quantitative.

The stability of the superoxide anion radical in dimethyl sulfoxide solutions containing a small proportion of water is consistent with the recent report (*11*) that disproportionation never involves reaction of two $O_2^{\dot{-}}$ molecules but only reaction of $O_2^{\dot{-}}$ with $HO_2^{\cdot}$. Since superoxide is a rather weak base ($pK_a = 4.8$ in H_2O), one would expect the relative concentration of the HO_2 species to be very low in aqueous DMSO. In this system the participation of water in the disproportionation reaction appears to be of a relatively high order.

The reduction of oxygen to superoxide in mammalian cells involves several enzymes which are intimately associated with the cell membranes (*3*, *4*). In cells other than erythrocytes and muscle cells, most of the oxygen is dissolved in the nonaqueous membrane interior rather than in the aqueous cytosol. Therefore, it is highly possible that enzymatic reduction of oxygen produces $HO_2^{\cdot}$, which is released from the enzyme into the aprotic membrane interior, where it is relatively stable. At the more polar membrane–water interface, however, the proton may dissociate, producing $O_2^{\dot{-}}$ in the immediate vicinity of the ester bonds of the phospholipids, which are therefore susceptible to nucleophilic cleavage.

The deleterious effects of superoxide on cell membranes has generally been assumed to result solely from peroxidation of the polyunsaturated fatty acids. The involvement of lipid peroxidation in membrane damage is amply documented by identification of oxidation products, and indirectly by demonstration of the protective effect of superoxide dismutase, catalase, and of the singlet oxygen scavengers histidine and mannitol (*12*). Peroxidation therefore likely involves singlet oxygen ($^1\Delta g$) which is generated by

spontaneous dismutation of superoxide and the subsequent reaction between superoxide and hydrogen peroxide (*13*). Generation of singlet oxygen and hydroxyl radical by the reaction described by Haber and Weiss (*14*) has been shown to be too slow to be of significance in biological systems (*15*, *16*).

Comparable membrane damage could also result from the deesterification of membrane phospholipids due to nucleophilic attack by superoxide, and deesterification may even be quantitatively the more significant effect of superoxide *in vivo*. The results of two recent studies are more easily explained by this second mechanism. Goldstein and Weissman (*17*) have shown that superoxide generated in aqueous solution increases the permeability of liposomes of egg phosphatidyl choline. Since egg phosphatidyl choline contains relatively few polyunsaturated fatty acid groups, the increased permeability is much more easily explained by a deesterification rather than peroxidation mechanism. Goldberg and Stern (*18*, *19*) have implicated superoxide anion as a mediator of drug-induced hemolysis of erythrocytes. Lipid peroxidation was not detected in their studies. It is therefore highly possible that the hemolysis was due to deesterification of membrane phospholipids by superoxide anion acting as a nucleophile. In a later study, Kellogg and Fridovich (*13*) clarified the role of oxygen in erythrocyte lysis, and proposed that superoxide and hydrogen peroxide gave rise to more reactive species which were responsible for lipid peroxidation. Since they did not demonstrate lipid peroxidation of the erythrocyte membranes, the role of superoxide as a nucleophile in this system remains to be investigated. Studies are in progress in this laboratory to investigate the involvement of superoxide-dependent deesterification in these and other systems dependent on the integrity of biomembranes.

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REFERENCES

1. I. FRIDOVICH, *Annu. Rev. Biochem.* **44**, 147 (1975).
2. J. S. VALENTINE AND A. B. CURTIS, *J. Amer. Chem. Soc.* **97**, 224 (1975).
3. R. A. PROUGH AND B. S. S. MASTERS, *Ann. N.Y. Acad. Sci.* **212**, (1973).
4. G. M. BARTOLI, T. GALEOTTI, G. PALOMBINI, G. PARISI, AND A. AZZI, *Arch. Biochem. Biophys.* **184**, 276 (1977).
5. T. PETERSON AND S. D. AUST, *Biochim. Biophys. Acta* **385**, 232 (1975).
6. M. V. MERRITT AND D. T. SAWYER, *J. Org. Chem.* **35**, 2157 (1970).
7. J. SAN FILIPPO, C.-I. CHERN, AND J. S. VALENTINE, *J. Org. Chem.* **40**, 1678 (1975).
8. R. A. JOHNSON AND E. G. NIDY, *J. Org. Chem.* **40**, 1680 (1975).
9. W. C. DANEN AND R. J. WARNER, *Tetrahedron Lett.* **11**, 989 (1977).
10. J. SAN FILIPPO, L. J. ROMANO, C.-I. CHERN, AND J. S. VALENTINE, *J. Org. Chem.* **41**, 586 (1976).
11. B. H. BIELSKI AND A. O. ALLEN, *J. Phys. Chem.* **81**, 1048 (1977).
12. E. F. KELLOGG AND I. FRIDOVICH, *J. Biol. Chem.* **250**, 8812 (1975).
13. E. W. KELLOGG AND I. FRIDOVICH, *J. Biol. Chem.* **252**, 6712 (1977).
14. F. HABER AND J. WEISS, *Proc. Roy. Soc. London* **A147**, 332 (1934).
15. J. J. VAN HEMMEN AND W. J. A. MEULING, *Biochim. Biophys. Acta* **402**, 133 (1975).

16. W. H. KOPPENOL, J. BULTER, AND J. W. VAN LEEUWEN, "International Symposium on Singlet Oxygen and Related Species in Chemistry and Biology," Pinawa, Manitoba, Canada, August 1977. Abstr. K9.
17. I. M. GOLDSTEIN AND G. WEISSMANN, *Biochem. Biophys. Res. Commun.* **75**, 604 (1977).
18. B. GOLDBERG AND A. STERN, *J. Biol. Chem.* **251**, 6468 (1976).
19. B. GOLDBERG AND A. STERN, *Arch. Biochem. Biophys.* **178**, 218 (1977).