Vol. 83, No. 3, 1978
August 14, 1978

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 927-932

THE ROLE OF THE SUPEROXIDE ANION IN THE XANTHINE OXIDASE-INDUCED AUTOXIDATION OF LINOLEIC ACID

Michael J. Thomas, Kathryn S. Mehl, and William A. Pryor*

Department of Chemistry
Louisiana State University
Baton Rouge, Louisiana 70803

Received April 14,1978

SUMMARY: The superoxide anion-radical ($O_2^{\cdot -}$) is an intermediate in the xanthine oxidase catalyzed autoxidation of linoleic acid. We have now found, however, that the rate of the xanthine oxidase catalyzed autoxidation of linoleic acid depends upon the initial concentration of conjugated diene hydroperoxide, the autoxidation product of linoleic acid. We propose that the reaction of diene hydroperoxide with $O_2^{\cdot -}$ produces alkoxy radicals that then initiate autoxidation. The rate constant for the diene hydroperoxide-$O_2^{\cdot -}$ reaction is $7 \times 10^3\ M^{-1}\ s^{-1}$.

The autoxidation of polyunsaturated fatty acids (PUFA)** has been studied extensively as a model for *in vivo* processes in which reactive free radicals cause pathology or cell death (1). A system that has been examined from this viewpoint is the autoxidation of PUFA initiated by xanthine oxidase (E. C. No. 1.2.3.2), an enzyme that is known to generate the superoxide anion radical (2). Superoxide itself does not initiate the autoxidation of PUFA (3a), and the mechanism(s) responsible for the initiation has (have) been difficult to identify. The first suggestion, and one that appeared to be quite reasonable, was that the hydroxy radical (HO·), formed from superoxide via eqs 1 and 2, is the critical initiator (4). However, it was subsequently demonstrated by

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + HO\cdot + HO^- \qquad (2)$$

several workers that eq 2 is much too slow to compete with the dismutation of

*To whom reprint requests should be addressed.

**Abbreviations used: DTPA, diethylenetriamine pentacetic acid; HPLC, high pressure liquid chromatography; LOOH, a mixture of 9-hydroperoxy-10,12-octadecadienoic and 13-hydroperoxy-9,11-octadecadienoic acids; PUFA, polyunsaturated fatty acid; SOD, bovine superoxide dismutase.

0006-291X/78/0833-0927$01.00/0

superoxide, eq 1, even at very high H_2O_2 concentrations, and it can be convincingly argued from this that eq 2 cannot be responsible for generating HO· radicals in the xanthine oxidase-PUFA system (5).

Other researchers have suggested that Fenton-type reactions, eqs 3 and 4, between chelated iron and H_2O_2 yield hydroxy radicals (3). Since biological systems have a plethora of metal ions and chelating compounds, this suggestion is quite reasonable.

$$O_2^{-}\cdot + \text{ADP-Fe(III)} \longrightarrow O_2 + \text{ADP-Fe(II)} \qquad (3)$$

$$\text{ADP-Fe(II)} + H_2O_2 \longrightarrow \text{ADP-Fe(III)} + HO\cdot + HO^{-} \qquad (4)$$

We here offer evidence for another mechanism for xanthine oxidase initiated autoxidation. We propose that PUFA autoxidation can be initiated by reaction of superoxide anion radicals with PUFA hydroperoxides, LOOH, that are present as impurities in PUFA, eq 5. This reaction produces alkoxy radicals,

$$O_2^{-}\cdot + \text{LOOH} \longrightarrow O_2 + LO\cdot + HO^{-} \qquad (5)$$

LO·, that initiate PUFA autoxidation, thus leading to more PUFA hydroperoxide molecules. We have demonstrated that hydroperoxidic impurities are necessary for autoxidation to be initiated under our conditions, and we have shown that eq 5 is sufficiently fast to compete with eq 1.

MATERIALS AND METHODS

Xanthine oxidase and diethylenetriamine pentacetic acid (DTPA), were obtained from Sigma Chemical Corp. Dicyclohexyl-18-crown-6 (purified by chromatography on alumina (6) before use) and sodium borohydride ($NaBH_4$) were purchased from Aldrich Chemical Co. Xanthine and linoleic acid were obtained from Calbiochem. Dimethyl sulfoxide (DMSO), which was used after drying over molecular sieves, and acetaldehyde (distilled before use) were obtained from Mallinckrodt Chemical Works. Potassium superoxide was obtained from Alpha Inorganics, 96%, Lot No. 11075. Bovine superoxide dismutase (E.C. No. 1.15.1.) was a gift from Dr. J. McCord.

Optical measurements were recorded on a Beckman Model 24 Spectrophotometer. High pressure liquid chromatography was carried out on a Waters Associates Model ALC-202 Liquid Chromatograph equipped with a Waters Model M-6000 chromatography pump. Analytical separations were performed with a Waters 30 cm x 3.9 mm μ-Porasil-packed column. Analysis of the products from superoxide reduction of methyl linoleate hydroperoxides was accomplished using 0.3% 2-propanol in hexane as eluant.

Diene Oxidations. The formation of diene hydroperoxide from linoleic acid was followed at 235 nm in a system consisting of 6.3×10^{-8} M xanthine oxidase, 48 mM acetaldehyde, 1 mM linoleic acid, and 0.1 mM DTPA, and 1.72 M ethanol. These experiments were carried out in 0.05 M phosphate buffer at pH 8.1, and with a total reaction volume of 3 ml. The kinetic experiments utilizing superoxide dismutase (SOD) contained SOD concentrations ranging from 0.4 to 0.02 nM in addition to the other compounds.

RESULTS AND DISCUSSION

Our evidence that the reaction of $O_2^{\cdot-}$ with LOOH, eq 5, is responsible for the initiation of autoxidation of linoleic acid comes from several types of experiments. Using the system described for diene oxidations (in Materials and Methods), we have found that: (1) Superoxide dismutase inhibits 97% of the autoxidation. (2) The initial rate of autoxidation, followed by monitoring the formation of LOOH at 235 nm, is proportional to the initial concentration of LOOH. (3) Linoleic acid purified by high pressure liquid chromatography (HPLC) undergoes slow autoxidation and the rate of the appearance of LOOH is identical, within experimental error, to that of spontaneous autoxidation in the absence of xanthine oxidase. (4) Sodium borohydride ($NaBH_4$) reduction of unpurified linoleic acid containing 26 mM LOOH yields a linoleic acid sample that still has a high absorption at 235 nm (due to conjugated dienol) but has a much slower rate of oxidation compared to the original unreduced acid sample. (5) Both unpurified and $NaBH_4$-treated linoleic acid do not inhibit xanthine oxidase, since they do not affect the xanthine oxidase-catalyzed conversion of xanthine to uric acid. Linoleic acid purified by HPLC caused only a 14% inhibition of uric acid formation.

Having established that both superoxide and LOOH are required for the initiation of autoxidation, we examined the products from reaction of LOOH with potassium superoxide. Peters and Foote (7) had previously shown that tetramethylammonium superoxide reacts with *tert*-butyl hydroperoxide by a free radical mechanism to give 90% *tert*-butyl alcohol (plus 10% acetone), eq 6.*

*Gibian and coworkers find that KO_2-18-crown-6 complex in toluene does not react with *tert*-butyl hydroperoxide (8), and that several superoxide reactions may involve proton abstraction (9). These observations do not contradict the conclusion of Peters and Foote (7) that the alkoxy radical is the free radical formed from reaction of KO_2 with hydroperoxide in polar solvents.

$$tert\text{-}C_4H_9\text{-}OOH + (CH_3)_4\overset{+}{N}\ O_2^{-\cdot} \longrightarrow tert\text{-}C_4H_9\text{-}OH + O_2 + (CH_3)_4\overset{+}{N}\ OH^- \quad (6)$$

We have found that LOOH undergoes a similar reaction. In a typical experiment, 4.4 μmole of LOOH in 90 μl of acetonitrile-hexane (2:1) was treated with approximately 3 μmole of potassium superoxide-dicyclohexyl-18-crown-6-complex in 150 μl DMSO. Analysis of the reaction by thin-layer chromatography and by HPLC showed that approximately half of the hydroperoxide was reduced and that the mixture of conjugated dienols that was formed is identical to that formed from the $NaBH_4$ reduction of LOOH.

Since it was the slow rate of reaction of superoxide with H_2O_2 that ruled out eq 2 as the source of the radicals that initiate autoxidation at physiological pH, it was necessary to determine whether the reaction of superoxide with LOOH, eq 5, was sufficiently rapid to be significant under these same conditions. The rate constant for eq 5 was determined by studying the effect of superoxide dismutase (SOD) on the rate of autoxidation, under the experimental conditions described above. Equations 1, 5, 7, 8, and 9 were used to derive a rate expression for the formation of LOOH, eq 10, in the presence of superoxide dismutase (R_{sod}). Equation 9 represents reactions, other than those listed, which may consume superoxide. Changes in the rates

$$\text{xanthine oxidase} + CH_3CHO \longrightarrow O_2^{-\cdot} \quad (7)$$

$$SOD + 2O_2^{-\cdot} \xrightarrow{\text{fast}} SOD + O_2 + H_2O_2 \quad (8)$$

$$O_2^{-\cdot} + X \longrightarrow \text{loss of } O_2^{-\cdot} \quad (9)$$

of the reactions described in eqs 1 and 9 are assumed to be relatively small under our experimental conditions, and the rate constants for eqs 1 and 9 combined are represented by the rate constant k_d. The expression describing the ratio of the rates of autoxidation in the absence (R_o) and in the presence (R_{sod}) of superoxide dismutase is given in eq 10. When the reciprocals

$$R_o/R_{sod} = 1 + 2k_8[SOD]/(k_5[LOOH] + k_d) \quad (10)$$

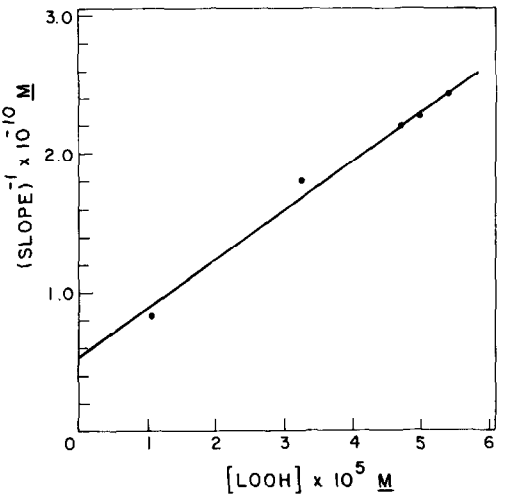

Fig. 1. A plot of the reciprocal of the slopes from R_o/R_{sod} versus [SOD]-plots (i.e., plots of eq 10) versus LOOH concentration.

of the slopes of a series of R_o/R_{sod} versus [SOD] plots (eq 11), carried out

$$(\text{Slope})^{-1} = \frac{k_5[\text{LOOH}]}{2k_8} + \frac{k_d}{2k_8} \qquad (11)$$

with different concentrations of LOOH, are plotted versus [LOOH], k_5 and k_d can be found from the slope and the intercept. Figure 1 shows a plot of Slope^{-1} versus [LOOH] and has a slope of 3.5×10^{-6} and an intercept of 0.52×10^{-10} M. Since $2k_8 = 2 \times 10^9$ M^{-1} s^{-1} (10), k_5 and k_d are calculated to be 7×10^3 M^{-1} s^{-1} and 0.1 s^{-1}, respectively. The value of k_d obtained is in acceptable agreement with previous, more direct measurements of k_1 (2).

These experiments show that superoxide reacts with LOOH, and, furthermore, that the initial rate of autoxidation of linoleic acid induced by xanthine oxidase is proportional to the initial concentration of LOOH that is present as an impurity in the linoleic acid. By analogy to the reaction of *tert*-butyl hydroperoxide with superoxide, the LOOH-superoxide reaction probably yields an alkoxy radical which initiates autoxidation. Under our conditions the LOOH-superoxide reaction is competitive with superoxide dispropor-

tionation, eq 1. Since $k_8 = 7 \times 10^3\ M^{-1}\ s^{-1}$, we predict that the superoxide-LOOH reaction will be competitive with eq 1 at physiological pH (≈7.4) at LOOH concentrations down to approximately 1 μM.

We suggest that the reaction of superoxide with PUFA and lipid hydroperoxides must be considered as a new and important source of the radicals that can initiate the autoxidation of PUFA *in vivo* (11). It might be presumed that eq 5 would be most important in lipophilic regions of the cell, such as membranes (1,9). The first LOOH molecules necessary to cause eq 5, and hence to initiate further conversion of PUFA to LOOH, could arise either from enzymatic conversion of PUFA to hydroperoxides (e.g., by lipoxidases or PG-synthetases) or by some spontaneous autoxidation process (11).

ACKNOWLEDGEMENT. This research was supported by a grant from the National Institutes of Health.

REFERENCES

1. Mead, J. F. (1976) in "Free Radicals in Biology" (W. A. Pryor, Ed.), Vol. I, pp. 51-68, Academic Press, New York, and references therein.
2. McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem., 244, 6049-6055.
3. (a) Fong, K.-L., McCay, P. B., and Poyer, J. L. (1976) Chem.-Biol. Interactions, 15, 77-89.
   (b) McCay, P. B., Floyd, R. A., Lai, E. K., Poyer, J. L., and Fong, K.-L. (1976) Biophys. J., 16, 66a.
   (c) McCord, J. M. and Day, E. D. (1978) FEBS Lett., 86, 139-142.
4. Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem., 245, 4641-4646.
5. (a) Bray, W. (1938) J. Amer. Chem. Soc., 60, 82-86.
   (b) George, P. (1947) Disc. Faraday Soc., 21, 196-205.
   (c) Dainton, F. S. and Rowbottom, J. (1953) Trans. Faraday Soc., 49, 1160-1173.
   (d) McClune, J. G. and Fee, J. A. (1976) FEBS Lett., 67, 294-298.
   (e) Halliwell, B. (1976) FEBS Lett., 72, 8-10.
   (f) Rigo, A., Stevanato, R., Finazzi-Agro, A. and Rotilio, G. (1977) FEBS Lett., 80, 130-132.
6. Pedersen, C. J. (1967) J. Am. Chem. Soc., 89, 7017-7036.
7. Peters, J. W. and Foote, C. S. (1976) J. Am. Chem. Soc., 98, 873-875.
8. Gibian, M. J. and Ungermann, T. (1976) J. Org. Chem., 41, 2500-2502.
9. Sawyer, D. T., Gibian, M. J., Morrison, M. M., and Seo, E. T. (1978) J. Am. Chem. Soc., 100, 627-628.
10. (a) Klug, D., Rabani, J., and Fridovich, I. (1972) J. Biol. Chem., 247, 4839-4842.
    (b) Rotilio, G., Bray, R. C., and Fielden, E. M. (1972) Biochim. Biophys. Acta, 268, 605-609.
11. Pryor, W. A. (1976) in "Free Radicals in Biology" (W. A. Pryor, Ed.), Vol. I, pp. 1-50, Academic Press, New York.