Mechanism of Lipoxidase Reaction. II. Origin of the Oxygen Incorporated into Linoleate Hydroperoxide[1]

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ABSTRACT

Two different series of experiments were performed to establish the origin of the oxygen molecule incorporated into hydroperoxide during the incubation of lipoxidase with linoleic acid. These showed, as previously assumed but never demonstrated, that the oxygen introduced into the hydroperoxide molecules comes from the gaseous phase and not from the aqueous phase. Furthermore, soybean lipoxidase does not catalyze the exchange between gaseous oxygen and water oxygen. Possibly, lipoxidase may be involved in the biosynthesis of hydroxy *trans,cis* conjugated octadecadienoates present in various seeds.

INTRODUCTION

As stated in a preliminary communication (1), it has always been assumed, but never demonstrated, that the oxygen molecule, incorporated into the hydroperoxide as a result of lipoxidase-catalyzed oxidation of linoleic acid, comes from the gas phase.

Publications before 1963 on the nature, mechanism of reaction and specificity of soybean lipoxidase reactions have been summarized by Tappel (2). In 1965, Hamberg and Samuelsson (3) suggested that lipoxidase attacks the ω-6 carbon atom specifically. They showed that lipoxidase-catalyzed oxidation of linoleic acid produced 13-hydroperoxyoctadeca-9,11-dienoic acid and 9-hydroperoxyoctadeca-10,12-dienoic acid in a ratio of 7:3. Following our development of an analytical procedure that permits the quantitative separation of methyl 13-hydroxystearate from methyl 9-hydroxystearate (4), an even higher specificity for soybean lipoxidase was discovered; i.e., the exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid (5).

The assumed source of the oxygen molecule in lipoxidase oxidation became suspect because of observations on hydrogenation of sorbic acid (6). During the homogeneous catalytic hydrogenation of this acid with pentacyanocobaltate, the hydrogen incorporated into the hydrogenated molecule originated in the H_2O molecule and not in the H_2 gas phase as predicted.

Two kinds of experiments were performed which demonstrated that lipoxidase does not catalyze oxygen exchange between the water and gas and that the oxygen incorporated into the hydroperoxide molecule by lipoxidase does indeed come from the gas phase.

Based on the data available from this series of investigations on the mechanism of reaction of lipoxidase, which correlate with recent advances in seed lipids chemistry, a biosynthetic role for lipoxidase is quite possible and is postulated.

EXPERIMENTAL

Two types of experiments were designed to determine where the oxygen incorporated into hydroperoxide originates: the first, incubation of lipoxidase with linoleic acid in a $H_2{}^{18}O$ buffer with $^{16}O_2$ in the gas phase; and the second, the same incubation but in normal $H_2{}^{16}O$ buffer with isotopic $^{18}O_2$ in the gas phase.

Incubation of Lipoxidase with Linoleic Acid

In $H_2{}^{18}O$ Buffer. Incubation was essentially as described previously (5), including treatment of the incubated products, reduction of the hydroperoxide with $NaBH_4$, methylation with diazomethane, reduction of the double bonds with 5% Pd on C, fractionation by column chromatography and analysis by mass spectrometry of the methyl hydroxystearate. The borate buffer was prepared from water enriched with 5.41 atom percent ^{18}O (Yeda Research and Development Co., Rehovoth, Israel).

In $^{18}O_2$ Gas Phase. A special flask (125 ml, round bottom) was designed (Fig. 1) that permitted us to introduce, through neck A, 80.0 ml of 0.05 M, borate buffer (pH 9), 180 mg linoleic acid (99.8% pure, Hormel Institute) dissolved in 10.0 ml 95% ethanol, 15 mg

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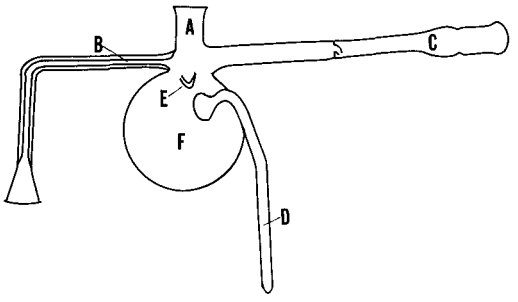

FIG. 1. Flask designed for incubation of lipoxidase with linoleic acid under $^{18}O_2$ gas: *A*, neck, 20 mm diameter; *B*, capillary tubing with joint leading to a Toepler pump; *C*, arm equipped with break seal for recovery of gas; *D*, a "cold finger" for O_2 condensation; *E*, glass hook from which to suspend glass basket with enzyme; *F*, round bottom flask, 125 ml.

crystalline lipoxidase (Nutritional Biochemical Corporation) suspended in a glass basket on glass hook E inside the flask and a small Teflon-coated magnet. The flask was then immersed in a −80C mixture of dry ice-methyl Cellosolve. After the contents of the flask were frozen, neck A was sealed. Neck B was then connected to a Toepler pump, which, in turn, was connected to a high-vacuum mercury-diffusion pump manifold. The whole system was then evacuated to 10^{-3} mm Hg. A Dewar flask filled with liquid N_2 was then raised around "cold finger" D to condense oxygen as it was introduced by the Toepler pump and to permit transfer of 100 ml oxygen into about a 45 ml headspace. The break seal on the $^{18}O_2$ reservoir (100 ml, 91.6 atom percent ^{18}O, Yeda, Rehovoth, Israel) was then broken with a magnet and the gas transferred into the reaction flask by 11 strokes of the Toepler pump. On the final cycle of the Toepler pump, the mercury was allowed to rise into the capillary joint and the tubing (neck B) was sealed off at 1 cm above the mercury.

Contents of the flask were thawed and brought up to room temperature by swirling carefully in a 25C water bath. After the basket of enzyme was released into the substrate, the mixture was stirred with a magnetic stirrer for 60 min. The sealed flask was again placed in the −80C mixture, frozen and connected through arm C to the high-vacuum manifold. After a 10^{-3} mm Hg pressure was reached, the seal on arm C was broken with a magnet and the gas phase over the reaction mixture was pumped through the Toepler pump and collected in another flask, also immersed in liquid N_2. This recovered gas was later analyzed by mass spectrometry. The reaction mixture was treated and carried through the same sequence of reactions as the products of the incubation of lipoxidase with linoleic acid in $H_2{}^{18}O$ buffer.

Analytical Procedures

Gas-liquid (GLC), thin-layer (TLC), and column chromatography, infrared (IR) and mass spectrometry were performed as described earlier (5). Ultraviolet (UV) spectra were taken on a Cory 14 recording spectrophotometer. The following analyses were performed before and after hydrogenation: GLC, TLC, IR and UV.

RESULTS AND DISCUSSION

The results of Experiment 1, in which lipoxidase was incubated with linoleic acid in a buffer with either enriched $H_2{}^{18}O$ or $H_2{}^{16}O$ under 1 atmosphere of $^{16}O_2$ gas, are presented in Table I. About 70–75% of the substrate in both incubations was recovered as 13-hydroperoxy-9,11-octadecadienoic acid and the rest as the original linoleic acid. No ^{18}O was de-

TABLE I

Analysis of Products from Incubation of Linoleic Acid in $H_2{}^{18}O$ and $H_2{}^{16}O$ with Lipoxidase and $^{16}O_2$ Gas

Product	Separation on column[a]	GLC determination[b]	IR a_{3640} cm^{-1}	Mass spectrometry analysis ^{16}O[c]	Mass spectrometry analysis ^{18}O[c]
In $H_2{}^{18}O$ buffer					
Methyl stearate	26.6	24.1	....	100	....
Methyl 13-ketostearate	19.8	19.1	....	100	....
Methyl 13-hydroxystearate	53.6	56.8	0.1651	100	....
In $H_2{}^{16}O$ buffer					
Methyl stearate	25.1	25.7	....	100	....
Methyl 13-ketostearate	18.2	15.6	....	100	....
Methyl 13-hydroxystearate	56.7	58.7	0.1665	100	....

[a] Weight %.

[b] Area %.

[c] Atom %.

tected in the final reduced product; i.e., methyl 13-hydroxystearate. Hence, lipoxidase cannot utilize oxygen atoms from water in forming hydroperoxide.

The analytical results of Experiment 2, in which lipoxidase was incubated with linoleic acid in $H_2{}^{16}O$ buffer and under $^{18}O_2$ gas, are given in Table II and Figure 2.

Oxygen pressures varied during these reactions because 100 ml oxygen (at STP) was introduced into a 45 ml headspace. Since about 15 ml oxygen was incorporated into the hydroperoxide molecules, there was a pressure drop from 2.2 atmospheres to 1.9 atmospheres during the reactions. In spite of this pressure change, there was no significant difference between the products produced in Experiments 1 and 2. Also, the control run with $^{16}O_2$ gas yielded the same product composition as the run with $^{18}O_2$. Analysis of the products showed that no ^{18}O was present in the methyl stearate fraction. Both methyl 13-hydroxystearate and methyl 13-ketostearate (hydrogenation byproduct, see below) contained more than 80 atom percent ^{18}O (84.0 and 81.8%, respectively). The difference between the ^{18}O enrichment in the final products and the level of ^{18}O in the gas used can be attributed mainly to the $^{16}O_2$, which remained dissolved in the buffer. In the mass spectrum (Fig. 2), all fragments containing hydroxyl oxygen (5) are seen to have shifted two mass numbers higher than the corresponding ^{16}O fragments.

As suspected, the methyl 13-ketostearate is a byproduct of the hydrogenation of methyl 13-hydroxy-9,11-octadecadienate. The following observations support this statement: No ketone fraction is present in the methylated incubation product of lipoxidase with linoleic acid; yet after catalytic hydrogenation of the incubation products, about 18% of the original linoleic acid is converted to methyl 13-ketostearate. In addition, the keto group has the same level of ^{18}O enrichment as the alcohol group in the methyl 13-hydroxystearate fraction.

Experiment 2 establishes that the oxygen molecule incorporated into the hydroperoxide comes from the gas phase.

IR spectrum of the methyl 13-hydroxystearates indicated a strong, sharp hydroxy peak at 3640 cm^{-1}. Introducing ^{18}O instead of ^{16}O into the molecule did not cause any change in wavelength of the hydroxy peak (Table I). The hydroxystearate from reduction of the hydroperoxide produced by lipoxidase oxidation of linoleic acid exhibited optical activity in contrast to the hydroxystearate from autoxidation of methyl linoleate.

TABLE II

Analysis of Products from Incubation of Linoleic Acid with Lipoxidase with $^{18}O_2$ or $^{16}O_2$ in $H_2{}^{16}O$ Buffer

Product	^{16}O[a]	^{18}O[a]	Total incubation product Composition[b]
With $^{16}O_2$ gas			
Methyl stearate	100	....	24.7
Methyl 13-ketostearate	100	....	18.9
Methyl 13-hydroxystearate	100	....	56.4
With $^{18}O_2$ gas			
Methyl stearate	100	....	24.1
Methyl 13-ketostearate	14.2	81.8	18.2
Methyl 13-hydroxystearate	14.1	84.0	57.7

[a] Atom %.
[b] Weight %.

Mass spectrometry analysis of the recovered gas after the $^{18}O_2$ incubation, indicated essentially the same composition of the original gas; i.e., 90.7 atom percent ^{18}O and 9.3 atom percent ^{16}O.

If lipoxidase had catalyzed the exchange of oxygen between the aqueous and the gaseous phases, some of the aqueous heavy oxygen would have been present in the gaseous oxygen and incorporated by the enzyme into the hydro-

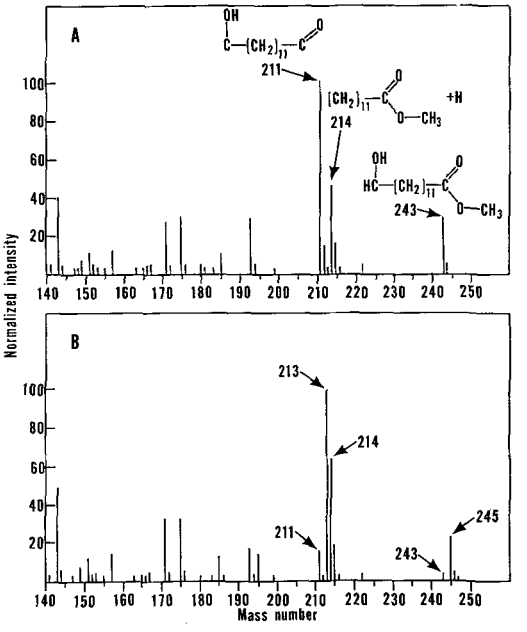

FIG. 2. Mass spectrometry fractionation pattern of methyl 13-hydroxystearate recovered from the reduced incubation products of lipoxidase with linoleic acid under $^{16}O_2$ gas (A) and under $^{18}O_2$ gas (B). The hydroxyl group containing fragments are two mass units higher in B than in A, owing to the ^{18}O incorporation.

peroxide. Since no isotopic ^{18}O was found in the methyl 13-hydroxystearate produced by Experiment 1, lipoxidase does not catalyze this exchange.

In recent publications by Ryhage, Samuelsson and Hamberg (7–9), essentially the same procedure was used to study the incorporation of oxygen in the conversion of 8,11,14-eicosatrienoic acid to prostaglandin E_1. They suggest that the introduction of a hydroxy group to C-15 of prostaglandin E_1, which involves also the isomerization of a double bond, is analogous to lipoxidase oxidation. In a review on seed lipids, Wolff (10) names the different hydroxy fatty acids in seeds containing exclusively 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid, 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid, or a mixture of both. The positions of the hydroxyl group (2,5,10), the optical activity of those compounds, the conjugated diene system, and the *trans* configuration of the unsaturated bond nearest the hydroxyl group, all point to the possibility that lipoxidase may be involved in the biosynthesis of these hydroxy *trans,cis* conjugated octadecadienoates.

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