Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 339-346

SOYBEAN LIPOXYGENASE-CATALYZED OXIDATIONS BY LINOLEIC ACID HYDROPEROXIDE : DIFFERENT REDUCING SUBSTRATES AND DEHYDROGENATION OF PHENIDONE AND BW 755C

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Received January 15, 1988

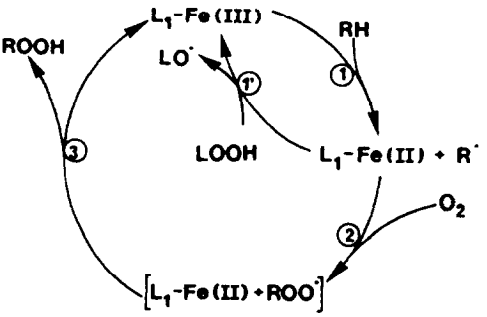
Summary : Phenidone is not a substrate for dioxygenation by soybean lipoxygenase-1 (L_1) but reduces $L_1Fe(III)$ into $L_1Fe(II)$, as shown by EPR spectroscopy. L_1 catalyzes the oxidation of phenidone by 13-HPOD, the hydroperoxide formed by dioxygenation of linoleic acid by L_1, with formation of 4,5-dehydrophenidone. Two moles of 13-HPOD are used per mole of phenidone dehydrogenated. Other pyrazoline derivatives such as BW 755C, but also, in a more general manner, different compounds containing phenol, aniline, hydrazine, hydroxylamine or hydrazide functions act as reducing substrates for decomposition of 13-HPOD by L_1.

Interest in the mechanism of lipoxygenases has been stimulated these last years by the discovery of the implication of lipoxygenases in the biosynthesis of leukotrienes which have an important role in inflammation and immediate hypersensitivity (1). Soybean lipoxygenase-1 (L_1) is particularly interesting for detailed mechanistic studies since it can be easily purified in large amounts and clear data are available on its catalytic cycle (see scheme 1 with RH = linoleic acid) (2). It catalyzes dioxygenation of linoleic acid to 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPOD). So far, very few compounds are known to act as substrates for the complete dioxygenation cycle by L_1 (scheme 1). They are all fatty acids possessing a cis,cis-1,4-pentadiene unit (2) or their analogs (3), and phenylhydrazones (4).

As a part of our current studies on compounds that can interact with L_1 active site and undergo a L_1-catalyzed oxidation by O_2 or other oxidizing agents, we show herein that (i) L_1 catalyzes the oxidation by 13-HPOD of phenidone and BW 755C, two classical inhibitors of lipoxygenases and cyclooxygenases (5,6), into the corresponding dehydrocompounds, and (ii) not only phenidone and BW 755C but also many compounds containing phenol, aniline,

ABBREVIATIONS : L_1 : soybean lipoxygenase-1, 13-HPOD : 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid, DETAPAC : diethylenetriamine-pentaacetic acid, BW 755C : 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline, NDGA : nordihydroguaiaretic acid, phenidone : 1-phenyl-3-pyrazolidone.

0006-291X/88 $1.50



Scheme 1 . Catalytic cycle of dioxygenation by L_1 (RH = linoleic acid, steps 1, 2, 3) and catalytic cycle of oxidations by L_1 and 13-HPOD (= LOOH) (RH = reducing agent as phenidone, steps 1 and 1').

hydrazine, hydroxylamine or hydrazide functions reduce L_1Fe(III) into L_1Fe(II) and are oxidized by 13-HPOD in the presence of L_1.

MATERIALS AND METHODS

BW 755C was a gift from Wellcome Lab. and ketoprofen from Rhône-Poulenc. 1-phenyl-3-hydroxy-pyrazole (4,5-dehydrophenidone), 1-phenyl-3-amino-2-pyrazoline and 1-phenyl-2-methyl-3-pyrazolidone were respectively prepared according to ref. 7, 8 and 9. N-hydroxyamphetamine, 4-methylbenzaldehyde-4'-bromophenylhydrazone and N'-phenylbenzoylhydrazide were prepared by previously described procedures (10,11,12). All other chemicals were purchased from commercial sources at the highest level of purity available. Linoleic acid (99 %, from Sigma) was purified under argon as reported previously (4). 13-HPOD (λmax = 234 nm, ϵ = 25 mM^{-1} cm^{-1}) was prepared enzymatically by a described procedure (13) and stored under argon in isopropanol at -18°C.

L_1 was purified from seeds (King Soy variety) by a described method (14,4) (λmax_1 = 280 nm, ϵ = 160 mM^{-1} cm^{-1}). Its specific activity was about 120 μmol.min^{-1}.mg^{-1}.

UV-visible measurements were done with an Uvikon 810 Kontron spectrophotometer. Reactions between L_1, 13-HPOD and various compounds were performed at 20°C in 50 mM tris-acetate buffer pH9 containing 0.1 mM DETAPAC in 3 ml cuvettes. Compounds were added from 10^{-2} M solutions in methanol and we verified that equal amounts of pure methanol had no significant effects on L_1.

Oxygen uptake was measured with a Gilson oxygraph equipped with a Clark electrode in a 1.2 ml thermostated (19°C) cell under conditions identical to those used for spectrophotometric assays.

HPLC analysis (after 10 min) of anaerobic incubations of 10 μM phenidone with 10 μM 13-HPOD and 0.5 μM L_1 in 3 ml buffer pH9 were done after acidification to pH5 by addition of 1 M citric acid to stop phenidone oxidation. Separations were made on a Nucleosil C18 reverse-phase column (Bischoff) (5 μm, 12.5 cm). For phenidone and its dehydroderivative, an isocratic elution mobile of a 35:65 mixture of CH_3CN : 0.1 M ammonium acetate buffer pH4.6 (1 ml per min) and UV detection at 254 nm were used. For 13-HPOD, elution mobile was a 70:30 CH_3CN : same buffer mixture and UV detection at 234 nm.

RESULTS

100 μM solutions of phenidone in 50 mM tris-acetate buffer pH9 consume dioxygen at a very low rate because of the slow autoxidation of this compound (15). In the presence of L_1, even in large amounts (0.2 μM), no increase of O_2

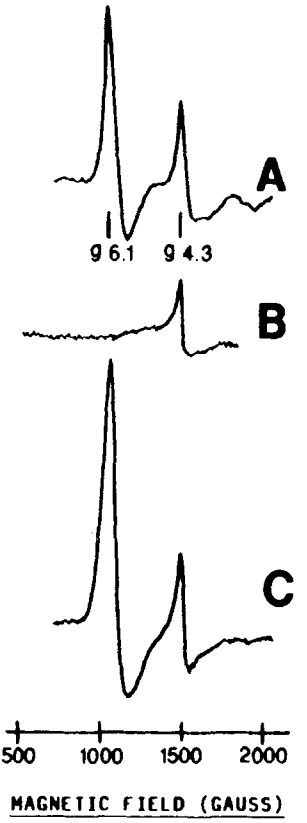


Figure 1 . Effect of phenidone on the EPR spectrum of ferric L_1 :

A : ferric enzyme (0.5 mM) obtained by addition of 1 mole of 13-HPOD per mole of L_1Fe(II), B : further treatment with 1 molar equivalent of phenidone, C : then, further treatment with 5 molar equivalents of 13-HPOD. Experiments were done under anaerobic conditions in 50 mM tris-acetate pH9 buffer, 0.1 mM DETAPAC. Spectra were recorded on a Brucker ER 220 D spectrometer at 9.34 GHz and 10 K, using an Oxford Instrument continuous flow cryostat, a Hall probe and a Hewlett-Packard frequency meter (100 KHz modulation frequency).

consumption occurs. This indicates that phenidone is not a substrate for the dioxygenase activity of L_1. However, we found that phenidone reduces readily the catalytically active ferric L_1 to its ferrous state. This was shown by the complete disappearance of the g = 6.1 EPR characteristic signal of L_1Fe(III), which was obtained by oxidation of resting L_1Fe(II) by one equivalent of 13-HPOD (2), upon addition of one equivalent of phenidone (Fig. 1). This reduction of L_1Fe(III) into L_1Fe(II) is reversible as a further addition of a few equivalents of 13-HPOD to the reaction mixture fully regenerates the L_1 ferric state. These data suggest a possible catalysis by L_1 of an oxidation of phenidone by 13-HPOD according to a shortened catalytic cycle consisting into two steps, the one-electron oxidation of phenidone by L_1Fe(III) and the reoxidation of L_1Fe(II) by 13-HPOD (steps 1 and 1' of scheme 1 with RH = phenidone). Accordingly, 13-HPOD reacts rapidly with phenidone in the presence of L_1 as judged by the fast disappearance of its UV peak at 234 nm upon

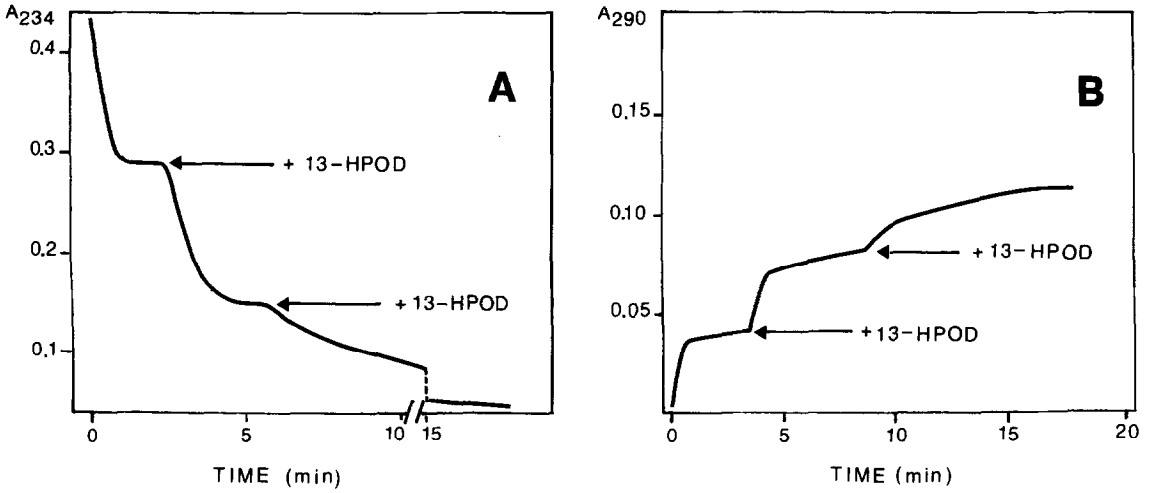


Figure 2 . Time courses of L_1-catalyzed oxidation of phenidone by 13-HPOD.

A : disappearance of 13-HPOD during 3 successive additions of 8 μM 13-HPOD to a solution containing initially 10.3 μM phenidone and 0.2 μM L_1. B : appearance of 4,5-dehydrophenidone (at 290 nm, ϵ = 18 mM^{-1} cm^{-1}) upon 3 successive additions of 13-HPOD (6.9 μM each) to a solution containing 9.3 μM phenidone and 0.2 μM L_1. Additions of L_1 together with 13-HPOD were necessary because of L_1 inactivation during the reaction.

reaction of 10 μM 13-HPOD with 10 μM phenidone and 0.2 μM L_1 (Fig. 2 and table 1). Under identical conditions but in the absence of either L_1 or phenidone, the rate of decomposition of 13-HPOD is negligible (table 1). The initial rate of disappearance of 13-HPOD is directly proportional to the concentrations of L_1 and of phenidone (only for concentrations below 10 μM for the latter

Table 1 . Dependence of initial rate of 13-HPOD consumption on concentrations of L_1 and phenidone.

L-1 (μM)	13-HPOD (μM)	Phenidone (μM)	Initial rates (μM/min)	
			aerobic	anaerobic conditions
0	10	10	<0.1	0
0.2	10	0	<0.1	<0.1
0.2	10	3	1.4	
0.2	10	5	2.5	
0.2	10	10	4.0	4.2
0.3	10	10	6.0	
0.4	10	10	8.0	
0.2	30	100	13	13
0.4	30	100	26	27

Conditions in Materials and Methods. Rates are determined at 234 nm using ϵ_{234} = 25 mM^{-1} cm^{-1}. Results are averages of four independent experiments for aerobic measurements and two independent experiments for anaerobic measurements.

compound). This rate is almost identical when the reaction is performed under anaerobic conditions (table 1).

HPLC analysis of incubates coming from reactions between phenidone, 13-HPOD and L_1 confirm the consumption of 13-HPOD and show the disappearance of phenidone with concomitant formation of 4,5-dehydrophenidone (data not shown). This compound is the major product of the reaction and is formed with a 75-80 % yield based on starting phenidone. From these HPLC experiments, it was estimated that 2 $\pm$ 0.2 moles of 13-HPOD are consumed per mole of phenidone under anaerobic conditions.

This stoichiometry of the L_1-catalyzed oxidation of phenidone by 13-HPOD was also determined by following, in UV-visible spectroscopy, either the consumption of 13-HPOD added progressively to an initial excess of phenidone, according to a technique described previously for L_1-catalyzed oxidation of N-alkylhydroxylamines by 13-HPOD (16), or the appearance of 4,5-dehydrophenidone (λ max = 290 nm) upon progressive additions of 13-HPOD to an initial excess of phenidone (Fig. 2). Both methods gave a stoichiometry of 2 moles of 13-HPOD consumed per mole of phenidone (2.1 and 2.05 respectively), in good agreement with the result obtained by HPLC.

The ability of various derivatives of phenidone to act as substrates for L_1-catalyzed oxidations by 13-HPOD according to the shortened catalytic cycle of scheme 1 (steps 1 and 1') was compared to that of phenidone itself. As shown in table 2, BW 755C and its analogue without the CF_3 group are also oxidized to the corresponding 4,5-dehydroderivatives (data not shown) and give rates of 13-HPOD consumption very similar to those of phenidone. For them also, these rates are negligible if L_1 is omitted. On the contrary, N-methylphenidone and 4,5-dehydrophenidone are inactive in this assay.

In a more general manner, **many compounds of various series were found to be oxidized by 13-HPOD when L_1 was present** (table 2). This is the case for compounds containing a phenol or a diphenol function, in agreement with a report (17) which appeared during the preparation of this manuscript. NDGA and pyrocatechol appear as the most reactive compounds in this series but simple phenols such as guaïacol and vitamin E are also active with lower rates. From all the compounds tested so far, para-aminophenol and N-hydroxyamphetamine gave the highest rates but several compounds containing an hydrazine, hydrazone, hydrazide or hydroxylamine (16) function were found to induce 13-HPOD consumption in the presence of L_1. It is noteworthy that well-known inhibitors of cyclooxygenase, like naproxen, indomethacin, ketoprofen and benoxaprofen appeared as inactive in this assay (i.e. their rate of L_1-catalyzed oxidation by 13-HPOD, if it exists, is below 1 μmol of 13-HPOD consumed per μmol of L_1 and per min).

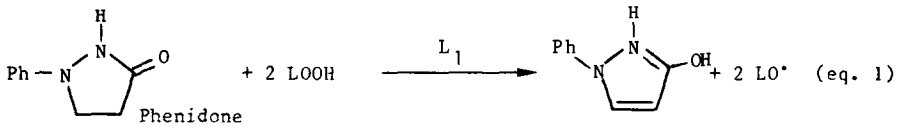
Table 2 . L_1-catalyzed reduction of 13-HPOD by various compounds.

Substrate	Initial rate[a] (min^{-1})	Substrate	Initial rate[a] (min^{-1})
Phenidone	68	Phenylhydrazine	10
BW 755C	68	2-hydrazinopyridine	52
1-phenyl-3-amino-2-pyrazoline	68		
1-phenyl-2-methyl-3-pyrazolidone	<1[b]	N-hydroxyamphetamine	170
1-phenyl-3-hydroxy-pyrazole (4,5-dehydrophenidone)	<1	4-methylbenzaldehyde-4'-bromophenylhydrazone	43
Phenol	<5		
Guaïacol	13		
Pyrocatechol	90	N'-phenylbenzoylhydrazide	8
Resorcinol	<5		
NDGA	140	Naproxen	<1
DL-α-tocopherol (Vitamin E)	13	Indomethacin	<1
Aniline	<1	Ketoprofen	<1
4-aminophenol	170	Benoxaprofen	<1
4-acetamidophenol (acetaminophen)	<1		

a) Initial rates of 13-HPOD disappearance (decrease of the 234 nm band), with 0.2 μM L_1, 30 μM 13-HPOD and 100 μM compound, are expressed in μmol per min and per μmol L_1. Consumption of 13-HPOD under the indicated conditions with compounds that gave rates >1 was verified by HPLC. b) Not significantly different from the rate of 13-HPOD consumption with L_1 alone under identical conditions (22).

DISCUSSION

Phenidone and BW 755C are not substrates for dioxygenation by L_1. However, they are substrates for a shortened catalytic cycle of L_1 (steps 1 and 1' of scheme 1) and are oxidized by 13-HPOD with formation of the corresponding dehydroderivatives. Whatever the detailed mechanism of dehydrophenidone formation may be, two moles of 13-HPOD (and so two short catalytic cycles of scheme 1) are required for the two-electron oxidation of one mole of phenidone into its dehydroderivative. The stoichiometry of eq. 1 is consistent with the previously reported need of one mole of LOOH (= 13-HPOD) for oxidation of $L_1Fe(II)$ with formation of LO• (2,18). The major fate of LO• is an intramolecular addition on the closest double bond leading to an epoxyallylic radical which has lost the conjugated diene system (2). This explains the disappearance of the 234 nm band observed in such reactions (Fig. 2A). Equation 1 correspond to a "hydroperoxidase-like" activity



of L_1 in the sense that an hydroperoxide acts as a cosubstrate and is reduced in the reaction. However, it is different from the classical hydroperoxidase activity of horseradish peroxidase or prostaglandin H synthase for which the

hydroperoxide is reduced to the corresponding alcohol and provides its two oxidizing equivalents for substrate oxidation (19). For instance, these two peroxidases catalyze the dehydrogenation of phenidone and BW 755C by alkylhydroperoxides but with formation of the alcohol derived from the hydroperoxide (20).

A priori, all compounds able to reduce $L_1Fe(III)$ to $L_1Fe(II)$ should be substrates for L_1-dependent oxidations by 13-HPOD. This is the case of linoleic acid itself in its anaerobic oxidation by 13-HPOD (2) and of N-alkylhydroxylamines (16) and catechols (17). Our results not only show that this is also the case of pyrazoline derivatives such as phenidone, but also indicate that many compounds containing various functions (table 2) behave similarly. Further work is necessary to determine the products of these reactions and the structural factors that are important for a compound to reduce $L_1Fe(III)$. However, it is already clear from our preliminary results that important differences exist between the structural factors necessary for a substrate to be oxidized either by L_1 or prostaglandin H synthase. For instance, acetaminophen is a good substrate for the latter (21) whereas it is inactive towards L_1.

This "peroxidase-like" activity of L_1 is interesting (i) for the detection of compounds able to enter the L_1 active site and to interact with its Fe(III) without being substrates for dioxygenation by L_1, (ii) because of possible cooxidations of compounds by L_1 in the presence of linoleic acid and O_2, the two precursors of 13-HPOD. Moreover, compounds that reduce efficiently the lipoxygenase-Fe(III) active state would tend to maintain the enzyme in its ferrous inactive state and would act as inhibitors. This was recently shown for NDGA (17). More work is necessary to know whether this phenomenon is involved in the inhibitory effects of phenidone or BW 755C.

ACKNOWLEDGMENTS : We thank I. Morgenstern-Badarau (Orsay, France) for her help in the EPR studies and Rhône-Poulenc Santé for a grant for one for us (C.C).

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