Inhibition of potato lipoxygenase by linoleyl hydroxamic acid: kinetic and EPR spectral evidence for a two-step reaction

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The reaction mechanism of an electrophoretically pure potato tuber lipoxygenase (ptLOX) was studied by EPR spectroscopy. An EPR spectrum of the 'native' ptLOX recorded at 4.5 ± 0.5 K showed signals of a high-spin (pseudo) axial Fe^{3+} with a g -value of approx. 6.3 ± 0.1 with a shoulder at $g = 5.9 \pm 0.1$, and a rhombic Fe^{3+} signal at $g = 4.35 \pm 0.05$. When the enzyme was treated with a 2-fold molar excess of 13(*S*)-hydroperoxyoctadecadienoic acid [13(*S*)-HPODE], a 3-fold increase in the integral intensity of the $g = 6.3$ signal was observed, indicating that 25% of the native ptLOX iron was in ferrous state. The positional isomer 9(*S*)-HPODE caused similar spectral changes. Therefore the catalytic centre of ptLOX appears to accommodate both positional isomers of linoleic acid hydroperoxides in a manner that ensures proper alignment of their hydroperoxy groups with the iron centre of the enzyme. Treatment of the Fe^{3+} -ptLOX form with a 3-fold molar excess of linoleyl hydroxamic acid (LHA) completely quenched the $g = 6.3$ signal.

Concurrently, a dramatic increase in the signal at $g = 4.35$ was detected, which was attributed to a newly formed LHA- Fe^{3+} -ptLOX complex. The spectral characteristics of the complex are similar to those of a 4-nitrocatechol- Fe^{3+} -ptLOX complex. From these observations, we conclude that LHA did not reduce Fe^{3+} to Fe^{2+} , but rather formed a LHA- Fe^{3+} -ptLOX complex. Formation of such a complex may be responsible for the inhibitory activity of LHA, at least in the initial stages of enzyme inhibition. A prolonged 15 min incubation of the complex at 23 ± 1 °C led to the partial quenching of the $g = 4.35$ signal. The quenching is attributed to the reduction of Fe^{3+} -ptLOX by LHA, with concomitant formation of its oxidation product(s). A kinetic scheme for the inhibition is proposed.

Key words: fatty acid hydroperoxides, inactivation, iron, redox reaction.

INTRODUCTION

The lipoxygenases (LOXs; EC 1.13.11.12) are a family of closely related non-haem iron enzymes (with an exception of a recently discovered manganese LOX from the take-all fungus *Gaumannomyces graminis*) [1]. They catalyse the dioxygenation of polyunsaturated fatty acids containing one or more 1,4-pentadiene moieties [2–4]. Overproduction of LOX products has been shown to accompany various pathological states and diseases of plants [5] and animals [6]. Therefore investigation of the mechanism of the formation of these products by LOXs and elaboration of methods of regulation of their biosynthesis are of utmost importance.

All iron LOXs known, including the one from potato (*Solanum tuberosum*) tuber (ptLOX) [7–10], contain a single ion of essential iron in either the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) form at their active centre. $\text{Fe}^{3+}/\text{Fe}^{2+}$ cycling is an essential part of the LOX catalytic mechanism [11,12]. Consequently, determination of the oxidation state of the iron, e.g. by EPR spectroscopy, is critical for understanding the kinetics, mechanism and regulation of LOX-catalysed reactions. To be catalytically active, the resting, EPR-silent Fe^{2+} form of LOX needs to be converted by an oxidizing agent [usually a fatty acid hydroperoxide (FAHP)] into an activated, EPR-visible Fe^{3+} form [10,11,13]. Thus far, only the interaction of 9(*S*)-10*E*,12*Z*-hydroperoxyoctadecadienoic acid [9(*S*)-HPODE] with Fe^{2+} -ptLOX has been studied by EPR [10].

No details of possible reactions of other FAHPs with Fe^{2+} -ptLOX have been reported, although the enzyme oxygenates a wide variety of other substrates [14–18], and therefore must undergo $\text{Fe}^{3+}/\text{Fe}^{2+}$ cycling utilizing the hydroperoxides formed *in situ* or by other enzymic systems, e.g. 15-LOX. To determine whether Fe^{2+} -ptLOX has strict requirements with regard to the structure of the FAHPs involved in its activation, it was necessary to investigate the reaction of ptLOX with 13(*S*)-9*Z*,11*E*-HPODE, the second most abundant product of the oxygenation of linoleic acid (LA) by ptLOX [19] and the major product of 15-LOX activity, also found widely in plants and mammals.

Down-regulation of LOX activity *in vitro* and *in vivo* is usually achieved by using synthetic and/or natural inhibitors of the enzyme. One of the better-known classes of LOX inhibitors is a group of compounds with the general formula R-C(O)NHOH , i.e. the hydroxamic acids [20–24], among which arachidonyl hydroxamic acid (AHA) attracts attention because of its low IC_{50} value (~ 0.1 μM) [24]. Despite this merit, AHA has two obvious disadvantages, namely low stability and high cost, which limit the use of this inhibitor in practical medicine.

To overcome these shortcomings of AHA, we have synthesized a more stable and less costly analogue, linoleyl hydroxamic acid (LHA) [25–27]. Being a derivative of LA, LHA bears the same ultimate structural similarity to the natural LOX substrates as does AHA, promising a similar affinity towards the enzyme. LHA-induced inhibition of LOX activity has been reported for

Abbreviations used: AHA, arachidonyl hydroxamic acid; $\text{C}_{12}\text{E}_{10}$, monododecyl ether of decaoxyethylene glycol; FAHP, fatty acid hydroperoxide; 9(*S*)-HPODE, 9(*S*)-hydroperoxyoctadecadienoic acid; 13(*S*)-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; LA, linoleic acid; LHA, linoleyl hydroxamic acid; LOX, lipoxygenase; ptLOX, potato tuber LOX; sLOX, soybean LOX.

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different types of LOX, including plant [25,26] and mammalian [27] enzymes, and has been studied both *in vitro* [25–27] and *in vivo* [28–30]. The IC_{50} values depend on the fatty acid substrate concentration, and are in the range 0.01–10 μ M. However, the mechanism of inhibition of LOX by LHA is not fully understood, and differs from enzyme to enzyme. For example, LHA has been shown to be a suicide substrate of soybean LOX (sLOX) [25], while acting in a concentration-dependent manner as a competitive inhibitor of a pig leucocyte 12-LOX [27] and of ptLOX [26]. Both of the latter enzymes were incapable of dioxygenating LHA. It is thus worthwhile to investigate the mechanism of LHA-induced inhibition of ptLOX – a model LOX functionally similar to mammalian 5-LOX [8] – in more detail.

It is conceivable that LHA, being a reductant, could reduce Fe^{3+} -LOX to a catalytically inactive Fe^{2+} form. It is also possible that LHA forms a complex with either Fe^{2+} - or Fe^{3+} -LOX, acting as a competitive non-reducing inhibitor. To discriminate between these possibilities, we conducted EPR studies of a model LHA/ptLOX reaction system, the results of which are presented here.

MATERIALS AND METHODS

Materials

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). LA was obtained through Nu-Chek Prep (Elysian, MN, U.S.A.). LHA was synthesized utilizing a procedure described previously [31]. The HPLC-grade solvents, manufactured by Burdick & Jackson, were purchased through VWR Scientific Products (Pittsburgh, PA, U.S.A.). Standard 5 mm EPR tubes were a product of Wilmad Glass (Buena, NJ, U.S.A.).

Purification of ptLOX and determination of its activity

The procedures for purification of the enzyme from potato tubers to electrophoretic homogeneity were essentially the same as described previously [32]. The final concentration of the native ptLOX stock solution was 0.6 mg of protein/ml ($\sim 6.4 \mu$ M). The stock solution was divided into two portions, the first of which was frozen 'as is' and stored at -80°C . The second portion of the purified enzyme was subjected to treatment with 2 mM sodium metabisulphite for 5 min on ice; the enzyme was then rapidly desalted to remove excess $Na_2S_2O_3$ and re-concentrated on an Amicon YM50 concentrating cartridge (Millipore, Bedford, MA, U.S.A.) in accordance with the manufacturer's recommendations in strictly anaerobic conditions under nitrogen. The final solution was aliquotted into small volumes and kept in liquid nitrogen. No loss of specific activity was observed after 6 months of storage.

The enzymic activity of ptLOX was measured as described previously [15–18,33] with 0.25 mM LA as substrate in a 0.1 M sodium phosphate buffer, pH 6.3, at $25.0 \pm 0.5^\circ\text{C}$. The buffer contained 0.04 % (w/v) monododecyl ether of decaoxyethylene glycol ($C_{12}E_{10}$) as solubilizer of the otherwise insoluble LA. The reaction was carried out in a standard 1 cm quartz spectrophotometric cuvette under constant mixing. The total volume of the reaction mixture was 2 ml. The reaction was started by addition of 1–10 μ l of the enzyme stock solution and monitored at 234 nm, which is the absorption maximum of the major reaction product, 9(*S*)-HPODE ($\epsilon_m = 23000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [34]). The values of absorbance (in absorbance units) were recorded automatically every 5 s for up to 30 min, and were plotted and analysed using a SigmaPlot program (SPSS, Inc.).

The specific activity of ptLOX, calculated from the steady-state portion of the kinetic curve, was found to be $\sim 45 \mu\text{mol}$ of product/min per ml.

Enzymic synthesis of 9(*S*)-HPODE and 13(*S*)-HPODE

ptLOX and sLOX were used to synthesize 9(*S*)-HPODE and 13(*S*)-HPODE respectively. The hydroperoxides were purified and analysed as described previously for linoleyl alcohol and 1-monolinoleoyl glycerol [17,18]. The hydroperoxides were stored in methanol at -80°C under argon.

EPR spectroscopy of the iron in the ptLOX catalytic centre

The original stock solution of purified ptLOX ($\sim 0.6 \text{ mg/ml}$) was concentrated to $\sim 15 \text{ mg}$ of protein/ml ($\sim 0.16 \text{ mM}$) on an Amicon YM50 cartridge under aerobic conditions. The $Na_2S_2O_3$ -treated enzyme preparation was concentrated similarly under a nitrogen atmosphere. The concentrated ptLOX samples tended to precipitate upon storage at 4°C and also during repetitive freezing/thawing; therefore they were placed immediately into the EPR tubes in 0.25 ml aliquots, treated (if necessary) with hydroperoxides and LHA as described below, then frozen and stored in liquid nitrogen until the EPR experiments. The experiments were performed on a Bruker ECS 106 Electron Spin Resonance spectrometer equipped with an ER 4116 DM Dual-Mode X-Band Resonator and a helium cryostat (Oxford Instruments, Witney, Oxon, U.K.) at $4.5 \pm 0.5 \text{ K}$. The spectra were recorded routinely at 9.62 GHz, 100 kHz modulation frequency, and power and gain setting of 10 mW and 80000 respectively. Neither the intensity nor the shape of the signal of the hydroperoxide-treated ptLOX changed over 3 months of storage in liquid nitrogen.

To convert the resting ferrous LOX (Fe^{2+} -ptLOX) into the catalytically active ferric form (Fe^{3+} -ptLOX), the native enzyme was treated with either 13(*S*)-HPODE or 9(*S*)-HPODE on ice. A small aliquot (typically 6 μ l) of a 4 mM stock solution of one of three hydroperoxides in acetonitrile was added to $\sim 0.25 \text{ ml}$ of the concentrated solution of ptLOX to bring the final concentration of the lipid hydroperoxide to approx. 0.3 mM, which was a 2-fold molar excess over the enzyme. The final concentration of acetonitrile never exceeded 2.5 % (v/v), which in a separate experiment was shown to have no apparent effect on the EPR signals of ptLOX. It was determined that a 1 min incubation (excluding the time required for mixing the reagents and freezing the sample in the EPR tube) was sufficient to achieve maximal conversion of ferrous ptLOX into its Fe^{3+} -form.

The EPR spectra of the LHA- Fe^{3+} -ptLOX complex were recorded essentially as described above, except that the native ptLOX was treated with a 1–3-fold molar excess of LHA either for $\sim 1 \text{ min}$ at 0°C , or for 1, 5 or 15 min at $23 \pm 1^\circ\text{C}$, and then frozen in liquid nitrogen.

All spectra were corrected for the baseline by subtracting a signal from an EPR tube filled with the same amount of the reaction solution without ptLOX. All buffer solutions were treated with ion-chelating resins to remove traces of transition metals, especially iron.

Computer analysis of the EPR spectra

This was performed using Bruker WinEPR System software (Bruker-Franzen Analytik, G.m.b.H., Karlsruhe, Germany). The experimental EPR spectra of Fe^{3+} -ptLOX and LHA- Fe^{3+} -ptLOX were integrated and de-convoluted, and the apparent ratio of the major components was determined.

Figure 1 Kinetic curve for LA oxidation by ptLOX (A) and the effects of 13(S)-HPODE on enzyme activity (B)

τ , lag period; V_{st} , steady-state rate of the reaction; a.u., absorbance units.

Figure 2 Inhibition of ptLOX by LHA

(A) Kinetic curves of LA oxidation by ptLOX without (curve 1) and with (curves 2–4) 20 μ M LHA. Experimental conditions: curve 2, 20 μ M LHA was mixed with LA prior to the addition of ptLOX; curve 3, LHA was added to a ptLOX/LA mixture to a final concentration of 20 μ M at the time indicated by the arrow; curve 4, ptLOX was preincubated with 20 μ M LHA for 1 min, and then the reaction was initiated by adding LA to a final concentration of 0.25 mM. a.u., absorbance units. (B) Residual activity of ptLOX with 0.25 mM LA in the presence of LHA. arb.u., arbitrary units (normalized).

RESULTS

Activation of ptLOX by hydroperoxides

During the oxidation of LA, purified ptLOX displayed a noticeable lag period, followed by a quasi-linear portion of the kinetic curve (Figure 1A). This segment of the curve was used to calculate the steady-state rate of the reaction (V_{st}), as described previously [17]. The duration of the lag period exceeded 1 min, which was far longer than the lag determined for soybean 15-LOX-1 [35]. Treatment of the enzyme with 0.02 mM 13(S)-HPODE prior to the addition of LA decreased the lag period to ~ 4 –6 s, while having no effect on V_{st} (Figure 1B). The positional isomer 9(S)-HPODE had the same effect. Preincubation of the stock solution (6.4 μ M) of ptLOX with 0.36 mM 13(S)-HPODE for 5–60 min prior to injection into the reaction mixture led to a slight (~ 10 –15%) time-dependent decrease in enzymic activity. A longer preincubation of ptLOX with or without the

hydroperoxide resulted in a gradual decline in enzymic activity, and therefore was avoided.

Inhibition of ptLOX by LHA

Inhibition of purified ptLOX by LHA was tested using a standard reaction mixture composed of 0.25 mM LA, 0.02 mM 13(S)-HPODE and 0.04% (w/v) $C_{12}E_{10}$, at pH 7.5, which is closer to the intracellular pH than to the enzyme's pH optimum for activity (~ 6.3). The inhibitory effect of LHA was tested under a variety of experimental conditions (Figure 2A). In all cases, the inhibitor caused a rapid loss of enzymic activity upon its addition to the reaction mixture. As judged from the steady-state reaction rate, addition of 40 μ M LHA to the reaction mixture prior to ptLOX resulted in ~ 5 -fold decrease in ptLOX catalytic activity (curve 2, Figure 2A), and this was also the case when the inhibitor was added during the reaction (curve 3, Figure 2A).

Figure 3 Swinbourne-Jenkins transformation of kinetic curve 2 from Figure 2(A)

a.u., absorbance units.

Figure 4 EPR spectra of native (curve A), $\text{Na}_2\text{S}_2\text{O}_5$ -treated (curve B) and 13(S)-HPODE-treated (curve C) ptLOX

A short 1 min preincubation of ptLOX with LHA in the absence of substrate and 13(S)-HPODE led to the highest (7–10-fold) degree of enzyme inhibition (curve 4, Figure 2A). Because of the length of time needed to mix all the reagents, it was not possible to test shorter periods of time. The IC_{50} value, determined from the concentration-dependence of inhibition of ptLOX by LHA upon oxidation of 0.25 mM LA at pH 7.5, was approx. 10 μM (Figure 2B). In a separate experiment it was shown that, unlike sLOX [25], ptLOX did not oxygenate 10–100 μM LHA. Catalytic amounts of SDS, shown formerly to noticeably accelerate the ptLOX-catalysed oxidation of various lipids [15–18,36], and 13(S)-HPODE also did not promote the ptLOX-induced oxygenation of LHA.

It was observed that, in the presence of LHA, the enzyme lost its activity in a time-dependent manner during the oxidation of LA (curves 2–4, Figure 2). The kinetic curves $\{P_t, t\}$ were plotted in Swinbourne-Jenkins co-ordinates $\{P_t; P_{t+5 \text{ min}}\}$ [37,38], and were numerically approximated with a straight line by using the equation $P_{t+5} = a + b \times P_t$ (Figure 3). The experimental curves deviated slightly from linearity at the beginning of the reaction, but the deviation was insignificant ($r^2 \geq 0.9998$) and, most likely, was a result of mixing/equilibrating the reactants. The slope of

Figure 5 Temperature-dependence of the EPR spectra of Fe^{3+} -ptLOX

Spectra a–g were recorded at temperatures of 109.0, 34.0, 25.5, 21.0, 17.5, 9.0 and 5.5 K respectively. arb.u., arbitrary units (normalized).

the approximating straight line, b , according to Swinbourne [37] and Jenkins [38], is equal to $e^{-k_{\text{in}} \times \Delta t}$, where k_{in} is a first-order rate constant of enzyme inactivation during the reaction; Δt in our case was set to be 5 (for more details on choosing values of Δt , see [37,38]). The calculated value of k_{in} was $0.3 \pm 0.1 \text{ min}^{-1}$.

EPR spectra of ptLOX

EPR spectroscopy of the freshly purified native ptLOX at a concentration of $\sim 0.16 \text{ mM}$ showed a low-intensity broad peak with a g -value of 6.3 ± 0.1 , with a shoulder at around $g = 5.9 \pm 0.1$, and a much narrower peak at $g = 4.35 \pm 0.05$ (Figure 4, spectrum A). Both peaks almost completely disappeared after treatment of the enzyme with 2 mM $\text{Na}_2\text{S}_2\text{O}_5$, giving the EPR-silent, 'resting' form of ptLOX (Figure 4, spectrum B), while oxidation of the native form of ptLOX with 13(S)-HPODE led to a 3-fold increase in the integral intensity of the EPR signal (Figure 4, spectrum C). The shoulder at $g = 5.9$ was still present, although less noticeable. The integral intensity of the $g = 4.35$ signal also rose, but to a much lesser extent. Prolonged incubation of the enzyme with 13(S)-HPODE at 0 °C caused a substantial drop in the intensity of the major signal at $g = 6.3$, as did repetitive freezing and thawing of the lipid/protein mixture. The intensities of the major $g = 6.3$ and $g = 4.35$ signals were temperature-dependent: the lower the temperature, the stronger the signal (Figure 5); therefore, to maximize the signal and to decrease the length of the experiments, they were conducted at a standard temperature of $4.5 \pm 0.5 \text{ K}$. It is noteworthy that the changes in the temperature at which the EPR spectra were recorded led to a noticeable variations in the relative intensities of the $g = 4.35$ and $g = 6.3$ signals. With the decrease in temperature from 25 K to 4 K, the relative intensity of the $g = 6.3$ signal rose more quickly than that of the $g = 4.35$ signal.

EPR spectra of LHA- Fe^{3+} -ptLOX and 4-nitrocatechol- Fe^{3+} -ptLOX complexes

As seen from Figure 6 (curve A), addition of LHA to Fe^{3+} -ptLOX in ~ 3 -fold molar excess followed by incubation at 0 °C for 1 min did not reduce the ferric enzyme, but rather resulted in the formation of a LHA- Fe^{3+} chelate complex. The narrow EPR signal of the newly formed complex had a g -value of ~ 4.35 .

Figure 6 EPR spectra of Fe^{3+} -ptLOX treated with LHA (curve A) or 4-nitrocatechol (curve B) for ≤ 1 min

The inset shows quenching of the $g = 4.35$ signal of the LHA- Fe^{3+} -ptLOX complex upon its incubation at room temperature. arb.u., arbitrary units (normalized).

Partial formation of the LHA- Fe^{3+} -ptLOX complex was observed when the LHA and ptLOX concentrations were the same (results not shown), while rapid and complete quenching of the $g = 6.3$ signal was achieved at a ≥ 2.5 -fold molar excess of LHA over ptLOX. A time-dependent decline of the intensity of the $g = 4.35$ signal was observed upon incubation of the LHA- Fe^{3+} -ptLOX complex at room temperature under anaerobic conditions (Figure 6, inset). The signal was noticeably quenched after a 15 min incubation. Similar treatment of Fe^{3+} -ptLOX with the non-reducing inhibitor 4-nitrocatechol resulted in a phenol-protein complex with an identical EPR signal (Figure 6, curve B). At this time, we cannot confirm whether or not LHA extracted Fe^{3+} and/or Fe^{2+} from the ptLOX active centre. Indirect evidence for the formation of a stable ternary LHA- Fe^{3+} -ptLOX complex was obtained in ultrafiltration experiments. A mixture of LHA and Fe^{3+} -ptLOX was subjected to separation on the Amicon YM50 filtration unit at 4°C , and an attempt was made to detect a low-molecular-mass LHA- Fe^{3+} complex in the ultrafiltrate by using UV spectrometry at 500 nm. No such complex was observed.

DISCUSSION

The results presented here suggest that both FAHPs 13(*S*)-HPODE and 9(*S*)-HPODE are capable of eliminating the lag period in the oxidation of LA by native ptLOX. This indicates that the catalytic centre of ptLOX could accommodate both the HPODEs in a way that ensures proper alignment of their hydroperoxy moieties and the ferrous iron of ptLOX. Substrate molecules can fit in the ptLOX catalytic cavity both in a direct and in a reverse orientation [17,18] unless their carboxy groups are modified with a bulky substituent [18], and the same could be the case with HPODEs.

Judging from the EPR spectra, the native preparation of ptLOX was a mixture of Fe^{2+} - and Fe^{3+} -ptLOX with a molar ratio of 3:1. This ratio may differ somewhat from the ratio found *in vivo*, which may be partly due to the use of ascorbic acid and $\text{Na}_2\text{S}_2\text{O}_5$ during enzyme purification from potato tuber

Figure 7 Proposed structure of a non-chelate LHA- Fe^{3+} -ptLOX complex (A; 'normal' orientation of the inhibitor) and of a chelate LHA- Fe^{3+} -ptLOX complex (B; 'inverse' orientation of LHA)

For more information on the structure of the catalytic cavity of ptLOX, see [41].

homogenates to prevent the ptLOX-catalysed oxidation of endogenous lipids and to inactivate the polyphenol oxidase system that is abundant in the crude extracts of potato tubers [39]. The ability of $\text{Na}_2\text{S}_2\text{O}_5$ to reduce Fe^{3+} -ptLOX supports this suggestion (spectrum B, Figure 4). The highly concentrated ptLOX solution (~ 15 mg of protein/ml) was found to be quite labile, since excessive treatment of the native enzyme with the hydroperoxides, in the form of either a prolonged preincubation at 0°C or a high concentration of the HPODEs applied, resulted in a decrease in the EPR signal, as did the repetitive freezing and thawing of the reaction mixture. It is noteworthy that a 1 h preincubation of a ~ 0.6 mg/ml solution of ptLOX with 0.36 mM 13(*S*)-HPODE did not change the ptLOX specific activity to any great extent (Figure 1B).

Unlike sLOX [25], Fe^{3+} -ptLOX did not catalyse the direct oxygenation of LHA, since (i) no oxygenation products of LHA were detected in the reaction mixture, and (ii) under anaerobic conditions, LHA apparently formed the LHA- Fe^{3+} -ptLOX chelate complex, which was spectrally similar to the reported ferric sLOX-4-nitrocatecholate complex (Figure 6 in the present study, and [40]). This implies the direct interaction of the hydroxamic moiety of LHA with the iron. Such an orientation of LHA, in turn, apparently precludes the pentadiene moiety from being sufficiently close to the iron centre to be oxygenated. The reported geometry of the catalytic cavities of various LOXs [41] apparently does not allow normally oriented LHA molecules to slide into the cavity deeply enough to ensure proper alignment of the hydroxamic moiety of LHA and the ptLOX iron (Figure 7A). Therefore a direct interaction of the ptLOX iron with the hydroxamic moiety of LHA requires the inhibitor to enter the enzyme's catalytic cavity in the 'C(O)NHOH-end-first', or 'reverse', orientation (Figure 7B). The latter reaction has to be fast and thermodynamically favourable, because no evidence of LHA dioxygenation, expected for normally oriented LHA, has been obtained in our experiments.

Upon anaerobic incubation of LHA- Fe^{3+} -ptLOX at room temperature for 1–15 min, the $g = 4.35$ signal was partly quenched (Figure 6, inset), which indicated that Fe^{3+} -ptLOX had

Scheme 1 Two-step inhibition of ptLOX by LHA

been gradually reduced by LHA to the EPR-invisible ferrous form. The most likely primary product of this reaction is a nitroxide derivative of LHA, which is usually formed upon reaction of ferric iron and a hydroxamic moiety. Since (i) the time needed for the formation of the initial $g = 4.35$ complex was less than 1 min, (ii) its quenching took more than 1 min to develop, and (iii) the inhibitory effect of LHA on the ptLOX-catalysed oxygenation of LA (compare curves 1 and 2, Figure 2) was seen almost instantaneously, it was concluded that the primary reaction of LHA- Fe^{3+} -ptLOX chelate complex formation was responsible for the immediate inhibition of the enzyme. At the same time, reduction of Fe^{3+} -ptLOX during incubation might play a role at the later stages of inhibition, which is evident from a comparison of kinetic curves 2 and 4 in Figure 2, and from Figure 6 (inset). It is noteworthy that, in the presence of LHA, the ptLOX-catalysed oxidation of LA stops long before substrate depletion occurs. A similar loss of enzymic activity was detected for the sLOX-catalysed oxygenation of LHA [25]. In an attempt to determine the mechanism of ptLOX inhibition during the reaction, kinetic curve 2 (Figure 2) was analysed in Swinbourne co-ordinates [37,38] (Figure 3). The linearity of the plot strongly suggested a (pseudo) first-order loss of ptLOX activity along the reaction of LA oxidation in the presence of LHA, with a k_{in} value of $\sim 0.3 \text{ min}^{-1}$. Such a loss is observed when an enzyme is strongly inhibited by the reaction product, or is inactivated irreversibly upon catalytic reaction ([39], and references cited therein).

As the hydroxamic acids form chelate complexes with both ferric and ferrous forms of iron, it is conceivable that LHA also reacts with Fe^{2+} -ptLOX, although this reaction could not be detected by means of EPR. The inhibitory effect of LHA was more pronounced if ptLOX had not been protected with either LA or 13(S)-HPODE (compare curves 2–4 in Figure 2A), which is most probably due to direct competition of LHA with the former two compounds for the substrate-binding centre of ptLOX (Scheme 1).

Our findings that Fe^{3+} -ptLOX, upon mixing with LHA, instantaneously forms the detected LHA- Fe^{3+} -ptLOX complex, which later is slowly converted into Fe^{2+} -ptLOX, is the first observation of such a transformation. This is in contrast with earlier publications, where it was reported that ferric sLOX was rapidly reduced by a number of hydroxamic acid derivatives [40,42,43], and there was no co-ordination observed before reduction [40]. One can expect that Fe^{3+} -ptLOX has a lower redox potential than sLOX, which is in line with the lower apparent rate of turnover of the potato enzyme. It appears that,

kinetically, the inhibition of ptLOX by LHA during oxygenation of LA is a two-step process, of which the first step is the instantaneous formation of a catalytically inactive LHA-ptLOX equilibrium complex, with the second step being a much slower (pseudo) first-order reaction of ptLOX inhibition or inactivation.

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REFERENCES

- Su, C. and Oliw, E. H. (1998) Manganese lipoxygenase. Purification and characterization. *J. Biol. Chem.* **273**, 13072–13079
- Yamamoto, S., Suzuki, H., Nakamura, M. and Ishimura, K. (1999) Arachidonate 12-lipoxygenase isozymes. *Adv. Exp. Med. Biol.* **447**, 37–44
- Silverman, E. S. and Drazen, J. M. (1999) The biology of 5-lipoxygenase: function, structure, and regulatory mechanisms. *Proc. Assoc. Am. Physicians* **111**, 525–536
- Kuhn, H. and Thiele, B. J. (1995) Arachidonate 15-lipoxygenase. *J. Lipid Mediators Cell Signalling* **12**, 157–170
- Reddy, P. S., Kumar, T. C., Reddy, M. N., Sarada, C. and Reddanna, P. (2000) Differential formation of octadecadienoic acid and octadecatrienoic acid products in control and injured/infected potato tubers. *Biochim. Biophys. Acta* **1483**, 294–300
- Cuendet, M. and Pezzuto, J. M. (2000) The role of cyclooxygenase and lipoxygenase in cancer chemoprevention. *Drug Metab. Drug Interact.* **17**, 109–157
- Galliard, T. and Phillips, D. R. (1971) Lipoxygenase from potato tubers. Partial purification and properties of an enzyme that specifically oxygenates the 9-position of linoleic acid. *Biochem. J.* **124**, 431–438
- Shimizu, T., Radmark, O. and Samuelsson, B. (1984) Enzyme with dual lipoxygenase activities catalyzes leukotriene A₄ synthesis from arachidonic acid. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 689–693
- Shimizu, T., Honda, Z., Miki, I., Seyama, Y., Izumi, T., Radmark, O. and Samuelsson, B. (1990) Potato arachidonate 5-lipoxygenase: purification, characterization, and preparation of 5(S)-hydroperoxyeicosatetraenoic acid. *Methods Enzymol.* **187**, 296–306
- Mulliez, E., Leblanc, J.-P., Girerd, J.-J., Rigaud, M. and Chottard, J.-C. (1987) 5-Lipoxygenase from potato tubers. Improved purification and physico-chemical characteristics. *Biochim. Biophys. Acta* **916**, 13–23
- de Groot, J. J., Veldink, G. A., Vliegthart, J. F., Boldingh, J., Wever, R. and van Gelder, B. F. (1975) Demonstration by EPR spectroscopy of the functional role of iron in soybean lipoxygenase-1. *Biochim. Biophys. Acta* **377**, 71–79
- Pistorius, E. K., Axelrod, B. and Palmer, G. (1976) Evidence for participation of iron in lipoxygenase reaction from optical and electron spin resonance studies. *J. Biol. Chem.* **251**, 7144–7148
- Slappendel, S., Veldink, G. A., Vliegthart, J. F., Aasa, R. and Malmstrom, B. G. (1981) EPR spectroscopy of soybean lipoxygenase-1. Description and quantification of the high-spin Fe(III) signals. *Biochim. Biophys. Acta* **667**, 77–86
- Nikolaev, V., Reddanna, P., Whelan, J., Hildenbrandt, G. and Reddy, C. C. (1990) Stereochemical nature of the products of linoleic acid oxidation catalyzed by lipoxygenases from potato and soybean. *Biochem. Biophys. Res. Commun.* **170**, 491–496
- Butovich, I. A., Tsys', E. V. and Mogilevich, T. V. (1992) Oxidation of linoleic acid and methyl linoleate by potato and soybean lipoxygenases: a comparative study. *Biokhimiya (Moscow)* **57**, 1012–1019

- 16 Butovich, I. A., Lukyanova, S. M. and Reddy, C. C. (1998) Oxidation of linoleyl alcohol by potato tuber lipoxygenase: possible mechanism and the role of carboxylic group in substrate binding. *Biochem. Biophys. Res. Commun.* **249**, 344–349
- 17 Butovich, I. A., Luk'yanova, S. M. and Reddy, C. C. (2000) Oxidation of linoleyl alcohol by potato tuber lipoxygenase: kinetics and positional, stereo, and geometrical (cis, trans) specificity of the reaction. *Arch. Biochem. Biophys.* **378**, 65–77
- 18 Butovich, I. A. and Reddy, C. C. (2001) Enzyme-catalyzed and enzyme-triggered pathways in dioxygenation of 1-monolinoleoyl-rac-glycerol by potato tuber lipoxygenase. *Biochim. Biophys. Acta* **1546**, 379–398
- 19 Chan, H. W. and Prescott, F. A. (1975) Specificity of lipoxygenases. Separation of isomeric hydroperoxides by high performance liquid chromatography. *Biochim. Biophys. Acta* **380**, 141–144
- 20 Summers, J. B., Mazdiyasni, H., Holms, J. H., Ratajczyk, J. D., Dyer, R. D. and Carter, G. W. (1987) Hydroxamic acid inhibitors of 5-lipoxygenase. *J. Med. Chem.* **30**, 574–580
- 21 Summers, J. B., Gunn, B. P., Mazdiyasni, H., Goetze, A. M., Young, P. R., Bouska, J. B., Dyer, R. D., Brooks, D. W. and Carter, G. W. (1987) In vivo characterization of hydroxamic acid inhibitors of 5-lipoxygenase. *J. Med. Chem.* **30**, 2121–2126
- 22 Summers, J. B., Kim, K. H., Mazdiyasni, H., Holms, J. H., Ratajczyk, J. D., Stewart, A. O., Dyer, R. D. and Carter, G. W. (1990) Hydroxamic acid inhibitors of 5-lipoxygenase: quantitative structure-activity relationships. *J. Med. Chem.* **33**, 992–998
- 23 Li, L., Zhu, Z., Joshi, B., Porter, A. T. and Tang, D. G. (1999) Apoptosis induction by a novel anti-prostate cancer compound, BMD188 (a fatty acid-containing hydroxamic acid), requires the mitochondrial respiratory chain. *Anticancer Res.* **19**, 51–60
- 24 Corey, E. J., Cashman, J. R., Kantner, S. S. and Wright, S. W. (1984) Rationally designed, potent inhibitors of leukotriene biosynthesis. *J. Am. Chem. Soc.* **106**, 1503–1504
- 25 Butovich, I. A., Bridnya, V. P. and Kukhar, V. P. (1991) Linoleate-hydroxamic acid: a suicide inhibitor of lipoxygenase. *Biokhimiya (Moscow)* **55**, 908–912
- 26 Butovich, I. A., Kharchenko, O. V., Bondarenko, L. B., Babenko, V. M. and Livarchuk, L. V. (1994) Linoleyl hydroxamic acid as 5-lipoxygenase inhibitor. *Biokhimiya (Moscow)* **59**, 597–600
- 27 Kharchenko, O. V., Cernjuk, V. N. and Butovich, I. A. (1999) Inhibitory effect of linoleyl-hydroxamic acid on the oxidation of linoleic acid by 12-lipoxygenase from porcine leukocytes. *Ukr. Biokhim. Zh.* **71**, 33–37
- 28 Moibenko, A. A., Grabovskii, L. A., Kotsiuruba, V. N., Bulakh, V. N., Tumanovskaia, L. V., Azarov, V. I. and Butovich, I. A. (1994) The mechanisms of the changes in coronary vascular resistance in anaphylactic shock. *Fiziol. Zh. im. I. M. Sechenova* **80**, 77–82
- 29 Marchenko, H. I., Kotsiuruba, V. M., Butovych, I. A., Sorochyns'kyi, A. E., Zrazhevs'ka, V. K. and Tumanovs'ka, L. V. (1994) The correction of disorders in arachidonic acid metabolism in coronary spasm of an immune origin. *Fiziol. Zh.* **40**, 81–87
- 30 Kukoba, T. V., Seredenko, M. M., Butovych, I. A. and Moibenko, O. O. (1998) The effect of linoleyl hydroxamic acid on lipid peroxidation processes and on the enzymatic activity of the antioxidant system in rats under hypoxia. *Fiziol. Zh.* **44**, 43–48
- 31 Butovich, I. A., Kukhar, V. P., Bridnya, V. P., Radchenko, O. A. and Kotlinskaya, A. (1989) Polyfluorosubstituted aldehydic carboxylic and hydroxamic acids as new inhibitors of lipoxygenases. *Dokl. Akad. Nauk Ukr. SSR Ser. B. Geol. Khim. Biol. Nauki* **5**, 55–58
- 32 Chen, X., Reddanna, P., Reddy, G. R., Kidd, R., Hildenbrandt, G. and Reddy, C. C. (1998) Expression, purification, and characterization of a recombinant 5-lipoxygenase from potato tuber. *Biochem. Biophys. Res. Commun.* **243**, 438–443
- 33 Butovich, I. A. and Kukhar, V. P. (1989) Potato 5-lipoxygenase. Kinetics of linoleic acid oxidation. *Ukr. Biokhim. Zh.* **61**, 106–108
- 34 Gibian, M. J. and Vandenberg, P. (1987) Product yield in oxygenation of linoleate by soybean lipoxygenase: the value of the molar extinction coefficient in the spectrophotometric assay. *Anal. Biochem.* **163**, 343–349
- 35 Schilstra, M. J., Veldink, G. A. and Vliegthart, J. F. (1994) The dioxygenation rate in lipoxygenase catalysis is determined by the amount of iron (III) lipoxygenase in solution. *Biochemistry* **33**, 3974–3979
- 36 Butovich, I. A. and Kukhar, V. P. (1991) Amphiphilic aliphatic acids – activators of 5-lipoxygenase. The adsorption-micellar regulatory mechanism. *Dokl. Akad. Nauk SSSR* **316**, 1486–1490
- 37 Swinbourne, E. S. (1960) Method for obtaining the rate coefficient and final concentration of a first-order reaction. *J. Chem. Soc.* **473**, 2371–2372
- 38 Jenkins, W. T. (1985) Use of Swinbourne plots to study potential suicide substrates: effects of ATP and ADP on yeast mitochondrial F1-ATPase. *Anal. Biochem.* **151**, 231–234
- 39 Butovich, I. A. (1986) Stability and catalytic properties of o-diphenol oxidase. 1. Oxidation of o-diphenols. *Ukr. Biokhim. Zh.* **58**, 10–16
- 40 Nelson, M. J., Batt, D. G., Thompson, J. S. and Wright, S. W. (1991) Reduction of the active-site iron by potent inhibitors of lipoxygenases. *J. Biol. Chem.* **266**, 8225–8229
- 41 Hughes, R. K., West, S. I., Hornostaj, A. R., Lawson, D. M., Fairhurst, S. A., Sanchez, R. O., Hough, P., Robinson, B. H. and Casey, R. (2001) Probing a novel potato lipoxygenase with dual positional specificity reveals primary determinants of substrate binding and requirements for a surface hydrophobic loop and has implications for the role of lipoxygenases in tubers. *Biochem. J.* **353**, 345–355
- 42 Falgoutret, J. P., Desmarais, S., Roy, P. J. and Riendeau, D. (1992) N-(4-chlorophenyl)-N-hydroxy-N'-(3-chlorophenyl)urea, a general reducing agent for 5-, 12-, and 15-lipoxygenases and a substrate for their pseudoperoxidase activities. *Biochem. Cell Biol.* **70**, 228–236
- 43 Chamulitrat, W., Mason, R. P. and Riendeau, D. (1992) Nitroxide metabolites from alkylhydroxylamines and N-hydroxyurea derivatives resulting from reductive inhibition of soybean lipoxygenase. *J. Biol. Chem.* **267**, 9574–9579

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