Requirement of monohydroperoxy fatty acids for the oxygenation of 15L$_S$-HETE by reticulocyte lipoxygenase

H. Kühn, R. Wiesner, H. Stender, T. Schewe, V.Z. Lankin*, A. Nekrasov* and S.M. Rapoport

Institute of Biochemistry, Humboldt University Berlin, Hessische Str. 3–4, DDR-104 Berlin, GDR and **All Union Cardiological Research Centre, Academy of Medical Sciences of the USSR, 3 Cherepkowskaya ul. 15 A, Moscow 12 1552, USSR*

Received 12 May 1986

The lipoxygenase from reticulocytes oxygenates 15L$_S$-HETE to 8-hydroperoxy-15-hydroxy-5,9,11,13-eicosatetraenoic acid and 5-hydroperoxy-15-hydroxy-6,8,11,13-eicosatetraenoic acid only in the presence of catalytic concentrations of monohydroperoxy fatty acids. During this reaction the hydroperoxy fatty acids are converted to more polar products including hydroxy fatty acids. From kinetic measurements of 15L$_S$-HETE oxygenation it was calculated that 1 mol monohydroperoxy fatty acid is consumed during the oxygenation of about 9 mol 15L$_S$-HETE.

Reticulocyte *Lipoxygenase* *Enzyme activation* *Oxygenation*

1. INTRODUCTION

Lipoxygenases convert polyenoic fatty acids to their corresponding hydroperoxy derivatives. Hydroperoxy fatty acids, the primary oxygenation products, are essential cofactors for this reaction [1–3] so that the lipoxygenase catalysis involves a self-activation. The mechanism of the activating process is not clarified as yet. De Groot et al. [4] proposed a reaction scheme of the lipoxygenase reaction in which the activation process of the native enzyme is accompanied by an oxidation of the enzyme bound ferrous iron to its ferric form [4]. However, the activation process was shown to be a more complex reaction since the ferric yellow enzyme, which is formed by the stoichiometric reaction of 1 mol enzyme with 1 mol of hydroperoxy fatty acid, also requires hydroperoxy fatty acid for its oxygenase activity [5]. Surprisingly we found that the products of the lipoxygenase-catalyzed oxygenation of 15L$_S$-HETE fails to activate the lipoxygenase. This observation permits the study of the chemistry and stoichiometry of the activation process without overlap with the lipoxygenase reaction per se.

The results presented here indicate that the activation process is connected with a chemical conversion of the activator and that a single activation of the enzyme is only sufficient for a limited number of substrate turnovers.

Abbreviations: 15L$_S$-HETE, 15L$_S$-hydroxy-5,8,11,13-(*Z,Z,Z,E*)-eicosatetraenoic acid; 13-HPODE, 13-hydroperoxy-9,11-(*Z,E*)-octadecadienoic acid; GC/MS, gas chromatography/mass spectroscopy; SP-HPLC, straight-phase high-pressure liquid chromatography; CP-HPLC, chiral-phase high-pressure liquid chromatography; 8,15-DiHETE, 8-hydroxy-15-hydroxy-5,9,11,13-(*Z,E,Z,E*)-eicosatetraenoic acid; 5,15-DiHETE, 5-hydroxy-15-hydroxy-6,8,11,13-(*E,Z,Z,E*)-eicosatetraenoic acid

2. MATERIALS AND METHODS

2.1. *Chemicals*

The commercial sources of the chemicals used were as follows: soybean lipoxygenase (grade IV), arachidonic acid (90% pure) and linoleic acid (90% pure) from Serva (FRG); [1-^{14}C]linoleic acid

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

(56 mCi/mmol) from Amersham (England). All solvents used were of analytical grade and were distilled prior to use.

2.2. *Assay*

The oxygenation of 15L_S-HETE was assayed spectrophotometrically by means of a Hitachi-557 spectrophotometer in 0.1 M phosphate buffer, pH 7.4, measuring the increase in absorbance at 270 nm (formation of conjugated trienes) or by recording repeated spectra in the range of 200–450 nm at 2°C.

2.3. *Preparations*

Reticulocyte lipoxygenase was purified to homogeneity as described [6]. 15L_S-HETE and 13-HPODE were prepared from the corresponding polyenoic fatty acids using soybean lipoxygenase and were purified by silica gel column chromatography. The 15L_S-HETE used as substrate for the oxygenase reaction was freed from traces of unconverted fatty acid and hydroperoxy derivatives by SP-HPLC preparation. Its chemical nature was confirmed by co-chromatography with authentic standards, in SP- and CP-HPLC, by UV- and IR-spectroscopy and by GC/MS. (The analytic data for 15L_S-HETE are available from the authors on request in the form of supplementary material.)

2.4. *Miscellaneous*

The molar absorption coefficients used were as follows: ϵ_{234} of 28000 $(M \cdot cm)^{-1}$ for 15-HETE and 13-HPODE [7], ϵ_{268} of 40000 $(M \cdot cm)^{-1}$ for 8,15-DiHETE and its 8-hydroperoxy derivative [8] and ϵ_{242} of 33000 $(M \cdot cm)^{-1}$ for 5,15-DiHETE [8]. Reduction of the hydroperoxy compounds was performed with sodium borohydride in water or triphenylphosphine in organic solvents. The fatty acid derivatives were methylated with diazomethane in ether. GC/MS analyses were performed as described elsewhere [9].

3. RESULTS

The lipoxygenase from reticulocytes converts 15L_S-HETE in the presence of catalytic amounts of monohydroperoxy fatty acids to a mixture of 5-hydroperoxy-15-hydroxy-6,8,11,13-eicosatetraenoic acid and 8-hydroperoxy-15-hydroxy-5,9,11,13-eicosatetraenoic acid. In fig.1 a representative HPLC analysis of the reduced reaction products of the oxygenation of 15L_S-HETE is shown. Product I which co-chromatographed with an authentic standard of 5,15-DiHETE amounts to 65% of the mixture of the reaction products. UV- and IR-spectroscopic data (not shown) as well as GC/MS analysis of the trimethylsilyl ethers confirmed the chemical nature of product I as 5,15-DiHETE. Typical mass ions at m/z 494 (M^+), 479 ($M^+ - 15$), 404 ($M^+ - 90$) and the onium ions at m/z 173 and 423 (OH group at C_{15}) as well as at m/z 203 and 393 (OH group at C_5) and their secondary ions at 333 (423 – 90) and 303 (393 – 90) [10] were found.

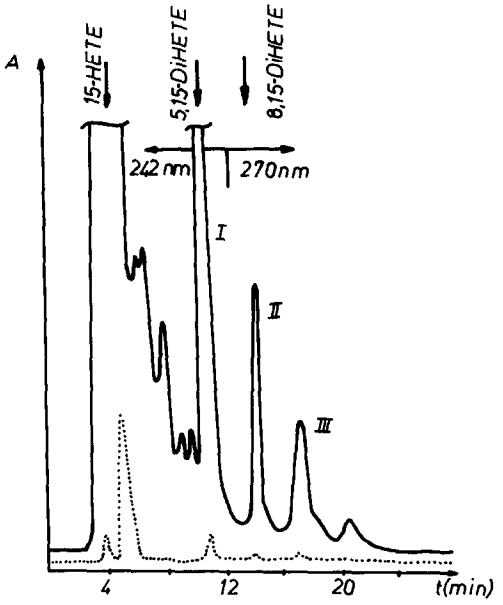

Fig.1. SP-HPLC analysis of the products of the oxygenation of 15L_S-HETE by reticulocyte lipoxygenase. Reticulocyte lipoxygenase (8 μg/ml) was incubated with 15L_S-HETE (35 μM) at 2°C in the presence of 1 μM 13-hydroperoxy-9,11-(*Z,E*)-octadecadienoic acid until the reaction had ceased. The incubation mixture was acidified to pH 3 and twice extracted with double volume of ethyl ether. The solvent was evaporated under reduced pressure, the products were dissolved in methanol, methylated and subjected to HPLC analysis on a Zorbax SIL column. The products were eluted isocratically with hexane/isopropanol (95:5, v/v). Absorbances at 242 and 270 nm were recorded. The retention times of the authentic standards are indicated by the arrows above the traces. The dotted line refers to the chromatogram recorded at lower sensitivity.

Products II and III amounting 20 and 15%, respectively, were two isomers of 8,15-DiHETE as known from UV-spectral properties and GC/MS data. Both compounds showed the typical fragmentation behaviour of 8,15-DiHETE with the most informative ions at m/z 479 ($M^+ - 15$), 404 ($M^+ - 90$), and the onium ions at m/z 173 and 423 (OH group at C_{15}) as well as at m/z 243 and 353 (OH group at C_8). Moreover the secondary ions at m/z 333 (423 – 90) and 263 (353 – 90) [11] were observed.

The oxygenation of 15Ls-HETE was followed kinetically by recording repeated spectra in the range of 200–350 nm (fig.2). Products with a peak of strong absorbance at 268 nm and shoulders at 260 and 278 nm were formed. These spectral properties are due to the formation of 8-hydroperoxy-15-hydroxy-5,9,11,13-eicosatetraenoic acid isomers. Moreover, it is seen from fig.2 that the absorbance at 240 nm decreased during the reaction. This decrease is due to the conversion of 15Ls-HETE. It should be stressed that this decrease did not reflect the real 15Ls-HETE turnover since the 5-hydroxyperoxy-15-hydroxy-6,8,11,13-eicosatetraenoic acid formed also absorbs in this region.

If HPLC-purified 15Ls-HETE was used as substrate no spectral changes were observed with either the lipoxygenase from reticulocytes (fig.3A, trace I) or soybeans (not shown). However, addition of small amounts of hydroperoxylinoleic acid led to a time-dependent increase in absorbance at 270 nm indicating the formation of conjugated

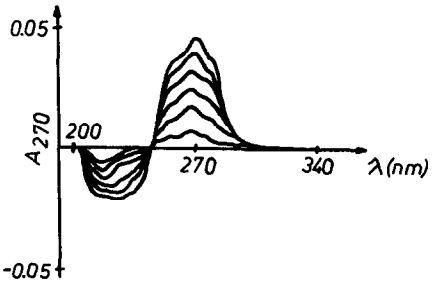

Fig.2. Conversion of 15Ls-HETE by reticulocyte lipoxygenase. Reticulocyte lipoxygenase (8 μg/ml) was incubated with 15Ls-HETE (35 μM) in the presence of 0.3 μM 13-hydroperoxylinoleic acid. Repeated spectra of the incubation mixture were recorded with a time interval of 2 min.

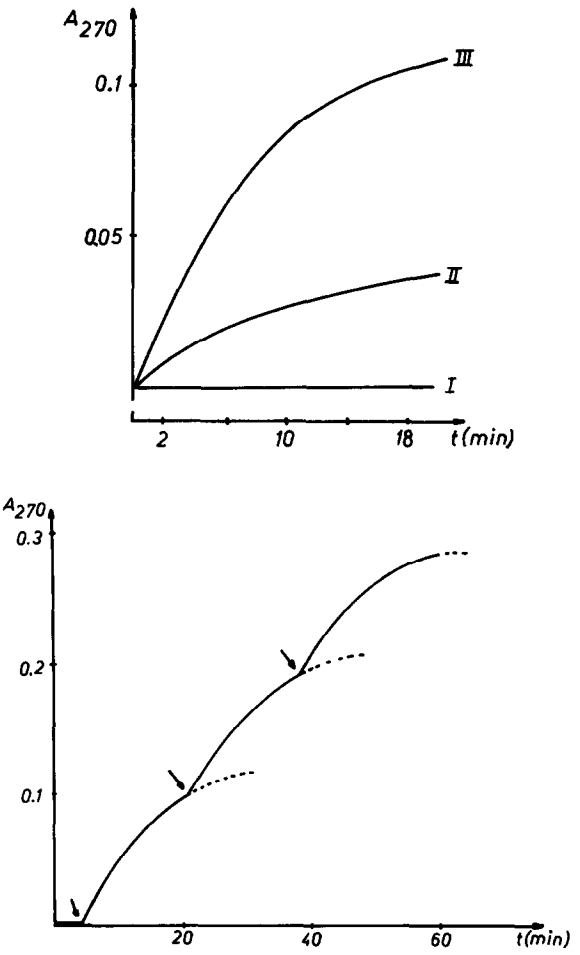

Fig.3. Requirement for hydroperoxylinoleic acid of the oxygenation of 15Ls-HETE. Reticulocyte lipoxygenase (8 μg/ml) was incubated with 15Ls-HETE (35 μM) in the presence of various concentrations of 13-HPODE. (A) Single addition of 13-HPODE: I, without; II, with 0.3 μM; III, 1.1 μM 13-HPODE. (B) Sequential addition of 13-HPODE. After the reaction had ceased further 13-HPODE (1.1 μM) was added as indicated by the arrows.

trienes (fig.3A, trace II). Comparable effects were observed if linoleic acid or arachidonic acid were substituted for hydroperoxylinoleic acid. At the high enzyme concentrations used, which were about one order of magnitude higher than that necessary for the oxygenation of polyenoic acids, the linoleic acid added as activator is rapidly oxygenated to its hydroperoxy derivative (not shown). The hydroperoxylinoleic acid formed acts

subsequently as activator for 15LS-HETE oxygenation.

Addition of higher concentrations of hydroperoxylinoleic acid led to the formation of higher amounts of reaction products (fig.3A). Other organic hydroperoxides such as *tert*-butyl hydroperoxide did not exert an activatory effect.

With 1.1 μM hydroperoxylinoleic acid the oxygenation of 15LS-HETE ceased after about 20 min (see fig.3B). Further addition of 15LS-HETE or lipoxygenase did not produce further increase in absorbance at 270 nm. However, after repeated addition of catalytic amount of hydroperoxy fatty acids the conversion of 15LS-HETE again proceeded with about the same rate as initially (fig.3B). The total substrate turnover was somewhat smaller after the second addition of hydroperoxylinoleic acid as known from increase in absorbance at 270 nm (fig.3B). To establish whether the hydroperoxy fatty acid is consumed during the oxygenation of 15LS-HETE, experiments with radioactively labelled linoleic acid as activator were performed. As seen from fig.4 (trace A) more than 90% of the linoleic acid added were converted to more polar products. Only small amounts of hydroperoxylinoleic acid, the real activator, remained.

If the lipoxygenase is incubated in the presence of hydroperoxy fatty acid but in the absence of 15LS-HETE only small amounts of the hydroperoxy fatty acid were converted (fig.4, trace B). Moreover, it was shown that preincubation of the hydroperoxy fatty acid with the enzyme for 20 min and subsequent addition of 15LS-HETE led to the same spectral changes as observed if activator and substrate were added simultaneously. These results clearly indicate that the hydroperoxy fatty acid acting as activator is consumed during the oxygenation of 15LS-HETE. During the activation process about 15% of the hydroperoxylinoleic acid were converted to its hydroxy derivative, a product which is not formed during the anaerobic linoleic acid supported hydroperoxidase reaction [12]. The chemical nature of the more polar products remains to be clarified. From the data presented in fig.3 it was calculated that 1.1 nmol hydroperoxylinoleic acid were consumed for the formation of about 3 nmol 8-hydroperoxy-15-hydroxy-5,9,11,13-eicosatetraenoic acids. As judged from the HPLC analysis this amount of conjugated triene formation corresponds to 9 nmol of reaction products. Therefore it may be concluded that 1 mol hydroperoxy fatty acid is consumed during about 9 catalytic cycles of the oxygenation reaction.

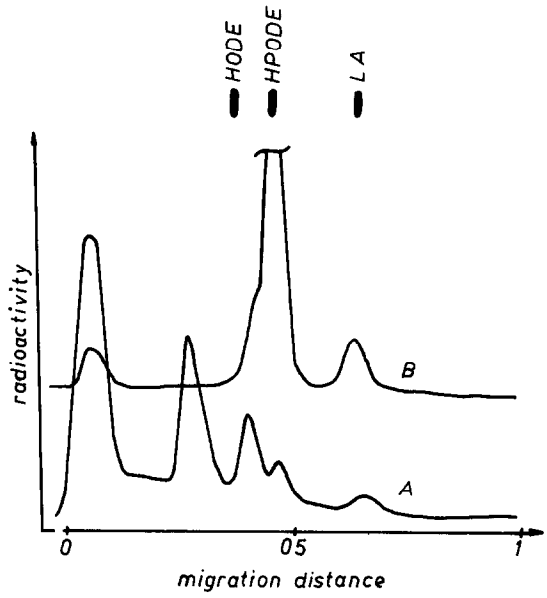

Fig.4. Conversion of hydroperoxylinoleic acid during the oxygenation of 15LS-HETE. Reticulocyte lipoxygenase (8 μg/ml) was incubated with 15LS-HETE (50 μM) in the presence of 1 μM [1-^{14}C]linoleic acid as activator (trace A). The absorbance at 270 nm was recorded and when the absorbance no longer increased, the reaction was stopped by acidification to pH 3. The sample was twice extracted with the double volume of diethyl ether, the solvent was evaporated under reduced pressure, the products were dissolved in methanol and aliquots were subjected to chromatography on precoated silica gel thin-layer plates (Silufol, Kavalier; Czechoslovakia). The chromatograms were developed with diethyl ether/hexane/acetic acid (70:50:1, by vol.). The radioactivity was scanned by means of a Bertholdt LB 2722 DC scanner. The migration distances of the reference compounds are indicated by the spots above the traces. LA, linoleic acid; HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid. (Trace B) Incubation of reticulocyte lipoxygenase (8 μg/ml) with 1 μM [1-^{14}C]hydroperoxylinoleic acid alone.

4. DISCUSSION

Lipoxygenases require hydroperoxy fatty acid as

activator not only for their reaction with polyenoic fatty acids but also for that with hydroxy fatty acids. The concentration of hydroperoxy fatty acids necessary for the oxygenation of 15Ls-HETE is about the same as that required to overcome the kinetic lag phase of the oxygenation of linoleic acid by the lipoxygenases from soybeans and reticulocytes [13,14]. At this low concentration of hydroperoxy fatty acids other lipoxygenase-catalyzed reactions such as the various types of hydroperoxidase reactions [15,16] which require high concentrations of hydroperoxy compounds are largely excluded.

For studies on the mechanism of the activation process a system with hydroxy polyenoic fatty acids as substrates is suitable since the oxygenation products do not exert the activatory effect as is the case if non-oxygenated polyenoic fatty acids are used as substrate.

It should be stressed that all experiments with this aim should be performed at 2°C since at higher temperatures hydroperoxy fatty acids are known to inactivate the reticulocyte lipoxygenase [17,18]. For this reason, at 25°C only one-fifth of the rate of 15Ls-HETE oxygenation was observed as compared with the rate measured at 2°C (not shown).

The approximate calculation that 1 mol of hydroperoxy fatty acid is consumed during the oxygenation of 9 mol of 15Ls-HETE indicates that a single activation of the enzyme is only sufficient for a limited number of catalytic cycles. During these substrate turnovers the lipoxygenase becomes deactivated and requires further activation to maintain the oxygenase reaction.

In preliminary studies with the soybean lipoxygenase it was found that this enzyme shows a similar activation behaviour as the lipoxygenase from reticulocytes. However, the reticulocyte system appears to be more suitable for the investigation of the activation process since the soybean enzyme exhibits a relatively low affinity for oxygenated fatty acids as substrates [8].

In conclusion, the oxygenation of 15Ls-HETE by the reticulocyte lipoxygenase appears to be an excellent system to further elucidate the mechanism of self-activation of lipoxygenases and related enzymes, such as cyclooxygenase [19,20].

ACKNOWLEDGEMENTS

The authors are greatly indebted to Dr L. Alder from the Department of Chemistry of the Humboldt University and Dr V.L. Sadovskaya from the All Union Institute of Applied and Molecular Biology and Genetics of Academy of Sciences of the USSR for the performance of the GC/MS measurements.

REFERENCES

[1] Haining, J.L. and Axelrod, B. (1957) J. Biol. Chem. 232, 193–202.
[2] Smith, W.L. and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1038–1047.
[3] Egmond, M.R., Brunori, M. and Fasella, P.M. (1976) Eur. J. Biochem. 61, 93–100.
[4] De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and Van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71–79.
[5] De Groot, J.J.M.C., Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1975) FEBS Lett. 56, 50–54.
[6] Schewe, T., Wiesner, R. and Rapoport, S.M. (1981) Methods Enzymol. 71, 430–441.
[7] Tappel, A.L., Boyer, P.D. and Lundberg, W.D. (1952) J. Biol. Chem. 199, 267–281.
[8] Van Os, C.P.A., Rijke-Schilder, G.P.M., Van Halbeek, H., Verhagen, J. and Vliegenthart, J.F.G. (1981) Biochim. Biophys. Acta 663, 177–193.
[9] Kühn, H., Wiesner, R., Schewe, T., Lankin, V.Z., Nekrasov, A. and Alder, L., Anal. Biochem. submitted.
[10] Maas, R., Brash, A. and Oates, J.A. (1982) in: Leukotrienes and Other Lipoxygenase Products (Samuelsson, B. and Paoletti, R. eds) pp.29–44, Raven, New York.
[11] Radmark, O., Lundberg, U., Jubitz, W., Malmsten, C. and Samuelsson, B. (1985) in: Leukotrienes and Other Lipoxygenase Products (Samuelsson, B. and Paoletti, R. eds) pp.61–76, Raven, New York.
[12] Salzmann, U., Kühn, H., Schewe, T. and Rapoport, S.M. (1984) Biochim. Biophys. Acta 795, 535–542.
[13] Funk, M.O., Kim, S.H.S. and Alteneder, A.W. (1981) Biochem. Biophys. Res. Commun. 98, 922–929.
[14] Ludwig, P., Silvestrini, C. and Colosimo, A. (1984) Abstracts of the 16th FEBS Meeting, Moscow, June 24–30, 1984, Abstr. No. III/129.

[15] Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1977) Prog. Fats Other Lipids 15, 131–165.
[16] Verhagen, J., Bouman, A.A., Vliegenthart, J.F.G. and Boldingh, J. (1977) Biochim. Biophys. Acta 486, 114–120.
[17] Verhagen, J., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1979) in: Adv. Biochem. Physiol. Plant Lipids (Appelquist, L.A. and Liljenberg, C. eds) pp.231–236, Elsevier, Amsterdam, New York.
[18] Härtel, B., Ludwig, P., Schewe, T. and Rapoport, S.M. (1982) Eur. J. Biochem. 126, 353–357.
[19] Rapoport, S.M., Härtel, B. and Hausdorf, G. (1984) Eur. J. Biochem. 139, 573–576.
[20] Hemler, M.F., Graff, G. and Lands, W.E.M. (1978) Biochem. Biophys. Res. Commun. 85, 1325–1351.
[21] Kulhacz, R.J., Miller, J.F. and Lands, W.E.M. (1985) Biochem. Biophys. Res. Commun. 130, 918–923.