C-11 H-ABSTRACTION FROM LINOLEIC ACID, THE RATE-LIMITING STEP IN LIPOXYGENASE CATALYSIS.

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Received August 27, 1973

Summary.
[11,11-$^2H_2$]-, [9,10,12,13-$^2H_4$]-, [9,10,11,11,12,13-$^2H_6$]- and unlabelled linoleic acids were incubated with pure lipoxygenase-1 from soya beans. The apparent rate constants of the overall reactions and the apparent Michaelis constants in air-equilibrated solutions at 25°C and pH 9.0 were obtained from Lineweaver-Burk plots. The apparent K_m-values were hardly affected by the type of substrate used. Substrates bearing ^2H instead of ^1H at C-11 gave rise to considerable isotope effects, $k_H/k_{^2H}$ values being 8.7 and 9.3 for dideutero- and hexadeutero linoleate, respectively. From the observed isotope effects it was concluded, that H-abstraction from C-11 is the rate-determining step in the overall reaction. All substrates used gave identical product distributions. No measurable exchange of deuterium with solvent hydrogen occurred during oxygenation.

Introduction.

In preyious studies on the stereochemistry of the oxygenation of unsaturated fatty acids catalysed by soybean lipoxygenase (fatty acid : oxygen oxido-reductase, E.C.1.13.11.12, formerly 1.13.1.13) it was observed, that incubation with substrate, labelled with ^3H at carbon atom n-8 in the L_S-configuration, leads to enrichment of ^3H relative to ^1H in unreacted substrate[1,2]. In addition to the stereochemical data provided by these experiments the observed tritium-enrichment in both the C_{20}[1)] and the C_{18}[2)] substrates demonstrated an important difference in the rate of hydrogen- vs. tritium-abstraction. However, a quantitative assessment of kinetic parameters using these ^3H- and ^{14}C-labelled substrates was hampered by the type of labelling. Therefore, in the present study a number of "carrier free" deutero-linoleic acids have been used to examine some aspects of the steady-state kinetics of the lipoxygenase reaction.

Materials and Methods.

Substrates.

Linoleic acid (purity higher than 99%), [11,11-$^2H_2$] linoleic acid (purity

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97.8%), [9,10,12,13-$^{2}H_4$] linoleic acid (purity 98%), [9,10,11,11,12,13-$^{2}H_6$] linoleic acid (purity 97.7%) were kindly provided by the Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. The isotope purity at C-11 of the dideutero- and hexadeutero-linoleic acids was: 95% C-$^{2}H_2$ and 5% C-^{2}H,H plus C-H_2. All substrates were stored under nitrogen at -25°C. Prior to use, the linoleic acids were freed from hydroperoxy acids, formed by autoxidation during storage.

Enzyme.

Pure lipoxygenase-1 from soybeans was used. Purification and properties of the enzyme are described by Finazzi Agrò et al.[3]. The specific activity of the enzyme was 106 μmole.min^{-1}.mg^{-1} (at substrate concentration 3.56 mM, product concentration approx. 5 μM, oxygen concentration approx. 250 μM, pH 9.0 and 25°C). The iron content of the enzyme was found to be 0.90 gramatom Fe per mole of enzyme (based on mol. weight: 98.600).

Kinetic measurements.

Substrates were dissolved in 3.0 ml 0.1 M sodiumborate buffer (pH 9.0), the concentration ranged from 16.7 to 500 μM. The reaction was started by adding 1 μl 0.1 M sodiumborate buffer (pH 9.0), containing 0.94 μg lipoxygenase to the linoleate solution, which was equilibrated with air at 25°C in a 10 mm cuvette. The solution was mixed rapidly and after about 15 sec the increase of absorbance at 234 nm with time was followed with a Unicam SP 1800 ultraviolet spectrophotometer, equipped with a Unicam AR 25 linear recorder.

Incubations on preparative scale.

The deuterated linoleic acids and unlabelled linoleic acid were dissolved in 0.1 M sodiumborate buffer (pH 9.0) to a final concentration of 3.56 mM. The solutions were cooled to 0°C and saturated with oxygen (the oxygen concentration was about 2.17 mM). Per mg C-11 deuterated linoleic acid 42 μg lipoxygenase-1 was added, while about 4 μg enzyme was added per mg C-11 unlabelled linoleic acid. The solutions were kept saturated with oxygen at 0°C during the incubations. The conversion was followed spectrophotometrically by adding 50 μl samples of the reaction mixture to 2.5 ml ethanol and measuring the absorbance at 234 nm. The extent of the reactions was calculated by using a molar extinction coefficient of 2.8×10^{4} l.M^{-1}.cm^{-1} for the hydroperoxy acids formed[4]. The reactions were stopped by acidification to pH 3.0 with 2M HCl. Products and unconverted substrate were isolated by extraction with diethylether. The combined extracts were washed once with double distilled water and dried over anhydrous sodiumsulphate. After treatment with diazomethane at 0°C the methyl esters of the products and unconverted substrate

were separated by tlc on 0.25 mm silica gel plates (20 x 20 cm, DC Fertig Platten, Kieselgel 60 F254, E. Merck, Darmstadt, W. Germany), developed with light petroleum (b.p. 60°-80^oC)- diethylether (3:2, v/v).

Product analyses.
The mixture of the hydroperoxy-octadecadienoates was dissolved in $^2HCCl_3$ and analysed by proton-magnetic resonance (p.m.r.) spectrometry with a Varian HA 100 spectrometer. To study the positional specificity of the enzyme, the mixture of the hydroperoxy-octadecadienoates was converted into a mixture of the corresponding hydroxy-stearates by catalytic hydrogenation (H_2/PtO_2) in methanol. The hydroxy-stearates were analysed by mass spectrometry with an AEI MS 9 mass spectrometer (70 eV, 80^oC) either as a mixture or after separation by tlc on 0.25 mm silica gel plates (20 x 20 cm, DC Fertig Platten, without fluorescence indicator, E. Merck) twice developed with the solvent system light petroleum (b.p. 60°-80^oC) - diethylether (3:2, v/v).

Results

The recordings of the increase of absorbance at 234 nm vs. time at substrate concentrations ranging from 16.7 to 50 µM showed a linear response between 15 and 45 sec after the addition of enzyme. From the slope of these lines, which could be extrapolated to t_0 (addition of enzyme), the apparent initial velocity of the reaction was calculated. When the substrate concentration was higher than 50 µM, the recordings of the increase of absorbance at 234 nm vs. time showed a sigmoidal response. Under these conditions the apparent initial velocity was calculated from the slope of the linear intercept between 1 and 2 min. after the addition of enzyme. The reciprocal of the apparent initial velocity, $^1/v$ (sec/µM) was multiplied by the initial enzyme concentration, e (3.1×10^{-3} µM) and the product, $^e/v$ (sec) was plotted against the reciprocal of the substrate concentration, $^1/S$ (μM^{-1}). The results, obtained from the incubations with linoleic acid and the deuterated linoleic acids are shown in fig. 1. By the method of least squares straight lines were obtained, which closely fitted the experimental points within the substrate concentration range from 16.7 to 50 µM. Allen[5)] reported, that the system lipoxygenase-linoleate obeys Michaelis-Menten kinetics at substrate concentrations lower than 150 µM in air-saturated solution at 25^oC and pH 9.0. Our results agree with this observation and show, that the deuterated substrates do not behave differently in this respect. The apparent K_m-values and rate constants k obtained from the Lineweaver-Burk plots are summarized in tabel I. The apparent K_m-values of the deuterated linoleic acids (approx. 20 µM) hardly differ from the apparent K_m-value found for linoleic acid (23 µM). These

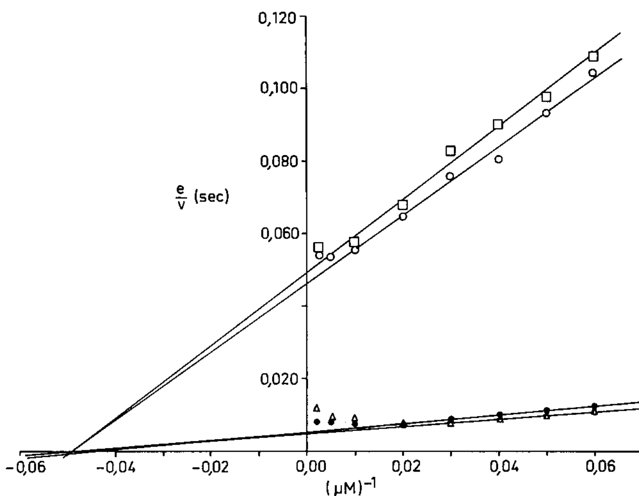

Fig. 1.
Lineweaver-Burk plots obtained from incubations of linoleic acid (●), [11,11-$^2H_2$] linoleic acid (o), [9,10,12,13-$^2H_4$] linoleic acid (Δ) and [9,10,11,11,12,13-$^2H_6$] linoleic acid (□) with soybean lipoxygenase-1 at pH 9.0 and 25°C. The oxygen concentration in the solutions is 250 μM. The points are the mean values of two or three determinations. The lines were calculated by the method of least squares from the experimental points between 16.7 and 50 μM substrate concentration.

values correspond with the K_m-values, previously reported by Allen[5] (24 μM) and Tappel et al.[6] (2×10^{-5} M) for the system soybean lipoxygenase-linoleate at pH 9.0 and 25°C. The apparent rate constants of the reactions with dideutero- and hexadeutero-linoleic acids are almost equal, namely 21.7 sec^{-1} and 20.2 sec^{-1}, but are considerably smaller than those observed for unlabelled linoleic acid and the tetradeutero-linoleic acid (which bears ^1H at C-11). These results indicate, that only substituting two ^2H atoms for ^1H atoms at C-11 (n-8) of linoleic acid reduces the reaction rate constant k, whereas substituting ^2H atoms at C-9, -10, -12, and 13 of linoleic acid for the ^1H atoms at these positions does not give rise to a substantial lowering of the apparent rate constant and does not affect the apparent K_m. The ratio of the rate constants of unlabelled linoleic acid and deuterated linoleic acid gives the kinetic isotope effect, $k_H/k2_H$, which is 8.7 ± 0.4 and 9.3 ± 0.4 for the dideutero- and hexadeutero-linoleic acids, respectively. By tlc-analysis, it was shown, that hydroperoxy acids are the only products formed from the deuterated substrates, whereas 70 eV mass spectra of the hydroxy-stearates, derived from the hydroperoxy acids revealed, that molecular oxygen was introduced only at C-13 (n-6) and at C-9 (n-10). The ratio of the deuterated 13- and 9-hydroxy-stearates was 97 to 3, respectively. The same ratio was obtained from mass spectra of unlabelled 13- and 9-hydroxy stearates. Analysis of the

Table I

SUBSTRATE	K_m (μM)	K (sec^{-1})	$K_H/K2_H$
LINOLEIC ACID	23.3 ± 1.4	188.2 ± 1.1	1.0
[9,10,12,13-$^2H_4$] LINOLEIC ACID	19.6 ± 0.9	181.5 ± 5.3	1.04 ± 0.03
[11,11-$^2H_2$] LINOLEIC ACID	20.5 ± 1.1	21.7 ± 0.9	8.7 ± 0.4
[9,10,11,11,12,13-$^2H_6$] LINOLEIC ACID	20.3 ± 1.4	20.2 ± 0.9	9.3 ± 0.4

The apparent Michaelis constants K_m and rate constants k obtained from the Lineweaver-Burk plots (fig. 1) are shown. The kinetic isotope effect $k_H/k2_H$ is calculated for dideutero-, tetradeutero- and hexadeutero-linoleic acids by taking the quotient of the rate constants k of the unlabelled linoleic acid and the deuterated linoleic acids, respectively.

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purified deuterated hydroxy-stearates also indicated, that some exchange of ^2H against ^1H took place. Although the catalytic hydrogenation was thought to be responsible for this, enzyme-catalysed exchange during oxygenation could not be excluded. Therefore, the purified (13- and 9-)hydroperoxy-octadecadien-oates were analysed directly by p.m.r. spectrometry. No indication was found for any exchange of deuterium label: the ratio of $^2H/H$ at C-11 (n-8) of the hydroperoxy-octadecadienoates (derived from the dideutero- and hexadeutero-linoleic acids) was the same as in the original substrates.

Discussion

The kinetic parameters, K_m and k, obtained from the experiments with the deuterated linoleic acids (tabel I), clearly demonstrate, that substitution of both hydrogen atoms at C-11 of linoleic acid only affects the rate constant k, but hardly influences K_m. Comparable results were obtained by Kepler et al.[7] from incubations of linoleic acid, [11,11-$^2H_2$] linoleic acid and [U-^2H] linoleic acid with the enzyme 12-cis, 11-trans linoleic acid isomerase. The kinetic isotope effect $k_H/k2_H$ approx. 9 in our experiments with the dideuter-ated- and hexadeuterated-linoleic acids consists of a primary- and a secondary

isotope effect. The secondary isotope effect, arising from the change in hybridization of C-11 from sp^3 to sp^2 is estimated to be slightly higher than 1, but not higher than 1.3 [8]. The value of the primary isotope effect would then fall within the range from about 7 to 9. The magnitude of this isotope effect justifies the conclusion, that the H-abstraction from C-11 of the substrate is the rate-limiting step in the overall reaction. Theoretically (Swain et al.[9]) a primary deuterium isotope effect ($k_H/k2_H$) of about 7 to 9 leads to a primary tritium isotope effect ($k_H/k3_H$) of about 16.5 to 24. This indicates, that incubation of lipoxygenase-1 with [$11L_S$-^3H,1-^{14}C] linoleic acid would give rise to strong enrichment of ^3H-label in unconverted substrate. Incubations with the dually labelled linoleic acid indeed showed this effect[2]; after almost complete conversion of the ^{14}C- labelled linoleic acid only a small amount of the ^3H- labelled substrate had reacted.
Since H-abstraction is the rate-limiting step in the overall oxygenation reaction, studies on isotope-enrichment in unconverted substrate do not show, whether or not the H-abstraction is an initial step in the oxygenation reaction. Recently, De Groot et al.[10] proved, that under anaerobic conditions lipoxygenase-1 abstracts hydrogen from linoleic acid, thereby producing detectable amounts of linoleic acid free radicals. Presumably, in the aerobic reaction also, molecular oxygen is not involved in the H-abstraction step. Therefore, evidence seems to accumulate, that hydrogen-abstraction from C-11 of linoleic acid is the initial- and rate-determining step in lipoxygenase reactions.

Acknowledgements

Thanks are due to Dr. H.J.J. Pabon, Mr. L. van der Wolf and Miss J.W. Bos of the Unilever Research Laboratory, Vlaardingen, The Netherlands, for the preparation of the deuterated linoleic acids, to the Department of Mass spectrometry of the Laboratory of Analytical Chemistry at the University of Utrecht, The Netherlands, and to the Department of p.m.r. spectrometry of the Laboratory of Organic Chemistry at the University of Utrecht, for the product analyses. This investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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