STEREOSPECIFICITY OF THE HYDROGEN ABSTRACTION AT CARBON ATOM n-8 IN THE OXYGENATION OF LINOLEIC ACID BY LIPOXYGENASES FROM CORN GERMS AND SOYA BEANS.

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SUMMARY

Stereospecifically tritium labeled linoleic acid has been incubated with the lipoxygenases from corn germs and soya beans at pH 6.6 and pH 9.0 respectively.
It has been concluded from the percentage retention of the tritium label in the main products formed, that these lipoxygenases have opposite stereospecificity in the hydrogen abstraction at carbon n-8: lipoxygenase from corn germs mainly removes the D_R(n-8) hydrogen, whereas lipoxygenase from soya beans removes the L_S(n-8) hydrogen. Stereospecificity of hydrogen abstraction and oxygen insertion are compared for both enzymes. The consequences of these findings for the reaction mechanism are discussed.

INTRODUCTION

Unsaturated fatty acids with a 1,4-pentadiene structure, the double bonds located at n-6 and n-9, are good substrates for lipoxygenase (linoleate: oxygen oxido-reductase, E.C. 1.13.1.13). The unsaturated acids are converted into optically active hydroperoxy acids with a conjugated double bond system, one of the hydrogen atoms at carbon atom n-8 being removed by the enzyme. Molecular oxygen is inserted into the substrate molecule at the n-6 or n-10 position, depending on the type of lipoxygenase or on the conditions of incubation. The positional and stereochemical specificities of different lipoxygenases have been studied by several authors [Tappel 1963[1], Hamberg & Samuelsson 1967[2], Veldink et al. 1970[3], Gardner & Weisleder 1970[4], Galliard & Phillips 1971[5], Chang et al. 1971[6], Veldink et al. 1972[7]].
Lipoxygenase from soya beans converts linoleic acid mainly into 13L_S* -hydroperoxy-9 cis,11 trans octadecadienoic acid at pH 9.0 in an oxygen atmosphere. Veldink et al.[3] reported the minor product in the oxygenation of linoleic acid, catalyzed by this enzyme preparation at pH 9.0, to be 9D_S-hydroperoxy-10 trans,12 cis octadecadienoic acid. They proposed that this acid was also formed enzymically. Gardner & Weisleder[4] revealed that corn germ

* L and D refer to the nomenclature according to the Fischer convention; subscripts S and R refer to the nomenclature according to Cahn, Ingold and Prelog 1956[9].

lipoxygenase produces at pH 6.9 $9D_S$-hydroperoxy-10,12-octadecadienoic acid as the main product. Recently Hamberg[8] reported the ratio between all four positional and stereochemical isomers of the hydroperoxy acids, which are formed on incubation of linoleic acid with lipoxygenases from different sources, under various conditions.

Hamberg & Samuelsson[2] demonstrated the removal of the L_S hydrogen from carbon atom n-8 of stereospecifically tritium labeled all cis-8,11,14-eicosatrienoic acid, when incubated with soya bean lipoxygenase at pH 9.0. The present study describes the stereospecificity of the hydrogen abstraction from carbon atom n-8 of linoleic acid, catalyzed by both corn germ (pH 6.6) and soya bean (pH 9.0) lipoxygenases.

EXPERIMENTAL

The stereospecifically tritium labeled linoleic acid was prepared from optically pure lactone of $5D_R$-hydroxy-dodecanoic acid*. (Tuynenburg Muys et al. 1963[10], Korver 1970[11]).

The free $5D_R$-hydroxy-dodecanoic acid, obtained after hydrolysis of the lactone, was methylated by treatment with diazomethane and tosylated (Schroepfer & Bloch 1965[12], Hamberg & Samuelsson 1967[13]). The tosylate of methyl $5D_R$-hydroxy-dodecanoate was reduced with $LiAl^3H_4$ and subsequently oxidized with CrO_3, which gave [$5L_S$-^{3}H] dodecanoic acid. This labeled acid was elongated by means of anodic coupling with monomethyl suberate into methyl [$11L_S$-^{3}H] stearate. The tritium labeled stearate was mixed with [1-^{14}C] labeled stearic acid. This mixture was saponified and the free stearic acid was incubated with the algae Chlorella vulgaris, strain 211-11h**. The dual labeled methyl linoleate was isolated from the lipids of the algae, according to the method of Bartels 1963[14]. After saponification, the labeled linoleic acid ($^3H/^{14}C$ ratio 0.54 $\frac{\text{counts.min}^{-1}}{\text{counts.min}^{-1}}$) was used as substrate for the lipoxygenase incubations. Details of the preparation of the stereospecifically tritium labeled linoleic acid will be published elsewhere.

Lipoxygenases

Partially purified lipoxygenase from corn germs (Zea mays, variety Caldera) was used (Veldink et al. 1972[7]). The specific activity, determined from oxygen uptake, was 0.3 units/E 280 nm.

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* this lactone was kindly provided by Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands.

** the algae were kindly provided by Unilever Research Laboratories, Colworth/Welwyn, United Kingdom.

Lipoxygenase from soya beans was purchased from Nutritional Biochemicals Co.; specific activity (oxygen uptake): 5.2 units/E 280 nm.

Prior to use the enzyme solutions were saturated with oxygen at 0°C. Enzyme activity was assayed with a Clark-oxygen electrode in a GME-Oxygraph model KM; UV-absorption spectra were recorded with a Unicam SP800-B spectrophotometer.

Incubations

The incubations were carried out at 0°C in an oxygen atmosphere. 4.8 mg 11L_S-^{3}H,1-^{14}C linoleic acid was incubated with 1.55 mg corn germ lipoxygenase in 5.0 ml 0.1 M potassium phosphate buffer solution (pH 6.6). Also 4.8 mg 11L_S-^{3}H,1-^{14}C linoleic acid was incubated with 0.26 mg soya bean lipoxygenase in 4.0 ml 0.1 M potassium borate buffer solution (pH 9.0). The rate of hydroperoxide formation was followed spectrophotometrically.

Isolation and separation of the reaction products

Aliquots of 1-1.2 ml were taken at 2, 15, 30 and 50 minutes of incubation time and these were added to 3 ml cold ethanol. Each sample was treated with 70 mg $NaBH_4$ at 0°C for 30 minutes. After dilution of the reaction mixture with aqua bidest and acidification to pH 2, the hydroxy acids were recovered by extraction with diethyl ether. Following methylation with diazomethane, the methyl esters were separated on silica gel G thin layer plates. The plates were developed with the solvent system light petroleum (b.p. 60°-80°C): diethylether 3:2 (v/v) and subsequently scanned with a Berthold model LB 2727 thin layer radioactivity scanner. The radioactive areas were scraped from the plates. Radioactive material was isolated by elution from the silica gel with diethyl ether. The positional isomers, methyl 9- and 13-hydroxy-octadecadienoates, cannot be separated adequately by TLC, therefore part of the mixture was hydrogenated in methanol with PtO_2 as catalyst and subjected to TLC on silica gel G. Methyl 9- and 13-hydroxy-stearates were separated by double development with the solvent system light petroleum (b.p. 60°-80°C): diethylether 3:2 (v/v).

Determination of the amount of tritium

^{3}H counts.min^{-1} relative to ^{14}C counts.min^{-1} were measured with a Packard Tricarb scintillation spectrometer. The ^{3}H content of the hydroxy-stearates was calculated from the measured ^{3}H/^{14}C ratio. After removal of the diethylether under a stream of nitrogen gas, the sample was dissolved in 16 ml toluene, containing 0.5% PPO and 0.03% dimethyl POPOP. This scintillation medium had low quenching properties and allowed reproducible countings of both isotopes.

RESULTS AND DISCUSSION

[11L_S-^{3}H,1-^{14}C] linoleic acid (17 µmol; 2.9 x 10^5 counts.min^{-1}. ^{3}H; 5.4 x 10^5 counts.min^{-1} ^{14}C; ratio 0.54) incubated with corn germ lipoxygenase at pH 6.6, was converted almost exclusively into 9-hydroperoxy-octadecadienoic acid. The hydroperoxy acid was reduced to hydroxy acid and the ^{3}H/^{14}C ratio was measured. The average out of 4 samples gave a 95% recovery of the tritium label in the mixture of 9- and 13-hydroxy-octadecadienoates. This mixture was hydrogenated and 9- and 13-hydroxy-stearates were separated by TLC. The percentage retention of the tritium label in both hydroxy stearates was calculated from the observed ^{3}H/^{14}C ratio in these products. The results are shown in scheme I. The corn germ lipoxygenase preparation converted linoleic acid into 95% of 9-hydroperoxy-octadecadienoic acid and 5% of 13-hydroxy-octadecadienoic acid. The main product appeared to contain 96% of the original tritium label.

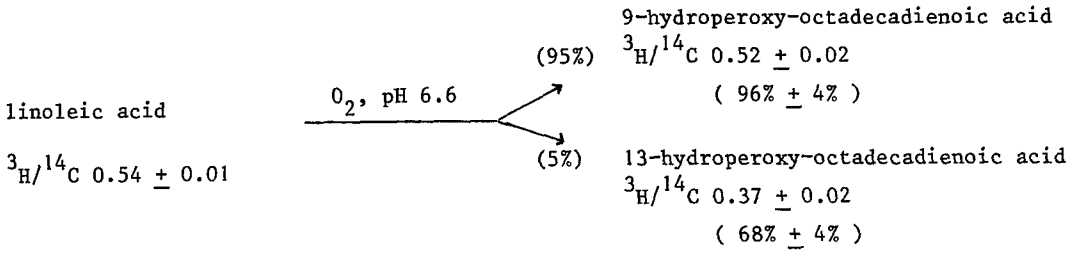
Because the substrate was stereospecifically L_S tritium labeled at carbon atom n-8, 96% of the D_R (n-8) hydrogen was removed by the enzyme. The hydrogen abstraction in the formation of the minor product proceded less selectively, compared to the main product: 68% retention of the tritium label in this product corresponded to 68% D_R (n-8) hydrogen abstraction.

Soya bean lipoxygenase catalyzes at pH 9.0 the conversion of linoleic acid into mainly 13-hydroperoxy-octadecadienoic acid. The results of an experiment with [11L_S-^{3}H,1-^{14}C] linoleic acid are also summarized in scheme I. Under the conditions used, 80% of the main product was formed. Only 15% of the original tritium label was retained in this product, so the enzyme removed 85% of the L_S (n-8) hydrogen atoms. A strong isotope effect became apparent by measuring the ^{3}H/^{14}C ratio in the non-converted linoleic acid. In the same experiment 20% of 9-hydroperoxy-octadecadienoic acid was formed. Scheme 1 shows that 59% of the tritium label was retained in this product. This means that the hydrogen abstraction was almost random, with a slight preponderance of the D_R over the L_S abstraction.

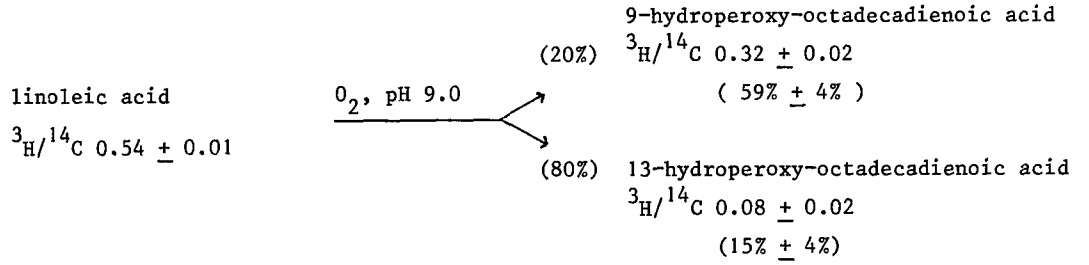
Comparing the stereochemistry of the hydrogen abstraction and oxygen insertion for the main reaction products, a close correlation is observed: L_S (n-8) hydrogen abstraction leads to 13L_S-hydroperoxy-octadecadienoic acid (soya bean lipoxygenase, pH 9.0) and D_R (n-8) abstraction leads to 9D_S-hydroperoxy--octadecadienoic acid (corn germ lipoxygenase, pH 6.6). The consequences of these findings may be visualized in fig. 1.

If the pentadiene system of the substrate molecule is oriented on the enzyme as a planar structure - as has been proposed earlier by Veldink et al.[3] - it may be seen that oxygen insertion and hydrogen abstraction take place at opposite sides of the plane.

Oxygenation catalyzed by corn germ lipoxygenase



Oxygenation catalyzed by soya bean lipoxygenase



Scheme 1

The oxygenation of unsaturated fatty acids, catalyzed by lipoxygenase, differs from oxygenation of olefins in which singlet oxygen is involved: introduction of singlet oxygen and hydrogen abstraction take place at the same side of a planar olefinic structure, as has been proposed by Foote[15]. The participation of ground state (triplet) oxygen in the enzymic formation of hydroperoxy acids is only probable, when the reaction is initiated by the abstraction of hydrogen as a radical. Initiation of the reaction by hydrogen abstraction has been concluded by Hamberg & Samuelsson[2]. They observed an isotope effect on incubation of L_S (n-8) tritium labeled eicosatrienoic acid, which indicates that hydrogen abstraction must precede or coincide with irreversible oxygen insertion.

However, that the hydrogen abstraction proceeds via a radical mechanism has still to be established definitely.

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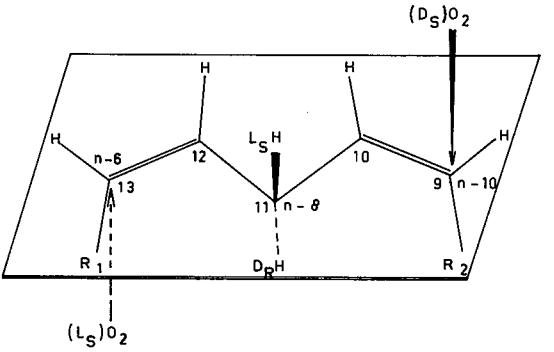

Fig. 1.

R_1 : $CH_3-(CH_2)_4-$ R_2 : $-(CH_2)_7-COOH$

A planar pentadiene system of linoleic acid has been constructed. In this model the L_S (n-8) hydrogen atom is pointing towards the reader, the D_R (n-8) hydrogen atom points downwards.
The approach of oxygen from above gives hydroperoxides with D-configuration; approach from below gives hydroperoxides with L-configuration.
Corn germ lipoxygenase (pH 6.6), main product $9D_S$-hydroperoxy-octadecadienoic acid: oxygen from above, D_R hydrogen abstraction from below.
Soya bean lipoxygenase (pH 9.0), main product $13L_S$-hydroperoxy-octadecadienoic acid: oxygen from below, L_S hydrogen abstraction from above.
These results indicate, that if the active sites of both enzymes are stereochemically identical, the differences in positional and stereochemical configuration of the hydroperoxides result from an alternate orientation of the substrate molecule on the enzymes.

References

1. A.L.Tappel (1963) in: The Enzymes, vol. 8 p 275, P.D.Boyer, H.Lardy & K.Myrbäck (Editors), Acad.Press, New York.
2. M.Hamberg & B.Samuelsson (1967), J. Biol. Chem. 242, 5329
3. G.A.Veldink, J.F.G.Vliegenthart & J.Boldingh (1970) Biochim. Biophys. Act. 202, 198
4. H.W.Gardner & D.Weisleder (1970) Lipids 5, 678
5. T.Galliard & D.R.Phillips (1971) Biochem. J. 124, 431
6. C.C.Chang, W.J.Esselman & C.O.Clagett (1971) Lipids 6, 100
7. G.A.Veldink, J.F.G.Vliegenthart & J.Boldingh (1972) Biochem. Biophys. Res. Comm. 47, 22
8. M.Hamberg (1971) Anal. Biochem. 43, 515
9. R.S.Cahn, C.K.Ingold & V.Prelog (1956) Experienta 12, 81
10. G.Tuynenburg Muys, B.van der Ven & A.P.de Jonge (1963) Appl. Microbiol. 11, 389
11. O.Korver (1970) Tetrahedron 26, 2391
12. G.J.Schroepfer Jr. & K.Bloch (1965) J. Biol. Chem. 240, 54
13. M.Hamberg & B.Samuelsson (1967) J. Biol. Chem. 242, 5336
14. C.T.Bartels (1969) Thesis, University of Utrecht p 33
15. C.S.Foote (1968) Acc. Chem. Res. 1, 104