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The Formation of *threo*-11-Hydroxy-*trans*-12 : 13-epoxy-9-*cis*-octadecenoic Acid by Enzymic Isomerisation of 13-L-Hydroperoxy-9-*cis*,11-*trans*-octadecadienoic Acid by Soybean Lipoxygenase-1

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The interaction of soybean lipoxygenase-1 with 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13-hydroperoxy-linoleate), the product of the enzymic dioxygenation of linoleic acid, yields either a yellow or a purple-coloured enzyme species depending on the amount of product used. With an excess of 13-hydroperoxy-linoleate a labile purple-coloured enzyme species is formed which reverts to a yellow-coloured form with concomitant conversion of the hydroperoxy compound. In this reaction 13-hydroperoxy-linoleate isomerises into *threo*-11-hydroxy-*trans*-12:13 epoxy-9-*cis*-octadecenoic acid as could be concluded from nuclear magnetic resonance and mass spectral data.

Experiments with 13-[$^{18}O_2$]hydroperoxy-linoleate showed a high retention (70%) of the two hydroperoxy oxygen atoms in the end product.

Recently, we described the changes in the optical, electron paramagnetic resonance and fluorescence spectra of soybean lipoxygenase-1 upon the interaction of the enzyme and 13-L-hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic acid (13-hydroperoxy-linoleate), the product of the enzymic dioxygenation of linoleic acid [1, 2]. Yellow and purple-coloured enzyme species are formed upon the addition of one equivalent or an excess of 13-hydroperoxy-linoleate respectively to the native enzyme. In the former case it has been reported that 13-hydroperoxy-linoleate is decomposed but the reaction products could not be identified due to the minute amount formed [3]. On the other hand the purple species is unstable: at room temperature it reverts to a yellow-coloured form with concomitant conversion of 13-hydroperoxy-linoleate. The main conversion product has been tentatively identified as 11-hydroxy-12:13-epoxy-9-*cis*-octadecenoic acid [1]. The electron paramagnetic resonance spectra of both yellow forms of the enzyme are identical; the yellow and the purple species contain iron in a Fe(III) state but in a different ligand symmetry [1].

The present report deals with the detailed structural analysis by NMR and mass spectrometry of the decomposition product. In addition, ^{18}O-labelled hydroperoxides were used to establish the fate of the oxygen atoms of the hydroperoxy group during the conversion of 13-hydroperoxy-linoleate.

Abbreviation. NMR, nuclear magnetic resonance.

Trivial name. 13-Hydroperoxy-linoleate.

Enzyme. Lipoxygenase or linoleate:oxygen oxidoreductase (EC 1.13.11.12).

MATERIALS AND METHODS

Soybean lipoxygenase-1 was isolated according to Finazzi-Agrò *et al.* [4]. Specific activity: 190 μmol O_2 min^{-1} · mg^{-1}. Linoleic acid (purity higher than 99%) was a gift from the Unilever Research Laboratories (Vlaardingen/Duiven, The Netherlands). 13-Hydroperoxy-linoleate was prepared as described previously [5].

$^{18}O_2$ (^{18}O: 99.89 atom%; ^{17}O: 0.017 atom%) was obtained from Miles Laboratories Inc. (Elkhart, Indiana, U.S.A.).

For the molar absorption coefficient of 13-hydroperoxy-linoleate at 234 nm the value of 25000 M^{-1} cm^{-1} was used [6].

Diazomethane was used for esterification. Hydroperoxy-octadecadienoates were converted into the corresponding hydroxystearates by catalytic hydrogenation (H_2/PtO_2 in CH_3OH).

Bis(trimethylsilyl)-trifluoroacetamide (Regis Chemical Co., Chicago, Illinois, U.S.A.) was used to trimethylsilylate hydroxyl functions.

Thin-layer chromatography was carried out on 0.25 or 0.50-mm pre-coated plates (silica gel 60F-254, 20 × 20 cm, E. Merck, A. G., Darmstadt, Germany).

Bands were located by viewing under ultraviolet light and spraying with phosphomolybdic acid.

Spectroscopy

70-eV mass spectra were recorded with an AEI MS9 mass spectrometer provided with a direct insertion probe. Ultraviolet absorption spectra were obtained on a Unicam SP1800 spectrophotometer equipped with a Unicam AR25 linear recorder.

NMR spectra were recorded at 90, 220 or 300 MHz with Varian instruments; tetramethylsilane in C^2HCl_3 was used as internal standard.

Preparation of ^{18}O-Labelled Linoleate Hydroperoxides

In a reaction vessel attached to a gas burette, 35 mg linoleic acid was incubated with lipoxygenase in an $^{18}O_2$ atmosphere in 50 ml 0,2 M sodium borate buffer, pH 10.0 for 45 min at 0 °C. After acidification and extraction with ether the hydroperoxides were purified by thin-layer chromatography on 0.50-mm plates in the system hexane/diethyl ether/acetic acid (60/40/1, by vol.). An aliquot of the ^{18}O-labelled hydroperoxides was esterified and hydrogenated. After thin-layer chromatography (hexane/diethyl ether, 3/2 by vol.) the methyl 13 and 9-hydroxystearates were collected together and trimethylsilylated.

Mass spectrometry demonstrated the presence of 6% of the 9-hydroxy isomer with characteristic peaks at *m/e* 229(+ 2) and 259(+ 2). Within the 13-hydroxy isomer fraction 92% of the molecules contained one ^{18}O and 8% was unlabelled. The percentages were calculated from the ratio of the intensities of the peaks at *m/e* 173(+ 2) and 315(+ 2). These values also reflect the oxygen isotope distribution in the original 13-hydroperoxy-linoleate fraction which accordingly contains 92% 13-[$^{18}O_2$]hydroperoxy-linoleate and 8% of non-labelled hydroperoxides.

RESULTS

Incubation of Lipoxygenase with 13-Hydroperoxy-linoleate

From a stock solution of 13-hydroperoxy-linoleate in 0.2 M sodium borate buffer, pH 10.0 (34.4 mM) a 50-µl portion was added to 1.6 ml of soybean lipoxygenase-1 (0.48 µmol) in the same buffer at 20 °C under aerobic conditions. After the purple to yellow conversion was completed another 50 µl of 13-hydroperoxy-linoleate was added. This procedure was repeated 8 times (total reaction time 65 min; total amount of 13-hydroperoxy-linoleate employed: 4 mg). The reaction mixture was then acidified and extracted with diethyl ether. The diethyl ether fractions were washed with water, dried and concentrated.

The residue was treated with diazomethane and subjected to thin-layer chromatography in the solvent system hexane/ether (3/2, by vol.). The major product (about 80%) had an R_F value of 0.21 (*cf.* R_F of methyl 13-hydroperoxy-linoleate = 0.62). A similar experiment was performed with 13-[$^{18}O_2$]hydroperoxy-linoleate.

Spectroscopy

The product at R_F 0.21 showed no ultraviolet absorption above 220 nm. After trimethylsilylation of this material thin-layer chromatography (hexane/ether 4/1, by vol.) gave two components (R_F 0.55; main component: 80–90% and R_F 0.47; 10–20%).

The mass spectrum of the R_F 0.55 material showed significant peaks at *m/e* 398 (M^+), 383 (M^+-15), 367 (M^+-31), 327 (M^+-71) and 285 (base peak). The spectrum is consistent with the trimethylsilyl derivative of methyl 11-hydroxy-12:13-epoxy-9-octadecenoate (Fig. 1A).

The configuration of the epoxy group and of the double bond was determined by NMR. Table 1 shows the assignment of significant resonance lines of methyl 11-hydroxy-12:13-epoxy-9-octadecenoate. The coupling constant of 2 Hz for the epoxy protons indicates a *trans* configuration whereas the coupling between the olefinic protons (11 Hz) points to a *cis* double bond. Spectrum simulation confirmed the *cis* double bond. The coupling of the protons at C-11 and C-12 suggests a *threo* configuration of the hydroxyl and epoxy functions ($J_{11,12}$ = 5 Hz) [7].

The mass spectrum of the R_F 0.55 material derived from 13-[$^{18}O_2$]hydroperoxy-linoleate is shown in Fig. 1 B. Comparison of the two mass spectra of Fig. 1 shows the presence of two additional sets of peaks in spectrum B differing by 2 and 4 *m/e* units from the corresponding peaks in Fig. 1A. This reflects the presence of both singly and doubly labelled epoxy-hydroxy compounds. The molar percentages of these species cold be calculated from the peak intensities of the M^+-15 fragment ions as is shown in Table 2. The 13-[$^{18}O_2$]hydroperoxy-linoleate predominantly yields the epoxy-hydroxy compound containing two atoms of ^{18}O $\left(\frac{64}{92} \times 100 = 69.6\%\right)$ in which both the epoxy and hydroxyl functions are labelled, and to a smaller extent molecules containing only one atom of ^{18}O $\left(\frac{28}{92} \times 100 = 30.4\%\right)$ The latter molecules contained ^{18}O in the epoxy group at C-12 and C-13 as is shown by the loss of ^{18}O in the fragmentation involving the elimination of the epoxy group.

Mass spectrometry of the minor amount of trimethylsilylated material with R_F 0.47 pointed to a mixture of epoxy-hydroxy compounds which *inter alia*

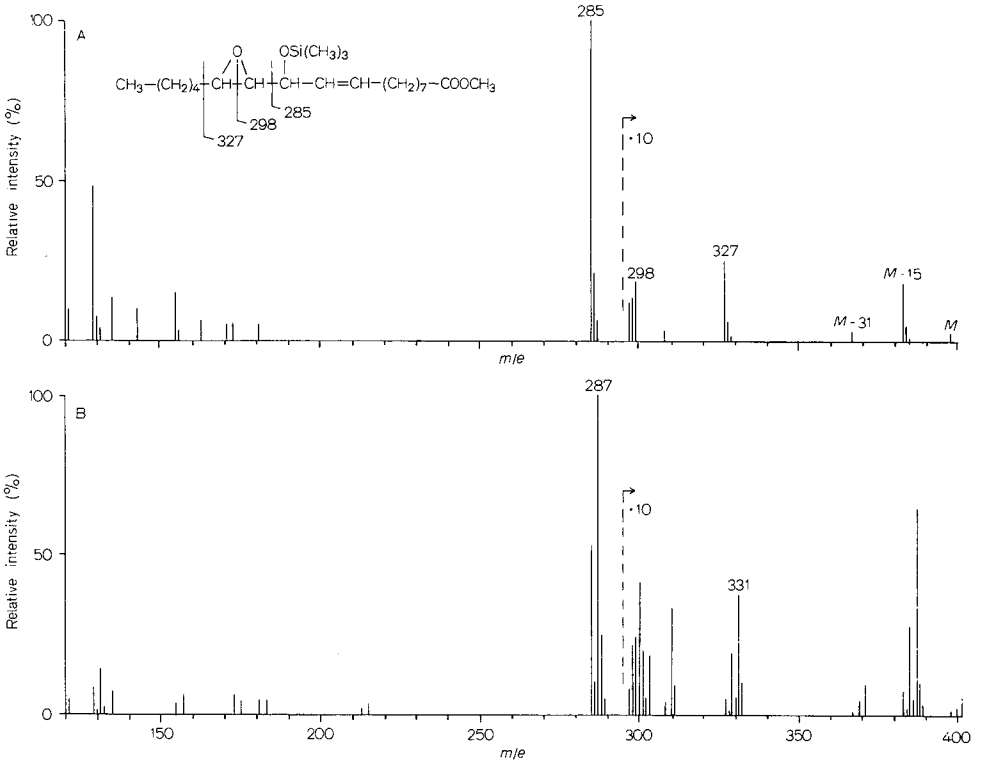

Fig. 1. *Mass spectra of the main product of the conversion of (A) 13-hydroperoxy-linoleate and (B) 13-[$^{18}O_2$]hydroperoxy-linoleate catalysed by soybean lipoxygenase-1.* Spectra were obtained using the methyl esters of the trimethylsilyl derivatives of the products

Table 1. *Relevant NMR parameters of methyl 11-hydroxy-12:13-epoxy-9-octadecenoate.*
Chemical shifts (δ) in C^2HCl_3 are quoted as ppm downfield from internal tetramethylsilane. Pattern. d.d = doublet of doublets; d.t = doublet of triplets

R = $CH_3-(CH_2)_3-$ R′ = $-(CH_2)_6-COOCH_3$

Chemical shift	Assignment	Pattern	Coupling constant	
ppm			Hz	
2.93	H-13	d,t	$J_{13,14}$	5
			$J_{12,13}$	2
2.78	H-12	d,d	$J_{11,12}$	5
4.25	H-11	d,d	$J_{10,11}$	8
5.46	H-10	d,d	$J_{9,10}$	11
5.60	H- 9	d,t	$J_{8,9}$	7.5

contains methyl 11-hydroxy-9:10-epoxy-12-octadecenoate derived from 9-hydroperoxy-linoleate (significant peaks at *m/e* 199 and 241).

Table 2. *^{18}O-isotopic composition of the trimethylsilyl derivative of methyl 11-hydroxy-12:13-epoxy-9-octadecenoate formed from 13-[$^{18}O_2$]hydroperoxy-linoleate (molar percentages calculated from the peak intensities of the M^+-15 fragment ions) and of the fragment ion M^+-$C_7H_{13}O$ (lacking the epoxy function)*

^{18}O	M^+-15	*m/e*	$M^+-[CH_3-(CH_2)_4-CH(O)CH]$	*m/e*
	%		%	
$0\times{}^{18}O$	8	383	35	285
$1\times{}^{18}O$	28	385	65	287
$2\times{}^{18}O$	64	387	0	

DISCUSSION

The conversion of 13-hydroperoxy-linoleate by various routes has been reported on several occasions. Hamberg and Gotthammar [8] found *threo*-11-hydroxy-12:13-epoxy-9-octadecenoic acid to be one of the thermal decomposition products of 13-hydroperoxy-linoleate; in about 10% of the molecules both of the hydroperoxide oxygens were retained. In a subsequent paper Hamberg [9] reported on the decompo-

sition of 13-hydroperoxy-linoleate through hemoglobin catalysis: both the *erythro* and *threo*-11-hydroxy-12:13-epoxy-9-octadecenoic acids were found. Gardner *et al.* [10] using systems containing Fe(II) or catalytic amounts of Fe(III) plus cysteine reported on the isolation of 11-hydroxy-*trans*-12:13-epoxy-9-*trans*(*cis*)-octadecenoic acid as one of the decomposition products of 13-hydroperoxy-linoleate.

In our case it is demonstrated that a similar conversion of 13-hydroperoxy-linoleate is mediated by soybean lipoxygenase-1. The outstanding feature of this reaction is the high retention (approx. 70%) of the two hydroperoxide oxygen atoms in the end product: *threo*-11-hydroxy-*trans*-12:13-epoxy-9-*cis*-octadecenoic acid.

It has been shown that both the native and the yellow form of the enzyme can bind 13-hydroperoxy-linoleate [11, 12] which in the latter case leads to the formation of an unstable purple-coloured enzyme species [1]. The instability is characterised by the conversion of the bound 13-hydroperoxy-linoleate. The high label retention indicates that the conversion is to an appreciable extent sterically conditioned leading to a type of cage reaction in which there is a reduced chance for exchange of the hydroxyl function with the medium. Therefore, the reaction is virtually an isomerisation. It should be mentioned that among the minor products formed in the purple-to-yellow transition 13-oxo-octadecadienoate and 13-hydroxy-octadecadienoate could also be detected, which is an indication for the involvement of the alkoxy radical. It has been reported earlier [2, 3] that the conversion of the native enzyme into the yellow species requires one equivalent of 13-hydroperoxy-linoleate. By using 13-hydroperoxy-[1-^{14}C]linoleate we found that the hydroperoxide was also decomposed in this case. Although, due to the minute amount of available material, the structure of the decomposition products could not be established, it is very likely that this reaction is similar to the one described for the purple enzyme species since thin-layer chromatography revealed the same R_F value for the main component.

We have also found that upon aerobic incubation of linoleic acid and soybean lipoxygenase-1 invariably small amounts of 11-hydroxy-12:13-epoxy-9-octadecenoic acid can be detected.

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