ON THE SPECIFICITY OF THE LIPOXIDASE CATALYZED OXYGENATION OF UNSATURATED FATTY ACIDS

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The enzyme lipoxidase catalyzes the oxidation of unsaturated fatty acids by molecular oxygen. The reaction seems to be specific for fatty acids possessing methylene group-interupted cis double bonds (Holman and Bergström, 1951). The mode of action of the enzyme has been visualized (Tappel, 1963) as an initial abstraction of one hydrogen atom from a methylene group between two cis double bonds, followed by an attack of one molecule of oxygen. This results in the formation of a hydroperoxide with two conjugated double bonds, where the isomerized double bond has attained the trans configuration. The two conjugated double bonds constitute a chromophore with λ_{max} at 234 mμ. It was early shown (Bergström, 1946) that the two main compounds obtained on aerobic incubation of sodium linoleate with lipoxidase were 9-hydroperoxyoctadeca-10,12-dienoic acid and 13-hydroperoxyoctadeca-9,11-dienoic acid. This finding has led many workers to anticipate that the lipoxidase catalyzed oxidation of unsaturated fatty acids is of a relatively unspecific nature, i.e. every pair of methylene group-interrupted cis double bonds should give rise to two different hydroperoxides.

That this is not the case is shown in the present report. A series of eight fatty acids, differing in chain length and location and degree of unsaturation have been incubated with lipoxidase. The obtained unsaturated hydroperoxy-fatty acids were reduced to the hydroxy compounds with sodium borohydride. After purification using silicic acid chromatography, the un-

saturated hydroxy compounds were hydrogenated, esterified with diazomethane and oxidized with chromic acid. The product consisted of the methyl esters of the saturated keto-fatty acids. These were analyzed by mass spectrometry, which allowed determination of the position of the keto group. Since there is no rearrangement of the initially introduced hydroperoxy group, the position of the keto group in the keto acids indicates the position of the hydroperoxy group.

In another experiment the unsaturated hydroxy acid fraction, obtained by reduction of the product from lipoxidase incubation with eicosa-8,11,14-trienoic acid, was subjected to oxidative ozonolysis. The degradation product was analyzed by gas chromatography and mass spectrometry.

EXPERIMENTAL

Incubations with lipoxidase (crystalline soy bean lipoxidase, ~20,000 units/mg, purchased from Fluka) were performed aerobically at 0° and pH 9.0. The fatty acids (4 mg) were converted into their ammonium salts by the addition of NH_4OH (0.019 M, 1 ml). The enzyme was dissolved in a buffer solution (1 mg of lipoxidase per 1 ml of 0.1 M borate buffer, pH 9.0). Incubations were started by the addition of 2.5 ml of enzyme solution. After 15 minutes an additional 0.5 ml of enzyme solution was added. The incubations were interrupted after 30 minutes by the addition of 15 ml of ethanol. The solution was immediately diluted with water, acidified to pH 3 and was extracted with ether. The ether was washed with water until the reaction was neutral and was evaporated at room temperature at a reduced pressure. The residue was dissolved in 1 ml of methanol, cooled to 0° and reduced with 10 mg of sodium borohydride. After 20 minutes at 0° and 40 minutes at room temperature the product was extracted with ether. The obtained material was purified using silicic acid chromatography. The hydroxy compounds, possessing the chromophore with λ_{max} at 234 mμ, could be detected by measuring the absorption of aliquots of the fractions.

The hydroxy compounds were hydrogenated under a stream of hydrogen gas using PtO_2 as catalyst. After treatment with diazomethane the compounds were oxidized with chromic acid (20 mg of chromium trioxide in 2 ml of 97.5% acetic acid) at 37° for 30 minutes. After this time, methanol was added in order to

destroy excess of chromic acid, and the product was extracted with ether. The compounds obtained were methyl esters of saturated monoketo acids. Mass spectrometric analysis of these keto esters were performed on the gas chromatographic effluent according to Ryhage (1964).

Oxidative ozonolysis was carried out as described earlier (Bergström et al., 1963). The material was esterified with diazomethane and acetylated. This derivative (2 mg) was dissolved in chloroform (4 ml) and treated with ozone at -14° for 6 minutes. After 10 minutes at room temperature the ozonide was oxidized with hydrogen peroxide (0.4 ml) in acetic acid (2 ml) at 50° for 18 hours. After that time the solution was taken almost to dryness. The residue was esterified with diazomethane and was analyzed by gas chromatography and mass spectrometry.

RESULTS AND DISCUSSION

The product obtained on incubation of eicosa-8,11,14-trienoic acid with lipoxidase was examined by oxidative ozonolysis in addition to analysis of the keto acids by mass spectrometry. The ozonolysis product consisted of two main compounds as judged by gas chromatography. These were identified by mass spectrometry as the methyl esters of α-acetoxyheptanoic acid and suberic acid. Isolation of these fragments indicates that the degraded compound was 15-acetoxyeicosa-8,11,13-trienoic acid. The compound obtained on lipoxidase incubation of eicosa-8,11,14--trienoic acid was therefore 15-hydroperoxyeicosa-8,11,13-trienoic acid. Analysis of the keto compound gave additional support for this structure. Three mass spectra were recorded on the ketoester peak of the gas chromatogram. The mass spectra were identical, and showed ions of high intensity at m/e 340, 309, 284, 269, 252, 237, 227 and 195. This fragmentation pattern is in accord only with methyl 15-ketoarachidate (Ryhage and Stenhagen, 1959). This finding independently showed that the incubation product consisted of 15-hydroperoxyeicosa-8,11,13-trienoic acid.

The structure of the compound obtained on incubation of arachidonic acid with lipoxidase was established by mass spectrometric analysis of the keto ester. As the keto compound was identified as methyl 15-ketoarachidate (giving a spectrum identical with that of the keto ester from 15-hydroperoxyeicosa--8,11,13-trienoic acid) the reaction product was 15-hydroperoxy-

eicosa-8,11,13-trienoic acid.

Similarly, the incubation product from eicosa-5,8,11,14,17--pentaenoic acid was identified as 15-hydroperoxyeicosa-5,8,11, 13,17-pentaenoic acid. The hydroperoxide formed on incubation of docosa-10,13,16-trienoic acid with lipoxidase was shown to be 17-hydroperoxydocasa-10,13,15-trienoic acid since the keto ester could be identified as methyl 17-ketodocosanoate.

On incubation of docosa-8,11,14-trienoic acid with lipoxidase no increment in the absorption at 234 mμ could be observed. As reference, eicosa-8,11,14-trienoic acid was used. Incubation of this acid with lipoxidase under the same conditions resulted in a rapid increase of the absorption at 234 mμ. This experiment therefore indicates that docosa-8,11,14-trienoic acid is not susceptible to lipoxidase catalyzed oxygenation.

The products obtained on the incubation of three unsaturated C_{18} acids were also analyzed as keto esters using mass spectrometry. The keto acids from linoleic acid could be identified as 9-ketostearic acid and 13-ketostearic acid, using synthetic specimens as references. The 9-keto acid constituted about 30% of the mixture as judged by mass spectrometry. Since no rearrangements could occur during the steps between the hydroperoxy acid and the keto acid the product obtained on incubation of linoleic acid with lipoxidase consists of about 30% 9-hydro-

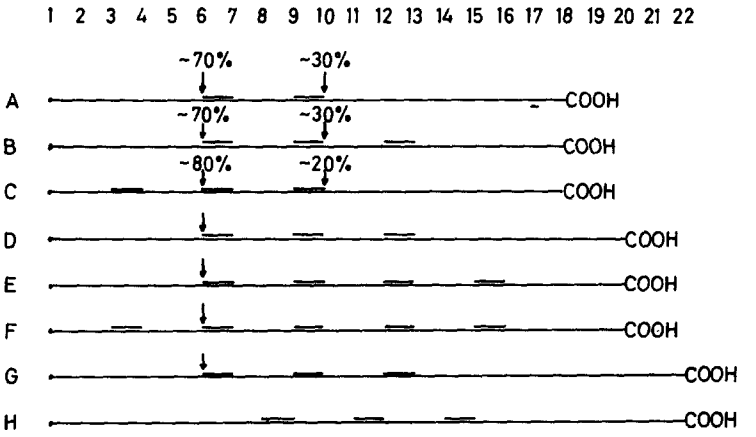

Fig. 1. Positions of the hydroperoxide groups introduced in the lipoxidase catalyzed oxygenation of unsaturated fatty acids. A, linoleic acid; B, γ-linolenic acid; C, α-linolenic acid; D, eicosa-8,11,14-trienoic acid; E, arachidonic acid; F, eicosa--5,8,11,14-pentaenoic acid; G, docosa-10,13-16-trienoic acid; H, docosa-8,11,14-trienoic acid.

peroxyoctadeca-10,12-dienoic acid and about 70% 13-hydroperoxy-octadeca-9,11-dienoic acid. Similarly, the product from γ-linolenic acid was shown to consist of about 30% 9-hydroperoxyoctadeca-6,10,12-trienoic acid and about 70% 13-hydroperoxyoctadeca--6,9,11-trienoic (fig. 1). On incubation of α-linolenic acid a product consisting of about 20% 9-hydroperoxyoctadeca-10,12,15--trienoic acid and about 80% 13-hydroperoxyoctadeca-9,11,15-trienoic acid was formed.

The results, which are summarized in fig. 1 revealed that the lipoxidase catalyzed introduction of oxygen into polyunsaturated fatty acids shows a high degree of specificity. Judging from the present study of eight different substrates, the following two general structures should be susceptible to attack by oxygen at the points indicated.

$$CH_3-(CH_2)_4-\underset{\omega-6}{\overset{\downarrow}{CH}} = CH-CH_2-CH = CH-R'$$

$$R''-CH = CH-CH_2CH = \underset{C-9}{\overset{\downarrow}{CH}}-(CH_2)_7-COOH$$

Thus acids with a diene system starting at position 6 counted from the methyl end (called ω-6) reacted to give ω-6 hydroperoxides. This reaction occurred irrespective of, if a double bond was present in ω-3 position as in α-linolenic acid or eicosa-5,8,11,14,17-pentaenoic acid.

A second point of attack by oxygen occurred at position 9, counted from the carboxyl end (C-9), provided that this carbon atom represented the beginning of the diene system. A Δ^6-double bond as in γ-linolenic acid did not seem to influence the reaction. The validity of the generalizations about the specificity of the reaction was further substantiated by the finding that docosa-8,11,14-trienoic acid did not react. This acid lacks both an ω-6 and a C-9 double bonded carbon with the proper location of the diene system.

Studies dealing with the stereochemical features of the removal of the hydrogen and the introduction of the oxygen will be reported later.

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