Biochimica et Biophysica Acta, 618 (1980) 153—162

BBA 57565

THE EFFECT OF MODIFICATION OF SULFHYDRYL GROUPS IN SOYBEAN LIPOXYGENASE-1 *

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(Received September 24th, 1979)

Key words: Lipoxygenase-1; Linoleic acid; Sulfhydryl group; Methylmercuric halide; 2-Hydroxy-ethylmercuric iodide; Co-oxidation; (Soybean)

Summary

Soybean lipoxygenase-1 was found to contain five free sulfhydryl groups and no disulfide bridges. Three sulfhydryl groups react readily with methylmercuric halides. This modification results in significant changes of the catalytic properties of the enzyme. Comparison of modified and native lipoxygenase-1 shows the following:

1. The catalytic constant of the oxygenation of linoleic acid is reduced by approximately 50%, whereas the affinity towards linoleic acid remains unaltered.
2. At high concentrations of substrate and low concentrations of enzyme the kinetic lag phase in the oxygenation is considerably longer.
3. The regio- and stereospecificities of the oxygenation are significantly lower.
4. Besides hydroperoxides, oxo-octadecadienoic acids (4%) are formed during the oxygenation.
5. The cooxidation capacity is considerably enhanced. Treatment of methylmercury-modified lipoxygenase-1 with NaHS results in the complete recovery of the sulfhydryl groups and of the catalytic properties.

Introduction

Lipoxygenase-1 from soybeans (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the dioxygenation of poly-unsaturated fatty acids,

* Part of the results of this investigation have been presented at the 14th World Congress of the International Society for Fat Research in Brighton (U.K.), 17—22 September, 1978.

containing a 1,4-*cis*,*cis*-pentadiene system to the corresponding conjugated (*n*-6)-L-hydroperoxy fatty acids [1]. The enzyme consists of a single polypeptide chain, has a molecular weight of approx. 100 000 and contains 1 gatom iron per mol. Although several reports on the amino acid composition have been published, the data on the number of cysteine and cystine residues are inconsistent. Stevens et al. [2] reported the presence of 4 cysteine and 2 cystine residues, whereas Stan and Diel [3] found 3 cysteine and 3 cystine residues. It has been supposed, that sulfhydryl groups in soybean lipoxygenase-1 are not essential for the enzymic oxygenation of linoleic acid, because no inhibition by *p*-chloromercuribenzoate or *N*-ethylmaleimide was observed [4—7]. However, it is conceivable that the sulfhydryl groups in the enzyme are inaccessible to these reagents. Therefore, small organic mercurials like CH_3HgI and CH_3HgCl [8,9] were used to reinvestigate the involvement of sulfhydryl groups in the catalytic activity of soybean lipoxygenase-1.

Materials and Methods

Materials

Soybean lipoxygenase-1 was isolated according to the method of Finazzi-Agrò et al. [10], as modified by Galpin et al. [11]. Linoleic acid (purity >99%) was obtained from Lipid Supplies (St. Andrews University, St. Andrews, U.K.). 13-L-Hydroperoxylinoleic acid was prepared by aerobic incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 and purified according to Verhagen et al. [12]. CH_3HgI and CH_3HgCl were purchased from ICN-Pharmaceuticals (New York, NY, U.S.A.), *p*-chloromercuribenzoate from Fluka, 5,5′-dithio-bis(2-nitrobenzoic acid) from Merck and 4,4′-dithiodipyridine ('Aldrithiol-4') from Aldrich. $^{14}CH_3HgI$ (3.1 Ci · mol^{-1}) was supplied by the Radiochemical Centre (Amersham, U.K.). Sephadex G25 and CM-Sephadex C50 were purchased from Pharmacia. $HOCH_2CH_2HgI$ was synthesized according to Zeller and Straub [13]. Crocin was isolated from saffran according to Friend and Mayer [14].

Assay of lipoxygenase activity

The enzymic activity was routinely measured polarographically in a Gilson Oxygraph equipped with a Clark oxygen electrode in a solution of 1.8 mM linoleic acid in air saturated 0.1 M sodium borate buffer (pH 9.0) at 25°C. Kinetic measurements of the oxygenation of linoleic acid were carried out spectrophotometrically (Cary 118 C) in a 1 cm pathlength cuvette in air saturated 0.1 M sodium borate buffer pH 10.0 at 25°C, by recording the increase of the absorbance at 235 nm. Rates of cooxidation of crocin were measured spectrophotometrically by recording the decrease of the absorbance at 440 nm ($A^{1\%}_{440} = 1369$ [14]) in 0.1 M sodium borate buffer (pH 10.0 at 25° C).

Modification of sulfhydryl groups in lipoxygenase-1

Modification reactions were carried out at room temperature by adding an appropriate amount of a mercury compound dissolved in dioxane (0.05 M) to a 10 μM solution of lipoxygenase-1 in 0.1 M sodium phosphate buffer (pH 7.0).

The extent of the modification was monitored by measuring the activity of aliquots taken from the reaction mixture.

The number of enzyme-bound CH_3Hg-groups were determined by incubating lipoxygenase-1 with $^{14}CH_3HgI$ (0.26 Ci · mol^{-1}) in 0.1 M sodiumphosphate buffer (pH 7.0) at room temperature. Final concentrations: 140 μM lipoxygenase-1; 1.4 mM methylmercuric iodide. After incubation for 24 h, which was found to be sufficient for completion of the modification, the mixture was dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) to remove the excess of methylmercuric iodide. After six days the outer buffer no longer contained radioactivity. The enzyme concentration in the dialyzate was determined by measuring the absorbance at 280 nm ($A_{280}^{0.1\%} = 1.6$). Samples were taken from the dialyzate to measure the radioactivity and to determine the number of sulfhydryl groups left after modification of the enzyme, as described under Analytical methods.

Modification of lipoxygenase-1 by $HOCH_2CH_2HgI$ was carried out by incubating 150 μM enzyme at room temperature with 1.5 mM $HOCH_2CH_2HgI$ in 0.1 M sodium phosphate buffer pH 7.0). After 45 h incubation, the activity of the enzyme (measured polarographically) had reached its minimum value (10%). The incubation mixture was then subjected to gel-filtration (Sephadex G25) to remove the excess mercurial. Subsequently, the free sulfhydryl groups left after modification of the enzyme were determined.

Analytical methods

The number of free sulfhydryl groups in lipoxygenase-1 were determined after denaturation by 1% sodium dodecyl sulphate or 6 M guanidine-HCl, using three different SH reagents, viz. Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid)) at pH 8.0 [15]. 'Aldrithiol-4' (4,4'-dithiodipyridine) at pH 5.0 and 7.5 [16] and *p*-chloromercuribenzoate at pH 7.0 [17]. The total number of half-cystines were determined in triplicate as cysteic acid, with an amino acid analyzer (Chromaspek, Rank Hilger), after 24 h hydrolysis of performic acid-oxidized lipoxygenase-1.

The radioactivity of the ^{14}C-labelled material was measured in a Packard Tricarb liquid-scintillation spectrometer. Aqueous samples were dissolved in 15 ml Insta Gel (Packard), whereas samples of the $^{14}CH_3HgI/CH_3HgI$ stock solution in dioxane were dissolved in 15 ml toluene, which contained 0.5% (w/v) 2,5-diphenyloxazole (PPO) and 0.03% (w/v) 1,4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP).

The regio- and stereospecificities of the methylmercury-modified lipoxygenase-1 were determined by incubating linoleic acid (1.8 mM) with modified enzyme (25 nM) in 0.1 M sodiumborate buffer (pH 9.0), in an oxygen atmosphere at room temperature. The course of the reaction was followed by measuring the absorbance at 234 nm in ethanol of small aliquots taken from the reaction mixture. After 45 minutes a maximum conversion (82%) of linoleic acid into hydroperoxide was reached ($\epsilon_{234} = 25\,000$ M^{-1} · cm^{-1}). The reaction was stopped by acidification of the incubation mixture to pH 2. The hydroperoxylinoleic acid isomers were purified and analyzed with respect to the positional and enantiomeric composition according to Van Os et al. [18].

Results and Discussion

Number of sulfhydryl and disulfide groups in lipoxygenase-1

After denaturation, soybean lipoxygenase-1 was found to contain five free sulfhydryl groups per mol (Table I). However, without denaturation none of the sulfhydryl groups were accessible to either 5,5′-dithio-bis(2-nitrobenzoic acid) or 4,4′-dithiodipyridine. From amino acid analysis, after performic acid oxidation and subsequent hydrolysis of the protein, a total number of five half-cystines per mol were found, indicating that no disulfide groups are present in soybean lipoxygenase-1. The variation in the reported numbers of half-cystines and free sulfhydryl groups in soybean lipoxygenase-1 [2—4,19—21] might be caused by differences in purity of the enzyme preparations used.

Modification of lipoxygenase-1 with organic mercury compounds

Preincubation of lipoxygenase-1 in 0.1 M sodium phosphate buffer (pH 7.0) with various organic mercury compounds leads to inhibition of the enzymic oxygenation of linoleic acid (Fig. 1). Residual activities of 10% and 20% were found in the standard polarographic assay, after preincubation with $HOCH_2CH_2HgI$ and $CH_3HgI(Cl)$, respectively. Rabenstein [22] reported, that at pH 7.0 the affinity of methylmercuric halides to sulfhydryl groups is much higher than to other functional groups in proteins. Therefore, the observed inactivation should be attributed merely to modification of sulfhydryl groups, taken into account that dioxane up to a concentration of 10% (v/v) had no effect on the enzymic activity. The more hydrophobic mercury compounds CH_3HgI and CH_3HgCl show a higher affinity for the enzyme than $HOCH_2CH_2HgI$. This is reflected in a higher rate of the irreversible inactivation by the former reagents and from the observation that addition of $HOCH_2CH_2HgI$, which gives the highest extent of inactivation, to enzyme already modified by $CH_3HgI(Cl)$ did not result in a further reduction of the activity.

The more polar and therefore more water-soluble compound $HOCH_2CH_2HgI$ was developed and successfully applied as a novel sulfhydryl modifying reagent. However, to lipoxygenase-1 the apolar reagents were superior, which suggests that the thiol groups are buried in a hydrophobic environment.

Incubation of lipoxygenase-1 with $^{14}CH_3HgI$ showed that 3.3 (±0.1) mol

TABLE I

NUMBER OF SULFHYDRYL GROUPS IN SOYBEAN LIPOXYGENASE-1

pCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, 4,4′-dithiodipyridine; SDS, sodium dodecyl sulfate.

Sulfhydryl reagent	pH	Denaturing reagent	Number of -SH groups
DTNB	8.0	1% SDS	4.64 ± 0.04 (S.D.)
DTNB	8.0	6 M guanidine-HCl	4.7 ± 0.1
4-PDS	5.0	6 M guanidine-HCl	5.04 ± 0.04
4-PDS	7.5	6 M guanidine-HCl	4.68 ± 0.01
4-PDS	7.5	1% SDS	5.0 ± 0.1
pCMB	7.0	1% SDS	4.65 ± 0.04

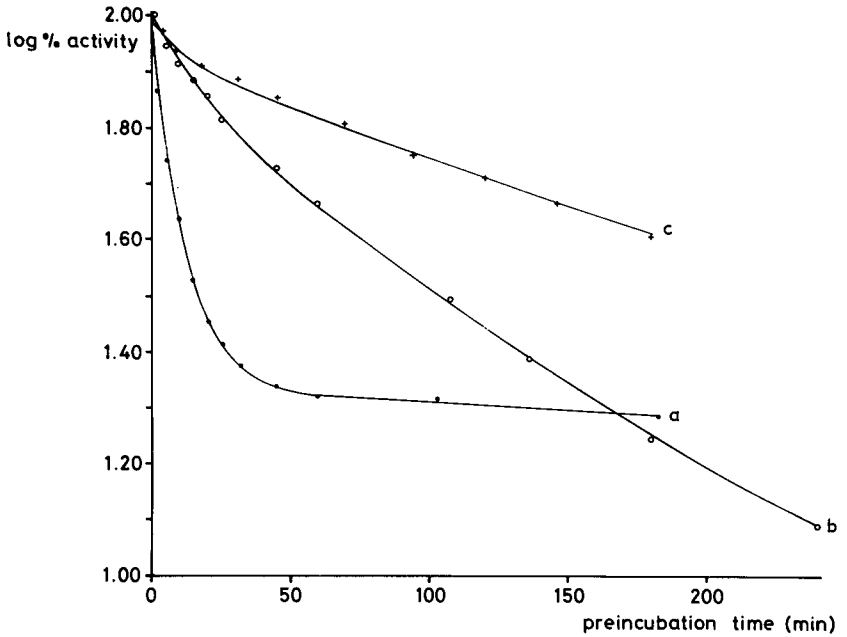

Fig. 1. Inactivation of lipoxygenase-1 by $CH_3HgI(Cl)$, $HOCH_2CH_2HgI$ and *p*-chloromercuribenzoate in 0.1 M sodium phosphate buffer (pH 7.0) at 20° C. Enzyme concentrations: 10 μM. Concentrations of the mercurials: CH_3HgI or CH_3HgCl: 0.8 mM, ●———● (a); $HOCH_2CH_2HgI$: 3.0 mM, ○———○ (b); *p*-chloromercuribenzoate: 5.0 mM, +———+ (c). Activities were measured polarographically in 0.1 M sodium borate buffer (pH 9.0) (linoleic acid concentrations 1.8 mM). Incubation of lipoxygenase-1 with *p*-chloromercuribenzoate ultimately leads to precipitation of the enzyme.

methylmercury are bound per mol enzyme. The number of free sulfhydryl groups in this modified enzyme was determined to be 1.5 (Table II), which indicates that three sulfhydryl groups per mol lipoxygenase-1 had reacted with methylmercuric iodide. Also with $HOCH_2CH_2HgI$ three sulfhydryl groups were found to be modified.

Catalytic properties of methylmercury-modified lipoxygenase-1

In the oxygenation of linoleic acid by soybean lipoxygenase-1 a kinetic lag period has been observed, the length of which is dependent on the concentra-

TABLE II

-SH CONTENT AND OXYGENATION RATE OF SOME LIPOXYGENASE-1 PREPARATIONS

The oxygenation rate was measured polarographically. Final concentrations: linoleic acid: 1.8 mM; enzyme 10 nM; buffer 0.1 M sodium borate (pH 10.0).

Enzyme	Number of -SH groups	v ($\mu M\ O_2 \cdot min^{-1}$)
Native lipoxygenase-1	4.6 ± 0.1 (S.D.)	232
Native lipoxygenase-1 + NaHS	4.50 ± 0.07	192
Methylmercury-modified lipoxygenase-1	1.52 ± 0.04	45
Methylmercury-modified lipoxygenase-1 + NaHS	4.53 ± 0.03	200

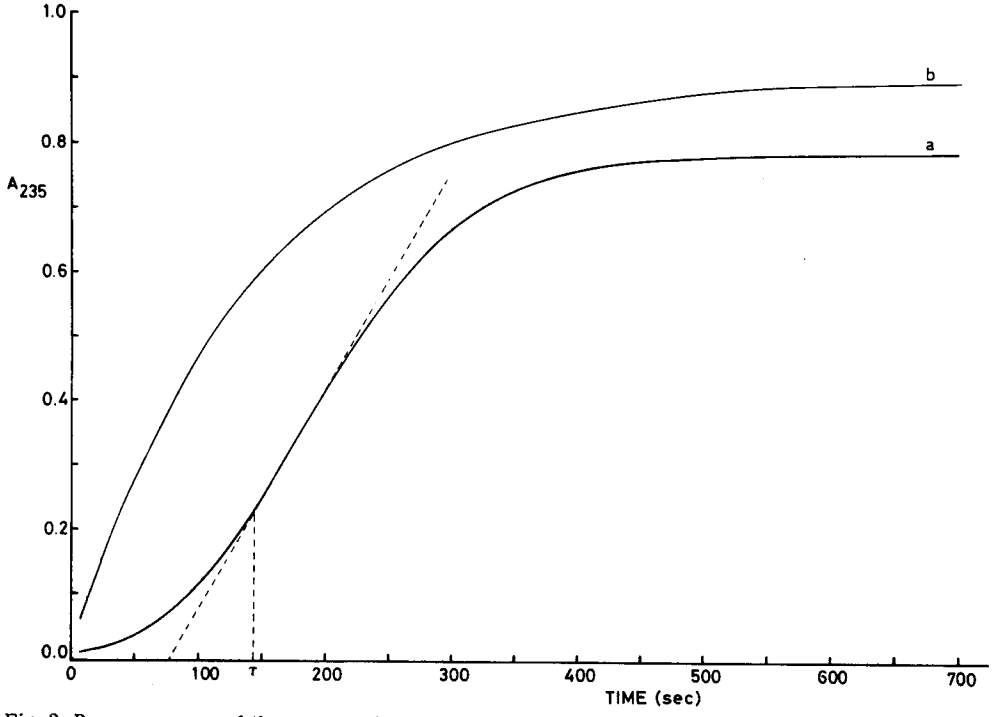

Fig. 2. Progress curves of the oxygenation of linoleic acid (40 μM) catalyzed by 2.5 nM methylmercury-modified lipoxygenase-1 (a) and by 1.25 nM native lipoxygenase-1 (b) at 25°C. In both cases the oxygenation capacity of the added enzyme was 0.03 μmol hydroperoxylinoleic acid produced per minute. The lag time is represented by τ.

tions of linoleic acid, enzyme and 13-L-ROOH [23—25]. It was found, that at pH 10.0 this lag period is much longer for the modified lipoxygenase-1 than for the native enzyme (Fig. 2). From Fig. 3 it appears, that the length of the lag period at a fixed concentration of linoleic acid is shortened upon increasing the concentration of modified enzyme (cf. Ref. 25). A plot of the reciprocal value of the lag time vs. the concentration of modified enzyme shows a linear relationship (Fig. 3). A linear increase of the lag time is observed upon increasing the linoleic acid concentration from 10 to 100 μM at a fixed concentration of modified enzyme (4 nM). The lag phase can be abolished by 13-L-ROOH, just like in the case of native lipoxygenase-1. Over a wide range of linoleic acid and modified enzyme concentrations the steady state of the oxygenation reaction is reached when 20—25% of the substrate has been converted into hydroperoxide. However, for the native enzyme only 6% of the substrate has to be converted to attain the steady state. It was found that the oxygen concentration has also an effect on the length of the lag phase e.g. upon saturating the incubation mixture with oxygen the lag period is considerably shortened for both the modified and the native enzyme.

Only in the absence of a lag phase (low linoleic acid concentrations and relatively high enzyme concentrations) the steady state rate of the oxygenation of linoleic acid appears to be a linear function of the concentration of modified lipoxygenase-1. To compare the kinetics of the oxygenation catalyzed by

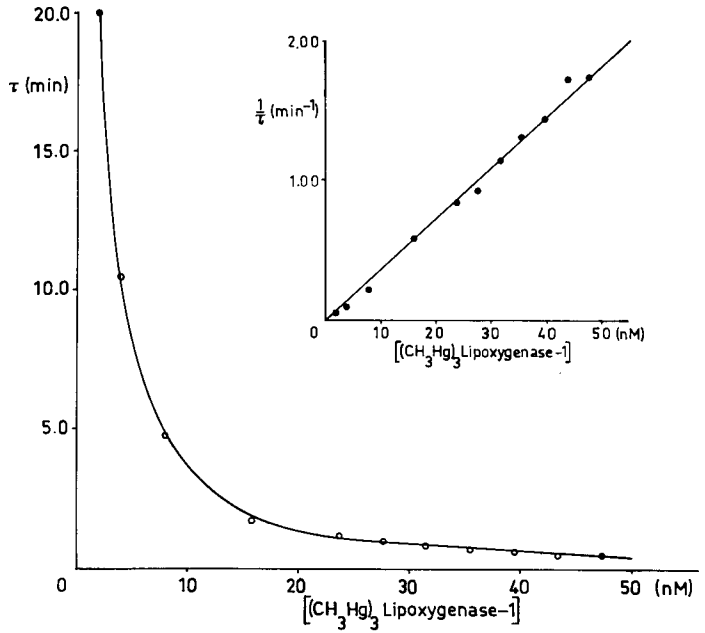

Fig. 3. The lag time (τ) in the oxygenation of linoleic acid as a function of the concentration of methylmercury-modified lipoxygenase-1. Reaction concentrations: linoleic acid concentration: 100 μM in 0.1 M sodium borate buffer (pH 10.0), t = 25°C, Insert: Reciprocal value of the lag time vs. the concentration of modified enzyme.

modified and native lipoxygenase-1, initial velocities of the formation of hydroperoxylinoleic acid were measured at various concentrations of linoleic acid. Fig. 4 shows double-reciprocal plots of the initial rate vs. the substrate concentration. With native enzyme (2 nM) a lag phase is absent at all substrate concentrations used, while with modified lipoxygenase-1 (2 nM) no lag phase is observed at linoleic acid concentrations below 8 μM. However, at linoleic acid concentrations over 8 μM the presence of a lag phase leads to a kinetic pattern reminiscent of substrate inhibition (Fig. 4, curved part). If the enzyme concentration is increased to such an extent, that the lag phase completely disappears and the resulting initial velocities are extrapolated to the original enzyme concentration (2 nM), simple Michaelis-Menten kinetics are found. The experimental data were fitted to the Michaelis-Menten equation by means of an iteractive least squares method.

It should be noted that the value of 3.3 ± 0.3 (S.D.) μM found for the K_m of native lipoxygenase-1 is much lower than the values reported previously [26–29]. This might be due to the fact that too large a Michaelis-Menten constant is found if a lag phase is present and steady state rates are taken as initial rates. The K_m for linoleic acid is not altered upon modification of the enzyme by $CH_3HgI(Cl)$. The value for the catalytic constant of modified lipoxygenase-1 (79 ± 2 (S.D.) s^{-1}) is approximately one-half that of the native enzyme (157 ± 6 (S.D.) s^{-1}). Furthermore, a decrease of the regio- and stereospecificities [30] of the enzymic oxygenation of linoleic acid (Table III) and the formation of

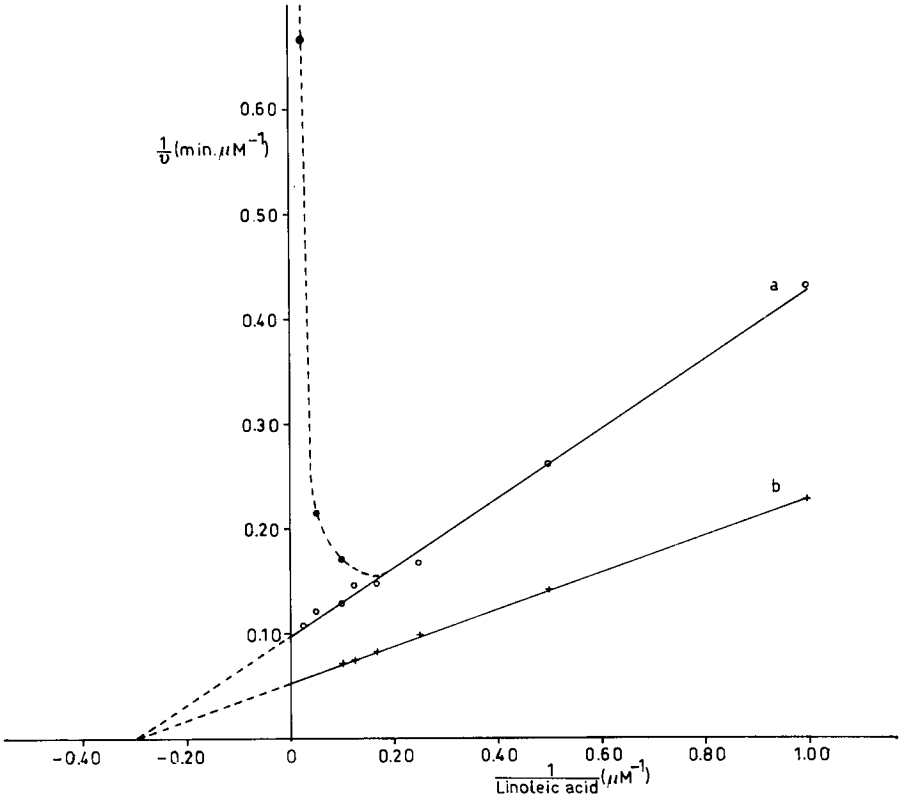

Fig. 4. Double-reciprocal plot of the linoleic acid concentration vs. the initial rate of the formation of hydroperoxylinoleic acid catalyzed by methylmercury-modified lipoxygenase-1 (a) and by native lipoxygenase-1 (b). Enzyme concentrations: 2 nM. Air-saturated 0.1 M sodium borate buffer (pH 10.0), t = 25°C. Solid lines: lag phase absent; broken line: lag phase present.

oxo-octadecadienoic acids (4% of the converted linoleic acid) have been observed.

Reactivation of methylmercury-modified lipoxygenase-1

To investigate a possible regeneration of the enzymic activity, experiments with several thiol compounds were carried out. Removal of the methylmercury-groups from the modified enzyme could not be achieved by treatment with β-mercaptoethanol, dithioerythritol or cysteine at pH 7.0. However, preincubation at pH 7.0 of modified 78 μM lipoxygenase-1 with 190 mM NaHS at room temperature resulted in a complete recovery of the activity within 30 min, as

TABLE III

PERCENTAGE COMPOSITION OF HYDROPEROXYLINOLEIC ACID ISOMER MIXTURES

Enzyme	13-L-ROOH	13-D-ROOH	9-L-ROOH	9-D-ROOH
Methylmercury-modified lipoxygenase-1	64.5	10.5	12.5	12.5
Native lipoxygenase-1, [18]	94.6	2.9	1.0	1.5

TABLE IV

COOXIDATION ACTIVITY

Reaction conditions: air-saturated 0.1 M sodium borate buffer (pH 10.0), t = 25°C. Oxygenation: 50 μM linoleic acid; cooxidation: 50 μM linoleic acid and 8 μM crocin.

Enzyme	Oxygenation (μmol O_2 · min^{-1} · mg^{-1})	Cooxidation (μmol crocin · min^{-1} · mg^{-1})	Ratio cooxidation/ oxygenation
Methylmercury-modified lipoxygenase-1	20.9	6.6	0.32
Native lipoxygenase-1	132	0.4	0.003
Pea lipoxygenase	0.18	0.04	0.22

compared with a control experiment in which 78 μM native enzyme retained 80% of its activity upon incubation with 190 mM NaHS for 30 min (Table II). After removal of the excess NaHS by ion-exchange chromatography (CM-Sephadex C50) the number of sulfhydryl groups in the reactivated enzyme were determined according to Ellman [15]. Table II shows that treatment of the modified enzyme with NaHS results in a complete recovery of the free sulfhydryl groups.

Comparison of the methylmercury-modified lipoxygenase-1 with 'type-2' lipoxygenases

It was found that upon modification of lipoxygenase-1 with methylmercuric halides the enzyme shows "type-2'-lipoxygenase characteristics e.g. an apparent substrate inhibition in the oxygenation of linoleic acid at pH 10.0 is observed, which is abolished upon lowering the linoleic acid-monomer concentration by adding Ca^{2+} (cf. Refs. 7, 31, 32). Furthermore, during the oxygenation of linoleic acid, oxo-octadecadienoic acids are formed approximately to the same extent (4%) as was found with pea lipoxygenase and soybean lipoxygenase-2. Interestingly, the modified enzyme displays cooxidizing properties as can be demonstrated with crocin as a co-substrate (Table IV) (cf. Refs. 33—35).

Acknowledgements

Thanks are due to Dr. C.P.A. van Os for determining the enantiomeric purity of the hydroperoxides and to Mrs. G.P.M. Rijke-Schilder for valuable technical assistance.

This investigation was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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