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STEADY-STATE KINETICS OF THE ANAEROBIC REACTION OF SOYBEAN LIPOXYGENASE-1 WITH LINOLEIC ACID AND 13-L-HYDROPEROXYLINOLEIC ACID

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Summary

The steady-state kinetics of the anaerobic reaction of soybean lipoxygenase-1 with linoleic acid and 13-L-hydroperoxylinoleic acid were studied. Initial rates of the formation of oxodienoic acids **, absorbing at 285 nm, were measured at pH 10. About 50% of the consumed 13-L-hydroperoxylinoleic acid was converted into oxodienoic acids regardless of the initial ratio of the two substrates.

A linear inhibition by both linoleic acid and 13-L-hydroperoxylinoleic acid was observed in the concentration range studied, which is on the upper side limited by the concentrations at which micelle- or acid-soap formation starts. A kinetic scheme is proposed based on one active site in lipoxygenase-1 which alternately binds the two substrates. Values for the kinetic constants were calculated by fitting simultaneously the complete set of data to the appropriate rate equation.

Introduction

Soybean lipoxygenase-1 (linoleate:oxygen oxido-reductase, EC 1.13.11.12) catalyzes the oxygenation of linoleic acid to 13-L-hydroperoxylinoleic acid. The latter, in turn, can react with the same enzyme under both aerobic and anaerobic conditions [1]. Under anaerobic conditions linoleic acid and 13-L-hydroperoxylinoleic acid are converted by lipoxygenase-1 in coupled reactions

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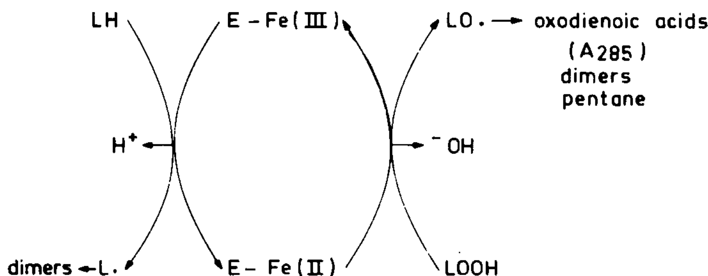
** Oxodienoic acids: 1 : 1 mixture of 13-oxo-octadeca, 9,11-dienoic acid and 13-oxo-trideca, 9,11-dienoic acid.



Scheme I. LH: linoleic acid; LOOH: 13-L-hydroperoxylinoleic acid.

to various products [2,3]. Scheme I shows a condensed version of the model as proposed by De Groot et al. [4]. According to this scheme the ferric enzyme is reduced by linoleic acid and the ferrous enzyme is then oxidized by the 13-L-hydroperoxylinoleic acid, which leads to the formation of oxodienoic acids absorbing at 285 nm. Previous studies [4,5] showed that these reactions can be performed separately. Therefore a mechanism for the anaerobic reaction involving a compulsory ternary complex of enzyme, linoleic acid and 13-L-hydroperoxylinoleic acid can be excluded. However, this does not necessarily rule out the existence of a second substrate binding site. To solve this problem it was decided to investigate the steady-state kinetics of the anaerobic reaction. If linoleic acid and 13-L-hydroperoxylinoleic acid compete for one binding site, the inhibition by these two substrates must be linear.

It was reported by Lagocki et al. [6] that lipoxygenase-1 reacts exclusively with the monomers of linoleic acid. Therefore the formation of micelles or acid-soaps at higher substrate concentrations can obscure the true nature of the inhibition [7]. In a preliminary study the concentration range in which both substrates occur in the monomeric form was determined [14]. Measurements were carried out at pH 10 instead of pH 9 because at pH 10 a wider range of concentrations of substrates in the monomeric form is available, while enzyme activity is still optimal.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans and purified according to Finazzi-Agrò et al. [8]. Spec. act. 185 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ (pH 10.0; t = 25°C). Linoleic acid (purity > 99%) was purchased from Lipid Supplies (St. Andrews University, St. Andrews, U.K.). 13-L-Hydroperoxylinoleic acid was prepared by incubation of linoleic acid with soybean lipoxygenase-1 in oxygen-saturated 0.1 M sodiumborate buffer pH 9.0. After acidification, extraction with diethylether and concentration the crude non-esterified 13-L-hydroperoxylinoleic acid was subjected to high performance liquid chromatography. A Varian Model 4100 liquid chromatography system was used with ultraviolet detection at 254 nm. (8 μl flow cell.) It was equipped with a Partisil-5 packed stainless steel preparative column (9.0 mm × 25 cm). Elution was performed with a mixture of hexane/ethanol/acetic acid (98.45 : 1.50 : 0.05, v/v/v). It

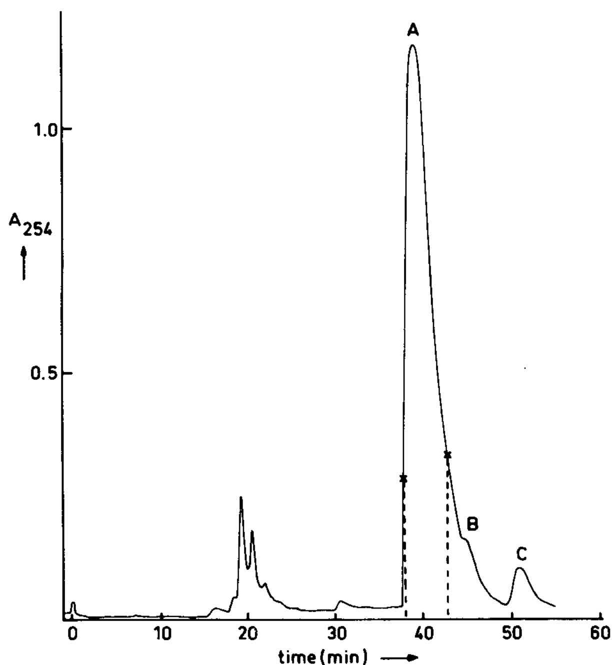


Fig. 1. High performance liquid chromatography of the hydroperoxides (non-esterified) produced during the aerobic incubation of lipoxygenase-1 with linoleic acid at pH 9.0. flow-rate: 200 ml/h. A: 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid. B: probably 13-L-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid [13]. C: 9-D-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid. Pure A was obtained by collection of the effluent between the indicated times.

was found that the addition of a small amount of acetic acid to the eluting solvent gives a considerable improvement of the separation of the isomers as compared to that reported previously [9]. The acetic acid was removed from the collected eluate by repeated washing with distilled water. Fig. 1 shows a typical chromatogram. Kinetic measurements were carried out in a 1-cm path-length cuvette provided with a rubber seal cap. Before filling, the cuvette was flushed with oxygen-free argon for 10 min. 13-L-Hydroperoxylinoleic acid in ethanol and linoleic acid in 1 M NH_4OH were then added in aliquots up to 5 μl and the cuvette completely filled (4.25 ml) with deaerated 0.1 M sodium borate buffer (pH 10.0) via the seal cap. Reactions were started by the addition of 5 μl (10 μg) enzyme solution in 0.1 M sodium acetate buffer (pH 5.0) and the change in absorbance at 285 nm was recorded with a Cary 118 C spectrophotometer. Mixing was facilitated by a glass bead in the cuvette. Spectra were recorded before and after reaction. Absorbances up to 3.5 corresponding to a concentration of 140 μM hydroperoxide or oxodienoic acid could be measured accurately. All assays were performed at 25°C. A molar absorption coefficient of 25 000 $M^{-1} \cdot cm^{-1}$ was used for 13-L-hydroperoxylinoleic acid at 234 nm and for the oxodienoic acids at 285 nm. Before each set of experiments the aerobic specific activity of the enzyme solution was measured. The initial velocities under anaerobic conditions were corrected afterwards for the small decrease in enzyme activity during storage. From the measured initial

absorbance change (v_{285} : ΔA_{285} per min) the initial rate of conversion of the two substrates (v : μM hydroperoxide or linoleic acid converted per min) was calculated according to the following equation:

$$v = v_{285} \cdot \frac{10^6}{25\,000} \cdot Q$$

Q = the initial concentration of the limiting substrate divided by the final concentration of the oxodienoic acids formed.

The initial velocities were then fitted to the rate equation derived for the proposed kinetic scheme (see Results and Discussion) via the method of least-squares. Although the system was not very sensitive to weighting an individual weighting factor of $1/v_{obs}^2$ was used, because a proportionality between v_{obs} and its standard deviation was observed. A variety of non-linear iterative least-squares procedures were used, which all led to the same minimum sum of squares. A procedure involving a Marquardt [10] algorithm was found to be most efficient. The 3-dimensional plot was obtained by means of a DISSPLA library routine. All calculations were performed on a CDC Cyber 73-28 computer.

Results and Discussion

The anaerobic reaction of lipoxygenase-1 with linoleic acid and 13-L-hydroperoxylinoleic acid proceeds until one of the two substrates is exhausted. The hydroperoxide is thus completely converted if linoleic acid is in excess. When the hydroperoxide is in excess the reaction is completed when the linoleic acid is depleted. We have found that a constant percentage of the consumed hydroperoxide is converted into oxodienoic acids independent of the initial molar ratio of the two substrates. A mean value of 50 ± 3.5% (S.D. 302 expts.) was obtained, which was constant during the course of the reaction.

Fig. 2 shows typical spectra before and after the reaction, at different initial substrate ratios.

Therefore, measurement of the rate of formation of oxodienoic acids, absorbing at 285 nm, is a reliable parameter for the anaerobic conversion of linoleic acid and 13-L-hydroperoxylinoleic acid by lipoxygenase-1.

In preliminary experiments at pH 9 a pseudo-non-linear inhibition of the anaerobic reaction by linoleic acid and 13-L-hydroperoxylinoleic acid was observed. It appeared that this non-linearity was caused by the formation of micelles or acid-soaps. In the present investigation kinetic measurements were carried out at pH 10.0. Only those total lipid concentrations were considered at which both substrates appeared to be present exclusively in the monomeric form as was determined by surface tension measurements [14].

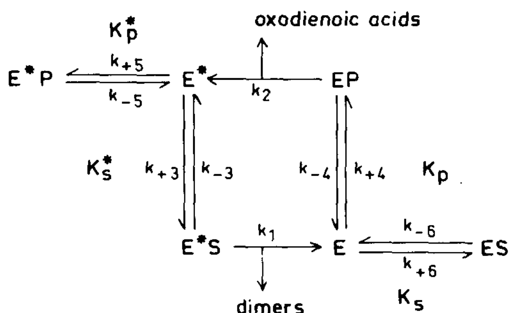
Substrate inhibition was found to occur for both linoleic acid and 13-L-hydroperoxylinoleic acid. Above the concentrations at which acid-soap formation starts, no further inhibition or much less inhibition than expected was observed (Fig. 3). The simplest kinetic scheme involving inhibition by both substrates, is based on one substrate binding site and is presented in Scheme II. It represents a substituted-enzyme mechanism ("ping-pong") with double sub-

Fig. 2. Ultraviolet spectra before (- - - - - -) and after (———) the anaerobic reaction of lipoxygenase-1 (23 nM) with linoleic acid and 13-L-hydroperoxylinoleic acid at pH 10.0. Molar ratios (μM/μM) of linoleic acid and hydroperoxide were respectively: A: 40/80; B: 40/40; C: 80/40.

strate inhibition. For this mechanism the following steady-state rate equation can be derived (assuming that the product-forming steps k_1 and k_2 are irreversible):

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_{\mathrm{m}}^{*s}}{s}\,(1 + p/K_p^*) + \frac{K_{\mathrm{m}}^{p}}{p}\,(1 + s/K_s) + 1\right]$$

where * indicates that the ferric form of the enzyme is concerned. The true



Scheme II. E*: E(Fe III); E: E(Fe II); S: linoleic acid; P: 13-L-hydroperoxylinoleic acid.

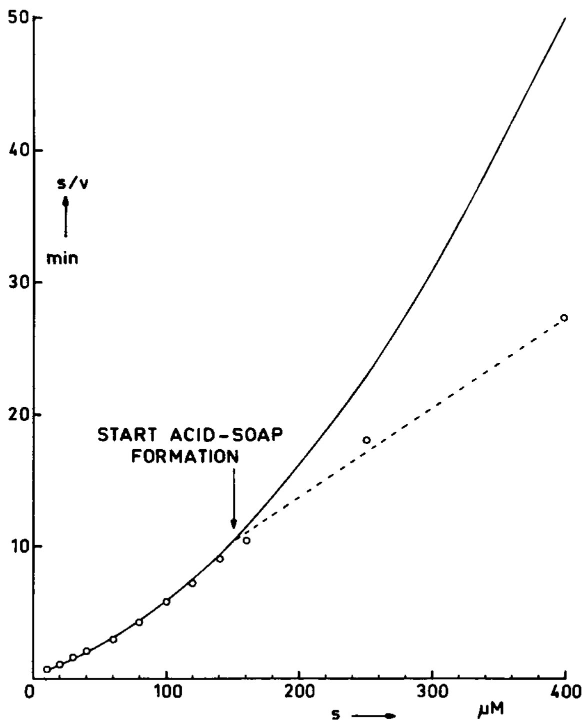


Fig. 3. The effect of acid-soap formation on the kinetics of the anaerobic reaction of lipoxygenase-1 (23 nM) with linoleic acid(s) and 13-L-hydroperoxylinoleic acid (20 μM) at pH 10. The solid line was computed from the rate equation by using the calculated kinetic parameters. (V = 143 μM · min^{-1}, K_m^{*s} = 55 μM, K_m^p = 73 μM, K_p^* = 153 μM and K_s = 111 μM.) The experimental values are represented by symbols. The broken line gives the relation between s/v and s assuming that the initial velocity remains constant above the concentration of acid-soap formation (enzyme acts only on monomers).

dissociation constants of the non-productive complexes of ferric enzyme and 13-L-hydroperoxylinoleic acid (p) and of ferrous enzyme and linoleic acid (s) are represented by K_p^* (= k_{-5}/k_{+5}) and K_s (= k_{-6}/k_{+6}), respectively.

K_m^{*s} and K_m^p are Michaelis-Menten constants, which are related to the true dissociation constants K_s^* (=k_{-3}/k_{+3}) and K_p (=k_{-4}/k_{+4}) according to the equations:

$$K_m^{*s} = \frac{k_2}{k_1 + k_2}\left(\frac{k_{-3}}{k_{+3}} + \frac{k_1}{k_{+3}}\right) \text{ and } K_m^p = \frac{k_1}{k_1 + k_2}\left(\frac{k_{-4}}{k_{+4}} + \frac{k_2}{k_{+4}}\right)$$

If the rapid-equilibrium assumption is made ($k_{-3} >> k_1$ and $k_{-4} >> k_2$) these equations reduce to:

$$K_m^{*s} = \frac{k_2}{k_1 + k_2} \cdot K_s^* \text{ and } K_m^p = \frac{k_1}{k_1 + k_2} \cdot K_p \ .$$

The measured initial velocities expressed in μM linoleic acid or hydroperoxide converted per min (160 expts.) were fitted to the rate equation via the method of least-squares.

An excellent fit of the experimental data to the proposed model was obtained. Table I lists the best-fit values for the kinetic constants with the

TABLE I

KINETIC PARAMETERS OF THE ANAEROBIC REACTION OF LIPOXYGENASE-1 WITH LINOLEIC ACID AND 13-L-HYDROPEROXYLINOLEIC ACID AT pH 10.0

t = 25°C. Values for the kinetic parameters were obtained by fitting the observed initial velocities to the rate equation. The experimental standard error was found to be 8%.

Parameter	μM S.E.
K_m^{*s}	55 ± 6
K_m^p	73 ± 8
K_s	111 ± 14
K_p^*	153 ± 25
V	143 μM · min^{-1} ± 11

associated standard errors. A value for k_{cat} of 104 ± 8 (S.E.) s^{-1} can be calculated from V, using an enzyme concentration of 23 nM. k_2 can be obtained from the equation:

$$k_{cat} = \frac{k_1 k_2}{k_1 + k_2}.$$

It is reasonable to assume that the rate constant for the hydrogen abstraction (k_1) has the same value in both the aerobic and anaerobic reaction. Since the hydrogen abstraction is the rate limiting step in the aerobic reaction [11],

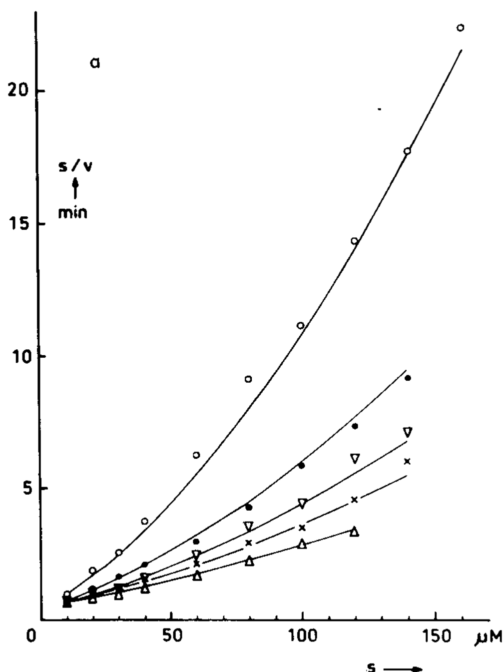


Fig. 4a. For legend see page 377

b

3

s / v

min

2

1

0

50

100 μM

s

Fig. 4b.

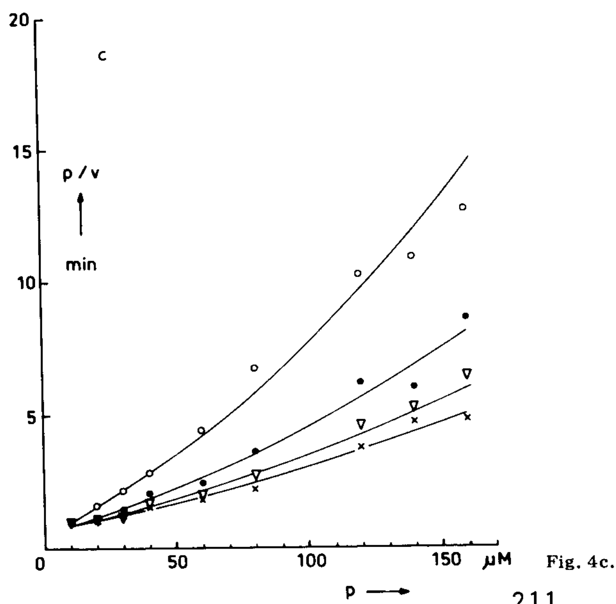


Fig. 4c.

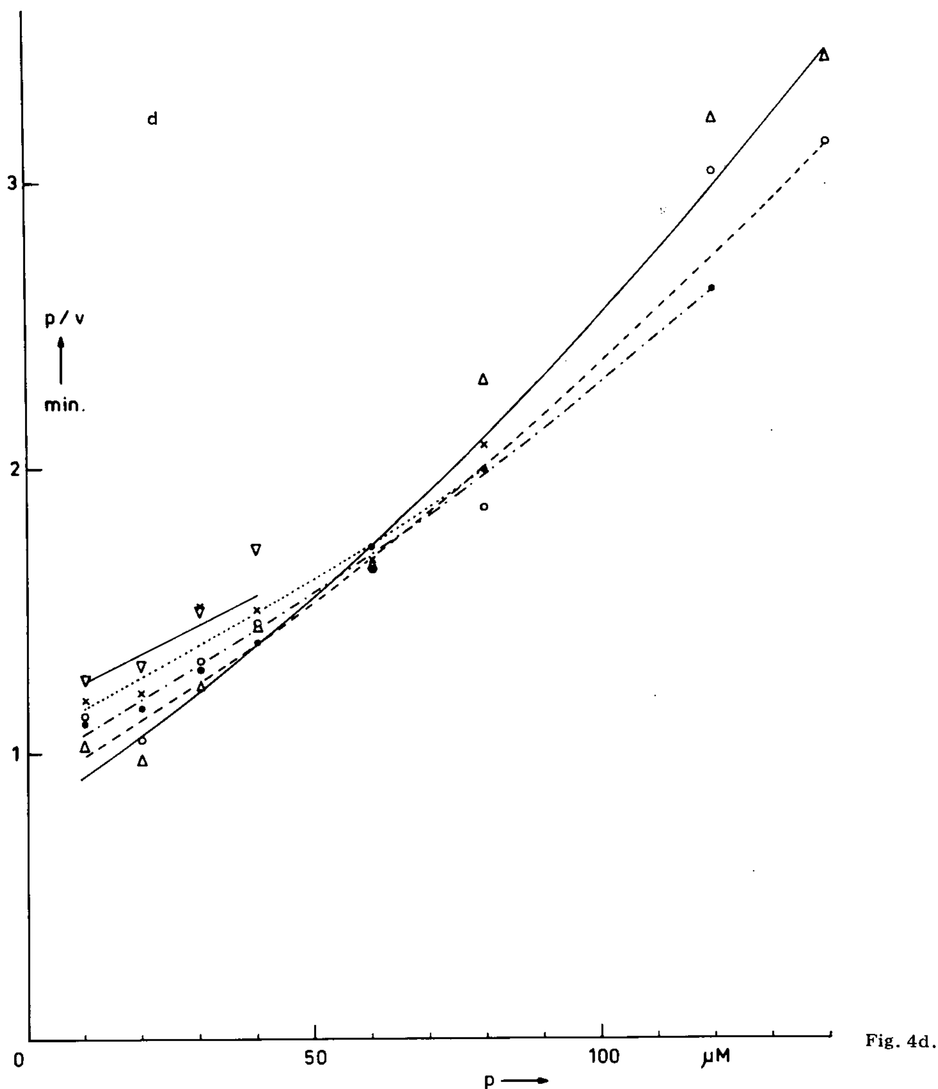


Fig. 4. Single-reciprocal plots of the initial velocities (v) as a function of the linoleic acid concentration (s) (a and b) or the 13-L-hydroperoxylinoleic acid concentration (p) (c and d). Enzyme concentration 23 nM, pH 10.0, t = 25°C. The curves were computed from the rate equation by using the calculated kinetic parameters. (V = 143 μM · min^{-1}, K_m^{*s} = 55 μM, K_m^p = 73 μM, K_s = 111 μM, K_p^* = 153 μM.) The observed initial velocities are represented by the following symbols. [The computed curves are represented by solid lines, unless otherwise indicated (b and d)]. (a) 13-L-Hydroperoxylinoleic acid concentrations: ○, 10 μM; ●, 20 μM; ▽, 30 μM; X, 40 μM; △, 60 μM. (b) 13-L-Hydroperoxylinoleic acid concentrations: ○———○, 80 μM; ●------●, 120 μM; ▽—·—·▽, 140 μM; X......X, 160 μM. (c) Linoleic acid concentrations: ○, 10 μM; ●, 20 μM; ▽, 30 μM; X, 40 μM. (d) Linoleic acid concentrations: △———△, 60 μM; ○------○, 80 μM; ●·—·●, 100 μM; X······X, 120 μM; ▽———▽, 140 μM.

its rate constant can be derived directly from the rate constant for the formation of hydroperoxide. A value of 309 ± 10 (S.E.) s^{-1} was found, which is in good agreement with previously reported values. (290 ± 4 s^{-1} [5] and 320 ± 16 s^{-1} [12].) A value for the rate constant (k_2) of the oxidation of ferrous enzyme

by hydroperoxide of 156 ± 16 (S.E.) s^{-1} was calculated. The rate of the latter reaction thus appears to be approximately one-half that of the reduction of the ferric enzyme by linoleic acid. This agrees well with the observations reported by Egmond et al. [5]. Knowing k_1 and k_2 and making the rapid equilibrium assumption, the true dissociation constants K_s^* and K_p can be calculated from K_m^{*s} and K_m^p respectively: $K_s^* = 163 \pm 22$ (S.E.) μM and $K_p = 110 \pm 14$ (S.E.) μM. When these values are compared with those for the dissociation constants of the non-productive complexes ($K_p^* = 153 \pm 25$ and $K_s = 111 \pm 14$) a striking similarity is seen in the affinity of the enzyme for both substrates. The enzyme in the ferrous state appears to have a slightly higher affinity for the two substrates than the enzyme in the ferric state.

Figs. 4a and b show plots of s/v against s and Figs. 4c and d plots of p/v against p. For clarity the plots of the lower and higher concentrations are presented separately (different scales). The substrate inhibition is clearly shown by the concave upwards curving of the plots. These figures demonstrate the fit of the experimental values to the curves computed from the rate equation and

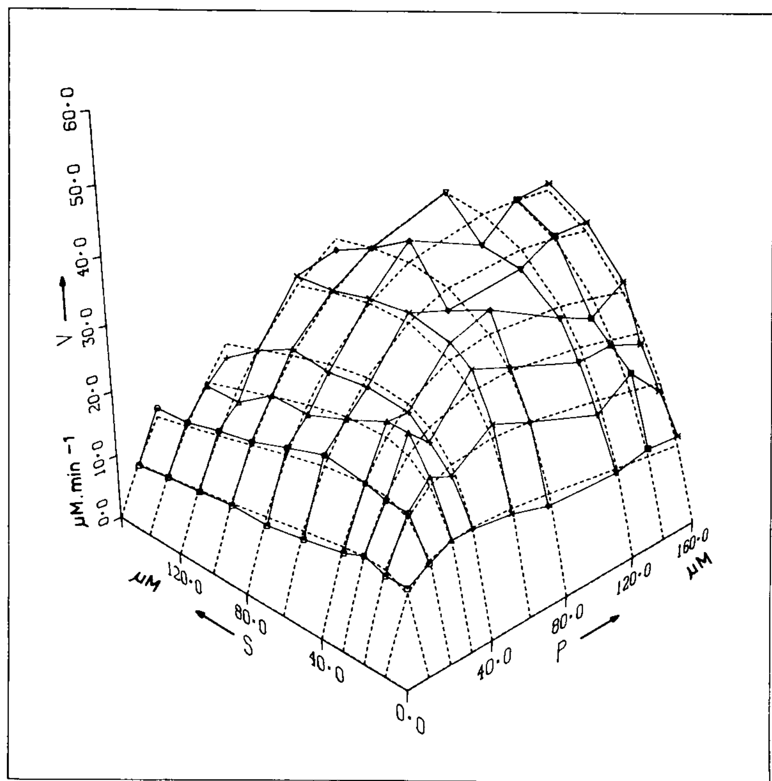


Fig. 5. Initial velocity (v) as a function of both the linoleic acid concentration (s) and the 13-L-hydroperoxylinoleic acid concentration (p). Viewpoint: $v = 75$ μM · min^{-1}, $s = -225$ μM, $p = -225$ μM. [enzyme]: 23 nM; buffer, 0.1 M sodiumborate (pH 10.0); $t = 25$°C. The experimental initial velocities (represented by symbols) are connected by solid lines. The broken lines represent the curves computed from the rate equation by using the calculated values for the kinetic parameters. ($V = 143$ μM · min^{-1}, $K_m^{*s} = 55$ μM, $K_m^p = 73$ μM, $K_p^* = 153$ μM and $K_s = 111$ μM.)

the calculated kinetic constants. Fig. 5 shows the relation between the initial velocity and the linoleic acid concentration as well as the hydroperoxide concentration.

The results can be summarized as follows:

(1) In the anaerobic reaction the conversion of 1 mol linoleic acid is strictly coupled to the conversion of 1 mol 13-L-hydroperoxylinoleic acid, which leads to the formation of 0.5 mol oxodienoic acids. Consequently, the extent of the formation of oxodienoic acids is determined exclusively by the concentration of the limiting substrate.

(2) The simplest mechanism which fits the experimental data and which is in agreement with previously reported observations [4,5], is described by a substituted enzyme mechanism with double substrate inhibition.

Acknowledgements

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