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Inactivation of Soybean Lipoxygenase 1 by 12-Iodo-*cis*-9-octadecenoic Acid†

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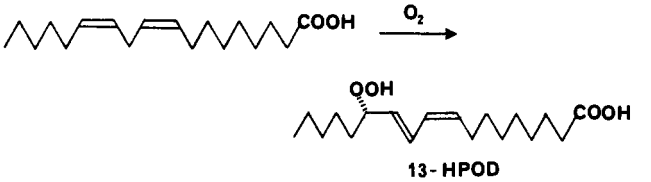
Received May 2, 1988; Revised Manuscript Received July 27, 1988

ABSTRACT: 12-Iodo-*cis*-9-octadecenoic acid (12-IODE) is a time-dependent, irreversible inactivator of soybean lipoxygenase 1. The rate of inactivation is independent of 12-IODE concentration above 20 μM and is half-maximal at about 4 μM. Inactivation by 12-IODE requires lipid hydroperoxide, which must be present even after the initial oxidation of the iron in the enzyme from ferrous to ferric. Inactivation by 12-IODE is also dependent on O_2. These findings suggest that 12-IODE is converted by the enzyme into a more reactive species, which is responsible for inactivation. No inactivation has been detected with 12-iodooctadecanoic acid, 12-bromo-*cis*-9-octadecenoic acid, 12-iodo-*trans*-9-octadecenoic acid, or a mixture of stereoisomers of 9,11-octadecadienoic acid.

Lipoxygenases catalyze the oxygenation of *cis,cis*-1,4-dienes to produce conjugated diene hydroperoxides. Enzymes of this type are widespread in the plant kingdom and have recently been found to be important in mammalian arachidonic acid metabolism (Needleman et al., 1986). One of the mammalian enzymes is a target for drug design, since it is involved in the biosynthesis of leukotrienes, which are produced in inflammation and anaphalaxis (Hammarström, 1983). Since the mammalian enzymes are difficult to purify, attempts to design mechanism-based inhibitors rely heavily on structural and mechanistic studies on the plant enzymes, particularly lipoxygenase 1 from soybeans (Veldink et al., 1977; Veldink & Vliegenthart, 1984). This enzyme is specific for fatty acids in which the 1,4-diene unit begins on the ω6 carbon, that is, the sixth carbon from the methyl terminus (Hamberg & Samuelsson, 1967; Holman et al., 1969). For example, the enzyme catalyzes the conversion of linoleic acid to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD):[1]



13-HPOD

Lipoxygenase 1 consists of a single polypeptide chain (M_r 94 500), the sequence of which has recently been determined (Shibata et al., 1987). The enzyme contains one atom of non-heme iron per molecule (Chan, 1973; Roza & Francke, 1973; Pistorius & Axelrod, 1973), which appears to be essential

† This work was supported by grants from the National Institutes of Health (GM37289) and Research Corp.
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[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 13-HOD, 13(*S*)-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid; 12-IODE, 12-iodo-*cis*-9-octadecenoic acid; *trans*-12-IODE, 12-iodo-*trans*-9-octadecenoic acid; 12-BrODE, 12-bromo-*cis*-9-octadecenoic acid; TLC, thin-layer chromatography; methyl 12-MsODE, methyl 12-[(methylsulfonyl)oxy]-*cis*-9-octadecenoate; GC/MS, gas chromatography/mass spectrometry; MS, mass spectrometry; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; IR, infrared; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.

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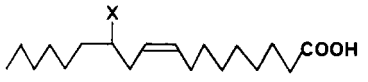
for catalytic activity (Pistorius & Axelrod, 1974). The iron in the purified enzyme is in the ferrous state (Slappendel et al., 1982; Cheesbrough & Axelrod, 1983) but can be rapidly oxidized to the ferric state by 1 equiv of 13-HPOD (DeGroot et al., 1975a,b; Pistorius et al., 1976; Slappendel et al., 1981). Furthermore, the enzyme is activated by 13-HPOD (Haining & Axelrod, 1958; Smith & Lands, 1972). These observations have led to the proposal that ferric lipoxygenase is the catalytically active form (DeGroot et al., 1975a; Pistorius et al., 1976). Additionally, it has been shown that the ferric enzyme can be reduced by linoleic acid in the absence of oxygen (DeGroot et al., 1975a; Egmond et al., 1977), and a catalytic cycle consistent with this observation has been proposed (DeGroot et al., 1975a).

As a possible approach to elucidating the mechanism by which lipoxygenase cleaves the C–H bond in normal substrates, we decided to investigate the action of the enzyme on compounds in which the ω6–ω7 double bond is replaced by a potential leaving group at ω7:



12-IODE, X=I
12-BrODE, X=Br

In this paper we report the unexpected discovery that one of these compounds, 12-iodo-*cis*-9-octadecenoic acid (12-IODE), is an efficient, irreversible inactivator of lipoxygenase 1. We also report our initial studies on the requirements for inhibition, which suggest that 12-IODE may act by a novel and interesting mechanism.

Experimental Procedures

Materials. Linoleic acid, ricinelaidic acid, ethyl 12-hydroxyoctadecanoate, glutathione, and glutathione peroxidase were obtained from Sigma Chemical Co. Methyl ricinoleate was purchased from Sigma or prepared from castor oil by the method of Swern and Jordan (1952). Soybean lipoxygenase was prepared by the method of Axelrod et al. (1981) with minor modifications (Clapp et al., 1985). 13-HPOD was prepared enzymatically from linoleic acid and reduced to 13-HOD with $NaBH_4$ (Gibian & Galaway, 1976).

Synthesis of 12-IODE. Methyl ricinoleate (methyl 12-hydroxy-*cis*-9-octadecenoate) was converted to its methanesulfonate (methyl 12-MsODE) by the general procedure of Crossland and Servis (1970). A solution of methyl 12-MsODE (0.82 g, 2.1 mmol) and NaI (0.45 g, 3.0 mmol) in 7.5 mL of acetone was refluxed under N_2. After 10.5 h a white precipitate (presumably sodium methanesulfonate) had formed, and the reaction mixture was partitioned between 25 mL of ethyl acetate and 25 mL of 1 M $NaHSO_4$. The organic phase was washed with 20 mL of 1 M NaCl and dried with $MgSO_4$. Evaporation of the solvent yielded a yellow oil, which showed one major product (R_f 0.61) by TLC in system I. This material was purified by flash chromatography (Still et al., 1978) on a 3 × 15 cm column of silica gel with hexanes/ethyl acetate, 96:4, as eluting solvent to give 470 mg of an oil that was homogeneous by GC/MS. Chemical ionization MS gave the expected M + 1 peak at m/z 423 for the methyl ester of 12-IODE, and the ^1H NMR spectrum showed all of the signals reported below for 12-IODE plus a singlet at δ 3.67 (3 H, OCH_3).

The methyl ester (119 mg) was saponified by stirring at 30 °C with a mixture of 1 M LiOH (1.2 mL) and THF (1.2 mL). After 9 h the mixture was acidified and partitioned between 5 mL of ethyl acetate and 3 mL of water. The aqueous layer was washed with 3 mL of ethyl acetate, and the combined organic layers were washed with 3 mL of 1 M NaCl, dried with $MgSO_4$, and concentrated to an oil (101 mg). TLC in system II showed only one product (R_f 0.42), but HPLC revealed ca. 2% of an impurity. Purification was carried out by HPLC in 1-mg portions on a Waters Radial Pak 8-μm C-18 cartridge eluted with acetonitrile/H_2O, 85:15, at a flow rate of 1.0 mL/min. The impurity elutes at 7.0 min and is probably 9,11-octadecadienoic acid. 12-IODE elutes at 8.2 min, and solvent evaporation yielded a colorless oil: IR (film) 2500–3500, 1710 cm^{-1}; ^1H NMR ($CDCl_3$, 250 MHz) δ 5.45 (m, 2 H, vinyl), 4.1 (m, 1 H, H-12), 2.65 (m, 2 H, H-11), 2.36 (t, J = 6.5 Hz, 2 H, H-2), 2.0 (m, 2 H, H-8). Decoupling at δ 2.65 resulted in simplification of the resonance at δ 4.1 and collapse of the upfield portion of the vinyl multiplet to an apparent AB system, J = 11 Hz. Decoupling at δ 2.0 caused collapse of the downfield portion of the vinyl multiplet to an apparent AB system, J = 11 Hz. Stock solutions of 50 mM 12-IODE in ethanol were stored at −20 °C and have been shown by HPLC to be stable for at least 3 months.

trans-12-IODE. Ricinelaidic acid (12-hydroxy-*trans*-9-octadecenoic acid) was converted to its methanesulfonate by treatment with 2.2 equiv of methanesulfonyl chloride and 2.9 equiv of triethylamine (Crossland & Servis, 1970). Refluxing the methanesulfonate overnight with 0.4 M NaI in acetone afforded *trans*-12-IODE. Purification by preparative-layer chromatography with system II yielded an oil that gave a 250-MHz ^1H NMR spectrum that was essentially identical with that of the cis isomer except for the appearance of the vinyl multiplet. Decoupling experiments identical with those described for the cis isomer gave a vinyl coupling constant of 15 Hz.

12-Iodooctadecanoic Acid. This material was prepared from ethyl 12-hydroxyoctadecanoate by the methods used for 12-IODE except that the final hydrolysis was carried out by refluxing ethyl 12-iodooctadecanoate (170 mg) with a mixture of 2 M HCl (0.70 mL) and dimethoxyethane (2.8 mL) for 4 h.

12-BrODE. A solution of methyl 12-MsODE (400 mg, 1.28 mmol) in 4.0 mL of 0.4 M NaBr in dimethylformamide was heated at 76 °C under N_2 for 3.5 h. Workup as described for methyl 12-IODE followed by preparative-layer chromatography in system I yielded 173 mg of an oil that was homogeneous by GC/MS. Chemical ionization MS gave the expected pair of M + 1 peaks at m/z 375 (^{79}Br) and 377 (^{81}Br), and ^1H NMR gave all of the signals reported below for 12-BrODE plus a singlet at δ 3.66 (3 H, OCH_3). The methyl ester was hydrolyzed as described for 12-IODE, and the product was purified by preparative-layer chromatography (system II, R_f 0.43): ^1H NMR (90 MHz, $CDCl_3$) δ 5.45 (m, 2 H, vinyl), 4.0 (m, 1 H, H-12), 2.6 (t, J = 7 Hz, 2 H, H-11), 2.35 (t, J = 7 Hz, 2 H, H-2).

9,11-Octadecadienoic Acid. Methyl 12-MsODE was subjected to elimination by heating with DBU as described by Gunstone and Said (1971), and the products were hydrolyzed as in the synthesis of 12-IODE. GC/MS and NMR indicate a 73:23 mixture of two isomers of 9,11-octadecadienoic acid plus an unidentified product (4%). Gunstone and Said (1971) report that the major isomer formed under these conditions is 9-cis,11-trans and the second is 9-cis,11-cis.

Analytical Methods. TLC was carried out on 250-μm layers of silica gel on glass (Aldrich Chemical Co.) with hexanes/ethyl acetate, 8:2 (system I), or hexanes/ethyl acetate/acetic acid, 75:25:2 (system II). Spots were visualized by exposure to iodine vapor. Preparative-layer chromatography was carried

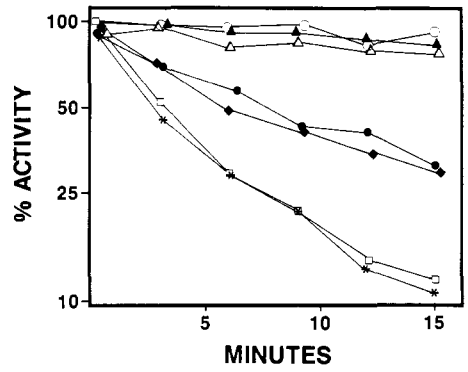


FIGURE 1: Time-dependent inactivation of soybean lipoxygenase by 12-IODE. At time zero, lipoxygenase (4.0 μL of a 9.4 μM solution; specific activity = 179) was added to 1.0 mL of 50 mM borate, pH 9.0, which contained the following: (*) 40 μM 12-IODE, 2 μM 13-HPOD; (□) 20 μM 12-IODE, 2 μM 13-HPOD; (◆) 4 μM 12-IODE, 2 μM 13-HPOD; (●) 4 μM 12-IODE, 0.5 μM 13-HPOD; (△) 4 μM 12-IODE; (▲) 2 μM 13-HPOD; (○) no additions. Each reaction mixture contained 1% (v/v) ethanol, and the final lipoxygenase concentration was 38 nM. The mixtures were maintained at 25 °C, and aliquots (50 μL) were withdrawn periodically and assayed for lipoxygenase activity in a total volume of 1.0 mL as described under Experimental Procedures.

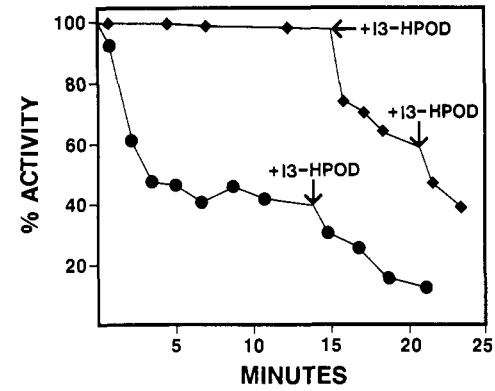


FIGURE 2: Inhibition of lipoxygenase by a large excess of 12-IODE and the effect of glutathione plus glutathione peroxidase. (●) At time zero lipoxygenase (2×10^{-5} μmol) was added to 1.0 mL of 40 μM 12-IODE in 50 mM borate, pH 9. At 14 min, 13-HPOD was added to a final concentration of 4 μM. (◆) A solution of 40 μM 12-IODE in 50 mM borate, pH 9.0, was preincubated at 25 °C for 5 min with 2.2 units of glutathione peroxidase and 250 μM glutathione. At time zero, 2×10^{-5} μmol of lipoxygenase was added. At 15 min, 13-HPOD was added to a final concentration of 12 μM, and a second identical aliquot was added at 20.5 min. The inactivation produced by these additions is of limited extent due to rapid consumption of the 13-HPOD by the glutathione/glutathione peroxidase system.

out on 20 × 20 cm plates coated with 2 mm of silica gel 60 PF-254 (EM Reagents), and zones were visualized by irradiation at 254 nm. HPLC was carried out on a Scientific Systems GS 400 instrument with a Waters Associates RCM-100 radial compression module and a Waters R403 refractive index detector or an ISCO V^4 UV–visible detector. NMR spectra were obtained on a Jeol FX90Q or a Bruker WM-250. GC/MS was carried out on a Finnigan 4021C system. Optical rotations were measured on a Perkin-Elmer 241 spectropolarimeter.

Enzyme Assays. Lipoxygenase activity was assayed at 25 °C in reaction mixtures containing 72 μM linoleic acid and 2 μM 13-HPOD in 50 mM borate buffer, pH 9.0. The formation of 13-HPOD was monitored spectrophotometrically at 234 nm; one unit is the amount of enzyme that catalyzes the formation 13-HPOD at an initial rate of 1 μmol/min. Lipoxygenase concentrations were determined spectrophotometrically with $\epsilon_{280}^{1\%} = 14.0$ (Axelrod et al., 1981). One unit of glutathione peroxidase is the amount of enzyme that catalyzes the consumption of NADH at an initial rate of 1 μmol/min in the coupled assay system described by Tappel (1978) with 50 μM 13-HPOD as substrate at pH 7.6, 25 °C. Rates were measured on a Perkin-Elmer 552 UV–visible spectrophotometer or a Hitachi 110A instrument.

Dialysis Experiments. Samples of enzyme (5 μg in 1.0 mL) that had been inactivated to >90% by 10 μM 12-IODE plus 2 μM 13-HPOD were dialyzed at 4 °C against 200 mL of 20 mM phosphate buffer, pH 7.0, or 20 mM borate buffer, pH 9.0. Fresh buffer was added after 1 and 2 h, and activity was measured at various times from 1 to 25 h. In no case was significant activity restored, and the residual activity decreased at about the same rate (ca. 50% per 25 h) as that of control samples of native enzyme.

RESULTS

The data in Figure 1 indicate that incubation of soybean lipoxygenase with 12-IODE and 13-HPOD results in time-dependent loss of enzymatic activity. At 4 μM 12-IODE and 0.5 μM 13-HPOD, loss of 50% of the initial activity occurs in 6–7 min. Under these conditions little inactivation occurs if 13-HPOD is absent. Thus, inactivation by 12-IODE, like the normal catalytic reaction, is stimulated by 13-HPOD. Inactivation is not stimulated by the corresponding hydroxy acid, 13-HOD, at 16 μM.

The semilogarithmic plots in Figure 1 do not give straight lines even though 12-IODE is present in 10^2–10^3-fold excess over enzyme; instead, a distinct upward curvature is observed in each experiment. While this situation precludes a simple pseudo-first-order treatment of the kinetics, several important observations can be made. At constant 13-HPOD (2 μM), the rate of inactivation is essentially the same at 20 and 40 μM 12-IODE, and the rate is about half-maximal at 4 μM 12-IODE. At 4 μM 12-IODE the rate of inactivation is increased only slightly by changing the 13-HPOD concentration from 0.5 to 2.0 μM. In another series of experiments at 40 μM 12-IODE the rate of inactivation was essentially identical at 2, 6, and 12 μM 13-HPOD.

The failure to observe pseudo-first-order kinetics in Figure 1 suggests that more than 1 equiv of 12-IODE may be required for complete inactivation. This was found to be the case in an experiment in which 5 μM lipoxygenase was incubated with 50 μM 12-IODE and 50 μM 13-HPOD. Under these conditions about 60% of the activity was lost over 10 min, and then inactivation ceased. No loss of activity occurred if 13-HPOD was omitted. These data imply that approximately 17 equiv of 12-IODE is required for complete inactivation.

If 12-IODE is present in very large (>10^3) excess over enzyme, significant inactivation occurs in the absence of 13-HPOD. Figure 2 shows the results of an experiment in which 20 nM enzyme was treated with 40 μM 12-IODE. Under these conditions about 60% of the initial activity is lost in the absence of 13-HPOD; additional activity is lost if 13-HPOD is added. As shown in Figure 2, the 13-HPOD-independent inactivation can be eliminated by preincubation of the inhibitor with glutathione peroxidase plus glutathione prior to adding lipoxygenase. Neither glutathione nor glutathione peroxidase alone protects the enzyme against inactivation by 12-IODE (see Figure 3).[2] These observations imply that the 13-HPOD-independent inactivation at high 12-IODE/enzyme is

[2] When 100 μM 12-IODE was incubated with 250 μM glutathione for 30 min at pH 9.0, no significant loss of thiol groups could be detected by the method of Ellman (1959). Thus, 12-IODE does not alkylate the thiol group of glutathione under these conditions.

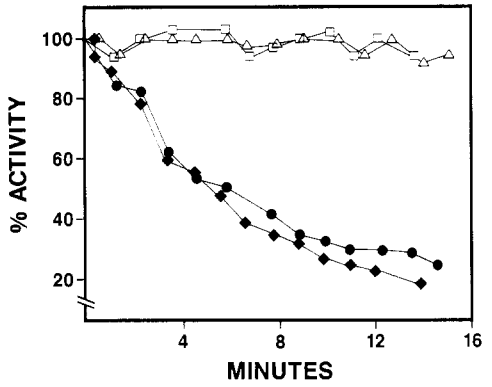


FIGURE 3: Requirement that 13-HPOD be present during inactivation by 12-IODE. (□) Lipoxygenase (14 μg; specific activity = 115) in 0.95 mL of 50 mM borate, pH 9, at 25 °C was treated with 5 μL of 1 mM 13-HPOD in ethanol. After 1 min, 0.1 unit of glutathione peroxidase (in 10 μL of 20 mM Tris, pH 7.6) and 30 μL of 10 mM glutathione were added. The mixture was incubated for 10 min at 25 °C and then treated with 5 μL of 6.8 mM 12-IODE in ethanol (t = 0 on plot). Three other experiments were carried out identically except that (△) 5 μL of ethanol was added in place of 12-IODE, (◆) glutathione was omitted, or (●) glutathione peroxidase was omitted.

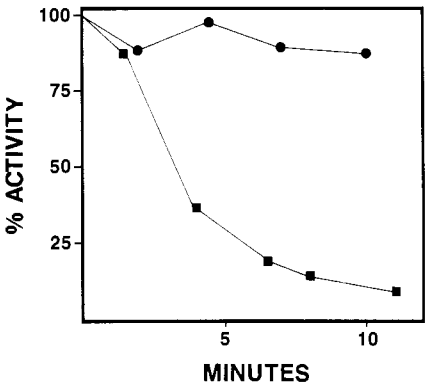


FIGURE 4: Effect of N_2 purging on inactivation of lipoxygenase by 12-IODE. (●) Anaerobic reaction. In a test tube sealed with a serum cap 2.5 mL of borate buffer (pH 9, 50 mM) was deaerated for 15 min by admitting N_2 through a needle immersed in the buffer and allowing it to escape through a second needle above the solution. Lipoxygenase (2.2 units) was then added by syringe followed by 1.5 μL of 4.1 mM 13-HPOD in ethanol and 2.0 μL of 50 mM 12-IODE in ethanol. Time zero on the plot is the time of 12-IODE addition. Aliquots (50 μL) were withdrawn by syringe and assayed for lipoxygenase activity as described under Experimental Procedures. (■) Aerobic reaction carried out in the same manner without deaeration of the buffer.

due to traces of lipid hydroperoxide or a lipoxygenase substrate (see Discussion) in the inhibitor preparation.

The dependence on 13-HPOD suggests that 12-IODE specifically inhibits the Fe^{3+} form of the enzyme. Figure 3 presents the results of an experiment designed to test whether 13-HPOD is required after the initial oxidation to the ferric state. Lipoxygenase (0.14 μM) was incubated for 1 min with 13-HPOD (5 μM) to oxidize the enzyme to the ferric form.[3] Glutathione peroxidase (0.1 unit) plus glutathione (0.3 mM) were then added to destroy excess 13-HPOD. After 10 min, 12-IODE was added to a final concentration of 34 μM. No significant inactivation was observed compared with a control to which no 12-IODE was added. Two additional controls were carried out exactly as above, except that glutathione was omitted from one and glutathione peroxidase from the other. In each of these controls inactivation occurred when 12-IODE was added. These results imply that the ferric enzyme is not inactivated by 12-IODE unless 13-HPOD is present.

To determine whether 12-IODE and 13-HPOD react nonenzymatically, 100 μM 12-IODE was incubated with 20 μM 13-HPOD in 50 mM borate, pH 9.0, at 25 °C. A second reaction was run without 13-HPOD. After 30 min the mixtures were extracted with ethyl acetate, and the products were converted to their p-bromophenacyl esters and analyzed by HPLC (Jordi, 1978). In the absence of 13-HPOD about 20% of the 12-IODE was converted to a mixture of four products. Two of these solvolysis products have been tentatively identified by coinjection with the standards 12-hydroxy-9-octadecenoic acid and 9,11-octadecadienoic acid; the other two presumably result from rearrangements that are characteristic of homoallylic systems (Story & Clark, 1972). No additional products were observed in the presence of 13-HPOD, and the solvolysis products were formed in the same yield as in the absence of 13-HPOD. Thus, no reaction between 12-IODE and 13-HPOD is detectable at concentrations considerably above those in our inactivation experiments. Furthermore, we have found that preincubation of 12-IODE and 13-HPOD in 50 mM borate, pH 9.0, for 10 min at 25 °C prior to adding enzyme has no effect on the rate of inactivation.

Lipoxygenase is readily inactivated by 0.5 μM cumene hydroperoxide. It has therefore not been possible to determine whether this compound will satisfy the peroxide requirement for inactivation by 12-IODE.

Since O_2 is a substrate for lipoxygenase, the action of 12-IODE on the enzyme under anaerobic conditions was investigated. Figure 4 shows that inactivation by 12-IODE can be nearly eliminated by purging the incubation medium with N_2 for 15 min prior to addition of enzyme. This result implies that the inactivation by 12-IODE is O_2 dependent.

The inactivation by 12-IODE was not reversed by dialysis at 4 °C for up to 25 h either at pH 7 or at pH 9 as described under Experimental Procedures.

The 12-IODE used in these experiments is probably racemic, since it exhibits no significant optical rotation, $[\alpha]^{22}_D < 0.2°$. The starting material, methyl ricinoleate, gave a rotation, $[\alpha]^{22}_D = 4.7°$, that was in good agreement with a previously reported value, $[\alpha]^{22}_D = 5.05°$ (Swern & Jordan, 1951), but loss of chirality probably occurs in the reaction of methyl 12-MsODE with sodium iodide, due to repeated S_N2 substitutions by iodide ion on the product (March, 1985; Cason & Correia, 1961). Optically inactive 12-BrODE was prepared by the same route using sodium bromide. No time-dependent inactivation was observed when 40 nM lipoxygenase was incubated with 80 μM 12-BrODE plus 2 μM 13-HPOD.

No significant time-dependent inactivation was detected when 40 μM 12-iodooctadecanoate, the saturated analogue of 12-IODE, was incubated with 70 nM lipoxygenase plus 7 μM 13-HPOD. No inactivation occurred when 43 μM *trans*-12-IODE was incubated with 72 nM lipoxygenase plus 6 μM 13-HPOD.

Elimination of HI from 12-IODE would produce 9,11-octadecadienoic acid. Attempts to detect the appearance of this compound spectroscopically (λ_{max} = 230 nm) during the inactivation by 12-IODE have been inconclusive. These experiments are complicated by the high absorbance of the

[3] The oxidation of ferrous lipoxygenase by 13-HPOD is known to be very rapid [see Egmond et al. (1977)], but the concentration dependence is not known. The following result indicates that 1 min is ample time for oxidation of the enzyme under the conditions of this experiment. Lipoxygenase (0.14 μM) and 5 μM 13-HPOD were incubated in the presence of 8 μM *N*-octylhydroxylamine, which has been shown to reduce ferric lipoxygenase back to ferrous lipoxygenase (Clapp et al., 1985). Consumption of 13-HPOD was monitored at 234 nm and found to be complete in 0.4 min. Since 13-HPOD was present in 36-fold excess over enzyme, these results indicate that the enzyme was oxidized and reduced 36 times in 0.4 min.

protein and 13-HPOD in the region of interest and by the possibility that some 13-HPOD is consumed during inactivation. If a small amount of 9,11-octadecadienoic acid is formed, it is probably not responsible for inactivation, since no inactivation occurred when lipoxygenase was incubated with 100 μM 9,11-octadecadienoic acid in the presence of 2 μM 13-HPOD.

Discussion

Our results demonstrate that 12-IODE is a time-dependent, irreversible inactivator of soybean lipoxygenase. The rate of inactivation is independent of 12-IODE concentration above 20 μM and is half-maximal at about 4 μM. Inactivation by 12-IODE is dependent on 13-HPOD, which stimulates the normal catalytic reaction, and on O_2. These findings raise the possibility that 12-IODE is a suicide inactivator (Walsh, 1984).

Our results indicate that lipid hydroperoxide is required for inactivation. Significant inactivation in the absence of added 13-HPOD is only observed if 12-IODE is present in $>10^3$-fold excess over enzyme. Under the latter conditions, inactivation appears to be due to traces of peroxide contamination, since it is eliminated by the presence of glutathione peroxidase plus glutathione. The contaminating material may be an autoxidation product of 12-IODE or of 9,11-octadecadienoic acid, produced by loss of HI from 12-IODE. Alternatively, the contaminant could be a substrate (e.g., linoleic acid), which is converted to a hydroperoxide by the small amount (ca. 5%) of ferric lipoxygenase that ESR studies have shown to be present in our enzyme preparations.

The lipid hydroperoxide dependence is most simply explained by the hypothesis that 12-IODE inactivates ferric lipoxygenase but not ferrous lipoxygenase. We have found, however, that if the enzyme is first oxidized to the ferric form by 13-HPOD and then treated with glutathione peroxidase plus glutathione to remove excess 13-HPOD, no inactivation occurs upon subsequent addition of 12-IODE. This result implies that 13-HPOD must contribute to the inactivation after the initial oxidation of the enzyme from ferrous to ferric.

The requirement for the *presence* of 13-HPOD raises the possibility that inactivation involves a reaction between 12-IODE and the purple complex that is formed when ferric lipoxygenase is treated with 13-HPOD (DeGroot et al., 1975b; Slappendel et al., 1983). This complex exhibits an ESR signal at $g = 4.3$, which indicates that the iron is in the ferric state and in a rhombic environment. In contrast, free ferric lipoxygenase gives an ESR signal at $g = 6.1$, which implies that the ferric ion is in an axial environment. The purple complex decomposes to free ferric lipoxygenase and a mixture of products derived from 13-HPOD (Garssen et al., 1976). This decomposition occurs slowly under aerobic conditions but much more rapidly under anaerobic conditions (Slappendel et al., 1983). The lability of the purple complex in the absence of oxygen could account for the failure of 12-IODE to inactivate under anaerobic conditions.

An alternative explanation for the 13-HPOD requirement is that 12-IODE reacts with ferric lipoxygenase to produce the ferrous enzyme plus a reactive intermediate derived from 12-IODE, which either reacts with the enzyme or dissociates. If this intermediate dissociates much more frequently than it reacts with the enzyme, then inactivation by 12-IODE would not be detectable unless 13-HPOD was present to reoxidize the enzyme to the ferric form. Our data indicate that about 17 equiv of racemic 12-IODE is required for complete inactivation. If it is assumed that only one enantiomer of 12-IODE is processed by the enzyme, then inactivation occurs in 1 out of every 8.5 turnovers, which predicts that about 12% of the activity should be lost in a single turnover. It is difficult to judge whether this small loss would have been detected in the experiment presented in Figure 3.

No inactivation is observed with 12-iodooctadecanoic acid or *trans*-12-IODE at concentrations 40-fold higher than that at which inactivation by 12-IODE is detectable. These results indicate that the cis 9–10 double bond in lipoxygenase substrates is also important for inactivation. A quantitative assessment of the importance of the stereochemistry of this double bond for substrate activity has recently been provided by Funk et al. (1987), who found that *trans*-9,*cis*-12-octadecadienoic acid is 0.03 times as active as linoleic acid.

The failure of 12-BrODE to inactivate suggests that some special property of the C–I bond contributes to inactivation by 12-IODE. One possibility is the susceptability of alkyl iodides to one-electron oxidation (Casanova & Eberson, 1973). Another is the ability of peracids to oxidize alkyl iodides to iodoso compounds, which rapidly lose IO^- to form carbocations (Macdonald et al., 1980). The latter possibility is especially attractive if the purple complex is the target of 12-IODE.

Further development of the mechanistic ideas presented here awaits the results of experiments with isotopically labeled preparations of 12-IODE currently being synthesized in our laboratory.

Acknowledgments

We are grateful to Dr. James Van Epp, Brown University, and Jimmy Spriggle, Bucknell University, for obtaining the GC/MS data reported here and to Dr. Van Epp for his assistance with the proton-decoupled NMR experiments.

Registry No. 13-HOPD, 33964-75-9; 12-IODE, 116784-49-7; methyl 12-MsODE, 57884-97-6; methyl 12-IODE, 116784-52-2; *trans*-12-IODE, 116784-50-0; 12-BrODE, 116784-51-1; lipoxygenase, 63551-74-6; ricinelaidic acid, 540-12-5; ethyl 12-hydroxyoctadecanoate, 74815-67-1; methyl ricinoleate, 141-24-2; ricinelaidic acid methanesulfonate, 78773-30-5; 12-iodooctadecanoic acid, 73640-30-9; 9-*cis*,11-*trans*-octadecadienoic acid, 2540-56-9; 9-*cis*,11-*cis*-octadecadienoic acid, 544-70-7.

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Diffusion-Dependent Rates for the Hydrolysis Reaction Catalyzed by Glyoxalase II from Rat Erythrocytes†

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Received April 20, 1988; Revised Manuscript Received July 27, 1988

ABSTRACT: Glyoxalase II from rat erythrocytes is a near optimal catalyst for the hydrolysis of *S*-D-lactoylglutathione in the sense that the magnitude of k_{cat}/K_m is limited, in large part, by the rate constant for diffusion-controlled encounter between substrate and active site. The experimental basis for this conclusion is derived from the dependencies of the kinetic properties of the enzyme on solution viscosity (pH 7, I_c = 0.1 M, 25 °C). When sucrose is used as a viscogenic agent, k_{cat}/K_m for *S*-D-lactoylglutathione (8.8×10^5 M^{-1} s^{-1}) decreases markedly with increasing solution viscosity. This effect appears not to be due to a sucrose-induced change in the intrinsic kinetic properties of the enzyme, since k_{cat}/K_m for the slow substrate *S*-acetylglutathione (3.7×10^4 M^{-1} s^{-1}) is nearly independent of solution viscosity. Quantitative treatment of the data using Stoke's law indicates that the rate of hydrolysis of *S*-D-lactoylglutathione will be ~50% diffusion limited when [substrate] $\ll K_m$; the encounter complex between enzyme and substrate partitions nearly equally between product formation and dissociation to form free enzyme and substrate. The same conclusion is reached when glycerol is used as a viscogenic agent, once the apparent activation effect of glycerol on the intrinsic activity of the enzyme is taken into account. Finally, the rate of formation of the encounter complex between substrate and active site may be governed to a significant extent by charge–charge interactions. This conclusion is based upon a Debye–Hückel treatment of the ionic strength dependency of k_{cat}/K_m for *S*-D-lactoylglutathione in which $k_{cat}/K_m \simeq 4 \times 10^7$ M^{-1} s^{-1} at zero ionic strength.

The widely distributed glyoxalase enzyme system composed of glyoxalases I (Glx I,[1] EC 4.4.1.5) and II (Glx II, EC 3.1.2.6) catalyzes the net glutathione (GSH) dependent conversion of methylglyoxal to D-lactate (eq 1). A proposed physiological function of the pathway is to rapidly remove from cells cytotoxic methylglyoxal that arises either as a normal or as an

† This work was supported by grants from the National Institutes of Health to D.J.C. (GM 31840) and to D.L.V. (GM 25295).
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[1] Abbreviations: Glx I, glyoxalase I; Glx II, glyoxalase II; GSH, glutathione; H_R and H_S, thiohemiacetals formed from GSH and phenylglyoxal in which the chirality at the thiohemiacetal carbon is *R* and *S*, respectively; I_c, ionic strength.

0006-2960/88/0427-8818$01.50/0