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THE DIFFERENTIAL EFFECT OF DISULFIRAM ON LIPOXYGENASES FROM *GLYCINE MAX*

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Key Word Index—*Glycine max*; Leguminosae; soybean; enzymology; lipoxygenase; disulfiram.

Abstract—A kinetic analysis of the effect of disulfiram on the isoenzymes of lipoxygenase from soybean has been carried out. The compound is an effective inhibitor of type-2 isoenzymes but has no effect on the type-1 isoenzyme under the conditions employed in this study. The inhibitory effect is reversible and therefore does not result from covalent modification of the enzyme. The inhibition is manifest as a prolongation of the lag phase commonly seen in progress curves for lipoxygenases rather than as a reduction of the catalyzed rate. A variety of structurally related and unrelated compounds have been investigated to identify the nature of the inhibitory effect. The antioxidant properties of disulfiram account for its ability to inhibit type-2 lipoxygenases. The inhibitory effect of antioxidants on type-2 lipoxygenase is only partly reversed when product hydroperoxide is included in the incubation mixture. These observations support the conclusion that free radical intermediates are integral to the catalytic mechanism of type-2 lipoxygenases.

INTRODUCTION

Plant mitochondria are able to consume oxygen in a pathway that is not inhibited by cyanide [1]. This pathway has been referred to as alternate respiration. Recently, the possibility that lipoxygenase (EC 1.13.11.12), a non-heme iron dioxygenase, present in or contaminating mitochondria, contributes to this oxygen consumption has been considered [2]. Towards this end, compounds have been sought that will inhibit either lipoxygenase or alternate respiration but not both. Disulfiram (tetraethylthioperoxydicarbonic diamide, tetraethylthiuram disulfide) selectively inhibits the cyanide-resistant respiratory activity in potato tuber mitochondria [3], but does not affect the lipoxygenase activity associated with the mitochondria of soybean shoot axes [4]. Since the source of lipoxygenase in mitochondrial preparations is obscure (i.e. intrinsic or contaminating) and its relation to the alternate pathway is not clear [5, 6], it seemed appropriate to check the known soluble lipoxygenases from soybean for disulfiram inhibition. This compound is known to interfere with certain enzyme activities by the formation of mixed disulfides with protein sulfhydryl groups [7]. Disulfiram also has the capacity to act as an antioxidant [8, 9].

Soybean lipoxygenase is known to exist as a mixture of isoenzymes [10]. While the type-1 enzyme (pH 9 active) has been extensively studied, the other isoenzymes (neutral active, type-2) have only been partly characterized. Since the type-1 lipoxygenase both contains reactive sulfhydryl groups [11] and is activated by the product of its reaction (a lipid peroxide) [12], the opportunities for a significant effect of disulfiram on catalysis by the enzyme appeared to exist. The purpose of the present investigation was to examine the two classes of lipoxygenase for inhibition by disulfiram as a further test of the validity of the use of this compound as a specific inhibitor of alternate respiration. In the process, some interesting kinetic characteristics of type-2 lipoxygenases were discovered.

RESULTS AND DISCUSSION

In order to evaluate the effects of disulfiram on lipoxygenase isoenzymes individually, the reported procedures for their separation were investigated [13–15]. It was found that while the type-1 enzyme could be readily purified, the remaining isoenzymes were not easily resolved. Differences in the source or condition of the soybeans used in the present study compared to those used in past investigations may account for our inability to separate these isoenzymes. Additionally, it should be noted that a more stringent criterion of purity (analytical isoelectric focusing) was applied to enzyme samples in the present study than has commonly been used in the past. For kinetic studies, the type-2 isoenzymes were examined together rather than as individuals. It is for this reason that the isoenzymes active at pH 7 but not at pH 9 are referred to as type-2 lipoxygenases in this paper. At least three such lipoxygenases have been identified in soybeans [16]. The kinetic results reported here are therefore for the lipoxygenases by class, with a comparison being made of the effects of disulfiram on the type-1 and type-2 enzymes.

Using standard kinetic assay procedures [17], it was found that disulfiram inhibited the neutral active lipoxygenases, but had no effect on the type-1 species. For example, using linoleic acid solubilized with Tween 20 as

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the substrate at pH 6.7, the activity of the type-2 enzymes (0.25 μg/ml) was completely suppressed by disulfiram present at a concentration of 50 μM. By contrast, the assay profile of the type-1 lipoxygenase at pH 9.2 using 60 μM linoleic acid as the substrate was unaffected by disulfiram at concentrations up to 200 μM. The kinetic profiles for the type-1 enzyme were likewise unaffected by disulfiram at pH 6.7 using the substrate for the type-2 isoenzymes. To further characterize the inhibitory effect of disulfiram on the neutral active lipoxygenases, kinetic studies were carried out at substantially higher enzyme concentrations. It was found that the observed inhibition was due to a prolongation of the initial slow portion of the progress curves (lag phase), rather than to an inhibition of the rate of reaction, as demonstrated in Fig. 1. As is evident in the figure, the effect was concentration-dependent, with the lag time increasing for increasing concentrations of disulfiram in the incubations.

Non-linear initial rate data have been a characteristic feature of type-1 lipoxygenase kinetic studies. The upward curvature typically observed when product formation is monitored has been attributed to a combination of product activation and substrate inhibition [12, 18]. Preformed hydroperoxide product when incorporated in the substrate solution produces a decrease in the lag. The effect is concentration-dependent and saturable indicating a specific enzyme–product interaction [19]. The interaction is thought to include a chemical component in which the enzyme is converted from a low activity iron(II) containing form into a more reactive iron(III) species as the hydroperoxide is reduced [20]. Substantially less is known about the kinetic mechanism of other lipoxygenases. Kinetic studies of the lipoxygenases active at neutral pH have been complicated by the limited solubility of the substrate in this pH range [21]. To study these isoenzymes, researchers have used various strategies to solubilize the substrate including the use of non-ionic detergents. The time course for catalysis observed under such conditions is curved, and the lag is reduced by the incorporation of 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [22]. This indicates a degree of mechanistic similarity for the various lipoxygenases.

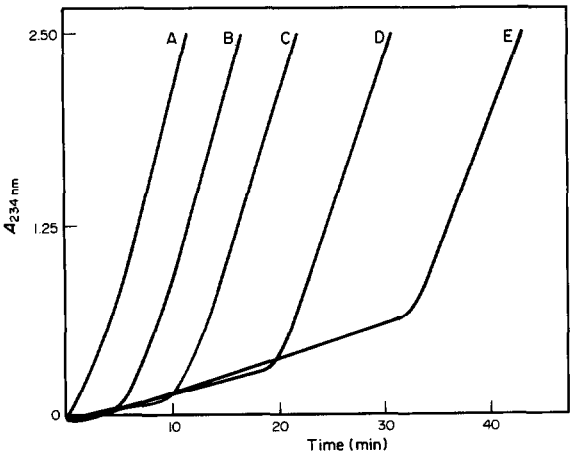


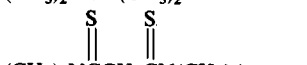
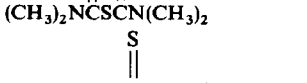
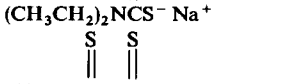
Fig. 1. Progress curves for type-2 lipoxygenases in the presence of various concentrations of disulfiram. (A) None; (B) 0.62 μM; (C) 1.25 μM; (D) 2.50 μM; (E) 5.00 μM. Linoleic acid, 1 mM; Tween 20, 0.1%; sodium phosphate buffer, 0.12 M, pH 6.7; lipoxygenase-2, 2.8 μg/ml; 25°.

Disulfiram has been previously used as a modifier of enzyme activities. Aldehyde dehydrogenase has, for example, been found to be very sensitive to this compound. The enzyme is 90% inactivated by two equivalents of disulfiram per tetrameric enzyme molecule [23]. This inactivation has been interpreted as resulting from the formation of a mixed disulfide between an enzyme sulfhydryl at or near the active site and the modifier [7]. The effect of disulfiram on neutral lipoxygenases appears not to be the result of a covalent interaction. Nearly full catalytic activity was expressed even after a prolonged disulfiram-induced lag period. Further, when the enzymes were treated with a large dose of the modifier and then dialysed without added thiol, the same activity was observed as for untreated control samples.

To characterize further the inhibitory effect of disulfiram on type-2 lipoxygenases, a variety of compounds having similar structural features were also checked for their effect on the enzymes. The results of a comparative investigation of these effects are collected in Table 1. The tetraethyl and tetramethylthiuram disulfides had roughly the same inhibitory effect, while diethyl dithiocarbamate was somewhat less effective. The monosulfide produced only a small increase in the lag and compounds not containing bridging sulfur were ineffective. These results were in sharp contrast with those for the inhibition of the alternate pathway in potato mitochondria [3]. In that case, tetramethylthiuram disulfide was at least 14 times less effective as an inhibitor than the tetraethyl compound, and diethyl dithiocarbamate was more than three orders of magnitude less effective. A distinction between the two pathways for oxygen consumption would appear to be straightforward. A comparison of the effect of disulfiram on oxygen consumption with that of diethyl dithiocarbamate would provide a means for discriminating between these two possibilities. It should be clear, however, that misleading conclusions might be drawn if disulfiram alone were used as an inhibitor in studies of the alternate pathway.

Having excluded covalent modification, alternative explanations for the effect of disulfiram on type-2 lipoxygenases were sought. Based upon the appearance of the progress curves in the presence of the inhibitor (Fig. 1), interference with product-mediated activation through an antioxidant effect appeared to be a likely prospect. The progress curves appeared to be divided into two distinct phases. A region of slow product formation preceded an abrupt activation presumably as a result of the complete consumption of the inhibitor. In support of this possibility was the observation that the amount of product formed before activation occurred was directly proportional to the amount of disulfiram added (correlation coefficient = 0.9997). These observations along with the known product activation of type-1 lipoxygenase led to the hypothesis that disulfiram was interfering with a product-mediated conversion of type-2 isoenzymes from a low to a high activity form. This hypothesis was tested by investigating the effect of the structurally unrelated antioxidant, BHT, and the effect of added hydroperoxide on the time course for catalysis. For comparison purposes, the lag times were measured [24] and are reported relative to that obtained for disulfiram at 2 μM, which has been assigned a value of 100. As shown in Table 1, a similar although less-pronounced prolongation of the lag time was observed when BHT was incorporated with the substrate. As expected, the lag was reduced when the

Table 1. Factors affecting the lag time in type-2 lipoxygenase progress curves

Compound	Concentration (μM)	Relative lag time	
		− product	+ product (25 μM)
	2	9	—
	2	8	—
None	0	13	3
	22	28	—
	2	81	—
	2	100	—
	2	(100)	66
BHT	5	48	32

product was included in the assay mixture in the absence of inhibitors. Interestingly, the inclusion of an activating concentration of product in the assay mixture only partly reversed the effect of either inhibitor, BHT or disulfiram. If the product of the reaction is unable to effect a complete activation of the antioxidant-suppressed enzymes, then it appears likely that the peroxy radical rather than the hydroperoxide is responsible for this effect in type-2 lipoxygenases. The peroxide could reduce the lag because it produces free radicals as the result of its decomposition.

Inhibition by antioxidants was one of the earliest observations made for plant lipoxygenases [25]. In fact, the inhibition of enzymatic polyunsaturated fatty acid oxygenation by phenolic compounds like nordihydroguaiaretic acid (NDGA) has commonly been used to identify lipoxygenase catalysis [26, 27]. Remarkably, this effect has not been thoroughly characterized. Commonly, incubations in the presence of NDGA are followed by examination of the mixtures for decreased levels of lipoxygenase products. The time course for the inhibition can apparently take two forms, the competitive inhibition of the catalytic rate or the prolongation of the lag phase. The observed effect depends both on the individual inhibitor being used and on the source and isoenzyme composition of the lipoxygenase. In one report, NDGA was found to be a competitive inhibitor of the lipoxygenase from broad beans, while the effect of BHT was to increase the lag for this enzyme [28]. In the case of the soybean enzyme, both possible results have been presented for the effect of NDGA. In one early study, the compound reportedly produced a prolongation of the lag phase [29]. In a conflicting report, NDGA was found to be a competitive inhibitor with no evidence for an induction period [30]. The results of the present study clarify the situation with regard to inhibition of the soybean isoenzymes. The type-2 lipoxygenases are clearly inhibited via an increased lag phase by antioxidant inhibitors at low concentration. The inhibition of the type-1 enzyme seen at higher concentrations of inhibitors is a separate effect, reflecting a fundamental difference in the mode of action of the isoenzymes.

There is considerable evidence from a variety of studies that lipoxygenase catalysis is a free radical process for those isoenzymes active at neutral pH. For example, the cooxidation of carotenes by these lipoxygenases is inhibited by free radical scavengers [31]. Also, the product distribution for type-2 enzymes at pH 6.6 has been found to contain practically equal quantities of the possible stereoisomers [32]. This finding is consistent with the release of a free radical from the enzyme that can be oxygenated to give either enantiomer. Finally, the existence of free peroxy radicals in type-2 lipoxygenase catalysis has been recently confirmed by oxygen isotope scrambling experiments [33]. The results of the present investigation reinforce this mechanistic formulation. The additional finding that the product does not overcome the inhibitory effect of the antioxidants indicates that the activation of these lipoxygenases may also be dependent on free radical intermediates. This provides a significant contrast with the type-1 lipoxygenase where the product hydroperoxide is the effective agent. This contrast is presented schematically in Fig. 2 as a plausible comparison of mechanisms. The observations presented here emphasize the idea that while similarities exist, important aspects of mechanism separate the various lipoxygenase isoenzymes.

EXPERIMENTAL

Chemicals. Disulfiram was recrystallized (EtOH) from 250 mg tablets of Antabuse (Ayerst Laboratories). Tetramethylthiuram monosulfide [34] and tetramethyldithiomalonamide [35] were prepared according to published procedures. Stock solns of the compounds were prepared in either EtOH or Me_2CO. Solns were

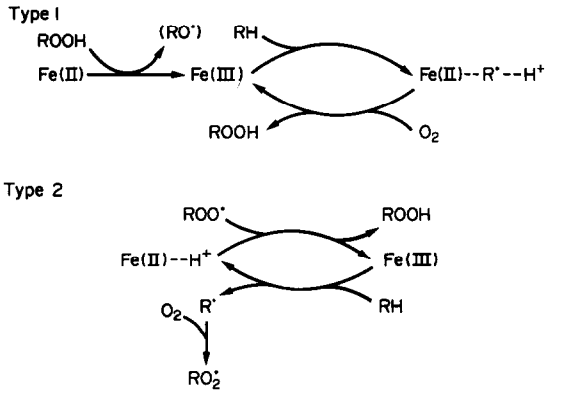


Fig. 2. Proposed mechanisms to account for the differences in lipoxygenase kinetic behavior.

made by transferring an aliquot of the stock soln into a test-tube. The solvent was removed by a stream of N_2 and the compounds were then dissolved in the appropriate amount of substrate soln. Linoleic acid was used as the substrate in the kinetic experiments as well as in the preparation of hydroperoxide. The 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid was prepared by a published procedure [36] except that the final purification was by flash chromatography using HOAc–Me_2CO–C_6H_{12} (1:25:74) as the eluant [37] rather than HPLC. The hydroperoxide was stored under N_2 at $-20°$. The product concn was 25 μM when included in incubations.

Purification of isoenzymes. Soybean lipoxygenases were purified by a modification of a published procedure [38]. Soybean lipoxygenase (75 mg, Sigma, type-I) was dissolved in 5 ml 0.05 M NaOAc butter, pH 5.5, containing 0.01 M NaCl and dialysed against 1 l. of the same buffer. The dialysate was applied to a column (2.5 cm × 70 cm) packed with CM-cellulose and equilibrated with 0.05 M NaOAc buffer, pH 5.5, containing 0.01 M NaCl. The column was flushed (100 ml/hr) with the equilibration buffer until the A_{280} of the eluate returned to the baseline. Elution of the isoenzymes was performed with a linear gradient formed with 1 l. of the equilibration buffer and 1 l. 0.05 M NaOAc buffer, pH 5.5, containing 0.25 M NaCl. Fractions of 16 ml were collected and protein elution was monitored continuously at 280 nm. Individual fractions were tested for lipoxygenase activity at pH 9.2 and 6.7. Protein concns were determined by dye binding analysis [39].

Isoelectric focusing of lipoxygenases was performed using a horizontal electrophoresis cell (Biorad). Lipoxygenases from CM-cellulose column chromatography were concd 10-fold (Minicon B15, Amicon) and 25 μl samples were applied to a 5% polyacrylamide gel (100 × 35 × 1 mm) containing 2% ampholytes, pH range 5–7 (LKB) The gels were focused at 6 W constant power for 2.5 hr at 10°. The gel was stained for protein with Coomassie brilliant blue R-250.

Kinetic experiments. Kinetic studies for lipoxygenase isoenzymes were carried out by following the formation of the conjugated hydroperoxide product by measuring increases in *A* at 234 nm. Due to the nature of the isoenzymes, kinetic experiments were carried out at pH 9.2 and 6.7 for type-1 and type-2 lipoxygenase, respectively. In experiments at pH 9.2, linoleic acid was dissolved in 0.1 M Na borate buffer at a concn below its critical micelle concn (60 μM). Substrate solns at pH 6.7 were prepared according to published procedures [17]. A stock soln containing 2.5×10^{-3} M linoleic acid dispersed with 0.25% Tween 20 was diluted with 0.2 M NaPi buffer to give 10^{-3} M linoleic acid soln. The stock soln of linoleic acid was kept under N_2 at $-20°$. Fresh aq. stock solns of linoleic acid were used throughout to prepare the final substrate solns. The reactions were carried out in a cuvette and utilized 3 ml of substrate and 25 μl of enzyme soln at pH 9.2 and 1 ml of substrate and 100 μl of enzyme soln at pH 6.7. The spectrophotometer was zeroed on the substrate-filled cuvette, preincubated at 25°, and at time zero the enzyme soln was added.

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