ENZYME INHIBITION BY ACETYLENIC COMPOUNDS

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Summary. Soybean lipoxidase and prostaglandin synthetase from sheep seminal vesicles are subject to powerful inhibition by 5,8,11,14-eicosatetraynoic acid, apparently by a slow but irreversible attack on the enzymes. It is postulated that inhibition may result from conversion of the acetylenic compound to a reactive allene by the enzymes studied, as has been proposed in the inhibition of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-N-acetylcysteamine.

Bloch and co-workers have demonstrated that 3-decynoyl-N-acetylcysteamine is an irreversible inhibitor of the interconversion of related thioesters of D-3-hydroxydecanoic, trans-2-decenoic, and cis-3-decenoic acids catalysed by β-hydroxydecanoyl thioester dehydrase from Eschericia coli (1,2). Arrest of the growth of cultures of E. coli by the acetylenic compound resulted from specific inhibition of the dehydrase, since growth was restored by addition of oleic acid to the cultures (3).

Earlier, Blain and Shearer studied the effect of a wide range of acetylenic compounds on soybean lipoxidase and found inhibition only by those acetylenes which are analogous in structure to the polyunsaturated fatty acid substrates (4). 5,8,11,14-Eicosatetraynoic acid was reported to be the most effective of the compounds studied in inhibiting the lipoxidase, by a competitive mechanism.

We have found that 5,8,11,14-eicosatetraynoic acid is an efficient and apparently irreversible inhibitor of prostaglandin synthetase in sheep seminal vesicles (5). On the other hand, the inhibition of prostaglandin synthetase

by polyethylenic fatty acids observed by Pace-Asciak and Wolfe (6) does not appear to be irreversible (5). We have now re-investigated the inhibition of soybean lipoxidase by the tetraacetylenic acid and found the effect to be irreversible. These studies indicate that while enzymes involved in the transformation of unsaturated substrates may generally be powerfully inhibited by both ethylenic and acetylenic analogs, only the acetylenic compounds appear to be responsible for irreversible attack.

EXPERIMENTAL

Materials. 1-^{14}C-labelled oleic, linoleic, and linolenic acids were obtained from Applied Science Laboratories. Tritium-labelled arachidonic acid was prepared by selective reduction of 5,8,11,14-eicosatetraynoic acid with tritium gas on Lindlar catalyst. Unlabelled oleic, linoleic, linolenic and arachidonic acids were obtained from the Hormel Institute. Authentic samples of prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\beta}$, A_1 and B_1, and sheep seminal vesicles were supplied by Dr. J. E. Pike, The Upjohn Company, Kalamazoo, Michigan. Soybean lipoxidase (50,000 U/mg) was obtained from Gallard-Schlesinger Chemical Manufacturing Corp., N.Y.

Enzymic reactions. (a) Soybean lipoxidase. Incubations with soybean lipoxidase were conducted at 24°C in 3 ml of 0.1 M borate buffer, pH 9, containing 2 μg of enzyme and 40 μg of linoleic acid substrate. Rate of increase in absorption at 234 mμ was followed using a Perkin-Elmer UV spectrophotometer Model 202, fitted with a time-drive drum and a temperature-controlled cell holder. Inhibitors were added as solutions in borate buffer.

(b) Prostaglandin synthetase. Incubations were carried out under O_2 at room temperature with 5 mg of an acetone powder preparation of the glands in 2 ml of 50 mM phosphate buffer, pH 7.4, containing 20 mM EDTA, 56 μg of reduced glutathione, 0.0005% di-t-butyl-p-cresol, and 25 μg of tritium-labelled arachidonic acid as substrate. Methylene chloride extracts of acidified aliquots were applied to 7 mm lanes in a 250 μ layer of silica gel G

and the chromatograms were developed to 19 cm with hexane:diethyl ether: acetic acid (25:75:1), air-dried ten minutes and then redeveloped to 10 cm in ethyl acetate:acetic acid (100:1).

Autoradiograms of the chromatograms were scanned with a photodensitometer (Photovolt Corp., Model 530) to determine the relative intensities of the spots produced by the substrate (R_F 0.8), the hydroxyacid by-products (R_F 0.65), and prostaglandin E_2 (R_F 0.15). The identity of PGE_2 was confirmed by treatment of the recovered material with alkali and observation of the UV spectrum and chromatographic properties of the PGB_2 formed. Authentic samples of prostaglandins and fatty acids were used as chromatographic reference materials.

Incubations of the seminal vesicle preparation with labelled oleic, linoleic and linolenic acids were also conducted.

The acetylenic inhibitor was added to incubation mixtures as a solution in borate buffer immediately before addition of substrate.

RESULTS

(a) Soybean lipoxidase. In the absence of inhibitors the conversion of linoleic acid to the UV-absorbing hydroperoxide proceeded to completion, as calculated from the molar absorbtivity of the product (28,000). When 25 μg of 5,8,11,14-eicosatetraynoic acid (4×10^{-5}M) was included in the incubation mixture the initial rate was reduced and the reaction ceased after about 40% conversion. A similar reduction in the initial rate was produced by 250 μg of oleic acid, a competitive inhibitor, but the reaction proceeded eventually to completion (Fig. 1). This indication of a slow but irreversible attack on the enzyme by the acetylenic acid was confirmed by preincubation of the lipoxidase with low concentrations of the acetylenic inhibitor. Complete inactivation of the enzyme was produced by 15 minutes preincubation with 1×10^{-6} M acetylenic acid. Four hours preincubation with 1×10^{-7} M acetylenic acid also produced complete inactivation. Stearolic acid produced inhibition similar to that by oleic acid.

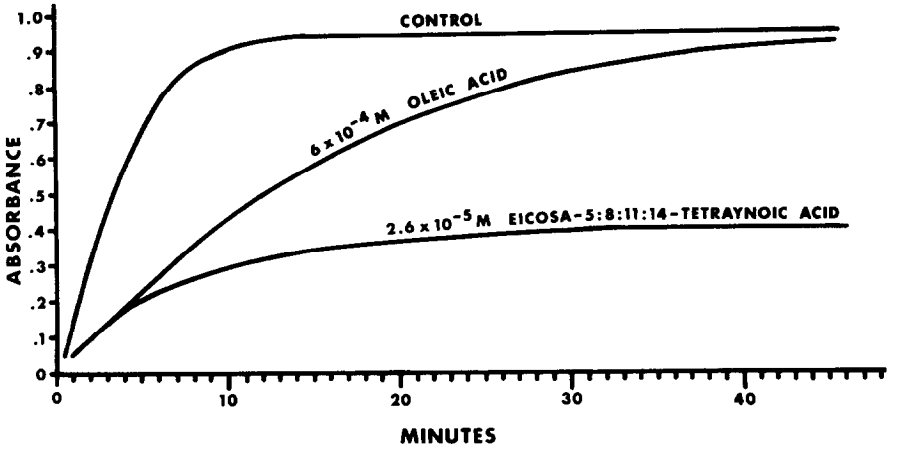

Fig. 1 Comparison of the rate curves for the conversion of linoleic acid to hydroperoxide by soybean lipoxidase with no inhibitor (control), with 6 x 10^{-4} M oleic acid, and with 2.6 x 10^{-5} M 5,8,11,14-eicosatetraynoic acid.

(b) Prostaglandin synthetase. In incubations of labelled linoleic or linolenic acid with the acetone powder preparation of seminal vesicles, the acids were completely converted to hydroxyacids in about two hours. When the incubation mixtures contained 4 x 10^{-5} M acetylenic acid the oxidation of the polyethylenic acids was totally inhibited. Oleic acid-1-^{14}C and 5,8,11,14-eicosatetraynoic acid-1-^{14}C were each recovered unchanged from incubations with the seminal vesicle preparation. The hydroxylation of linoleic acid was not prevented by 10^{-3} M cyanide or by conducting the incubation at pH 9.

Under the conditions employed the acetone powder preparation completely converted the tritiated arachidonic acid to PGE_2 (87%) and other products (13%) in 50 minutes. In the presence of 5 x 10^{-5} M unlabelled linoleic or linolenic acid, the initial rate of prostaglandin synthesis was reduced but proceeded eventually to completion. It is therefore concluded that although the polyethylenic acids are inhibitors of prostaglandin synthesis their effect does not proceed to total inhibition as is the case with the acetylenic acid (Fig. 2).

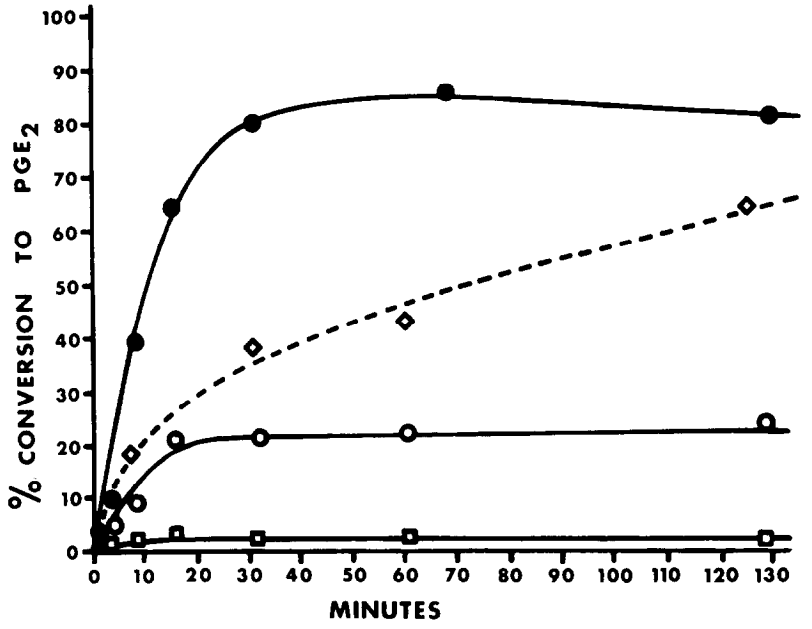

Fig. 2 Rate curves for conversion of 25 μg of ^{3}H-arachidonic acid to PGE_2 with: ●——● no inhibitor; ○——○ 4×10^{-6}M; and □——□ 4×10^{-5}M 5,8,11,14-eicosatetraynoic acid; ◇- - -◇ 4×10^{-5}M linoleic acid.

In the presence of 2.5 μg of the acetylenic inhibitor (4×10^{-6} M) conversion of ^{3}H-arachidonic acid commenced at a somewhat reduced rate, and ceased at 20% conversion to PGE_2, with 5% of other products. With 4×10^{-5} M inhibitor, conversion to PGE_2 was limited to 2.5%, with 5% of other products. Addition of further labelled substrate to these inhibited reaction mixtures did not induce the formation of additional products, but further conversion was obtained on addition of a large amount of fresh enzyme preparation.

CONCLUSIONS

The rate curves obtained for soybean lipoxidase and prostaglandin synthetase inhibited by the acetylenic acid are indicative of a slow but irreversible reaction of the inhibitor with the enzymes. Bloch has concluded that the inhibition of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-N-acetyl-cysteamine occurs by conversion of the acetylenic inhibitor to an allene, the 2,3-decadienoyl thioester, which then reacts irreversibly with

a histidine moiety at the active site of the enzyme (7). Such an abstraction of a proton from the α-carbon atom is postulated as an essential feature in the conversion of the normal substrates. It is therefore of interest that in each of the enzymic reactions we have studied, abstraction of a proton from a methylene group adjacent to an ethylenic bond of the substrate is involved. Similar action on acetylenic compounds analogous to the normal substrates might therefore be expected to yield allenes in each case. Such a mechanism for the inhibitions would help to explain the slow rate of the inactivations observed. It may also mean that while competitive inhibition by acetylenic compounds may be observed over a wide range of reactions involving ethylenic substrates, irreversible inhibition may be confined to those instances where an allene can be produced.

The substrate specificity, the inhibition by the acetylenic acid and lack of inhibition by cyanide or alkaline conditions indicate that the oxidation of linoleic and linolenic acids by the seminal vesicle preparation is effected by a lipoxidase rather than by the non-specific catalysis of heme compounds. It seems probable that these hydroxylations are effected by the system responsible for the initial attack on arachidonic acid in its conversion to prostaglandins.

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