Acetylene-Allene Acyl Thioester Isomerase from Hog Liver

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Summary: A hog liver enzyme that catalyzes the conversion of 3-decynoyl-N-acetylcysteamine to 2, 3 decadienoyl-N-acetylcysteamine has been purified to homogeneity. This enzyme also catalyzes the isomerization of 3-cis or 3-trans enoyl CoA derivatives. Its properties are similar to those of the isomerase required for the β-oxidation of oleic acid.

Introduction: Certain acetylenic substrate analogs have been found to inactivate various enzymes irreversibly (1-3). For example, β-hydroxydecanoyl thioester dehydrase isomerizes 3-decynoyl-NAC* to 2,3 decadienoyl-NAC, a highly reactive allene, which rapidly combines with an active site residue thereby inactivating the enzyme (4). However, the conversion of an acetylenic to an allenic compound by an enzyme has so far been only inferred but not demonstrated by direct isolation of the isomerization product. We have now purified an enzyme which catalyzes the conversion of 3-acetylenic fatty acyl thioesters to 2,3 dienoyl fatty acyl thioesters, without inactivation of the enzyme. In this instance, the allenic thioester product accumulates. The homogeneous acetylene-allene isomerase also isomerizes Δ^3-cis or Δ^3-trans acyl CoA compounds to Δ^2-trans acyl CoA derivatives, the same reactions that are catalyzed by an enzyme first described by Stoffel (5). The latter enzyme is believed to be essential for the β-oxidation of oleic acid by liver mitochondria (6).

Materials and Methods: All enzyme purification steps, to be described elsewhere, were carried out at 4°. Hog liver was obtained from the Corsair Packing Co., Pawtucket, R.I. Electrofocusing was performed on an LKB 110 ml column using 1% pH 3-10 ampholytes. Gels were scanned at 550 nm with a Gilford 240 using the 10 cm gel scanning attachment. Equilibrium centrifugation was done according to Yphantis (7) in a Spinco model E ultracentrifuge equipped with photoelectric scanning optics. The partial specific volume of the enzyme was determined according to Edelstein and Schachman (8). For characterization of the allenic thioester, spectral analyses were performed with a Varian T-60 NMR spectrometer, a Cary 118 spectrophotometer and a Perkin-Elmer Infracord spectrometer. Mass spectra were obtained with an Associated Electrical Industries MS-9 double focusing instrument. Optical rotations were measured with a Perkin-Elmer 141 polarimeter.

Enzyme Assay: The enzyme was assayed spectrophotometrically at 25° on a Cary 118 or

*The abbreviation used is NAC, N-acetylcysteamine.

Gilford 240 by measuring an increase in absorbance at 263 nm with time for both allenic and Δ^2-trans acyl thioester products. For 2, 3 dienoyl acyl thioester, λ_{max}=263 nm (ε =4000) and for Δ^2-trans acyl thioesters, λ_{max}=263 nm (ε =6700). Substrate concentrations varied from 1×10^{-3} to 5×10^{-5} M depending on the chain length, thioester moiety and unsaturation of the compound used. The assay buffer was 0.05M potassium phosphate buffer, pH 7.4. Between 0.1-1.0 µg of enzyme was routinely used to start the reaction. 3-Acetylenic and Δ^3-olefinic thioesters were synthesized by a modification of the mixed anhydride procedure (9).

Inhibition Experiments: 3-Decynoyl-NAC (1×10^{-4}M) or 2, 3 decadienoyl-NAC (1×10^{-4}M) were preincubated with 1×10^{-6} M enzyme in 0.05 M potassium phosphate at pH 7.4 for various lengths of time. After dialysis to remove the potential inhibitors, the enzyme was assayed with 1×10^{-4} M 3-decynoyl-NAC or 7×10^{-5} M 3-cis-decenoyl CoA as substrates.

Isolation of the Allenic Product: 3-Decynoyl-NAC (35 mg) in 2.26 ml of acetonitrile was dissolved in 200 ml of 0.05 M potassium phosphate buffer, pH 7.4 at 25°, 40 µg of the isomerase (pI 7.01, see below) in the same buffer was added and the reaction was allowed to proceed to equilibrium as determined spectrophotometrically. The solution was then saturated with sodium chloride, extracted three times with diethyl ether and the extract dried over magnesium sulfate. Filtration and evaporation of solvent yielded 33 mg of a yellow oil. This oil was applied to a Unisil column (1.5 ml) and eluted with methylene chloride: methanol (200: 1 v/v). 25 mg of a clear viscous oil was obtained after analysis of fractions by TLC on silica gel and UV spectroscopy.

Results and Discussion: Fractionation of hog liver extracts by procedures to be described separately yielded 150-500 fold purified enzyme shown to be homogeneous by polyacrylamide gel electrophoresis at pH 8.7 (10), SDS gel electrophoresis (11) and equilibrium ultracentrifugation (Fig. 1). Electrofocusing separated the enzyme into four differently charged forms, (pI = 6.57, 6.84, 7.01 and 7.27) which had the same subunit molecular weight (45000 ± 1000) as determined by SDS gel electrophoresis. Equilibrium ultracentrifugation of the pI 7.01 enzyme indicated a molecular weight of 91000 ± 2000 suggesting a dimeric

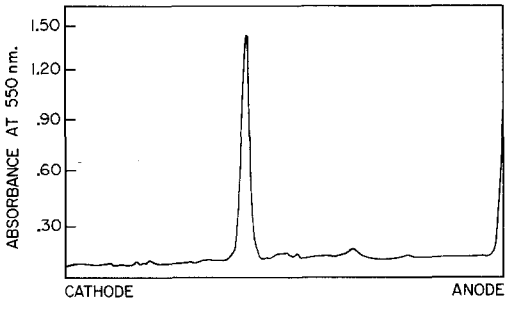

Fig. 1: SDS gel (7.5%) of purified isomerase, pI 7.01 form. 8.5 µg of SDS treated enzyme was subjected to electrophoresis, stained with .25% Coomassie Brilliant Blue R-250 and scanned at 550 nm in a Gilford 240 spectrophotometer equipped with a linear transport attachment.

structure for the native enzyme. All forms of the isomerase had coincident acetylene and olefin isomerization activity through the last four purification steps.

Failure of 3-Decynoyl and 2,3 Decadienoyl Thioesters to Inhibit Isomerase: Preincubation of isomerase with 3-decynoyl-NAC or 2,3-decadienoyl-NAC for various lengths of time as described in methods failed to diminish enzyme activity as judged by subsequent assay with 3-cis-decenoyl-CoA or 3-decynoyl-NAC. Likewise, the pantetheine and coenzyme A thioesters of 3-decynoic acid were not inhibitory, but were active substrates. Since model reactions indicate that a variety of nucleophiles undergo Michael addition with 2,3 dienoyl acyl thioesters (12), it is possible that weak binding of the allenic product and/or orientation effects prevent covalent reaction with the liver isomerase.

Characterization of Allenic Acyl Thioester Product: A large scale incubation of 3-decynoyl-NAC with the pI 7.01 enzyme yielded a pure product having properties identical with synthetic 2,3 decadienoyl-NAC (12). Other products were not detected by TLC or ultraviolet spectroscopy. The compound's characteristic spectral properties are: ultraviolet: λ_{max}=263 nm (ε=3500) (in ethanol); infrared: ν_{max}=5.12 μ (C=C=C stretch); NMR ($CDCl_3$): δ 5.9-6.0 (2H multiplet) for allenic protons; mass spectrum (70 ev): m/e = 269 parent ion. The allenic product was optically active $[\alpha]_D^{25}$ = +55° (c = 13.3 mg/ml in CH_2Cl_2) and irreversibly inhibited β-hydroxydecanoyl thioester dehydrase at a faster rate than racemic 2,3 decadienoyl-NAC. Only dextrarotatory 2,3 decadienoic acid had previously been shown to be an inhibitor of β- hydroxydecanoyl thioester dehydrase (13).

Table 1. Comparison of V_{max} and K_m for various C_{10}-acetylenic thioesters and 3-cis-decenoyl-NAC.

Substrate	$V_{max} \times 10^2$, μmoles/min	$K_m \times 10^4$, M
3-Decynoyl-NAC	5.0	2.1
3-Decynoyl-pantetheine	2.5	1.7
3-Decynoyl-CoA	1.2	1.0
3-cis-Decenoyl-NAC	0	

The isomerase (.2 μg) used was chromatographically homogeneous but had not been subjected to electrofocusing. V_{max} and K_m were determined from double reciprocal plots.

Table 2. Comparison of k_{cat} and K_m for C_6-olefinic and C_6-acetylenic derivatives.

Substrate	k_{cat}, sec^{-1}	$K_m \times 10^5$, M
3-trans-Hexenoyl-CoA	10.0	9.2
3-cis-Hexenoyl-CoA	17.8	1.4
3-Hexynoyl-CoA	24.2	4.3

The pI 7.01 form of the isomerase was used in each assay. k_{cat} and K_m were determined from double reciprocal plots.

Substrate Specificity and Mechanism: The multiple forms of the isomerase exhibited a broad chain length specificity (C_6-C_{12}) for both 3-acetylenic and 3-cis or trans-olefinic acyl thioester substrates. While 3-acetylenic thioesters of coenzyme A, pantetheine and N-acetylcysteamine were good substrates, the corresponding olefinic-NAC derivatives were not. In fact, 3-cis-decenoyl-NAC was not a substrate and inhibited acetylene-allene isomerase activity competitively ($K_i = 1 \times 10^{-4}$M) (Table 1). In comparison with olefinic substrates with the same chain length and thioester moiety (CoASH), the acetylene has a greater k_{cat} and similar K_m making it the most active substrate for the enzyme (Table 2). Finally, the isomerization of 2,2 di-deutero-3-decynoyl-NAC to the corresponding allene proceeded with a deuterium isotope effect of $k_H/k_D = 3.5$ at 25°. This suggests that the rate limiting step in the isomerase reaction is abstraction of a proton at C_α.

If the enzyme described here should prove to be identical with the isomerase isolated by Stoffel et al., an enzyme believed to function in the β-oxidation of oleic acid (5,6), then it would be remarkable that this enzyme prefers an unnatural substrate over a normal metabolite. In summary, it is shown that an allenic product, in spite of its great susceptibility to nucleophilic attack, will not necessarily inactivate the enzyme that generates it. In this respect, the properties of the enzyme described here differ strikingly from those of β-hydroxydecanoyl thioester dehydrase (4).

Acknowledgments

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