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Allenic Thioester Hydration Activity of Beef Liver Crotonase[1]

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The conversion of 3-decynoyl-*N*-acetylcysteamine to 2,3-decadienoyl-*N*-acetylcysteamine catalyzed by a homogeneous mammalian liver enzyme has recently been described (F. M. Miesowicz and K. E. Bloch, *Biochem. Biophys. Res. Commun.* **65**, 331 (1975). The allenic thioester product, however, does not accumulate in crude liver extracts suggesting that it undergoes further enzymatic transformation. Evidence is presented here that crotonase (enoyl Coenzyme A hydratase) catalyzes the hydration of allenic thioesters to the corresponding β-keto thioesters which are normal intermediates in the β-oxidation of fatty acids.

INTRODUCTION

Acetylenes, allenes, and polyacetylenes occur naturally in higher plants and fungi (*2*). The biosynthesis of natural acetylenes appears to involve a variant of fatty acid biosynthesis from the ubiquitous intermediates, malonyl-CoA and acetyl-CoA (*2*). It is therefore possible that acetylenic acids are also catabolized by the usual β-oxidation pathway. Partial β-oxidation of any long-chain molecule having an acetylenic bond at an odd numbered carbon atom would eventually yield a 3-acetylenic thioester. At this stage 3-acetylenic thioesters may be metabolized further by isomerization, hydration and thiolytic cleavage, i.e., by the same sequence of enzymatic reactions which operate in the β-oxidation of the more common saturated and olefinic fatty acids (*3*).

Recently, the isolation and purification of an enzyme which catalyzes the conversion of 3-acetylenic fatty acyl thioesters to 2,3-dienoyl fatty acyl thioesters was reported (*1*). This enzyme also isomerizes Δ^3-*cis*- or *trans*-acyl CoA derivatives to the corresponding Δ^2-*trans*-acyl CoA thioesters. If, analogously, crotonase mediated the hydration of the 2,3-dienoyl fatty acyl thioesters in addition to α,β-unsaturated acyl thioesters, β-keto fatty acyl thioesters would be formed directly. We present evidence here that beef liver crotonase does indeed catalyze the hydration of 2,3-decadienoyl-NAC.[3]

MATERIALS AND METHODS

Crystalline bovine crotonase (enoyl Coenzyme A hydratase, EC 4.2.1.17) was prepared as reported by Stern et al. (*4*) with the minor modifications described by Steinman and Hill (*5*). Beef liver was obtained from Acme Boneless Beef Co., Sheldon,

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[3] Abbreviation used: NAC, *N*-acetylcysteamine.

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Mass. Crotonyl-CoA was purchased from P–L Biochemicals, Inc. and *p*-chloromercuribenzoate from Calbiochem. 2,3-Decadienoyl-NAC was prepared by isomerization of 3-decynoyl-NAC with sodium ethoxide in ethanol as described (*6*). Ultraviolet and infrared spectra were recorded with a Cary 118 spectrophotometer and Perkin–Elmer 457 A, respectively.

Enzyme Assay

Enzyme activity was determined spectrophotometrically on a Cary 118 or Gilford 240 instrument. Crotonase was assayed as described (*4*, *5*). Allene hydrase activity was followed by the decrease in absorbance at 263 nm with 3×10^{-4} *M* 2,3-decadienoyl-NAC ($\lambda_{max} = 263$ nm; $\varepsilon = 4000$) in 0.05 *M* potassium phosphate–1% *p*-dioxane (pH 7.4).

For enzymatic assays the 2,3-decadienoyl-NAC concentrations had to be kept near the K_m. At higher concentrations this substrate is insoluble in phosphate buffer. Addition of about 20 μg of crystalline crotonase initiated the reaction.

Inhibition Experiments

2,3-Decadienoyl-NAC (3×10^{-4} *M*) was preincubated for 1 hr with 2×10^{-7} *M* crotonase in 0.05 *M* potassium phosphate buffer–1% *p*-dioxane (pH 7.4) at 0°C. Enzyme activity was then measured with crotonyl-CoA as the substrate and compared with an appropriate control (containing no allenic thioester). In a separate experiment, crotonase was preincubated for 10 min at 0°C with the irreversible inhibitor *p*-chloromercuribenzoate (PCMB) ($7–9 \times 10^{-4}$ *M*) (*7*).

Product Analysis

Beef liver crotonase (2 mg) was added to 3.6×10^{-4} *M* 2,3-decadienoyl-NAC in 200 ml of 0.05 *M* potassium phosphate buffer–1% acetonitrile (pH 7.5). 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 was dried over sodium sulfate. Filtration and evaporation of solvent yielded a yellow oil which was applied to a precoated silica gel plate (E. Merck Darmstadt). The plate was developed with methanol:hexane:ether (2:5:5). Two bands were obtained (R_f, 0.66 and 0.25). Both bands were scraped off, dissolved in ether, and dried over sodium sulfate. The resulting oils were subjected to infrared and ultraviolet analysis. One band ($R_f = 0.66$) corresponded to authentic unreacted allenic-NAC and the other ($R_f = 0.25$) to reaction product.

RESULTS AND DISCUSSION

Evidence for the Involvement of Crotonase in Allene Thioester Hydration

Enzyme preparations of increasing purity were assayed in parallel with crotonyl-CoA and 2,3-decadienoyl-NAC. The two activities copurified (Table 1) suggesting that crotonase metabolizes both α,β-unsaturated and 2,3-dienoyl acyl thioesters. The much lower reactivity observed for the allenic NAC derivative (Table 1) is not unexpected, since crotonase is quite selective with respect to the thiol ester moiety. Crotonyl-NAC

TABLE 1

COMPARISON OF ENZYME ACTIVITY FOR CROTONYL-CoA AND 2,3-DECADIENOYL-NAC AT VARIOUS STAGES OF CROTONASE PURIFICATION (4, 5)

Step	Specific activity[a] of crotonyl-CoA I	Specific activity[a] of 2,3-decadienoyl-NAC II	Ratio of I/II
Crude homogenate	2.2	0.001	2200
Acid–heat step	7.4	0.003	2470
Acetone precipitation	14.7	0.007	2100
Ammonium sulfate fractionation	76.4	0.028	2730
DEAE-52 eluate	95.5	0.030	3180
Crystallization	922.7	0.173	5330

[a] Specific activity is defined as μmoles per minute per milligram of protein at 25°C. Protein was determined by the method of Warburg and Christian (*8*), except for the crystallized enzyme where $E_{1cm}^{0.1\%}$ (280 nm) = 0.576 (*9*) was used.

thioester is not hydrated (*10*). Also, the V_{max} for olefinic CoA thioesters decreases with increasing chain length of the acyl moiety (C_4 to C_{16}) (*11*).

The value for the ratio of activities after the final 12fold purification is not necessarily inconsistent with the proposed catalysis of both reactions by a single enzyme. One explanation may be that since the activities at all times were measured at vastly different protein concentrations (about one thousandfold). Possibly, in the final purification step (Table 1) the crotonase assay falls into a nonlinear region of the protein versus activity relationship, whereas the allene activity does not. The result of this type of kinetic situation would be the high crotonase/allene activity ratio observed for the final step.

Since the crystalline preparation of crotonase was homogenous as judged by SDS gel electrophoresis ($R_f = 0.53$, subunit molecular weight = 26,200 ± 1000) with 20 μg of protein (*12*), and polyacrylamide gel electrophoresis at pH 8.7 (*13*) with 50 μg of protein, the ability to act on allenic thioester is clearly an intrinsic property of the enzyme. Finally *p*-chloromercuribenzoate (PCMB) inhibited crotonyl-CoA and allenic-NAC activities equally (Table 2).

TABLE 2

INHIBITION OF ENZYME ACTIVITY BY PCMB WITH 2,3-DECADIENOYL-NAC AND CROTONYL-CoA SUBSTRATES[a]

Trial	Control	Activity remaining (%)	
		Crotonyl-CoA (PCMB inhibited)	2,3-Decadienoyl-NAC (PCMB inhibited)
1	100	33.5	37.6
2	100	20.7	18.3

[a] For experimental details see Materials and Methods.

Characterization of Hydration Product Formed from Allenic Thioester

If crotonase catalyzes the conversion of 2,3-decadienoyl-NAC and crotonyl-CoA by the same mechanism, i.e., by direct hydration, the product expected from the allenic thioester is the enoyl form of β-keto decanoyl-NAC. The product formed in the crotonase–allenic-NAC reaction does indeed exhibit the peak at 303 nm characteristic for enols of thioesters (*14*, *15*). Moreover, the ir spectrum of the reaction product showed a new carbonyl band at 1725 cm^{-1}, the same frequency found for the β-keto group of β-keto palmitoyl-NAC (*16*).

Crotonase preincubated with a 1500-fold molar excess of 2,3-decadienoyl-NAC had not lost any activity when assayed with crotonyl-CoA. Thus, the allene serves as a crotonase substrate without irreversibly inhibiting the "normal" crotonase activity of the enzyme. The K_{eq} for the crotonase–allenic-NAC reaction was 3.6 and the K_m for 2,3-decadienoyl-NAC was 3.3×10^{-4} *M*.

As the present experiments show, the substrate specificity of crotonase is sufficiently broad for catalyzing the hydration of allenic as well as of olefinic thioesters. Also, as previously reported, the enzyme that isomerizes acetylenic to allenic thioesters is apparently identical (*1*) with the 3-*cis* → 2-*trans*-olefin isomerase required for the metabolism of Δ^9-olefinic acids. Therefore it appears that certain allenes and acetylenes can be handled by the enzymes of the normal β-oxidation pathway. Possibly, only the recently isolated acetylene–allene acyl thioester isomerase (*1*) and the usual β-oxidation enzymes (*3*) are required for the degradation of acetylenes and allenes in mammalian systems.

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