Inactivation of Pig Heart Thiolase by 3-Butynoyl Coenzyme A, 3-Pentynoyl Coenzyme A, and 4-Bromocrotonyl Coenzyme A†

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ABSTRACT: The unsaturated thio esters, 3-pentynoyl-, 3-butynoyl-, and 4-bromocrotonyl-CoA, have been investigated as affinity labels of pig heart thiolase and their properties compared with a known affinity label, 2-bromoacetyl-CoA. Incubation of thiolase with low concentrations of all of these compounds produced a rapid and irreversible loss of enzyme activity. Inactivation by 3-pentynoyl- and 4-bromocrotonyl-CoA obeys first-order kinetics. Acetoacetyl-CoA affords excellent protection against inactivation by all acyl-CoA esters tested whereas acetyl-CoA protects only against inactivation by 4-bromocrotonyl- and 2-bromoacetyl-CoA. In contrast to the 3-acetylenic CoA esters, 2-butynoyl- and 4-pentynoyl-CoA are relatively weak inactivating agents. This observation combined with studies on the pH dependence of inactivation and the low reactivity of 3-pentynoylglutathione with free thiol in a model system suggests that the 3-acetylenic esters may not directly inactivate thiolase. Rather it is proposed that a basic group on the enzyme may catalyze isomerization to the highly reactive 2,3-dienoyl thio ester which is the species responsible for inactivation. The observation that $NaBH_4$ inactivates thiolase in the presence of acetoacetyl- or acetyl-CoA is consistent with the presence of an amine at the active site and suggests that the catalytic reaction proceeds through a ketimine intermediate.

Thiolase catalyzes the thiolytic cleavage of the carbon chain of β-ketoacyl-CoA esters according to the general reaction

$$RCH_2\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}SCoA + CoASH \rightleftharpoons RCH_2\overset{O}{\overset{\|}{C}}SCoA + CH_3\overset{O}{\overset{\|}{C}}SCoA$$

The following general mechanism for thiolase has been proposed (Lynen, 1953; Gehring and Lynen, 1972)

$$RCH_2\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}SCoA \xrightleftharpoons[1]{EnzSH\ AcCoA} RCH_2\overset{O}{\overset{\|}{C}}SEnz \xrightleftharpoons[2]{CoASH\ EnzSH} RCH_2\overset{O}{\overset{\|}{C}}SCoA$$

Evidence in favor of this mechanism involves: (1) the fact that the enzyme possesses an essential thiol (Lynen, 1953; Chase and Tubbs, 1966; Gehring *et al.*, 1968); (2) the enzyme can catalyze an exchange between acetyl-CoA and acetoacetyl-CoA (reaction 1; Gehring *et al.*, 1968); (3) acetyl-CoA covalently labels thiolase according to reaction 2 (Gehring *et al.*, 1968). Furthermore, kinetic studies have confirmed that a covalent enzyme–substrate intermediate is formed prior to thiolytic cleavage by CoASH (Goldman, 1954).

The only affinity labels so far described for thiolase are 2-bromoacetyl-CoA (Chase and Tubbs, 1966) and 2-bromoacetylpantetheine (Gehring *et al.*, 1968) which covalently label the essential thiol and are obviously very useful in studying reaction 2. We felt that in order to study reaction 1 further, it would be necessary to develop affinity labels more closely resembling the substrate (acetoacetyl-CoA) rather than the product (acetyl-CoA). Toward this end, we have synthesized 4-bromocrotonyl-, 3-butynoyl-, and 3-pentynoyl-CoA. All of these agents irreversibly inactivate thiolase at low concentrations. The data for the inactivation of thiolase by 3-acetylenic acyl-CoA esters can be rationalized by proposing that thiolase contains a basic group capable of isomerizing the 3-acetylene to the 2,3-diene and the 2,3-dienoyl-CoA ester is subject to nucleophilic attack by the enzyme.

Experimental Section

2-Butyn-1-ol was obtained from Chemical Procurement Laboratories Inc., College Point, N. Y. 3-Pentyn-1-ol and 4-pentyn-1-ol were obtained from K & K Laboratories Inc., Plainview, N. Y. D-Pantethine was obtained from Sigma Chemical Co., St. Louis, Mo., and CoASH from P-L Biochemicals Inc., Milwaukee, Wisc. All other compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis.

Carboxylic Acids. 2-Butynoic, 3-butynoic, 3-pentynoic, and 4-pentynoic acids were prepared by oxidation of the corresponding alcohols (Heilbron *et al.*, 1949). The acids were initially obtained as oils; however crystallization from petroleum ether (bp 63–75°) was facilitated by treating an ethereal solution of the crude acid with activated charcoal. 2,3-Butadienoic acid was obtained in 40% yield by isomerization of 3-butynoic acid (250 mg) in 10 ml of 18% (w/v) potassium carbonate at 40° for 3 hr (Eglington *et al.*, 1954). The product was identified by its characteristic ir absorption at 5.1 μ. 4-Bromocrotonic acid was prepared by refluxing crotonic acid with *N*-bromosuccinimide in the presence of benzoyl peroxide (Bradshaw *et al.*, 1969). Structures of the acids were confirmed by their ir and nmr spectra.

Acyl Chlorides. The chlorides of 4-bromocrotonic acid, 2-

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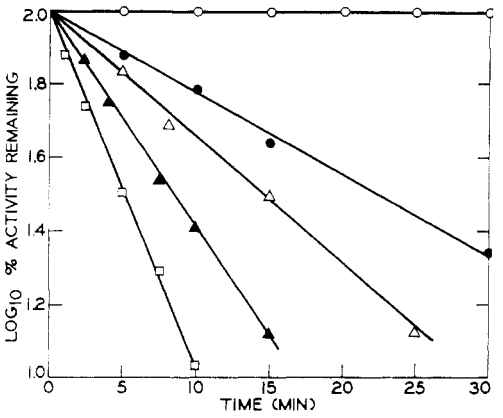


FIGURE 1: Inactivation of thiolase by 3-pentynoyl-CoA. Thiolase was inactivated with the following concentrations of 3-pentynoyl-CoA: (○) none; (●) 4 μM; (△) 8 μM; (▲) 16 μM; (□) 32 μM.

butynoic acid, 3-butynoic acid, 2,3-butadienoic acid, 3-pentynoic acid, and 4-pentynoic acid were prepared by reaction of 50 mg of acid with 1 ml of redistilled oxalyl chloride. The reaction mixture was heated at 40–50° for 30 min and excess oxalyl chloride was removed with a nitrogen stream. Anhydrous ether (1 ml) was added and subsequently removed in a stream of nitrogen. This procedure was repeated 2–3 times to ensure complete removal of oxalyl chloride. The residual acyl chloride was finally dissolved in 0.5 ml of anhydrous ether and used immediately for the acylation of thiols.

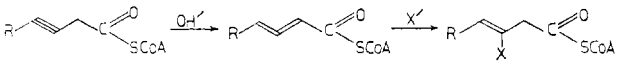
Thiol Esters. Acyl esters of CoASH, pantetheine, and glutathione with 2-butynoic, 3-butynoic, 2,3-butadienoic, 3-pentynoic, 4-pentynoic, or 4-bromocrotonic acid were prepared by reaction of thiol with excess acyl chloride. Acylation was performed at 0° in 0.1 M $KHCO_3$. The ethereal solution of acyl chloride and bicarbonate solution of thiol were mixed by a nitrogen stream and the pH was maintained at 7.5–8.0 by addition of 1 M $KHCO_3$. Thiol disappearance was followed using the nitroprusside reagent of Toennies and Kolb (1951). When acylation was complete the reaction mixture was taken to pH 2 with 1 M HCl, extracted 6–8 times with an equal volume of ether and excess ether was removed by a nitrogen stream. The solution of thiol ester was finally brought to pH 6 with 0.1 M $KHCO_3$ and stored at −20°.

Bromoacetyl-CoA and bromoacetylglutathione were prepared by transhaloacetylation (Chase and Tubbs, 1969).

N-Acetylcysteamine esters of 4-bromocrotonic, 3-butynoic, and 3-pentynoic acids were prepared by reaction of excess *N*-acetylcysteamine with acyl chloride. The acyl chloride (2 mmoles) in anhydrous ether (4 ml) was added dropwise with stirring to 5 mmol of *N*-acetylcysteamine in 0.1 M $KHCO_3$ (10 ml) under a stream of nitrogen. The pH was maintained at 7.5–8.0 with 0.1 M $KHCO_3$. After 15–20 min, acylation of thiol was complete and the reaction mixture was acidified and extracted with 3 × 50 ml of ether. The aqueous solution was evaporated under reduced pressure and taken up in a small volume of ethanol. The precipitated salts were removed by centrifugation and the supernatant solution was subjected to repeated thin-layer chromatography on silica gel using petroleum ether (63–75°)–acetone–methanol (75:25:1) as solvent. Acyl-*N*-acetylcysteamine derivatives were located by hydroxylamine–ferric chloride spray (Whittaker and Wijesundra, 1952) and by their ability to decolorize dilute aqueous potassium permanganate.

Pantetheine was obtained by the reduction of D-pantethine with $NaBH_4$. *N*-Acetylcysteamine was prepared by the method of Martin *et al.* (1959). Acyl thio ester concentrations were determined by the ferric hydroxamate method of Lipmann and Tuttle (1945).

SCHEME I



3-pentynoyl-CoA, R = CH_3; 3-butynoyl-CoA, R = H

Preparation of Thiolase. Thiolase was prepared from pig heart by the method of Stern (1955) and had a final specific activity of 47 U/mg of protein at 23° with saturating acetoacetyl-CoA and CoASH.

Inactivation of Enzyme. Thiolase (50 μg) was incubated at 23° in a final volume of 0.2 ml of 0.1 M Tris-HCl (pH 8.1), 2.5 mM $MgCl_2$, and 5 mM KCl containing other additions as required. Inactivation was commenced by the addition of a small volume of thio ester and aliquots were removed at appropriate intervals for assay of enzyme activity.

Enzyme activity was determined at 23° in 1.0 ml of 0.2 M Tris-HCl (pH 8.1), 5 mM $MgCl_2$, and 10 mM KCl containing 20 μM acetoacetyl-CoA and 50 μM CoASH. Acetoacetyl-CoA cleavage was monitored by the subsequent decrease in extinction at 303 nm (Lynen *et al.*, 1952).

Results

Inactivation of Thiolase. The usual substrates for thiolase are β-keto acyl-CoA esters. However, the β-keto functional group may not be essential for binding at the active site since we have observed that unsaturated acyl-CoA esters, such as crotonyl-CoA, competitively inhibit thiolase with respect to acetoacetyl-CoA. This prompted us to investigate the action of a number of unsaturated acyl-CoA esters that could potentially react with functional groups at the active site. 4-Bromocrotonyl-CoA was chosen because the electron-withdrawing properties of the conjugated olefin will render the bromo group very susceptible to nucleophilic displacement (see Table III). The choice of 3-acetylenic substrates was based on studies of the inactivation of decenoyl-dehydrase by 3-decynoyl-*N*-acetylcysteamine (Brock *et al.*, 1967; Helmkamp *et al.*, 1968; Helmkamp and Bloch, 1969). It has been shown (Endo *et al.*, 1970; Morisaki and Bloch, 1972) that under basic conditions, 3-acetylenic acyl thio esters can isomerize to 2,3-dienoyl thio esters which are very susceptible to nucleophilic attack by a Michael-type addition (Scheme I).

On incubation of thiolase with 4-bromocrotonyl-, 3-pentynoyl-, 3-butynoyl-, or 2-bromoacetyl-CoA, the enzyme was progressively and irreversibly inactivated. Figure 1 shows the time dependent inactivation of thiolase in the presence of 3-pentynoyl-CoA. The rate of inactivation obeyed first-order kinetics. The kinetics of inactivation of thiolase by 4-bromocrotonyl-CoA were similar. For both 3-butynoyl- and 2-bromoacetyl-CoA, the rate of inactivation was concentration dependent; however, only the initial loss of activity followed first-order kinetics. The concentration range in which these two esters are effective is 1–10 μM. Since the concentration of active site thiol is 6 μM, assuming a protomer molecular weight of 44,000 (Gehring and Riepertinger, 1968), it is apparent that these two esters are active on a molar ratio of active site to inactivating agent of unity. This could explain why the kinetics are not first order.

The complex between thiolase and the 3-acetylenic acyl-CoA esters was very stable. Thus, when 50 μg of thiolase (in 1 ml)

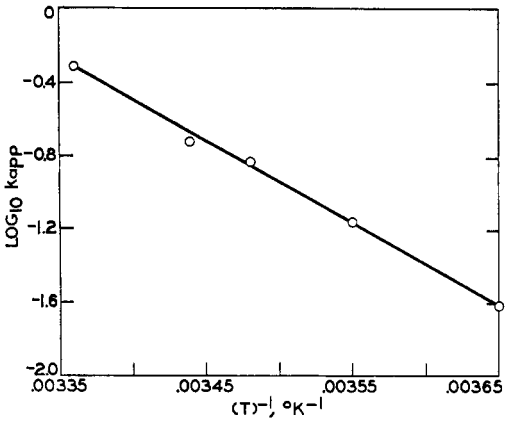


FIGURE 2: Influence of temperature on the inactivation of thiolase by 3-pentynoyl-CoA. k_{app} for inactivation was determined at various temperatures using 50 μM 3-pentynoyl-CoA.

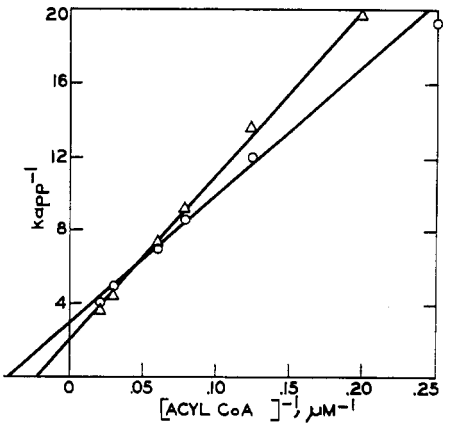


FIGURE 3: Reciprocal plot of k_{app} *vs.* acyl-CoA concentration for the inactivation of thiolase by 3-pentynoyl-CoA (○) or 4-bromocrotonyl CoA (△).

was completely inactivated by 3-pentynoyl-CoA, the enzyme could be dialyzed at 0° against 1 l. of 0.1 M potassium phosphate (pH 7), containing 0.1 mM dithiothreitol (changed every 24 hr) for 168 hr with no recovery of activity. Under identical conditions, the 4-bromocrotonyl-CoA-inactivated enzyme recovered about 15% of its original enzyme activity. Untreated enzyme lost only 10% of its original activity.

Figure 2 shows an Arrhenius plot for the effect of temperature on the inactivation of thiolase by 3-pentynoyl-CoA. From the slope of this plot the activation energy of inactivation was calculated as 20.9 kcal. Most chemical reactions that proceed at a reasonable rate and have half-lives in the order of minutes or hours usually have an activation energy of the order of 20 kcal.

For an active-site directed reagent (I), the formation of the inactive covalent complex with the enzyme (EI) is generally assumed to proceed through a noncovalent intermediate (E·I)

$$E + I \overset{K_I}{\rightleftharpoons} E\cdot I \xrightarrow{k_3} EI$$

In this situation, the apparent first-order rate constant for inactivation (k_{app}), K_I, and k_3 are related by the equation (Kitz and Wilson, 1962)

$$\frac{1}{k_{app}} = \frac{k_3}{K_I}\frac{1}{(I)} + \frac{1}{k_3}$$

In this situation, a reciprocal plot of k_{app} and the inhibitor concentration should be linear. Figure 3 shows a plot of this type for the inactivation of thiolase by 3-pentynoyl- or 4-bromocrotonyl-CoA. Both of these agents conform to the predicted result for an active-site-directed alkylating agent. For 3-pentynoyl-CoA K_I and k_3 were estimated as 2.5×10^{-5} M and 0.33 min^{-1}, respectively, and for 4-bromocrotonyl-CoA, K_I and k_3 were estimated at 1.25×10^{-5} M and 0.50 min^{-1}, respectively. The estimated values for the enzyme–inhibitor dissociation constant (K_I) for both 3-pentynoyl- and 4-bromocrotonyl-CoA are similar to the K_M for acetoacetyl-CoA (2×10^{-5} M) in the catalytic reaction.

Inactivation by 3-butynoyl-CoA does not conform to this pattern since a reciprocal plot of k_{app} *vs.* 3-butynoyl-CoA concentration is sigmoidal rather than linear. This could indicate that the inactivation by 3-butynoyl-CoA is cooperative. However, the kinetics of inactivation of thiolase by 3-butynoyl-CoA are only partially first order, which means that estimates of k_{app} are probably inaccurate.

The structural specificity for inactivation was tested by using a wide variety of derivatives (Table I). Within a given series the potency of inactivation decreases markedly in the order: acyl-CoA ester > acylpantetheine ester > acyl-*N*-acetylcysteamine ester > free acid. This is in agreement with the known specificity of thiolase for β-ketoacyl thio esters as substrates (Lynen *et al.*, 1956; Stern and Ochoa, 1956) where

TABLE I: Structural Requirements for the Inactivation of Thiolase.[a]

Compound	Concn (M)	% Inactivation 2 min	5 min	10 min
4-Bromocrotonyl-CoA	2.5×10^{-5}	30	50	72
	10^{-4}	95	100	100
4-Bromocrotonyl-pantetheine	2×10^{-4}	34	56	67
	10^{-3}	92	100	100
4-Bromocrotonyl-*N*-acetylcysteamine	2×10^{-4}	10.7	28.6	35.7
4-Bromocrotonic acid	5×10^{-3}	0	0	0
3-Butynoyl-CoA	5×10^{-6}	61	74	75
	10^{-5}	90	95	95
3-Butynoyl-*N*-acetylcysteamine	2×10^{-4}	3	6	12
3-Butynoic acid	5×10^{-3}	0	0	0
3-Pentynoyl-CoA	2.5×10^{-5}	49	69	90
	10^{-4}	100	100	100
3-Pentynoyl-pantetheine	2×10^{-4}	30	50	70
	10^{-3}	90	100	100
3-Pentynoyl-*N*-acetylcysteamine	2.5×10^{-4}	10	23.2	36
3-Pentynoic acid	5×10^{-3}	0	0	0
2-Butynoyl-CoA	5×10^{-5}	16.6	31.2	44
	10^{-4}	46.5	60	74.2
4-Pentynoyl-CoA	10^{-4}	2	12.5	25
	5×10^{-4}	36	54	64
2,3-Butadienoyl-CoA	5×10^{-6}	56	63	75
	10^{-5}	100	100	100
Crotonyl-CoA	5×10^{-4}	0	0	0

[a] Inactivation experiments were performed as described in the Methods section.

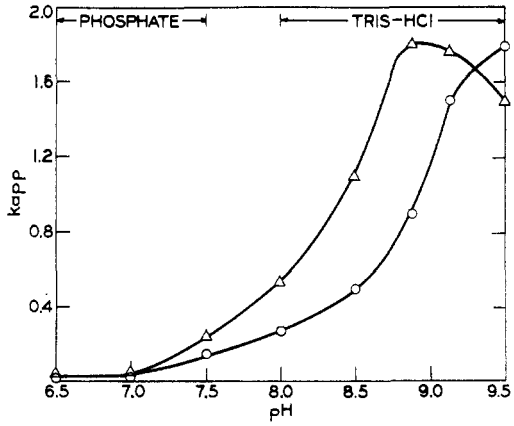


FIGURE 4: Effect of pH on the rate of inactivation of thiolase by 4-bromocrotonyl-CoA and 3-pentynoyl-CoA. k_{app} for inactivation was determined in 0.1 M buffer at the appropriate pH containing 5 mM KCl and 2.5 mM $MgCl_2$, using either 60 μM 4-bromocrotonyl-CoA (△) or 60 μM 3-pentynoyl-CoA (○) as the inactivating agent.

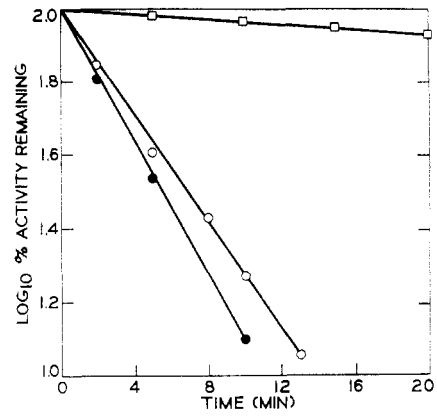


FIGURE 6: Effect of acetoacetyl- and acetyl-CoA on the inactivation of thiolase by 3-pentynoyl-CoA. The rate of inactivation of thiolase was measured under the following conditions: (○) 15 μM 3-pentynoyl-CoA; (●) 15 μM 3-pentynoyl-CoA and 300 μM acetyl-CoA; (□) 15 μM 3-pentynoyl-CoA and 100 μM acetoacetyl-CoA.

the activity as substrates decreases markedly as the structural resemblance to the CoA moiety decreases. β-Ketoacylpantetheines exhibit about the same affinity as CoA esters (Gehring *et al.*, 1968); however, their rate of reaction is markedly reduced (Stern and Ochoa, 1956). It might have been expected that 4-bromocrotonate would inactivate thiolase through nonspecific reaction with enzyme thiols. However, 4-bromocrotonate is a very poor thiol reagent (see Table III) and inactivation was obtained only with very high concentrations (>10 mM). Gehring *et al.* (1968) have reported a similar result with iodoacetic acid which was 100 times less potent than iodoacetamide.

The difference in potency between 3-pentynoyl- and 3-butynoyl-CoA can be explained by the chain-length specificity of thiolase from pig heart. Thus, for pig heart thiolase, acetoacetyl-CoA is the preferred substrate and there is a marked decrease in affinity for the C_5 compound, β-ketovaleryl-CoA (Stern and Ochoa, 1956).

The location of the acetylenic bond at the 3,4 position in the acetylenic acyl-CoA is an important factor in determining the potency of the agents as inactivating agents. Thus, 2-butynoyl- and 4-pentynoyl-CoA are much less potent than the respective 3-acetylenic acyl-CoA esters. This is consistent with the proposed mechanism of inactivation by 3-acetylenic acyl-CoA esters (Scheme I). The allenic compound, 2,3-butadienoyl-CoA, effectively inactivated thiolase and its potency was similar to 3-butynoyl-CoA. The olefin bonds in 2,3-butadienoyl-CoA must be especially susceptible to nucleophilic attack, since crotonyl-CoA which possesses a simple conjugated olefinic bond has no ability to inactivate thiolase.

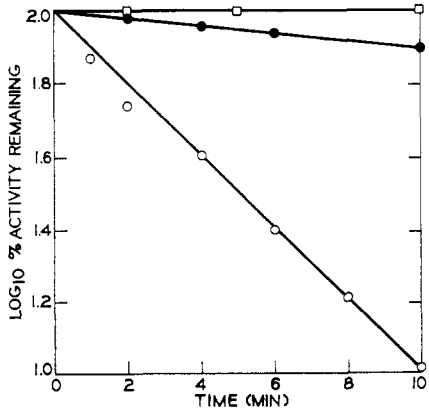


FIGURE 5: Effect of acetoacetyl- and acetyl-CoA on inactivation of thiolase by 4-bromocrotonyl-CoA. The rate of inactivation of thiolase was measured under the following conditons: (○) 25 μM 4-bromocrotonyl-CoA; (●) 25 μM 4-bromocrotonyl-CoA and 300 μM acetyl-CoA; (□) 25 μM 4-bromocrotonyl-CoA and 100 μM acetoacetyl-CoA.

pH Dependence of Inactivation. Figure 4 shows the influence of pH on k_{app} for the inactivation of thiolase by either 4-bromocrotonyl- or 3-pentynoyl-CoA. Neither agent inactivates below pH 7; however, as the pH becomes more alkaline the rate of inactivation increases. The maximum rate of inactivation with 4-bromocrotonyl-CoA occurs at approximately pH 8.9 and the pK for inactivation is estimated at 8.4. Above pH 8.9, the rate of inactivation decreases slightly. For the inactivation of thiolase by 3-pentynoyl-CoA, the pH curve is shifted toward more alkaline pH and the estimated pK for inactivation is increased to approximately 8.9. The difference between the estimated pK's for inactivation by 4-bromocrotonyl- and 3-pentynoyl-CoA suggests that a more basic group on the enzyme may be required for inactivation by 3-pentynoyl-CoA compared to 4-bromocrotonyl-CoA.

Influence of Acetoacetyl-CoA and Acetyl-CoA on Inactivation. If the agents used in the present work are inactivating thiolase by binding at the enzyme's active site then it would be expected that the presence of the normal substrate, acetoacetyl-CoA, should prevent inactivation. Figures 5 and 6 show that acetoacetyl-CoA (100 μM; 5 × K_M) affords excellent protection against inactivation by either 4-bromocrotonyl-CoA (25 μM) or 3-pentynoyl-CoA (15 μM), indicating that both of these agents are active-site-directed affinity labels. Acetoacetyl-CoA (100 μM) also gave complete protection against the rapid inactivation produced by either 5 μM 3-butynoyl-CoA or by 5 μM 2-bromoacetyl-CoA.

In contrast to the substrate protection experiment, the protection by the enzyme product, acetyl-CoA, indicates a difference between the mechanism of inactivation by bromoacyl-CoA esters and 3-acetylenic acyl-CoA esters. Thus, acetyl-CoA (300 μM) gave effective protection against inactivation by 4-bromocrotonyl-CoA (Figure 5) or 2-bromoacetyl-CoA (5 μM; not shown), whereas it afforded virtually no protection against inactivation by either 3-pentynoyl-CoA (Figure 6) or 3-butynoyl-CoA (5 μM; not shown). Indeed, in a number of cases acetyl-CoA actually enhanced the rate of inactivation of thiolase by the 3-acetylenic CoA esters.

TABLE II: Influence of Thiols on the Inactivation of Thiolase by Bromoacyl-CoA Esters and 3-Acetylenic Acyl-CoA Esters.[a]

Inactivating Agent (μM)	k_{app} (min^{-1})	k_{app} + CoASH (min^{-1})	k_{app} + DTT[b] (min^{-1})
4-Bromocrotonyl-CoA (25)	0.129	0.120	0
2-Bromoacetyl-CoA (10)	0.50	0.04	0
3-Butynoyl-CoA (10)	1.15	0.082	0
3-Pentynoyl-CoA (25)	0.128	0.01	0

[a] Thiolase (50 μg) was dissolved in 0.2 ml of 0.1 M Tris-HCl containing 5 mM KCl, 2.5 mM $MgCl_2$, and the appropriate concentration of CoASH (0.8 mM) or dithiothreitol (1 mM). The inactivation reaction was commenced by addition of the indicated concentration of acyl-CoA ester. The k_{app} for inactivation was determined in the presence or absence of thiol. [b] DTT = dithiothreitol.

TABLE III: Model Reactions with Glutathione.[a]

Alkylating Agent (mM)	Pseudo-First-Order Rate Constant (min^{-1})
2-Bromoacetic acid (10)	0.178
4-Bromocrotonic acid (10)	0.238
3-Pentynoic acid (10)	0
2-Butynoic acid (10)	0
2-Bromoacetylglutathione (2)	2.53
4-Bromocrotonylglutathione (2)	11.5
3-Pentynoylglutathione (2)	0.034
2-Butynoylglutathione (2)	0.92

[a] Glutathione (1 mM) was reacted at 24° with the appropriate alkylating agent in 1 ml of 0.1 M Tris-HCl (pH 8), containing 5 mM KCl and 2.5 mM $MgCl_2$. At various times, 0.1-ml samples were removed and the thiol content was immediately titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

Influence of Thiols on Inactivation. It has been established clearly that thiolase contains an essential thiol located at the active site (Gehring and Harris, 1968, 1970). Both 4-bromocrotonyl-CoA and 2-bromoacetyl-CoA will react readily with thiols. Similarly, if the 3-acetylenic acyl-CoA esters inactivate thiolase *via* the formation of the 2,3-diene, then a thiol could react with this species as shown in Scheme I. In the event that all of these agents inactivate thiolase by reacting with the essential enzyme thiol, then the inclusion of excess exogenous thiol should protect against inactivation. Table II shows that the inclusion of high concentrations of CoASH (0.8 mM; 16 × K_M) protects against inactivation by 2-bromoacetyl-, 3-butynonyl-, and 3-pentynoyl-CoA. This effect is also obtained with dithiothreitol, showing that protection does not require that the exogenous thiol can recognize the enzyme active site. The protection is not due to reaction of CoASH with the alkylated enzyme, since once the enzyme has been inactivated by any of these agents the addition of CoASH (0.8 mM) or dithiothreitol (1 mM) does not lead to the recovery of enzyme activity.

Dithiothreitol protected against inactivation by 4-bromocrotonyl-CoA whereas CoASH did not (Table II). However, if the conditions of the experiment were modified such that CoASH (0.8 mM) and 4-bromocrotonyl-CoA (25 μM) were allowed to react at 24° for 5 min prior to the addition of thiolase, then there was no loss of enzyme activity. Obviously under these conditions CoASH is the only available thiol with which 4-bromocrotonyl-CoA can react. Under the conditions where CoASH failed to protect, the concentration of CoASH (0.8 mM) is 130 times greater than the predicted concentration of active-site thiol (6 μM using a protomer molecular weight of 44,000; Gehring and Riepertinger, 1968). Since 4-bromocrotonyl-CoA reacts preferentially with the enzyme (as detected by the unchanged rate of inactivation) it must be concluded that the enzyme thiol is a superior nucleophile to the thiol in CoASH. Presumably, the ability of dithiothreitol to protect under conditions when CoASH fails to protect must reflect the fact that it is a better nucleophile than CoASH.

Model Reactions with Thiols. The key step in the inactivation of thiolase by the 3-acetylenic acyl-CoA esters may involve their conversion to the 2,3-diene intermediate (Scheme I). It is known that reactions of this type are catalyzed by basic conditions (Eglington *et al.*, 1954; Endo *et al.*, 1970). Since the inactivation is performed at pH 8, it is feasible that the medium is sufficiently basic to catalyze this isomerization and hence the observation that 3-acetylenic acyl-CoA esters inactivate thiolase would not be evidence for the location of a basic group at the active site.

In order to investigate this point, model reactions with the thiol of glutathione were performed employing the same buffer used in the inactivation experiments. Table III shows that, as expected, 4-bromocrotonic acid and 2-bromoacetic acid reacted with glutathione and conversion to their respective acylglutathione esters markedly enhanced their reactivity. 3-Pentynoic acid had no detectable reaction with glutathione, whereas 3-pentynoylglutathione did react; however, it is obvious that 3-pentynoylglutathione is not as reactive as 4-bromocrotonylglutathione. This is in contrast to the ability of 4-bromocrotonyl- and 3-pentynoyl-CoA to inactivate thiolase where the rate constant for inactivation (k_3) was similar for both derivatives. The low reactivity of the 3-acetylenic thio ester in the model reaction suggests that it is unlikely that it is capable of reacting directly with thiolase. Rather, it seems reasonable to propose that a basic group on the enzyme catalyzes conversion to the highly reactive 2,3-dienoyl thio ester and this is the inactivating species.

It was not possible to study the reactivity of 2,3-butadienoylglutathione since this derivative was hydrolyzed under the conditions used in the present work. However, it is known that allenic acids and their esters are subject to nucleophilic attack (Adams and Ulich, 1920; Kurtz *et al.*, 1959; Morisaki and Bloch, 1972). The enhanced reactivity of conjugated unsaturated bonds is shown by the fact that 2-butynoylglutathione reacts readily with glutathione.

Inactivation by Sodium Borohydride in the Presence of Acetoacetyl-CoA or Acetyl-CoA. A number of enzymes probably use a protonated ketimine derivative as an electron sink during carbon–carbon bond cleavage (*i.e.*, aldolase type reactions; Westheimer and Cohen, 1938; Rose *et al.*, 1965; Morse and Horecker, 1968). Kornblatt and Rudney (1971) have shown that an intermediate of this type might be involved in the catalytic mechanism of yeast thiolase since the enzyme is inactivated by $NaBH_4$ in the presence of acetoacetyl-CoA. Table IV shows the results of similar studies with heart thiolase. It is apparent that $NaBH_4$ alone has no effect on the

TABLE IV: Inactivation of Thiolase with Sodium Borohydride.[a]

Additions	% Inactivation 1 hr (24°)	24 hr (0°)	48 hr (0°)
None	0	19.3	26.7
10 mM $NaBH_4$	0	14.1	18.0
25 μM acetoacetyl-CoA	0	0	15.5
10 mM $NaBH_4$ + 25 μM acetoacetyl-CoA	39.7	74.4	92.5
25 μM acetyl-CoA	9.5	32.1	55.0
10 mM $NaBH_4$ + 25 μM acetyl-CoA	46.7	86.5	100

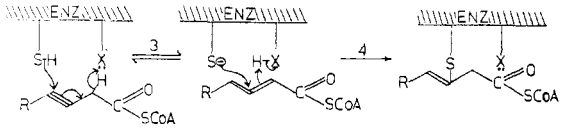
[a] Thiolase (100 μg) was incubated in 0.2 ml of 50 mM potassium phosphate (pH 6.8), containing the additions shown in the legend. After the reaction, the mixture was diluted with buffer and an aliquot was assayed for enzyme activity.

enzyme; however, in the presence of either acetoacetyl-CoA or acetyl-CoA, the enzyme was inactivated. Incubation of thiolase with acetyl-CoA alone also caused a pronounced inactivation of the enzyme; however, $NaBH_4$ markedly potentiated this effect. In contrast to most cases where $NaBH_4$ has been used to reduce protonated ketimine intermediates and where inactivation usually occurs within minutes (Horecker *et al.*, 1961), the inactivation of thiolase by $NaBH_4$ is very slow.

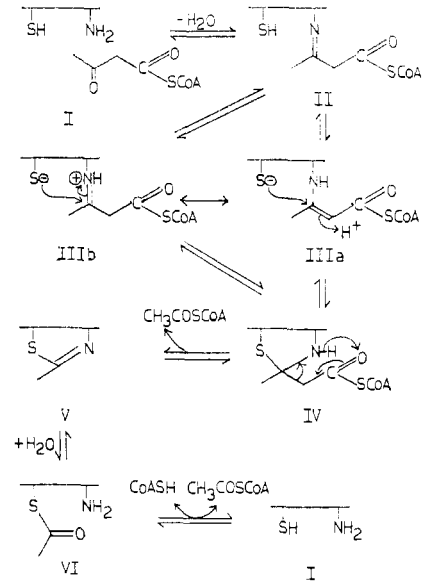
Discussion

The present results clearly demonstrate that 3-acetylenic CoA esters are potent site specific inactivating agents of thiolase from pig heart. 3-Acetylenic derivatives have also been shown to inactivate decenoyl dehydrase (Helmkamp *et al.*, 1968; Helmkamp and Bloch, 1969) and lactate oxidase (Walsh *et al.*, 1972). For decenoyl dehydrase, the inactivating species derived from 3-decynoyl-*N*-acetylcysteamine has clearly been shown to be the 2,3-dienoyl thio ester (Endo *et al.*, 1970; Morisaki and Bloch, 1972) and inactivation probably proceeds through addition of a histidine nitrogen to the allenic thioester (Morisaki and Bloch, 1972). A similar mechanism has been proposed for the inactivation of lactate oxidase by 2-hydroxy-3-butynoic acid where the nucleophile is probably the nitrogen of the enzyme's flavine coenzyme (Walsh *et al.*, 1972). The present data for the inactivation of thiolase by 3-acetylenic acyl-CoA esters indicates that a similar mechanism may be involved. Examination of the relative potency of acetylenic acyl-CoA esters as inactivating agents of thiolase (Table I) reveals that 3-acetylenic thio esters are extremely potent, 2-acetylenic thio esters are moderate and 4-acetylenic thio esters only weak inactivating agents. The ability to inactivate thiolase may be compared to the ease with which the corresponding acids isomerize to the 2,3-diene configuration. Jones *et al.* (1954) have shown that under basic conditions, 2-acetylenic and 3-acetylenic acids are in equilibrium with the 2,3-dienoic acids. In contrast, 4-acetylenic acids only give rise to 2,4-dienoic acids.

SCHEME II



SCHEME III



The inactivation by 3-pentynoyl-CoA appears to be dependent upon a basic group on the enzyme, as illustrated by the fact that the p*K* for inactivation of thiolase by 3-pentynoyl-CoA is 8.9, whereas for 4-bromocrotonyl-CoA the p*K* for inactivation is 8.4. Furthermore, model studies of the reaction of 3-pentynoylglutathione with the thiol in glutathione show that the basic conditions (0.1 M Tris-HCl, pH 8) employed in the inactivation reaction are not sufficient to explain the rapid inactivation of the enzyme. We have not been able to show that 2,3-butadienoyl-CoA is a better inactivating agent of thiolase than 3-butynoyl-CoA. However, it must be noted that both of these agents are such potent inactivating agents that they are active at molar ratio of unity for the concentrations of the inactivating agent and the active-site thiol.

In the event that thiolase contains a basic group (X:) at the active site, then a reasonable mechanism for the inactivation of thiolase can be proposed (Scheme II). An analogous mechanism can be deduced for the inactivation of decenoyl dehydrase by 3-decynoyl-*N*-acetylcysteamine using the imidazole moiety of the histidine at the active site. At present the suggestion that the 3-acetylenic acyl-CoA esters react with the thiol at the active site of thiolase is conjecture; however, the observation that exogenous thiols provide protection against inactivation shows that thiols are suitable nucleophiles. The failure of acetyl-CoA to protect against inactivation by the 3-acetylenic acyl-CoA esters is unexpected since acetyl-CoA has been shown to acetylate the active-site thiol (Gehring and Harris, 1970). It is possible that the 3-acetylenic CoA esters can react directly with the *S*-acetyl-enzyme or with an alternative nucleophile on the enzyme. Clarification of this point must await synthesis of labeled 3-acetylenic acyl-CoA esters.

The reaction catalyzed by thiolase is analogous to the well-known Claisen condensation reactions of organic chemistry (Hauser and Hudson, 1942). These reactions are usually catalyzed by bases such as sodium alkoxides, sodium amide, or triphenylmethylsodium (Hudson and Hauser, 1939). The proposal that the catalytic mechanism of thiolase involves a basic group is in complete accord with these facts. The observation that $NaBH_4$ inactivates the enzyme in the presence of acetoacetyl-CoA or acetyl-CoA suggests that the group involved

could be an amine and that the catalytic reaction proceeds through a ketimine intermediate. Following the general principles established for Claisen reactions by Hauser and Hudson (1942) it is possible to postulate a mechanism for thiolase that satisfies all existing data (Scheme III). The reaction would probably proceed through the enamine intermediate (IIIa) since it is known that structures of this type (*i.e.*, ethyl β-aminocrotonate) exist almost exclusively in the enamine conformation (Glickman and Cope, 1945). Interestingly, if the mechanism proceeds through IIIa the addition of the enzyme thiol proceeds by a Michael addition in a reaction analogous to that involved in the proposed mechanism for inactivation of thiolase by the 3-acetylenic acyl-CoA esters (Scheme II; reaction 4). Furthermore, the participation of the enamine intermediate could explain why thiolase has a high affinity for unsaturated fatty acyl-CoA esters. Finally, if the reaction proceeds through the enamine (IIIa), then it would be expected that $NaBH_4$ inactivation would be relatively slow since the proposed ketimine (II) does not exist as the protonated form. Schellenberg (1963) has clearly demonstrated that the reduction of protonated ketimines is very much faster than the reduction of neutral ketimines.

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