Long Chain Enoyl Coenzyme A Hydratase from Pig Heart*

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SUMMARY

A long chain enoyl-CoA hydratase, which in addition to crotonase is present in pig heart muscle, has been isolated and partially purified. This enzyme appears to be located in the mitochondria and is more tightly membrane-bound than crotonase. It catalyzes the hydration of medium and long chain trans-Δ^2,3-enoyl-CoA substrates to their corresponding L-3-hydroxy derivatives but it is nearly inactive toward crotonyl-CoA. The highest V_max was observed with Δ^2,3-octenoyl-CoA, while longer chain substrates gave progressively decreasing values. However, the same K_m value of 24 μM was obtained for all even numbered Δ^2,3-enoic acid derivatives containing 8 to 14 carbons. The pH optimum of octenoyl-CoA, while longer chain substrates gave progressively decreasing values. However, the same K_m value of 24 μM was obtained for all even numbered Δ^2,3-enoic acid derivatives containing 8 to 14 carbons. The pH optimum of this enzyme was found to be 8.5.

Inhibition studies showed that this hydratase, in contrast to bovine liver crotonase, is not significantly inhibited by acetoacetyl-CoA but is strongly inhibited by long chain enoyl-CoA substrates. This inhibition can be at least partially prevented by the presence of bovine serum albumin. The enzyme is also inhibited by sulfhydryl inhibitors, as, for example, p-chloromercuribenzoate or N-methylmaleimide, which at concentrations of 1 mM inhibited the enzyme to the extent of 100% and 69%, respectively.

Enoyl-CoA hydratase (EC 4.2.1.17), or crotonase, is one of a group of mitochondrial enzymes which together catalyze the β oxidation of fatty acids. Crotonase catalyzes the reversible hydration of trans-Δ^2,3-enoyl-CoA substrates to their corresponding L-3-hydroxy derivatives (1). Although this enzyme acts on substrates of different chain lengths, it is significantly more active with short chain than with long chain enoyl-CoA substrates. For example, the maximal rate of hydration is approximately 150 times higher with crotonyl-CoA as the substrate than with Δ^3,4-hexadecenoyl-CoA (2). It has recently been observed that acetoacetyl-CoA in its enolate form binds to crotonase and thereby competitively inhibits the hydration of substrates (2). This finding, together with the fact that crotonase acts only slowly on long chain substrates, led to the suggestion that crotonase may serve as a regulatory enzyme in fatty acid oxidation (2). This suggestion is based on the assumption that crotonase is the only hydratase involved in β oxidation.

The present report describes the partial purification and characterization of a long chain enoyl-CoA hydratase from pig heart. This enzyme is virtually inactive with crotonyl-CoA, but it shows comparable activities toward different longer chain enoyl-CoA compounds. It appears to be located in the mitochondria and is more tightly associated with membranes than is crotonase.

EXPERIMENTAL PROCEDURES

Materials

Coenzyme A, acetyl-CoA, NAD^+, and NADH were obtained from P-L Biochemicals, Inc. N-Methylmaleimide, bovine serum albumin, and cytochrome c were purchased from Sigma Chemical Co. Sodium p-chloromercuribenzoate and α-iodoacetamide were bought from Calbiochem. Ethyl chloroformate, triethylamine, 2-decenoic acid, and 2-octenoic acid were obtained from Aldrich Chemical Co. 2-Hexadecenoic acid was purchased from Miles Laboratories, Inc., L-3-Hydroxyacyl-CoA dehydrogenase and crotonic anhydride were bought from Boehringer and Soehne and Eastman Kodak Co., respectively. All other chemicals were of reagent grade. trans-Δ^2,3-Hexenoic acid, trans-Δ^2,3-dodecenoic acid, and trans-Δ^2,3-tetradecenoic acid were synthesized by reacting malonic acid in the presence of pyridine with α-butyraldehyde, 2-decanal, and n-dodecanal, respectively, according to a procedure by Linestad et al. (3).

Methods

Preparation of Substrates—The following substrates were prepared according to published procedures: crotonyl-CoA (4) and acetoacetyl-CoA (5). The CoA derivatives of Δ^2,3-hexenoic acid and its longer chain homologs were synthesized by reacting their mixed anhydrides, prepared by the method of Wieland and Köppe (6), with CoA. In a standard preparation, Δ^3,4-enoic acid (0.2 mmole) was dissolved in absolute tetrahydrofuran (2 ml) and allowed to react first with triethylamine (0.2 mmole) and then with ethyl chloroformate (0.2 mmole). After 10 min at 25° the solution was rapidly filtered and added dropwise over a period of 10 min to a solution of CoA (7 μmoles) dissolved in a mixture (5 ml) of water and tetrahydrofuran (2:2, v/v) which had been adjusted to pH 8 by the addition of NaHCO_3 (40 mg). After a total reaction time of 20 min the pH of the reaction mixture was adjusted to 3 and the tetrahydrofuran was removed by evaporation under vacuum. The longer chain enoyl-CoA compounds (C_9 and longer) were purified by precipitation at pH 1 and by subsequently washing them with peroxide-free ether. The resulting precipitates were then redissolved in water by adjusting the pH to 3. In order to precipitate Δ^3,4-decenoyl-CoA it was necessary to saturate the solution with NaCl in addition to adjusting the pH to 1. The shorter chain Δ^3,4-enoyl-CoA substrates were used without further purification after excess enoic acid had been removed by extraction with peroxide-free ether.
The concentrations of $\Delta^2$-enoyl-CoA substrates were determined in several ways: (a) by use of the method of Ellman (7) after cleaving the thioester bond with hydroxylamine at pH 7; (b) by determining the decrease in absorbance at 233 nm after cleaving the thioester bond with hydroxylamine at pH 7; and (c) by determining the decrease in absorbance at 233 nm as a result of the crotonase-catalyzed hydration of the substrates. However, the third method is of limited value because the equilibrium constant has only been reported for crotonyl-CoA and $\Delta^3$-hexenoyl-CoA (1, 8, 9). A molar extinction coefficient of $\epsilon_{260} = 6700$ cm$^{-1}$ M$^{-1}$ was used for calculating the substrate concentrations.

**Enzyme Assays**—The hydratase activity was routinely measured by following the decrease in absorbance at 263 nm on a Gilford recording spectrophotometer, model 240 (direct method). A standard assay contained 50 $\mu$moles of potassium phosphate (pH 8), 50 $\mu$g of bovine serum albumin, 15 $\mu$moles of $\Delta^2$-enoyl-CoA, and 2 to 4 $\mu$g of partially purified long chain enoyl-CoA hydratase in a total volume of 0.6 ml. Under these conditions the rates were linear for approximately 2 min. In some instances a combined assay was used in which the hydratase was coupled with the NAD$^+$-dependent $\Delta^4$-hydroxyacyl-CoA dehydrogenase. When an excess of dehydrogenase was used, the reduction of NAD$^+$ was dependent only on the rate of hydration of the $\Delta^2$-enoyl-CoA substrate. A standard assay contained 50 $\mu$moles of Tris-HCl (pH 9.0), 60 $\mu$moles of KCl, 50 $\mu$g of bovine serum albumin, 60 $\mu$moles of NAD$^+$, 15 $\mu$moles of $\Delta^2$-enoyl-CoA, 10 $\mu$g of $\Delta^3$-hydroxyacyl-CoA dehydrogenase, and 2 to 4 $\mu$g of partially purified long chain enoyl-CoA hydratase in a total volume of 0.6 ml.

**Purification of Long Chain Enoyl-CoA Hydratase**—Pig heart (225 g) was cut into small pieces and blended together with 600 ml of 0.05 M potassium phosphate (pH 7.0) and 5 mm mercaptoethanol for 5 min. The resulting suspension was centrifuged for 30 min at 27,000 $\times$ g and the precipitate discarded. The supernatant (homogenate) was fractionated with (NH$_4$)$_2$SO$_4$ and the protein fraction which precipitated between 30 and 75% saturation was collected by centrifugation and dissolved in 0.01 M potassium phosphate (pH 7.0) and 5 mm mercaptoethanol. This solution was extensively dialyzed against 0.01 M potassium phosphate (pH 7.0) and 5 mm mercaptoethanol and was then applied to a DEAE-cellulose column (5 X 45 cm) which had been previously equilibrated with 0.01 M potassium phosphate (pH 7.0) and 5 mm mercaptoethanol. The column was washed with 0.01 M potassium phosphate (pH 7.0), 5 mm mercaptoethanol, and 0.1 M NaCl until all ultraviolet-absorbing material ceased to be eluted. The column was then developed with a gradient made up of 1.6 liters each of 0.01 M potassium phosphate (pH 7.0)-5 mm mercaptoethanol-0.1 M NaCl and 0.01 M potassium phosphate (pH 7.0)-5 mm mercaptoethanol-0.6 M NaCl. Fractions of 25 ml were collected and assayed for hydratase activity with the CoA derivatives of crotonic acid, $\Delta^3$-decanoic acid and either $\Delta^3$-hexadecenoic acid or $\Delta^3$-tetradecenoic acid. The fractions containing long chain hydratase free of apparent crotonase activity were pooled and the protein was precipitated with (NH$_4$)$_2$SO$_4$ and finally dissolved in a minimal volume of 0.01 M potassium phosphate (pH 7.0) and 5 mm mercaptoethanol. Samples of such partially purified hydratase were stored for several months without change in activity.

**Subcellular Fractionation of Pig Heart Homogenate**—For subcellular fractionations small pieces of pig heart were forced through a meat grinder, suspended in 0.25 M sucrose, and homogenized in a Teflon-pestled Potter-Elvehjem type homogenizer attached to a motor spun at 1,200 rpm. The homogenate was filtered through a glass wool plug and fractionated in a similar manner as described by Schneider and Hogboon for rat liver homogenates (10). The following five fractions were obtained under the indicated conditions: nuclei at 500 X g for 10 min; heavy mitochondria at 5,000 X g for 20 min; light mitochondria at 24,000 X g for 10 min; microsomes at 122,000 X g for 60 min, and the 122,000 X g supernatant or soluble fraction. The enoyl-CoA hydratase activities were determined with crotonyl-CoA, $\Delta^2$-decenoyl-CoA, and $\Delta^2$-hexadecenoyl-CoA as substrates as described above. Succinate-cytochrome c reductase was assayed as described by Stotz (11). L-3-Hydroxyacyl-CoA dehydrogenase was measured spectrophotometrically at 340 nm. The assay mixture contained 50 $\mu$moles of potassium phosphate (pH 7.0), 60 $\mu$moles of NADH, 15 $\mu$moles of acetocetyl-CoA, and an aliquot of the fraction to be measured in a total volume of 0.6 ml.

**Results**

**Intracellular Localization of Long Chain Enoyl-CoA Hydratase**—The pig heart homogenate was separated into five subcellular fractions which were assayed with respect to five enzymatic activities. Since the 24,000 X g pellet, named light mitochondria, was virtually devoid of any of these five activities, there will not be further reference to it. As shown in Fig. 1, succinate-cytochrome c reductase, which is a membrane-bound mitochondrial activity, was found in the 5,000 X g and also in the 500 X g pellet, a finding which shows that the nuclear fraction contained a significant portion of the mitochondria. Crotonase and L-3-hydroxyacyl-CoA dehydrogenase, both of which are soluble mitochondrial enzymes, were found chiefly in the 122,000 X g supernatant, a finding which suggests that most of the mitochondria were broken. This observation is not surprising in view of the rigorous treatment required for homogenizing the tough pig heart muscle. In contrast, the $\Delta^2$-hexadecenooyl-CoA hydratase activity was mostly (72%) associated with the mitochondrial fragments present in the 5,000 X g and 500 X g fractions, while the $\Delta^2$-decanoyl-CoA activity was evenly distributed between the 122,000 X g supernatant and the mitochondria-containing fractions. It is likely that the $\Delta^3$-decanoyl-CoA hydratase activity reflected the combined actions of crotonase and the long chain enoyl-CoA hydratase. Thus it is concluded that the long chain enoyl-CoA hydratase is located in the mitochondria and is more tightly membrane-bound that are either crotonase or L-3-hydroxyacyl-CoA dehydrogenase. Of interest is the observation that a higher percentage of crotonase than of L-3-hydroxyacyl-CoA dehydrogenase was found in the 122,000 X g supernatant. If it is assumed that all crotonase activity was originally located inside the mitochondria, it can be concluded that either L-3-hydroxyacyl-CoA dehydrogenase is more tightly associated with the mitochondrial membrane.
membrane than is crotonase or that another acetoacetyl-CoA reductase, which would have to be more tightly membrane-bound than L-3-hydroxyacyl-CoA dehydrogenase, is present in pig heart mitochondria. Finally, the enzymes of fatty acid oxidation which were assayed in pig heart homogenate had the following specific activities: L-3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA as substrate, 0.46 unit per mg; crotonyl-CoA hydratase, 0.28 unit per mg; Δ\(^{2,3}\)-decanoyl-CoA hydratase, 0.28 unit per mg; and Δ\(^{2,3}\)-hexadecenoyl-CoA hydratase, 0.11 unit per mg.

**Isolation and Partial Purification of Long Chain Enoyl-CoA Hydratase**—Long chain enoyl-CoA hydratase was separated from crotonase and partially purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose as summarized in Table I. Assuming that all activity observed with Δ\(^{2,3}\)-hexadecenoyl-CoA was solely due to the long chain hydratase, a 8.5-fold purification was achieved. Significant amounts of long chain enoyl-CoA hydratase activity appeared to remain membrane-bound as evidenced by their presence in the 27,000 \(\times g\) precipitate of the crude heart extract and in the 30% ammonium sulfate precipitate. Also, upon dialysis of the 30 to 75% ammonium sulfate fraction, a precipitate formed at 30% ammonium sulfate fraction. These observations, in addition to the result from the subcellular fractionation experiment, strongly suggest that the long chain enoyl-CoA hydratase is associated with the mitochondrial membrane and is only partially solubilized upon homogenization in a blender. The separation of long chain enoyl-CoA hydratase and crotonase achieved by DEAE-cellulose chromatography is shown in Fig. 2. The long chain hydratase (Peak II) was eluted with approximately 0.45 m NaCl, and the resulting material was essentially free of crotonyl-CoA hydratase, thiolase, and 3-hydroxyacyl-CoA dehydrogenase activities. Peak I represents crotonase but possibly also long chain enoyl-CoA hydratase which has a tendency to remain associated with other proteins. For example, when during the isolation of this hydratase, the ammonium sulfate fractionation was not performed, the crotonase-free long chain hydratase was eluted at the position of the second large protein peak. The material present in the fractions corresponding to Peak II was isolated and chromatographed on Sephadex G-200. Since the hydratase was eluted as a sharp peak directly with the void volume (data not shown), it can be concluded that either long chain enoyl-CoA hydratase is a very large protein or that it tends to form aggregates. Evidence for the latter possibility was obtained from the observation that a fraction of the partially purified long chain enoyl-CoA hydratase precipitated when centrifuged for 30 min at 90,000 \(\times g\). Only 60% of the hydratase remained in solution upon centrifugation but treatment of the precipitate with phosphate buffer containing 1% Triton X-100 resulted in solubilization of 22% of the original activity. The percentage of the precipitated hydratase increased when the enzyme was kept for prolonged periods in dilute solution. For this reason and because of an observed increase in substrate inhibition with increasing purity, the enzyme was not further purified.

**Catalytic Properties of Long Chain Enoyl-CoA Hydratase**—The long chain enoyl-CoA hydratase catalyzes the hydration of Δ\(^{2,3}\)-enoyl-CoA substrates to their corresponding L-3-hydroxy derivatives as evidenced by the ability of the hydration products to serve as substrates for L-3-hydroxyacyl-CoA dehydrogenase (see Table II). The reduction of 1 mole of NAD\(^+\) per mole of Δ\(^{2,3}\)-decanoyl-CoA was observed when taking the 85% completion of the dehydrogenation at pH 9 (12) into account. The direct measurement of the hydration of Δ\(^{2,3}\)-decanoyl-CoA at 263 nm showed that at equilibrium the ratio of 3-hydroxydecanoyl-CoA to Δ\(^{2,3}\)-decanoyl-CoA was 2.8 (see Table II) from which an equilibrium constant of \(K = 5.1 \times 10^{-2}\) m was calculated. This value is similar to that found for the hydration of crotonyl-CoA by crotonase (1, 9).

Fig. 3 shows the relationship between the rate of hydration...
TABLE II

Product determination

The hydration of Δ²,3-decenoyl-CoA was followed spectrophotometrically at 263 nm as described under "Experimental Procedures," except that 50 pmoles each of Tris-HCl (pH 9) and KCl were substituted for the normally used phosphate buffer. The reduction of NAD⁺ was measured by coupling the hydratase with the L-3-hydroxyacyl-CoA dehydrogenase assay as described under "Experimental Procedures."

| Δ²-Decenoyl-CoA added (µM) | Δ³-Decenoyl-CoA hydrated (µM) | NAD⁺ reduced (µM) |
|---------------------------|-------------------------------|-------------------|
| 7.1                       | 5.3                           | 6.3               |
| 7.1                       | 10.4                          | 11.5              |
| 14.2                      | 14.2                          |                   |

![Fig. 3](image)

Fig. 3. The rate of hydration of Δ²,3-decenoyl-CoA as a function of its concentration. Assays were performed by the direct method as described under "Experimental Procedures."

of Δ²,3-decenoyl-CoA and its concentration. Of interest is the substrate inhibition which became effective at even lower substrate concentrations when no bovine serum albumin was present in the reaction mixture. In Fig. 4 the initial velocities versus concentrations were plotted on reciprocal coordinates for several Δ²,3-enoyl-CoA substrates. In contrast to crotonase, this enzyme showed similar activities toward all substrates tested except for crotonyl-CoA (not shown) toward which it was nearly inactive. The activity with crotonyl-CoA as a substrate was 1% of the activity found with Δ²,3-decenoyl-CoA. Since crotonase and long chain enoyl-CoA hydratase were well separated on DEAE-cellulose (see Fig. 2), it is not very likely that the residual activity with crotonyl-CoA was due to a contamination by crotonase.

The kinetic parameters for several Δ²,3-enoyl-CoA substrates of different chain lengths are listed in Table III. All Kᵣ values were the same (24 µM) with the exception of the Kᵣ for Δ²,3-hexenoyl-CoA which was nearly twice as large (45 µM) as all others. The highest maximal velocity was observed with Δ²,3-octenoyl-CoA as the substrate while longer chain substrates gave progressively decreasing values. It appears possible that the decrease in activity with increasing chain length of the substrate was due to the above mentioned substrate inhibition which becomes more pronounced with longer chain substrates.

![Fig. 4](image)

Fig. 4. The rates of hydration of several Δ²,3-enoyl-CoA substrates as a function of their concentrations. Assays were performed by the direct method as described under "Experimental Procedures" with 2.3 µg of hydratase per assay and with the following substrates: Δ²,3-hexenoyl-CoA (C₆), Δ²,3-octenoyl-CoA (C₈), Δ²,3-decenoyl-CoA (C₁₀), Δ²,3-dodecenoyl-CoA (C₁₂), and Δ²,3-tetradecenoyl-CoA (C₁₄).

TABLE III

Substrate specificity

The kinetic constants were obtained from the plots shown in Fig. 4.

| Substrate               | Kᵣ (µM) | V_max (µmol/min/mg) | Relative V_max (%) |
|-------------------------|---------|---------------------|--------------------|
| Δ²,3-Hexenoyl-CoA (C₆)  | 45      | 1.52                | 78                 |
| Δ²,3-Octenoyl-CoA (C₈)  | 24      | 1.95                | 100                |
| Δ²,3-Decenoyl-CoA (C₁₀) | 24      | 1.44                | 74                 |
| Δ²,3-Dodecenoyl-CoA (C₁₂)| 24     | 0.82                | 42                 |
| Δ²,3-Tetradecenoyl-CoA (C₁₄)| 24   | 0.66                | 34                 |

The activity of this enzyme is strongly pH-dependent as shown in Fig. 5. The optimal pH was found to be approximately 8.5, a value which is similar to values of 9 and 9.4 reported for crotonase (1, 8). With decreasing pH the activity decreases and the shape of this part of the pH curve resembles a titration curve with a midpoint at pH 6.5. A slight but definite stimulation of the hydratase activity was observed when the phosphate buffer was replaced by Tris-HCl as shown in Fig. 5.

Inhibition Studies—The sensitivity of the long chain enoyl-CoA hydratase toward substrates, especially longer chain ones, has already been mentioned. As shown in Fig. 6A, Curve 1, Δ²,3-hexenoyl-CoA at a concentration of 30 µM totally inhibited the enzyme. The initial change of absorbance appears to be due mostly to a nonspecific interaction of the substrate with the protein because a similar change in absorbance was observed when only the substrate and bovine serum albumin were present in the assay mixture. Increasing amounts of bovine serum albumin resulted in increased protection against substrate inhibition (see Fig. 6A, Curves 2 and 3). For comparison an identical experiment was performed with Δ²,3-hexenoyl-CoA as substrate. As shown in Fig. 6B even this short chain substrate inhibited the enzyme, although less pronouncedly than the long chain derivative, and again bovine serum albumin prevented the inhibition. Since the substrate
inhibited by acetoacetyl-CoA (2), prompted a similar investigation with the long chain enoyl-CoA hydratase. When acetoacetyl-CoA was present in the assay mixture in a 6-fold excess (0.7 mM) over \( \Delta^2 \)-decanoyl-CoA, a slight inhibition (10%) was observed. However, in contrast to bovine liver crotonase, acetoacetyl-CoA at the same concentration as acetoacetyl-CoA caused a similar inhibition (17%). It is possible that the observed inhibition was of the same nature as the above mentioned inhibition of long chain hydratase by CoA derivatives of \( \Delta^3 \)-enoic acids. Thus it is suggested that the small inhibition of this enzyme by acetyl-CoA and acetoacetyl-CoA is of no physiological significance.

Finally, the effect of sulphydryl inhibitors on the activity of the long chain enoyl-CoA hydratase was investigated. A 69% (85%) inhibition was observed when the hydratase was pre-incubated for 15 min at pH 7 in the presence of 1 mM (5 mM) N-methylmaleimide and then assayed with \( \Delta^2 \)-octenoyl-CoA as the substrate. An identical experiment performed with \( p \)-chloromercuribenzoate as the inhibitor showed an increase in the inhibition from 13% to 100% when the inhibitor concentration was raised from 0.1 to 1 mM. A 16% inhibition by \( \alpha \)-iodoacetamide at 5 mM concentration was observed, but because this experiment was performed at pH 7 the observed degree of inhibition may not be optimal. Thus it is concluded that at least one thiol group is essential for the full activity of long chain enoyl-CoA hydratase, although these findings cannot be taken as evidence for the participation of a sulphydryl group in the catalytic event.

**DISCUSSION**

In discussions of fatty acid oxidation it has always been assumed that the hydration of all \( \Delta^3 \)-enoyl-CoA intermediates is catalyzed by only one enzyme, namely crotonase (13). However, some evidence has recently been obtained which would indicate that at least in some \( \beta \) oxidation systems more than one hydratase must be present. Waterson et al. found that the purified crotonase from *Clostridium acetobutylicum* catalyzed only the hydration of crotonyl-CoA and \( \Delta^2 \)-hexenoyl-CoA but that crude extracts from the same organism were active with \( C_6 \) to \( C_{16} \) derivatives, a finding which led them to suggest that in this organism a long chain enoyl-CoA hydratase may be present in addition to crotonase (14). Wit-Peters et al. have reported that during the purification of crotonase from calf liver, the ratio of hydratase activities determined with crotonyl-CoA and \( \Delta^2 \)-hexadecenoyl-CoA increased from 5.3 to 420 (15). Thus they suggested that in addition to crotonase a long chain enoyl-CoA hydratase must be present in the homogenate. However, Waterson and Hill found that the ratio of hydratase activities obtained with crotonyl-CoA, \( \Delta^2 \)-decanoyl-CoA, and \( \Delta^2 \)-hexa-decenoyl-CoA remained constant during the purification of crotonase from bovine liver (2). Although the presence of a separate long chain enoyl-CoA hydratase in bovine liver remains in question, the present report clearly shows that a separate long chain enoyl-CoA hydratase exists in pig heart. Even though the functional role of this long chain enoyl-CoA hydratase has not yet been established, it appears reasonable to assume that it participates in the \( \beta \) oxidation of fatty acids, especially since this enzyme is localized in the mitochondria.

Assuming that both crotonase and long chain enoyl-CoA hydratase are involved in fatty acid oxidation, a comparison of their chain lengths' specificities is of interest. Since the pig heart crotonase has not yet been purified and characterized, the data obtained with the bovine liver enzyme are used for comparative purposes. These data show that crotonase is highly active with crotonyl-CoA but acts much more slowly on longer chain substrates. The difference between the rates of hydration with crotonyl-CoA and longer chain enoyl-CoA as substrates is most pronounced at low substrate concentrations because the \( K_m \) for crotonyl-CoA is 10 times smaller than that for \( \Delta^2 \)-hexenoyl-CoA, the second best substrate (2). In contrast, the long chain enoyl-CoA hydratase is nearly inactive with crotonyl-CoA but is fully active with all longer chain enoyl-CoA substrates. Hence it appears that the optimal rate of hydration of all \( \Delta^3 \)-enoyl-CoA intermediates in fatty acid oxidation would be achieved if crotonase and the long chain enoyl-CoA hydratase act in a concerted manner. A similar situation exists with respect to the mitochondrial thiolytic activity which is due to a short chain specific thiolase only active on acetoacetyl-CoA (16) and a general purpose enzyme which is more active on long- than on short chain substrates (17). Since a short and
long chain-specific acyl-CoA dehydrogenase is also expected to be present in pig heart (18), it is possible that the enzymes of \( \beta \) oxidation in heart tissue are arranged in two groups, one of which is concerned with the degradation of long chain fatty acids to medium or short chain fatty acyl-CoA intermediates which are then further degraded by the group of short chain-specific enzymes. This is certainly a highly speculative model which would additionally require the presence of a second \( \Delta^3 \)-hydroxyacyl-CoA dehydrogenase in heart tissue. The reason for choosing such an arrangement is based on the assumption that the enzymes of fatty acid oxidation exist in the mitochondria in a highly ordered manner and on the observation that the long chain enoyl-CoA hydratase is much more tightly membrane-bound than is crotonase.

The effect of acetoacetyl-CoA on the rate of hydration of \( \Delta^3 \)-decenoyl-CoA by long chain enoyl-CoA hydratase was studied because it has been reported that bovine liver crotonase is inhibited by acetoacetyl-CoA (2). On the basis of the observed inhibition and substrate specificity, it has been suggested that crotonase functions as a regulatory enzyme in fatty acid oxidation (2). According to this proposal, acetoacetyl-CoA would inhibit the hydration of \( \Delta^3 \)-hexadecenoyl-CoA or \( \Delta^3 \)-tetradecenoyl-CoA but not the hydration of shorter chain substrates to such an extent that these steps would become rate-limiting. Experiments performed with whole mitochondria, especially beef heart mitochondria, appear to lend support to this proposal because the oxidation of palmitic acid but not of octanoic or butyric acid was reduced by acetoacetyl-CoA. Inhibition experiments performed with the long chain enoyl-CoA hydratase from pig heart showed that this enzyme was only slightly and unspecifically inhibited by acetoacetyl-CoA. Thus it appears highly unlikely that the proposed regulatory mechanism is effective in pig heart, if one assumes that the long chain hydratase is involved in \( \beta \) oxidation. An additional reason for a re-evaluation of the proposed regulatory schema of fatty acid oxidation is the observed strong inhibition of \( \Delta^3 \)-hydroxyacyl-CoA dehydrogenase by acetoacetyl-CoA with a \( K_I \) of 7.7 \( \mu \)M, while the \( K_I \) for the inhibition of crotonase by acetoacetyl-CoA is 30 \( \mu \)M (2).

The long chain enoyl-CoA hydratase was found to be inhibited by sulfhydryl inhibitors. Although this finding does not constitute proof for the participation of a sulfhydryl group in the catalytic event, it suggests that at least one thiol group is positioned near or at the catalytic site or that a sulfhydryl group is required for maintaining the enzyme in an active conformation. Studies by Hill and co-workers (19, 20) with crotonase have led to the suggestion that this enzyme, despite its inactivation by thiol inhibitors, does not possess a sulfhydryl group which participates in the catalytic event, but that the chemical modification of one specific thiol group results in a sterical restriction at the catalytic site which is the cause of the inhibition. It is possible that the long chain enoyl-CoA hydratase is affected by the sulfhydryl inhibitors in a similar manner.

1 J. Schifferdecker and H. Schulz, unpublished result.

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