Enoyl Coenzyme A Hydratase (Crotonase)

Catalytic Properties of Crotonase and Its Possible Regulatory Role in Fatty Acid Oxidation*

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SUMMARY

The substrate specificity of bovine liver crotonase has been examined with seven \( \Delta^3 \)-trans-enoyl-CoA substrates, containing an even number of carbon atoms. The \( V_{\text{max}} \) for this series decreases progressively from a value of about 340,000 moles per min per mole of enzyme for crotonyl-CoA, the \( \text{C}_4 \) derivative, to 2,300 for the \( \text{C}_6 \) derivative.

The action of several CoA derivatives on crotonase has been tested. None were found to stimulate the enzyme and only one derivative, acetoacetyl-CoA, was found to be markedly inhibitory. Evidence was obtained that the enolate form of acetoacetyl CoA was the inhibitory species and acted as a competitive inhibitor with a \( K_i \) of \( 1.6 \times 10^{-4} \text{ M} \). a value about ten times lower than the \( K_m \) for the best substrate, crotonyl-CoA. The interaction of acetoacetyl-CoA with crotonase was studied by ultraviolet difference spectroscopy and it was found that 6 molecules of inhibitor were bound per molecule of enzyme, or an average of one per subunit. This suggests that there are six active sites per molecule of native enzyme. The binding constant for the inhibitor was about equal to the kinetically determined value for \( K_i \).

The catalytic properties of crotonase have been compared with the turnover numbers and substrate specificities of the other enzymes acting in \( \beta \)-oxidation of fatty acyl-CoA derivatives. This comparison suggests that crotonase, by virtue of its substrate specificity and its sensitivity to feedback inhibition by acetoacetyl CoA, may play a regulatory role in fatty acid oxidation. The effects of acetoacetyl-CoA on the rate of oxidation of butyric, octanoic, and palmitic acids by heart muscle or liver mitochondria were those expected if crotonase is acting, at least in part, to regulate fatty acid oxidation.

Enzyme and Reagents—Crotonase, five times recrystallized, was prepared as reported earlier (3) and was shown to be homogeneous as described elsewhere (4).

Crotonase, or enoyl-CoA hydratase (EC 4.2.1.17) is the only enzyme in the mitochondrial \( \beta \)-oxidation pathway for fatty acids which catalyzes the reversible stereospecific hydration of \( \Delta^3 \)-trans-enoyl-CoA substrates to the corresponding \( L(+) \)-\( \beta \)-hydroxyacyl-CoA derivatives. We wish to report here studies on the substrate specificity of bovine liver crotonase and its inhibition by acetoacetyl-CoA. It has been found that the rate of hydration of \( \Delta^3 \)-trans-enoyl-CoA substrates decreases markedly with increasing chain length. Because acetoacetyl-CoA is a potent competitive inhibitor of crotonase, it is possible that under conditions which allow acetoacetyl-CoA to accumulate, oxidation of long chain fatty acids would be reduced by virtue of inhibition of the crotonase-catalyzed step. Studies with intact mitochondria seem to support this view. For these reasons it is possible that crotonase may play a central role in fatty acid oxidation. Preliminary accounts of portions of this work have been presented previously (1, 2).

Experimental Procedure

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All other chemicals were reagent grade and were used without further purification.

Crotonyl-CoA was prepared by the method of Simon and Shemin (6). All other \( \Delta^3 \)-enoyl-CoA substrates were prepared by the mixed anhydride method of Goldman and Vagelos (7). The \( \Delta^2 \)-enoic acid (35 \( \mu \)moles) and triethylamine (35 \( \mu \)moles) were mixed with 1.5 ml of tetrahydrofuran. After 10 min 35 \( \mu \)moles of ethyl chloroformate in 0.5 ml of tetrahydrofuran were added, and the reaction was allowed to proceed at 0°
for 20 min. Precipitated triethylamine hydrochloride was removed by filtration through a Pasteur pipette fitted with a glass wool plug. The filtered solution was then evaporated to dryness, and the residue was dissolved in 1.6 ml of tetrahydrofuran. Reduced coenzyme A (20 mg) was dissolved in 1.6 ml of tetrahydrofuran-water mixture (7:3, v/v) and adjusted to pH 8 by dropwise addition of 1 M sodium bicarbonate. The mixed anhydride solution was then added to the CoA in 0.5 ml aliquots at 3- to 5-min intervals at room temperature. The pH was kept at 8 and distilled water was added as necessary to maintain a clear solution. After 20 min the reaction was complete as judged by disappearance of -SH groups. The solution was then adjusted to pH 5 with 10% perchloric acid and most of the tetrahydrofuran was removed by evaporation. The C10 to Cn acyl-CoA derivatives precipitated on adjusting the solution to pH 3 with 10% perchloric acid and were freed from residual unesterified acid by ether extraction. The C10 to C18 enoyl-CoA derivatives are soluble in acid, and were purified from residual CoA by ion exchange chromatography on Whatman DE-52 using a lithium chloride gradient as previously described (8).

Preparation of Mitochondria—Rat heart mitochondria were a gift from Dr. Salih Wakil; they were obtained from the Institute for Enzyme Research, University of Wisconsin, and were kept frozen at -15°C in 0.25 M sucrose until used. The mitochondria were then diluted with 0.25 M sucrose to the concentrations desired for assay.

Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9). Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9). Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9). Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9). Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9). Rat liver mitochondria were prepared by a procedure similar to that described by Johnson and Lardy (9).

Measurement of Hydrazide Activity—The activity of crotonase with each enoyl-CoA substrate was measured spectrophotometrically at either 280 or 263 nm with a Cary model 15 recording spectrophotometer equipped with a water-jacketed cell holder maintained at 25°C. Assay mixtures contained 0.033 M Tris-HCl, pH 7.5, 5 × 10⁻⁴ M EDTA, 0.1% egg albumin, and substrate at the concentrations indicated under "Results." For routine assays, crotonyl-CoA was used at a concentration of 2 × 10⁻⁴ M. Initial velocity measurements were made with 1-cm cells under conditions where absorbance changes were linear with enzyme concentration. The decrease in absorbance for a 1 ml solution of substrate was 0.700 and 4.400 at 263 nm and 280 nm, respectively (11). Concentrations of all substrates were measured spectrophotometrically assuming a molar extinction coefficient of 19,200 at 232 nm.

Difference Spectra with Crotonase and Acetoacetyl-CoA—These measurements were made by methods essentially identical with those reported earlier (12, 13), employing tandem cuvettes with an over-all width of 2 cm (1 cm per compartment). The concentration of acetoacetyl-CoA was measured spectrophotometrically at 260 nm (pH 7) assuming the molar extinction coefficient (ε₂₆₀ cm⁻¹) = 15,400. The concentration of the enolate tautomer of acetoacetyl-CoA was measured spectrophotometrically at 307 nm assuming a molar extinction coefficient of 25,000. This value was obtained by spectrophotometric titration with either alkali or various divalent cations (14, 15). According to the data of Stern (16), the enolate tautomer at pH 7.5 represents about 4% of the total acetoacetyl-CoA in solution.

Mitochondrial Fatty Acid Oxidation—The assay for fatty acid oxidation is similar to that described by Bressler and Friedberg (16). Reactions were performed at 37°C with shaking in 25-ml flasks sealed with Kontes serum stoppers fitted with polyethylene center-well inserts. The cupts were suspended at a level below that of the water level of the bath to minimize condensation effects.

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Results

Action of Crotonase on Δ³⁻⁻Enoyl-CoA Substrates—Fig. 1 shows the relationship between the initial velocity and the substrate concentration for seven Δ³⁻⁻enoyl-CoA substrates, each containing an even number of carbon atoms. Clearly, the rate of hydration decreases with increasing chain length. Crotonyl-CoA is the best substrate and the turnover number with this substrate has been calculated to be 340,000 moles per min per mole of enzyme. In contrast the turnover number with hexadecenoyl-CoA is 2,300 moles per min per mole of enzyme. It is noteworthy that this latter value falls within the range of turnover numbers displayed by the other enzymes of fatty acid oxidation as will be discussed later.

The V₅₀ and Kₘ values for each substrate were calculated from double reciprocal plots and are summarized in Table 1. The relationships between chain length of the substrates and these kinetic parameters are shown in Fig. 2. It is noteworthy that V₅₀ decreases regularly with increasing chain length from C₂ to C₁₈. The Kₘ value for crotonyl-CoA is about 10 to 20 times smaller than the Kₘ values for substrates containing 6 or
FIG. 1. Velocity of hydration as a function of substrate concentration. Crystalline bovine crotonase (1 to 10 ng) was added to reaction mixtures (0.5 ml) containing 3.3 x 10⁻⁵ M Tris-HCl, pH 7.5, 5 x 10⁻⁷ M egg albumin, 5 x 10⁻⁷ M potassium EDTA, and each of the enoyl-CoA substrates at the concentrations indicated. Initial rates of hydration were measured spectrophotometrically at 263 nm as described in the text. The total number of carbon atoms in the enoyl moiety of each substrate is noted as C through C₁₁ by the appropriate number.

TABLE I

| Substrate specificity of bovine liver crotonase |
|-----------------------------------------------|
| The kinetic parameters obtained for each enoyl-CoA substrate were obtained from double reciprocal plots of the data from Fig. 1. |

| Δⁿ⁺-Enoyl-CoA substrate (chain length) | Kᵣᵣ | Vᵣᵣ | Relative rates |
|---------------------------------------|------|------|----------------|
| Crotonyl-CoA (C₄)                     | 2.0 x 10⁻⁵ | 340,000 | 148 |
| Hexenoyl-CoA (C₅)                     | 2.4 x 10⁻⁴ | 151,000 | 67 |
| Octenoyl-CoA (C₆)                     | 2.8 x 10⁻⁴ | 83,500 | 36 |
| Decenoyl-CoA (C₇)                     | 3.0 x 10⁻⁴ | 38,000 | 17 |
| Dodecenoyl-CoA (C₈)                   | 4.0 x 10⁻⁴ | 16,000 | 7 |
| Tetradecenoyl-CoA (C₉)                | 4.2 x 10⁻⁴ | 5,000 | 2 |
| Hexadecenoyl-CoA (C₁₀)                | 5.0 x 10⁻⁴ | 2,300 | 1 |

more carbon atoms, although the Kᵣᵣ values for the C₅ to C₁₀ substrates vary no more than about 2-fold from one another.

It has been found that the relative rates of hydration of crotonyl-CoA, decenoyl-CoA, and hexadecenoyl-CoA (Table I) remain constant at each step in the purification of crotonase prepared by the method of Stern (3). This observation is consistent with the view that crotonase is the sole hydratase acting in fatty acid oxidation (17).

Crotonase Inhibition—Because of the interesting effects of acetyl-CoA and related nucleotides on the hydration of crotonyl-coenzyme A (1, 18), these and other compounds which are structurally related to crotonyl-CoA have been tested as inhibitors of crotonase. With crotonyl-, octenyl-, or hexadecenoyl-CoA as substrate (initial concentrations well below the Kᵣᵣ values), the following compounds gave neither marked inhibition nor activation (18): CoA, acetyl-CoA, butyryl-CoA, pantetheine, ATP, ADP, AMP, GTP, UTP, CTP, adenosine, and crotonate. All compounds were tested at concentrations between 0.12 and 0.20 mM. Acetoacetetyl-CoA was found to be the only good inhibitor for crotonase, although neither ethyl acetacetoate nor acetacetoate were inhibitory. As shown in Fig. 3, acetoacetyl-CoA is an effective competitive inhibitor for crotonase with crotonyl-CoA as substrate. The apparent Kᵣᵣ calculated from these data is 3 x 10⁻⁵ M, although, as noted below, the enolate species of acetoacetyl-CoA is the actual inhibitory species and the Kᵣᵣ for the enolate is about 25 times smaller. As expected, the same Kᵣᵣ value was obtained for acetoacetyl-
CoA with substrates varying in chain length from 4 to 16 carbon atoms.

In view of the potent inhibitory effects of acetoacetyl-CoA, the inhibition by equilibrium mixtures of crotonyl-CoA and β-hydroxybutyryl-CoA were tested with longer chained enoyl-CoA substrates. These equilibrium mixtures proved to be good inhibitors as shown in Fig. 4 with octenoyl-CoA as substrate. The apparent \( K_i \) for the equilibrium mixture was calculated to be \( 2 \times 10^{-3} \) M, or about equal to the \( K_m \) for crotonyl-CoA (Table I).

**Interaction of Crotonase with Acetoacetyl-CoA**—The nature of the interaction of crotonase with acetoacetyl-CoA has been examined by ultraviolet difference spectroscopy. Fig. 5 shows the difference spectrum of the inhibited enzyme, which was obtained by measuring the absorbance of mixtures of acetoacetyl-CoA and crotonase against solutions of inhibitor and enzyme at the same concentrations but in different cells in the reference beam of the spectrophotometer. This spectrum, aside from the shoulders at 278 and 292 nm resulting from the perturbation of aromatic residues, is indistinguishable from that of the enolate form of acetoacetyl-CoA (14). This suggests that the enolate tautomer of acetoacetyl-CoA is the inhibitory species and has a marked affinity for the enzyme. At pH 7.5, 25°, the enolate form represents about 4% of the total acetoacetyl-CoA in solution (15). Recalculation of the \( K_i \) for acetoacetyl-CoA (Fig. 3) based on the concentration of the enolate gives a \( K_i \) of \( 1.6 \times 10^{-6} \) M, a value about 10 times lower than the \( K_m \) for the best substrate, crotonyl-CoA (Table I).

Because of the large spectral change associated with the binding of the enolate tautomer of acetoacetyl-CoA, it was possible to titrate crotonase with acetoacetyl-CoA. Fig. 6 shows the titration expressed as the amount of enzyme-inhibitor complex formed as a function of inhibitor concentration. The experimentally determined values correspond very closely to the theoretically calculated curve for a noncooperative reaction with a dissociation constant of \( 1.7 \times 10^{-3} \) M. Thus, the dissociation constant for acetoacetyl-CoA is very similar to its kinetically determined \( K_i \) (3 \( \times 10^{-5} \) M). After correcting for the concentration of the enolate tautomer, the dissociation constant for acetoacetyl-CoA is very similar to its kinetically determined \( K_i \) for the enolate form (1.6 \( \times 10^{-6} \) M). This shows that crotonase not only has approximately the same affinity for acetoacetyl-CoA over a million-fold range in protein concentration, but also...
Fig. 8. The effect of acetoacetyl-CoA on the rate of oxidation of butyryl, octanoate, and palmitate by heart muscle mitochondria. The reaction mixtures are described under "Experimental Procedure." Butyrate (0.1 μmole, 180,000 cpm), octanoate (0.1 μmole, 160,000 cpm), and palmitate (0.1 μmole, 210,000 cpm) were present at an initial concentration of 10⁻⁶ M. Each mixture contained 14.15 mg of mitochondrial protein. The initial concentration of acetoacetyl-CoA, when present, was 10⁻⁶ M. The open symbols refer to assays without acetoacetyl-CoA and the shaded symbols to assays in the presence of acetoacetyl-CoA. □ and ○, palmitate; △ and ▲, octanoate; ○ and □, butyrate.

Fig. 9. Palmitate oxidation by heart muscle mitochondria as a function of acetoacetyl-CoA concentration. Assays were performed as in Fig. 8 with 12 mg of beef heart mitochondria and 100 μmoles of palmitate (210,000 cpm) per reaction mixture. The initial concentration of palmitoyl-CoA was 10⁻⁶ M and those for acetoacetyl-CoA as indicated. Incubation was for 60 min.

suggests that the kinetic inhibition constant is a true dissociation constant.

The titration data in Fig. 6 can be used to determine the number of molecules of inhibitor bound per molecule of enzyme. When these data are expressed as shown in Fig. 7, it has been found that 6 ± 0.1 molecules are bound per molecule of crotonase. This suggests that there is one independent binding site, presumably the catalytic site, on each of the 6 subunit polypeptide chains in the enzyme.

Effect of Acetoacetyl-CoA on Mitochondrial Fatty Acid Oxidation—Because acetoacetyl-CoA is a potent inhibitor of crotonase and it is a major end product of fatty acid oxidation, the effect of acetoacetyl-CoA on the mitochondrial oxidation of fatty acids was examined. Fig. 8 shows the rate of oxidation of butyrate, octanoate, and palmitate by beef heart muscle mitochondria in the presence and absence of acetoacetyl-CoA. The rates are expressed as the amounts of 14CO₂ derived from the 14C-carboxyl labeled acids. Clearly, the rate of oxidation of butyrate and octanoate is unaffected by acetoacetyl-CoA although the rate of palmitate oxidation is markedly depressed by acetoacetyl-CoA. The effect of concentration of acetoacetyl-CoA on palmitate oxidation is shown in Fig. 9. Acetoacetyl-CoA has similar effects in liver mitochondria as shown in Fig. 10, although the amount of inhibition of palmitate oxidation is not as great as found with heart mitochondria.

DISCUSSION

Substrate Specificity of Crotonase—Earlier studies with crotonase suggested that it had a broad substrate specificity. Crotonyl-CoA, hexenoyl-CoA, and the β-hydroxyacyl-CoA derivatives containing 4, 6, 8, 9, and 12 carbon atoms were found to be good substrates (21, 22). It appears to have a strict requirement for thiol esters of CoA, and although it can hydrate crotonylpantetheine (22), the rate of hydration is only about 0.01 that of crotonyl-CoA. Crotonase also displays a broad specificity in terms of the stereochemistry of the Δ₃ double bond, since it hydrates the trans- as well as cis-enoyl-CoA derivatives (23). The studies reported here extend our knowledge of the substrate specificity of crotonase and show that it acts on Δ₃-trans-enoyl-CoA substrates ranging in chain length from 4 to 16 carbon atoms, although the rate of hydration falls markedly as chain length is increased (Fig. 1, Table I). In view of the possible importance of this observation it was essential to consider whether the longer chain enoyl-CoA substrates formed micelles which could influence the observed kinetic parameters.
Although the critical micelle concentrations for the enoyl-CoA derivatives used here are unknown, it is unlikely that micelles influenced the rate studies for the following reasons. First, no micelle formation would be expected to alter substrate binding, and thus $K_m$ more than the rate of hydration ($V_{\text{max}}$). Just the opposite was found: $K_m$ varied only slightly from $C_4$ to $C_{16}$, whereas $V_{\text{max}}$ decreased progressively with increasing chain length. Secondly, no abrupt transitions were found in the kinetic data as a function of substrate concentration. If monomer-micelle transitions occurred they should be reflected by nonlinear double reciprocal plots. Finally, the hydratase of fatty acid oxidation, the electron transport flavoprotein, or the thiokinases did not show any evidence of saturability or cooperativity, whereas $V_{\text{max}}$ decreased progressively with increasing chain length.

Inhibition by Acetoacetyl-CoA.—Spectroscopic examination of the reaction of acetoacetyl-CoA with crotonase indicated several important points. First, the difference spectrum obtained in Fig. 6 is almost indistinguishable from that of the enolate form of acetoacetyl-CoA and it can be concluded that this is the species interacting with crotonase. It is noteworthy that the enolate is a potent competitive inhibitor and structurally resembles both crotonyl- and $\beta$-hydroxybutyryl-CoA. For these reasons, the enolate can be considered a transition state analog for crotonase (26). Thirdly, titration of the enzyme with acetoacetyl-CoA shows that an average of 1 molecule of inhibitor is bound per enzyme subunit. Since the inhibitor is competitive it is likely that there is one active site per subunit. In addition, there is no indication of cooperativity in the binding of acetoacetyl-CoA, in accord with the fact that crotonase shows no regulatory effects of acetoacetyl-CoA on crotonase activity. In view of the properties of the other enzymes in fatty acid oxidation, it is evident that crotonase and acetoacetyl-CoA may play an important role in the regulation of $\beta$-hydroxyacyl-CoA dehydrogenase.

A consideration of the properties of the enzymes in fatty acid oxidation which are listed in Table II illustrates this point. As noted earlier, the chain length specificities of all of the oxidizing enzymes are compared as a function of the chain length for their substrates, as shown in Fig. 11. The turnover numbers for all of the enzymes of fatty acid oxidation are compared as a function of the chain length for their substrates, as shown in Fig. 11. The turnover numbers for each of the enzymes, except crotonase, fall between about 3.5 to 9 pmoles of substrate per min per g of liver, as indicated in the figure by the bars with horizontal stripes. These turnover numbers for the other enzymes, except crotonase, fall between about 3.5 to 9 pmoles of substrate per min per g of liver, as indicated in the figure by the bars with horizontal stripes. These activities are catalyzed by multiple enzymes, each specific for short, medium, or long chain length substrates, which have equivalent activities in crude extracts (17).

### Table II

| Enzyme(s)                        | $V_{\text{max}}$ as a function of acyl-CoA chain length, $C_4$ to $C_{16}$ |
|----------------------------------|--------------------------------------------------------------------------|
| Fatty acyl thiokinases           | Uniform*                                                                 |
| Acyl-CoA dehydrogenases          | Uniform*                                                                 |
| Enoyl-CoA hydratase              | $1:9:7:17:36:67:114$                                                    |
| $\beta$-Hydroxacyl-CoA dehydrogenase | $1:1:1:1:1:1$                                                        |
| $\beta$-Ketoacyl-CoA thiolase     | $1:1:1:1:1:1:3:9:3$                                                     |

* These activities are catalyzed by multiple enzymes, each specific for short, medium, or long chain length substrates, which have equivalent activities in crude extracts (17).

**Fig. 11.** The turnover of fatty acyl-CoA intermediates in liver. The respective activities of each of the enzymes of fatty acid oxidation were obtained from the literature and are expressed in logarithmic form versus substrate chain length as indicated on the bar graph. The bars with horizontal stripes correspond to the range of turnover of the respective substrates of either the acyl-CoA dehydrogenases, electron transport flavoprotein, $\beta$-hydroxyacyl-CoA dehydrogenase, or thiolase. The activation reaction catalyzed by the thiokinases (not shown) appears to be 2 to 3 times the turnover rate of the other enzymes except crotonase. The differential turnover of enoyl-CoA substrates of varying chain length as determined in this report is represented by the open bars. The theoretical rates of hydration in the presence of a 10-fold excess of acetoacetyl-CoA over hexadecenoyl-CoA are indicated by the closed bars. These activities catalyzed by multiple enzymes of limited substrate specificity were found to occur in relatively equivalent amounts in liver, and thus display no overall chain length specificity in terms of approximate in vivo turnover. The turnover values for each activity and the literature sources are as follows: thiokinases, approximately 30 (27); acyl-CoA dehydrogenases, 3.5 to 15 (28-31); electron transport flavoprotein, approximately 20 (32); $\beta$-hydroxyacyl-CoA dehydrogenase, 5 to 30 (17, 24); and thiolase, 9 to 10 (25, 33). It is important to emphasize that the variation of these minimal values represents the values of different reports rather than being due to chain length specificities. The values used for crotonase (open vertical bars) as described in this report vary progressively from 8.5 units per g for the turnover of hexadecenoyl-CoA to 1,300 units per g for the hydration of crotonyl-CoA, in accord with the data of Table I and the values for crotonyl- and hexenoyl-CoA reported earlier (22).
values, taken from published data, represent minimal turnover numbers and, although obtained under conditions of substrate saturation, are not $V_{\text{max}}$ values for each enzyme. The values for crotonase are shown as the open vertical bars, and although they are calculated from $V_{\text{max}}$ values determined in this study, they are about equal to those reported in crude liver extracts for $C_4$ and $C_6$ enoyl-CoA substrates. From these considerations, it is striking that the turnover for the $C_4$ to $C_{14}$ enoyl-CoA substrates is considerably greater than those for all other acyl-CoA substrates of the same chain length. Only hexadecenoyl-CoA is hydrated at a rate about equal to those for transformation of other acyl-CoA substrates. Fig. 11 also shows the expected turnover numbers for each of the substrates of crotonase in the presence of a 10-fold excess of acetoacetyl-CoA over the enoyl-CoA substrate. These values are indicated by the solid vertical bars. This shows that accumulation of acetoacetyl-CoA would reduce the rate of hydration of enoyl-CoA substrates with chain lengths between $C_4$ and $C_14$, but under these conditions the crotonase-catalyzed reaction is not rate limiting. In contrast, acetoacetyl-CoA accumulation would markedly depress the turnover of $C_14$ and $C_6$ enoyl-CoA substrates and under these conditions would limit the rate of fatty acid oxidation. Thus, by a combination of its cascading substrate specificity and its marked susceptibility to acetoacetyl-CoA, crotonase would be rate limiting in its action on long chain substrates, which are used in initiation of fatty acid oxidation, whereas it would continue to hydrate shorter chained ($C_4$ to $C_{12}$) substrates at rates equal to or greater than those for the other reactions in $\beta$ oxidation. These conditions would be expected to allow the short and medium chain length acyl-CoA intermediates of fatty acid oxidation to be metabolized to acetyl-CoA at a steady rate and they would not be expected to accumulate in the mitochondria. Fig. 12 shows schematically the reactions of fatty acid oxidation and the possible effects of acetoacetyl-CoA on the hydration of long and intermediate chain enoyl-CoA substrates. It should be noted that crotonyl-CoA and $\beta$-hydroxybutyryl-CoA also

![Fig. 12. The regulation of $\beta$ oxidation. The proposed regulatory role of crotonase as a consequence of its cascading substrate specificity and sensitivity to feedback inhibition is indicated at different stages of fatty acid oxidation.](http://www.jbc.org/Downloadedfrom)

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Fig. 12: The regulation of $\beta$ oxidation. The proposed regulatory role of crotonase as a consequence of its cascading substrate specificity and sensitivity to feedback inhibition is indicated at different stages of fatty acid oxidation.
inhibit hydration of longer chain enoyl-CoA substrates (Fig. 4) about as well as acetoacetyl-CoA. Were these CoA intermediates to accumulate to an appreciable extent, then there would be an enhancement of the inhibition of hydration of longer chain enoyl-CoA substrates by acetoacetyl-CoA alone.

Although additional studies will be required to assess whether acetoacetyl-CoA aids in regulation of fatty acid oxidation through its inhibitory action on crotonase, several factors are consistent with this view. First, the rate of oxidation of palmitate by heart and liver mitochondria is depressed by acetoacetyl-CoA, but the rates of butyrate and octanoate oxidation are unaffected (Figs. 8, 9, and 10). It was impossible to assess whether the acetoacetyl-CoA was indeed inhibiting oxidation at the level of the crotonase-catalyzed step in the mitochondria, but the effects observed are in qualitative agreement with the proposed regulatory scheme (Fig. 12). Secondly, acetoacetyl-CoA is an end product of fatty acid oxidation and its inhibitory effects may be considered as a type of feedback inhibition as found in other regulatory processes in metabolism. Third, the concentration of acetoacetyl-CoA required to inhibit hydration of enoyl-CoA substrates is very low. It can be considered a transition state analog inhibitor of crotonase since its $K_I$ is about 10 times less than the $K_m$ for crotonyl-CoA and about 500 times less than the $K_m$ for hexadecenoyl-CoA. Although the normal intramitochondrial concentration of acetoacetyl-CoA is unknown, its inhibitory effects on oxidation of palmitate are readily apparent when intact mitochondria are exposed to levels as low as 0.1 mM (Fig. 9). It is reasonable to assume that the intramitochondrial concentrations may well be lower than this in the experiments shown in Figs. 8 to 10 and are unlikely to exceed the concentrations of palmitate. Finally, the scheme proposed in Fig. 12 could also possibly account for the fact that fatty acids of intermediate chain length do not accumulate in mitochondria. The rate of oxidation of long chain (greater than C14) substrates will be controlled at the crotonase step in the presence of acetoacetyl-CoA. This could limit entry of long chain fatty acids into the oxidation pathway. But once fatty acid CoA intermediates shorter than C14 are formed, they could be expected to be degraded at a steady state. Further studies will be required to test these points.

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