Enoyl Coenzyme A Hydratase (Crotonase)

CATALYTIC PROPERTIES OF CROTONASE AND ITS POSSIBLE REGULATORY ROLE IN FATTY ACID OXIDATION*

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ROBERT M. WATERSON† AND ROBERT L. HILL
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

SUMMARY

The substrate specificity of bovine liver crotonase has been examined with seven Δ^3-1-trans-enoyl-CoA substrates, containing an even number of carbon atoms. The Vmax for this series decreases progressively from a value of about 340,000 moles per min per mole of enzyme for crotonyl-CoA, the C3 derivative, to 2,300 for the C6 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 × 10^-4 M, a value about ten times lower than the K_m for the best substrate, crotonoyl-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 β 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.

which catalyzes the reversible stereospecific hydration of Δ^3-trans-enoyl-CoA substrates to the corresponding L(+)-β-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 Δ^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

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

Crotonase A, acetyl-CoA, acetoacetyl-CoA, and DPN were obtained from P and L Laboratories. Tetrahydrofuran (Malinkrodt) was distilled fresh daily over sodium borohydride. Crotonic anhydride and ethyl chloroformate were products of Eastman. Pure trans-a,β-unsaturated free fatty acids from 6 to 16 carbon atoms in length were a gift from Dr. Salah Wakil. Bovine serum albumin, ATP, and dl-carnitine were obtained from Sigma.

L-14C-Labeled butyric, octanoic, and palmitic acids were obtained from New England Nuclear. Unlabeled butyric and octanoic acids were obtained from Sigma, and palmitic acid was a product of the Hormel Institute. Labeled acids were diluted with unlabeled material to achieve a specific activity of approximately 1 μCi per μmole. Palmitic acid was solubilized by the addition of a small amount of ammonium hydroxide and heating as reported earlier (5).

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 trans-Δ^3-enoyl-CoA substrates were prepared by the mixed anhydride method of Goldschmidt and Vaegler (7). The Δ^3-enolic acid (35 μmoles) and triethylamine (35 μmoles) were mixed with 1.5 ml of tetrahydrofuran. After 10 min 35 μmoles 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 N_2 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 3 with 10% perchloric acid and most of the tetrahydrofuran was removed by evaporation. The C_10 to C_18 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 C_4 to C_6 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—Heavy fast heart mitochondria were a gift from Dr. Sallie Wakil; they were obtained from the Institute for Enzyme Research, University of Wisconsin, and were kept frozen at -15° 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). Rate weighing from 150 to 200 g were decapsulated, and the livers were removed and placed in cold 0.25 M sucrose (Mann, special enzyme grade). All subsequent preparative procedures were carried out at 0-4°. The liver was cut into small pieces and washed several times with cold 0.25 M sucrose until the wash solution remained clear. The minced material was then put through a previously chilled garlic press to remove fibrous tissue and collected in 0.25 M sucrose (10 ml per g). This extract was gently homogenized (3 to 4 passes) and centrifuged at 1200 × g for 2 to 4 min at 0°. The supernatant was added to the assay mixture and the reaction was initiated by the addition of DPN. The reaction was terminated by the addition of perchloric acid.

Measurement of Hydrase Activity—The activity of crotonase with each enoyl-CoA substrate was determined spectrophotometrically at 25° and 1 cm reaction cuvette. The reactions were carried out in a water-jacketed cell holder maintained at 25°. The concentration of acetoacetyl-CoA was measured spectrophotometrically at 260 nm (pH 7) assuming the molar extinction coefficient (ε_260) of 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 Friedman (16). Reactions were performed at 37° with shaking in 25-ml flasks sealed with Kontes serum stoppers fitted with polyethylene center-well inserts. The cups were suspended at a level below that of the water level of the bath to minimize condensation effects.

Reaction mixtures were composed of Krebs-Ringer phosphate (0.2 ml of a fresh, five times aerated solution), 20 mg of bovine serum albumin, 10 μmoles of ATP, 6.25 μmoles of DPN, 4 μmoles of dl-carnitine, 1 μmole of coenzyme A, 100 μmoles of 1-C fatty acid substrate and varying amounts of mitochondria (10 to 20 mg of protein) per 1.0 ml. Reaction was initiated by injection of the mitochondrial solutions into the system and subsequently terminated by the injection of 0.5 ml of 2 × HSO_4 into the reaction mixture. Following acidification 0.2 ml of 10-X hyamine hydroxide (Packard) was injected into the cup insert. The ^{14}C_0 derived from fatty acid oxidation was then collected for subsequent liquid scintillation measurements by shaking for 1 hour at 37°. Control assays were carried out in similar fashion without the addition of mitochondria. The plastic inserts were removed following CO_2 trapping, wiped carefully, and placed in 10 ml of scintillation fluid (4 g of 2,5-diphenyl oxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene per liter of toluene) and counted in a Packard model 3520 Tri-Carb liquid scintillation counter.

RESULTS

Action of Crotonase on Δ^3-Enoyl-CoA Substrates—Fig. 1 shows the relationship between the initial velocity and the substrate concentration for seven Δ^3-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 V_max and K_m values for each substrate were calculated from double reciprocal plots and are summarized in Table I. The relationships between chain length of the substrates and these kinetic parameters are shown in Fig. 2. It is noteworthy that V_max decreases regularly with increasing chain length from C_4 to C_18. The K_m value for crotonyl-CoA is about 10 to 20 times smaller than the K_m 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 \times 10^{-5} M Tris-Cl, pH 7.5, 5 \times 10^{-7} M egg albumin, 5 \times 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_i through C_{16} by the appropriate curve.

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.

<table>
<thead>
<tr>
<th>\Delta^2-trans-Enoyl-CoA substrate (chain length)</th>
<th>( K_m ) (moles/liter)</th>
<th>( V_{max} ) (moles/min/mole enzyme)</th>
<th>Relative rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crotonyl-CoA (C_4)</td>
<td>2.0 \times 10^{-5}</td>
<td>340,000</td>
<td>148</td>
</tr>
<tr>
<td>Hexenoyl-CoA (C_6)</td>
<td>2.4 \times 10^{-4}</td>
<td>151,000</td>
<td>67</td>
</tr>
<tr>
<td>Octenoyl-CoA (C_8)</td>
<td>2.8 \times 10^{-4}</td>
<td>83,500</td>
<td>36</td>
</tr>
<tr>
<td>Decenoyl-CoA (C_{10})</td>
<td>3.0 \times 10^{-4}</td>
<td>88,000</td>
<td>17</td>
</tr>
<tr>
<td>Dodecenoyl-CoA (C_{12})</td>
<td>4.0 \times 10^{-4}</td>
<td>16,000</td>
<td>7</td>
</tr>
<tr>
<td>Tetradecenoyl-CoA (C_{14})</td>
<td>4.2 \times 10^{-4}</td>
<td>5,000</td>
<td>2</td>
</tr>
<tr>
<td>Hexadecenoyl-CoA (C_{16})</td>
<td>5.0 \times 10^{-4}</td>
<td>2,300</td>
<td>1</td>
</tr>
</tbody>
</table>

more carbon atoms, although the \( K_m \) values for the C_6 to C_{16} 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-CoA (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 \( K_m \) 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. Acetoacetyl-CoA was found to be the only good inhibitor for crotonase, although neither ethyl acetoacetate nor acetoacetate were inhibitory. As shown in Fig. 3, acetoacetyl-CoA is an effective competitive inhibitor for crotonase with crotonyl-CoA as substrate. The apparent \( K_s \) calculated from these data is 3 \times 10^{-5} M, although, as noted below, the enolate species of acetoacetyl-CoA is the actual inhibitory species and the \( K_s \) for the enolate is about 25 times smaller. As expected, the same \( K_s \) value was obtained for acetoacyl-
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°C, 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 the reaction as calculated from these data is $0.7 \times 10^{-6}$ 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 butyrate, 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^{-4} M. Each mixture contained 14.15 mg of mitochondrial protein. The initial concentration of acetoacetyl-CoA, when present, was 10^{-5} 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 nmol of palmitate (210,000 cpm) per reaction mixture. The initial concentration of palmitoyl-CoA was 10^{-4} M and those for acetoacetyl-CoA as indicated. Incubation was for 60 min.

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 Oxida-
tion—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 14C-CO₂ 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 Δ^3 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 Δ^3, 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, 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 C4 to C16, 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, $\beta$-hydroxyacyl dehydrogenase (24) and thiolase (25) act equally well on long or short chained CoA derivatives and if micelles were formed with substrates for these enzymes, they had little apparent effect on kinetics of the enzymes.

**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 acetoacetil-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 evidence for cooperativity in the binding of acetoacetyl-CoA, in accord with the fact that crotonase has equivalent activities in crude extracts (17).

**Crotonase and Acetoacetyl-CoA in Regulation of Fatty Acid Oxidation**—When the substrate specificity of crotonase and the inhibitory effects of acetoacetyl-CoA on crotonase are considered in view of the properties of the other enzymes in fatty acid oxidation, it is evident that crotonase and acetoacyt-CoA may play an important role in the regulation of fatty acid oxidation. A consideration of the properties of the enzymes in fatty acid oxidation which are listed in Table II illustrates this point. As noted earlier (17), and confirmed in these studies, crotonase appears to be the only hydratase of fatty acid oxidation. In contrast, two of the other four enzymes, the thiolases and the acetyl-CoA dehydrogenases exist in multiple forms, each form having different chain length specificities but with equivalent optimal activities. The other two enzymes, the $\beta$-hydroxyacyl dehydrogenase and thiolase, exist as a single species just as crotonase, but, unlike crotonase, they act almost equally as well on all substrates irrespective of chain length. Thus, crotonase is unique among the enzymes of fatty acid oxidation because its rate of hydration of substrates differing in chain length from C4 to C16 varies about 100-fold. At present the other enzymes in fatty acid oxidation, including the electron transport flavoprotein, are not recognized to possess regulatory properties as evidenced by their catalytic behavior or sensitivity to metabolites. Finally, the uniqueness of crotonase in fatty acid oxidation is most evident when 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 dashes. These numbers are from the references listed in Fig. 11. The turnover of fatty acyl-CoA intermediates in liver.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>$V_{\text{max}}$ as a function of acyl-CoA chain length, C1: C2: C3: C4: C5: C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acyl thiokinases</td>
<td>Uniform*</td>
</tr>
<tr>
<td>Acyl-CoA dehydrogenases</td>
<td>Uniform*</td>
</tr>
<tr>
<td>$\beta$-Hydroxyacyl-CoA dehydrogenase</td>
<td>1:1:1:1:1:1</td>
</tr>
<tr>
<td>$\beta$-Ketoacyl-CoA thiolase</td>
<td>1:1:1:1:1:1:1</td>
</tr>
</tbody>
</table>

*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).
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 \( C_{12} \) 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 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 \( C_{12} \), 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 \( C_{16} \) 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

![Diagram of fatty acid oxidation](http://www.jbc.org/Downloadedfrom http://www.jbc.org)
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 C16) 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 C16 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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