Purification and Characterization of Fatty Acid β-Oxidation Enzymes from *Caulobacter crescentus*

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Acetoacetyl coenzyme A (acetoacetyl-CoA) thiolase, an enzyme required for short-chain fatty acid degradation, has been purified to near homogeneity from *Caulobacter crescentus*. The relative heat stability of this enzyme allowed it to be separated from β-ketoacyl-CoA thiolase. The purification scheme minus the heating step also permitted the copurification of crotonase and 3-hydroxyacyl-CoA dehydrogenase. These activities are in a multienzyme complex in *Escherichia coli*, but a similar complex was not observed in *C. crescentus*. Instead, separate proteins differing in enzymatic activity were detected, analogous to the β-oxidation enzymes that have been isolated from *Clostridium acetobutylicum* and from mitochondria of higher eucaryotes. In these cells, as appears to be the case with *C. crescentus*, the individual enzymes form multimers of identical subunits.

Caulobacter crescentus is a gram-negative bacterium that exhibits a sequence of morphological changes at the cell surface during each cell cycle (27). These events involve the temporally and spatially defined biogenesis of a single flagellum, a stalk, pili, and DNA bacteriophage receptors. Several of the protein components of these surface structures are membrane associated and are targeted to specific cell surface locations. Because portions of the cell membrane are retained from one cell cycle to another, the possibility exists that the structure and biosynthesis of the membrane are involved in the positioning of these proteins. To determine whether the cell membrane has a role in this process, we are investigating membrane lipid metabolism.

The phospholipid composition of the *C. crescentus* membrane is unusual in that it is predominantly composed of phosphotidylglycerol and cardiolipin (4). Neither phosphotidylethanolamine nor phosphotidylserine is synthesized by *C. crescentus* (4). The fatty acid composition is a mixture of saturated and monounsaturated 16- and 18-carbon fatty acids (15). These fatty acids are synthesized by an anaerobic pathway in a manner similar to that in *Escherichia coli* (15). The biogenesis of the membrane is being studied in wild-type cells and in mutants altered in phospholipid (3) and fatty acid (10, 11) synthesis. Some of these mutants are auxotrophic for glycerol-3-phosphate and lack glycerol-3-phosphate dehydrogenase activity (3); others require oleic acid for growth (10, 11). A common feature of these mutants is that they all block membrane lipid synthesis in the absence of supplement, coincident with the disruption of surface differentiation events.

To determine how membrane lipid turnover might contribute to the expression of cell surface differentiation events in *C. crescentus*, we have begun an investigation of both long- and short-chain fatty acid catabolism in this organism (22). Long-chain fatty acids are transported into the cell and degraded by constitutive β-oxidation enzymes. Five enzymes in the long-chain fatty acid β-oxidation pathway have been identified in *C. crescentus* (22). These include acyl

coenzyme A (acyl-CoA) synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase (referred to here as crotonase), 3-hydroxyacyl-CoA dehydrogenase, and β-ketoacyl-CoA thiolase. Long-chain fatty acids are degraded by these five β-oxidation enzymes in *E. coli* (Fig. 1). Short-chain fatty acid catabolism in *E. coli* is known to require three β-oxidation enzymes (crotonase, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA dehydrogenase) as well as two additional enzymes involved in the degradation of β-keto short-chain fatty acids (acetyl-CoA transferase and acetoacetyl-CoA thiolase) (Fig. 2; 5, 12, 20, 24, 29).

We demonstrate here that there are two distinct thiolases in *C. crescentus*: β-ketoacyl-CoA thiolase, which utilizes long-chain fatty acids, and acetoacetyl-CoA thiolase, which is specific for β-keto short-chain fatty acid substrates. We describe the purification of the *C. crescentus* acetoacetyl-CoA thiolase. The addition of a heat denaturation step enabled the separation of a heat-labile β-ketoacyl-CoA thiolase from a heat-stable acetoacetyl-CoA thiolase, which was further purified.

Crotonase and 3-hydroxyacyl-CoA dehydrogenase were also purified by a similar procedure. Although these two enzymes copurify, each activity could be attributed to a separate protein. Therefore, *C. crescentus* differs from *E. coli* (1, 25, 26, 28), in which both of these enzyme activities are attributed to a single polypeptide.

MATERIALS AND METHODS

Materials. [^{14}C]oleic acid (53.8 mCi/mmol) was obtained from Dupont, NEN Research Products. Oleic acid, silver nitrate, and sucrose were obtained from Fisher Scientific Co. Crotonyl-CoA, acetoacetyl-CoA, Sephacryl S-200, and proteins for molecular weight standards (ovalbumin, bovine serum albumin [BSA], malate dehydrogenase, and lactate dehydrogenase) were obtained from Pharmacia, Inc. Cytochrome *c* was obtained from Schwarz/Mann, and *C. crescentus* flagellin was kindly provided by Sandra Reuter, of our laboratory. ATP, NADH, CoA, catalase, and ammonium sulfate were obtained from Boehringer Mannheim Biochemicals. Sodium dodecyl sulfate was obtained from Pierce Chemical Co. Acrylamide, *N,N*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and Coo-

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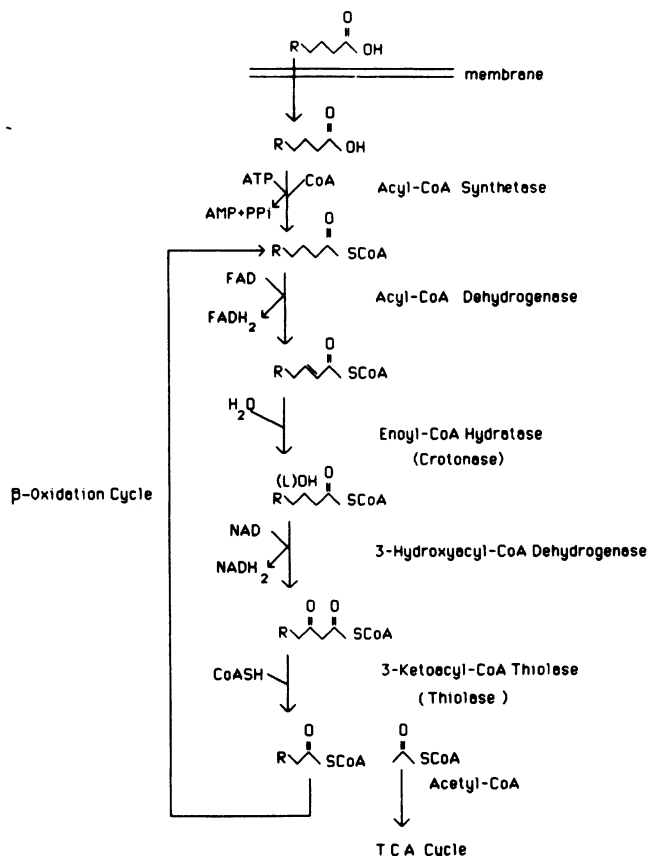† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

FIG. 1. Schematic of the enzymatic pathway for fatty acid β oxidation. Abbreviations: SCoA, S-CoA; CoASH, CoA-SH; TCA, tricarboxylic acid.

FIG. 2. Schematic of the pathway for short-chain fatty acid degradation (20). Abbreviations are as for Fig. 1.

massie brilliant blue R-250 were supplied by Bio-Rad Laboratories. Triton X-100 was obtained from Amersham Corp. Glutaraldehyde was obtained from Eastman Kodak Co. Palmitoyl-CoA and all other reagent-grade chemicals were obtained from Sigma Chemical Co. β-Keto-octanoyl-CoA was kindly provided by H. Schulz.

Enzyme assays. Acyl-CoA synthetase was assayed as described by Kameda and Nunn (13), using $[1-^{14}C]$oleic acid as the substrate. Acyl-CoA dehydrogenase was assayed as described by Binstock et al. (1), with palmityl-CoA as the substrate. The role of crotonase in the β oxidation of fatty acids is the stereospecific hydration of $trans$-β-unsaturated fatty acyl-CoA derivatives to the 3-hydroxyacyl-CoA form (Fig. 1). Crotonase (enoyl-CoA hydratase) activity was measured by monitoring the decrease in A_{263} due to the hydration of the Δ2,3 double bond of the substrate crotonyl-CoA, as described by Binstock and Schulz (2). 3-Hydroxyacyl-CoA dehydrogenase catalyzes the oxidation of the fatty acid 3-hydroxyacyl-CoA form that had been hydrated by crotonase in the previous step (Fig. 1). The 3-hydroxyacyl-CoA dehydrogenase activity was assayed by using a short-chain substrate, acetoacyl-CoA, and measuring its reduction by NADH, the reverse of the reaction in vivo (32). 3-Ketoacyl-CoA thiolase catalyzes the introduction of a second CoA group, causing the cleavage of acetyl-CoA from the remainder of the fatty acid (Fig. 1). This enzyme was assayed by using β-keto-octanoyl-CoA as the substrate and measuring the decrease in A_{303}. 3-Ketoacyl-CoA thiolase was assayed as described by Feigenbaum and Schulz (6)
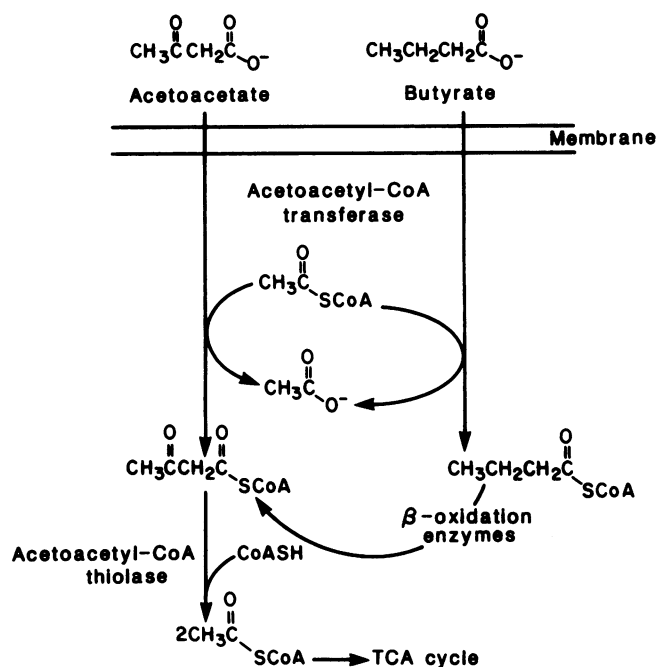
except that N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer was substituted for Tris. Acetoacetyl-CoA thiolase was also assayed by measuring the decrease in A_{303}, using acetoacetyl-CoA as the substrate, as described by Feigenbaum and Schulz (6).

Purification of acetoacetyl-CoA thiolase, crotonase, and 3-hydroxyacyl-CoA dehydrogenase. Cultures of $C.$ $crescentus$ SC451 (pro) were grown in minimal medium (15) supplemented with 1 mM proline and 1 mM oleic acid. Cultures were harvested in early log phase by centrifugation at 15,000 × g for 10 min. The cells were kept frozen at −20°C until use. During the enzyme purification, all manipulations were carried out at 4°C except for the FPLC (fast protein liquid chromatography) columns, which were run at room temperature. The frozen cells (7.5 g) were suspended in 21 ml of 10 mM KH_2PO_4 (pH 7.5), and the cells were disrupted in a French press as described by Maloy et al. (17). The disrupted cells were centrifuged at 15,000 × g for 30 min, and the supernatant was saved. DNase I (0.3 mg/ml) was added to the supernatant, and the mixture was incubated for 50 min on ice and then for 10 min at 37°C. For purification of acetoacetyl-CoA thiolase, the DNase I-treated cell extract was incubated for 5 min at 63°C. This heat inactivation step was omitted for purification of crotonase and 3-hydroxyacyl-CoA dehydrogenase. After this step, the two extracts were processed in the same manner. The extracts were centrifuged at 100,000 × g at 4°C for 1 h. Ammonium sulfate (3.4 g) was added to the supernatant (14 ml) to 40% saturation. After being mixed for 15 min on ice, the mixture was centrifuged at 5,000 × g for 15 min, and the precipitate was dissolved in buffer A (20 mM KH_2PO_4 [pH 7.5], 1 mM dithiothreitol [DTT], 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine). The supernatant (13 ml) was mixed with 2.67 g of ammonium sulfate (40 to 70% saturated), stirred for 15 min on ice, and centrifuged at 5,000 × g for 15 min. The resulting precipitate was dissolved in 1 ml of buffer A and dialyzed overnight against 1 liter of

TABLE 1. Purification of acetoacetyl-CoA thiolase

Step	Total U (nmol/min)	Yield (%)	Sp act (nmol/min per mg)	Fold purification
Crude extract	113,609	100	728	1
DNase	106,440	94	689	1
63°C + high spin	55,028	48	1,763	3
Ammonium sulfate (40–70%)	41,880	37	5,289	7
Sephacryl S-200	20,621	18	10,286	14
Superose 6	5,176	5	14,223	20
Mono Q	1,639	2	40,975	56

TABLE 3. Ammonium sulfate fractionation of β-oxidation enzymes in extracts[a] of *C. crescentus*

Ammonium sulfate fraction	Activity (nmol/min per mg)				
	Acyl-CoA synthetase	Acyl-CoA dehydrogenase	Crotonase	3-HOADH[b]	Acetoacetyl-CoA thiolase
0–40	57.0	53	1,363	249	174
40–70	13.6	11	7,318	568	2,958
>70	1.4	<1.0	2,146	188	473

[a] A DNase-treated crude cell extract was fractionated by ammonium sulfate precipitation as described in Materials and Methods.

[b] 3-HOADH, 3-Hydroxyacyl-CoA dehydrogenase.

buffer A. The dialysate was concentrated (Immersible CX-10; Millipore Corp.) to 0.5 ml and applied to a Sephacryl S-200 column (62 by 1.5 cm) preequilibrated with buffer A. The enzymes were then eluted with buffer A. Crotonase and 3-hydroxyacyl-CoA dehydrogenase enzyme activities coeluted in a broad peak, and the fractions with the highest specific activities were pooled. The combined eluate (7 ml) was dialyzed overnight against 1 liter of buffer B (50 mM KH$_2$PO$_4$ [pH 7.5], 50 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM PMSF, 2 mM benzamidine). The dialysate was concentrated to 0.25 ml and applied to a Pharmacia Superose 6 gel permeation column that had been preequilibrated with buffer B, and the enzymes were eluted with buffer B. The flow rate was 0.5 ml/min, and 0.25-ml fractions were collected. The enzyme activities were separated on this gel permeation column. This permitted the calculation of the Stokes radius for crotonase and 3-hydroxyacyl-CoA dehydrogenase. The fractions with the highest specific activities for each of the three enzymes were pooled (2.5 ml) and dialyzed overnight against 1 liter of buffer C (25 mM HEPES [pH 7.5], 10 mM MgCl$_2$, 1 mM DTT, 2 mM benzamidine, 1 mM PMSF, 5% glycerol). The dialysate (2.5 ml) was then applied to a Pharmacia Mono Q column (anion exchanger) that had been equilibrated with buffer C and washed with buffer D (buffer C containing 1 mM NaCl). Enzymes were eluted by applying a shallow 20-ml gradient to 100 mM NaCl, followed by a 10-ml gradient of 120 to 200 mM NaCl. The flow rate was 1 ml/min, and 0.5-ml fractions were collected. The elution profile of the enzymes from the Mono Q column is shown in Fig. 5. The purification procedures for the acetoacetyl-CoA thiolase and for crotonase and 3-hydroxyacyl-CoA dehydrogenase are summarized in Tables 1 and 2.

Protein concentrations were determined by either the procedure of Lowry et al. (16) or the Bio-Rad protein assay. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis, using the buffer system of Laemmli (14) and slab

gels of 10% polyacrylamide with a 3% polyacrylamide stacking gel. After electrophoresis, the gels were stained with silver by the procedure of Morrissey (19).

Sucrose gradient centrifugation. Linear 5 to 20% sucrose gradients (4.8 ml) were prepared in buffer A as described by Martin and Ames (18). Samples (0.15 ml) of both crude cell extracts and purified enzyme fractions were mixed with protein standards and layered onto the sucrose gradients. The protein standards catalase ($s_{20,w}$ = 11.3S), lactic dehydrogenase ($s_{20,w}$ = 7.3S) malate dehydrogenase ($s_{20,w}$ = 4.3S), and cytochrome c ($s_{20,w}$ = 1.7S) were assayed as described by Haga et al. (8). Centrifugation was carried out at 45,000 rpm for 20 h at 4°C in a Beckman SW50.1 rotor. Fractions (0.14 ml) were collected, and the positions of the protein standards and the β-oxidation enzymes were determined by assays for the individual enzyme activities. The position of purified acetoacetyl-CoA thiolase was determined by SDS-polyacrylamide gel electrophoresis because the purified enzyme lost 90% of its activity when applied to a sucrose gradient and could not be assayed directly.

RESULTS

Purification of acetoacetyl-CoA thiolase. The five enzymes involved in fatty acid β oxidation were assayed after ammonium sulfate fractionation of a crude extract to determine whether they could be separated early in the purification procedure. The acyl-CoA synthetase and acyl-CoA dehydrogenase activities were predominantly in the 0 to 40% ammonium sulfate fraction, whereas the acetoacetyl-CoA thiolase, crotonase, and 3-hydroxyacyl-CoA dehydrogenase activities were predominantly in the 40 to 70% ammonium sulfate fraction (Table 3).

E. coli contains two thiolases for the degradation of β-keto fatty acids: an acetoacetyl-CoA thiolase specific for short-

TABLE 2. Purification of crotonase and 3-hydroxyacyl-CoA dehydrogenase

Step	Crotonase				3-Hydroxyacyl-CoA dehydrogenase			
	Total U (nmol/min)	Yield (%)	Sp act (nmol/min per mg)	Fold purification	Total U (nmol/min)	Yield (%)	Sp act (nmol/min per mg)	Fold purification
Crude extract	774,080	100	1,832		87,420	100	217	
DNase	774,080	100	1,878	1	87,420	100	219	1
High-speed spin	774,080	100	3,665	2	87,420	100	470	2
Ammonium sulfate (40–70%)	436,620	56	7,660	4	46,657	53	483	2
Sephacryl S-200	282,258	36	14,651	8	16,929	19	876	4
Superose 6	72,500	9	40,277	22	3,325	3	1,847	9
Mono Q	36,840	5	182,202	100	1,317	1.5	9,811	45

TABLE 4. Effect of heat on thiolase specific activity with short-
and long-chain fatty acid substrates

Sample	Activity (nmol/min per mg)	
	Acetoacetyl-CoA	β-Keto-octanoyl-CoA
Crude extract	217	562
DNase	209	551
Heat (63°C)	222	<5
High-speed spin	500	<5

chain fatty acids and a 3-ketoacyl-CoA thiolase that de-
grades medium- and long-chain fatty acids (5, 6). Two
separate thiolase activities were detected in C. crescentus
when partially purified extracts of C. crescentus were heated
to 63°C for 5 min and then assayed by using both short- and
long-chain fatty acids as substrates (Table 4). The heating
step eliminated 3-ketoacyl-CoA thiolase activity from the
extract. The heat-stable acetoacetyl-CoA thiolase was then
purified to apparent homogeneity by the procedure shown in
Table 1.

After the ammonium sulfate fractionation, the 40 to 70%
saturated fraction was dialyzed and concentrated as de-
scribed in Materials and Methods. Two gel permeation
columns (Sephacryl S-200 and Superose 6) were used in
tandem to further purify the enzyme. The final step in the
purification of acetoacetyl-CoA thiolase involved high-reso-
lution anion-exchange chromatography on a Pharmacia
Mono Q column eluting with a shallow NaCl gradient. The
acetoacetyl-CoA thiolase activity eluted from the column at
an NaCl concentration of 175 mM. Acetoacetyl-CoA thio-
lase was purified 56-fold by this procedure. Although the fold
purification is not high, analytical SDS-polyacrylamide gel
electrophoresis of the Mono Q fraction of acetoacetyl-CoA
thiolase with (Fig. 3B) and without (Fig. 3A) heat treatment
showed that the enzyme from heated cell extracts had been
purified to near homogeneity. The C. crescentus ace-
toacetyl-CoA thiolase has an apparent subunit molecular
weight of 45,000.

**Purification of crotonase and 3-hydroxyacyl-CoA dehydro-
genase.** Pawar and Schulz (25) have presented evidence that
crotonase and 3-hydroxyacyl-CoA dehydrogenase are part

of a single multifunctional polypeptide. Crotonase isolated
from Clostridium acetobutylicum, on the other hand, has
been shown to be a single polypeptide that forms a tetramer
of identical subunits (31). Also, both enzyme activities have
been attributed to separate proteins in the mitochondria of
higher organisms (7, 9, 30). Therefore, further purification of
these two activities from C. crescentus cell extracts was
carried out in an attempt to determine whether they are
separate proteins.

The procedure used to purify acetoacetyl-CoA thiolase
was used to purify crotonase and 3-hydroxyacyl-CoA dehy-
drogenase except that the heating step was omitted (Table
2). Crotonase and 3-hydroxyacyl-CoA dehydrogenase co-
eluted from a Sephacryl S-200 column, but the enzymes
eluted separately from the Superose 6 column. The elution
profiles of crotonase and 3-hydroxyacyl-CoA dehydrogenase
allow us to calculate the Stokes radius for both enzymes
(Fig. 4). The Superose 6 column was calibrated with stan-
dard proteins before the application of the Sephacryl S-200
fraction, as described in Materials and Methods. Crotonase
had a Stokes radius of 2.7 nm, and the value for 3-hydroxy-
acyl-CoA dehydrogenase was 2.2 nm. These small values
imply that unless the enzymes either have a peculiar shape
that retards them on the Superose 6 column or interact with
the column matrix, they should have a molecular mass no
larger than that of BSA (68 kilodaltons [kDa]), which was the
first protein standard to elute from the column. The separa-
tion of these enzyme activities on the Pharmacia Superose 6
column provided the first evidence that they are separate
polypeptides.

Anion-exchange chromatography on a Pharmacia Mono Q
column of pooled peak fractions from the Superose 6 column
separated acetoacetyl-CoA thiolase activity (eluted with an
NaCl concentration of 175 mM) from the crotonase and
3-hydroxyacyl-CoA dehydrogenase activities that coeluted
with 110 mM NaCl (Fig. 5). Analytical SDS-polyacrylamide
gel electrophoresis of the fraction containing the crotonase
and 3-hydroxyacyl-CoA dehydrogenase activities revealed
fewer than five major protein bands. None of these, how-
ever, comigrated with the 78-kDa component of the E. coli
β-oxidation complex that had been purified by the procedure
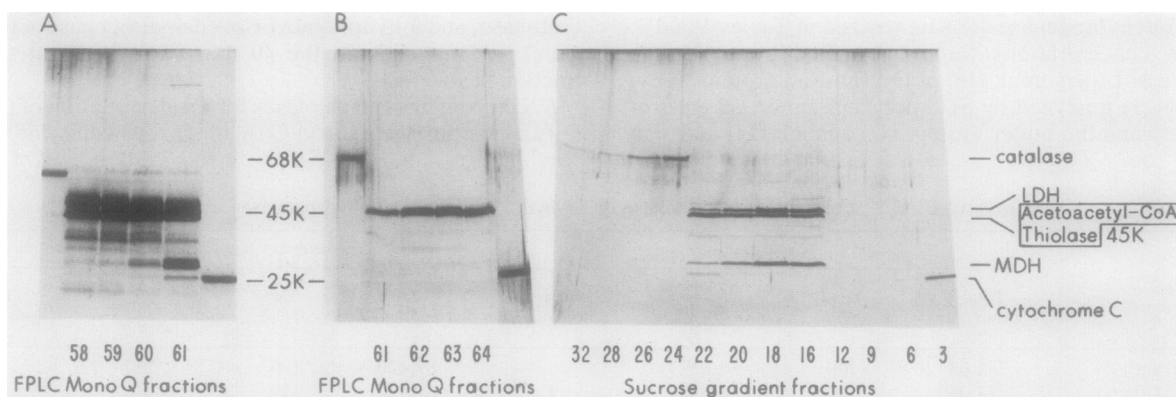of Feigenbaum and Schulz (6) (data not shown). The E. coli

FIG. 3. SDS-polyacrylamide gel electrophoresis of acetoacetyl-CoA thiolase after Mono Q column chromatography and sucrose density
gradient centrifugation. (A) Acetoacetyl-CoA thiolase purified from unheated cell extracts; (B) acetoacetyl-CoA thiolase purified from heated
cell extracts (Table 1). The internal protein standards in panels A and B were BSA (left lane) and flagellin (right lane), respectively. (C)
Sucrose density gradient fractions of the Mono Q-purified acetoacetyl-CoA thiolase from heated cell extracts combined with four internal
protein standards: catalase, lactate dehydrogenase, malate dehydrogenase, and cytochrome c. The gels were silver stained by the procedure
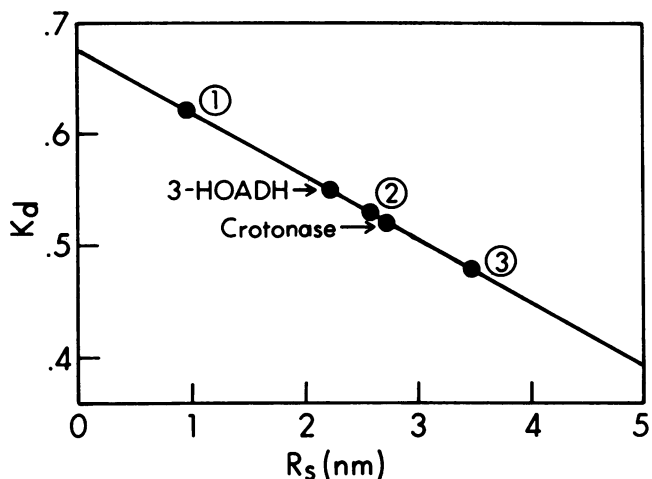of Morrissey (19).

FIG. 4. Distribution coefficient (K_d) versus Stokes radius for crotonase and 3-hydroxyacyl-CoA dehydrogenase (3-HOADH) based on the standard curves for cytochrome c (1), ovalbumin (2), and BSA (3). The K_d values were obtained by determining the elution rate of the two β-oxidation enzyme activities and the standard proteins from a high-resolution Superose 6 gel permeation column as described in Materials and Methods.

78-kDa subunit has been reported to contain both crotonase and 3-hydroxyacyl-CoA dehydrogenase activities (25).

The purification procedure shown in Table 2 resulted in a 100-fold purification of crotonase, with an overall yield of 5%. The enzyme was active over a wide range of pH, from 5 to 10, with optimum activity at pH 8. The crotonase Mono Q fraction required BSA (200 μg/ml) for maximum activity. This fraction retained almost complete activity up to 3 to 4 days after storage at 4°C. The 3-hydroxyacyl-CoA dehydrogenase was purified 45-fold. The enzyme tolerated a broad pH range from 5 to 9 but was inactivated by incubation for 5 min at temperatures above 43°C and was relatively unstable during the purification procedure. This instability contributed to the 1.5% yield.

Sedimentation behavior of the β-oxidation enzymes in crude extracts and in partially purified fractions. We determined the distribution of the fatty acid β-oxidation enzyme activities

and acetoacetyl-CoA thiolase in sucrose density gradients (Fig. 6). The sedimentation patterns of the acyl-CoA synthetase and acyl-CoA dehydrogenase activities in crude extracts were measured in one gradient (Fig. 6A), and those of the 3-hydroxyacyl-CoA dehydrogenase, crotonase, and acetoacetyl-CoA thiolase activities in crude extracts were measured in another (Fig. 6B). The sedimentation pattern of the partially purified Mono Q fraction of crotonase is shown in Fig. 6C. In each gradient, the internal protein standards were cytochrome c, lactate dehydrogenase, and catalase. The Mono Q fraction of acetoacetyl-CoA thiolase was also sedimented in a sucrose density gradient. This highly purified fraction did not contain crotonase activity (Fig. 5). Although the bulk (>90%) of the activity was lost after the purified thiolase was centrifuged through the sucrose gradient, the presence of the enzyme in each fraction was detected by SDS-polyacrylamide gel electrophoresis (Fig. 3C). The crude activity of the acetoacetyl-CoA thiolase (Fig. 6B) exhibited the same sedimentation coefficient, 7.5S, as determined for the purified acetoacetyl-CoA thiolase containing a single 45-kDa protein (Fig. 3C). In crude extracts, three enzyme activities, crotonase, acyl-CoA dehydrogenase, and acetoacetyl-CoA thiolase, were found to cosediment (Fig. 6A and B). Acyl-CoA synthetase and 3-hydroxyacyl-CoA dehydrogenase showed broad peaks of activity but were partially separated from the other three enzyme activities. The 3-hydroxyacyl-CoA dehydrogenase activity consistently exhibited a slowly sedimenting peak and shoulder trailing into the region of the gradient containing crotonase, acyl-CoA dehydrogenase, and acetoacetyl-CoA thiolase activities. Although the 3-hydroxyacyl-CoA dehydrogenase activity was eluted from the Mono Q column with the same salt concentration as did crotonase, the fact that the enzymes could be separated on a high-resolution Superose 6 gel permeation column (Fig. 4) and by sucrose density gradient centrifugation (Fig. 6B) suggests that, unlike the complex of crotonase, β-ketoacyl-CoA thiolase, and 3-hydroxyacyl-CoA dehydrogenase observed in *E. coli* (1, 25, 26), the *C. crescentus* 3-hydroxyacyl-CoA dehydrogenase appears to be a separate protein and not part of a large multifunctional protein.

In crude extracts, crotonase, acyl-CoA dehydrogenase, and acetoacetyl-CoA thiolase cosedimented at an $s_{20,w}$ of
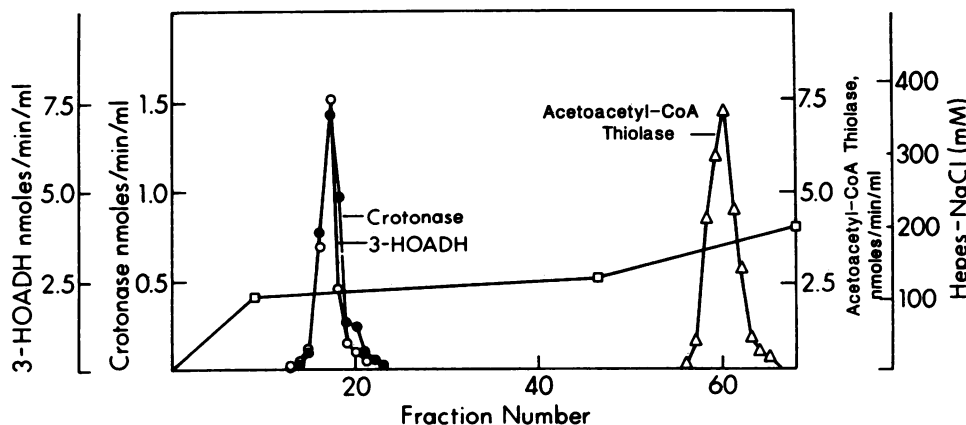

FIG. 5. High-resolution anion-exchange chromatography of β-oxidation enzymes on Pharmacia Mono Q. The enzymes were eluted from a shallow NaCl gradient (□) as described in Materials and Methods. Enzyme activity is expressed as 10^4 nmol/min per ml for crotonase activity (●), 10^2 nmol/min per ml for 3-hydroxyacyl-CoA dehydrogenase (3-HOADH) activity (○), and 10^3 nmol/min per ml for acetoacetyl-CoA thiolase activity from unheated cell extracts (△).
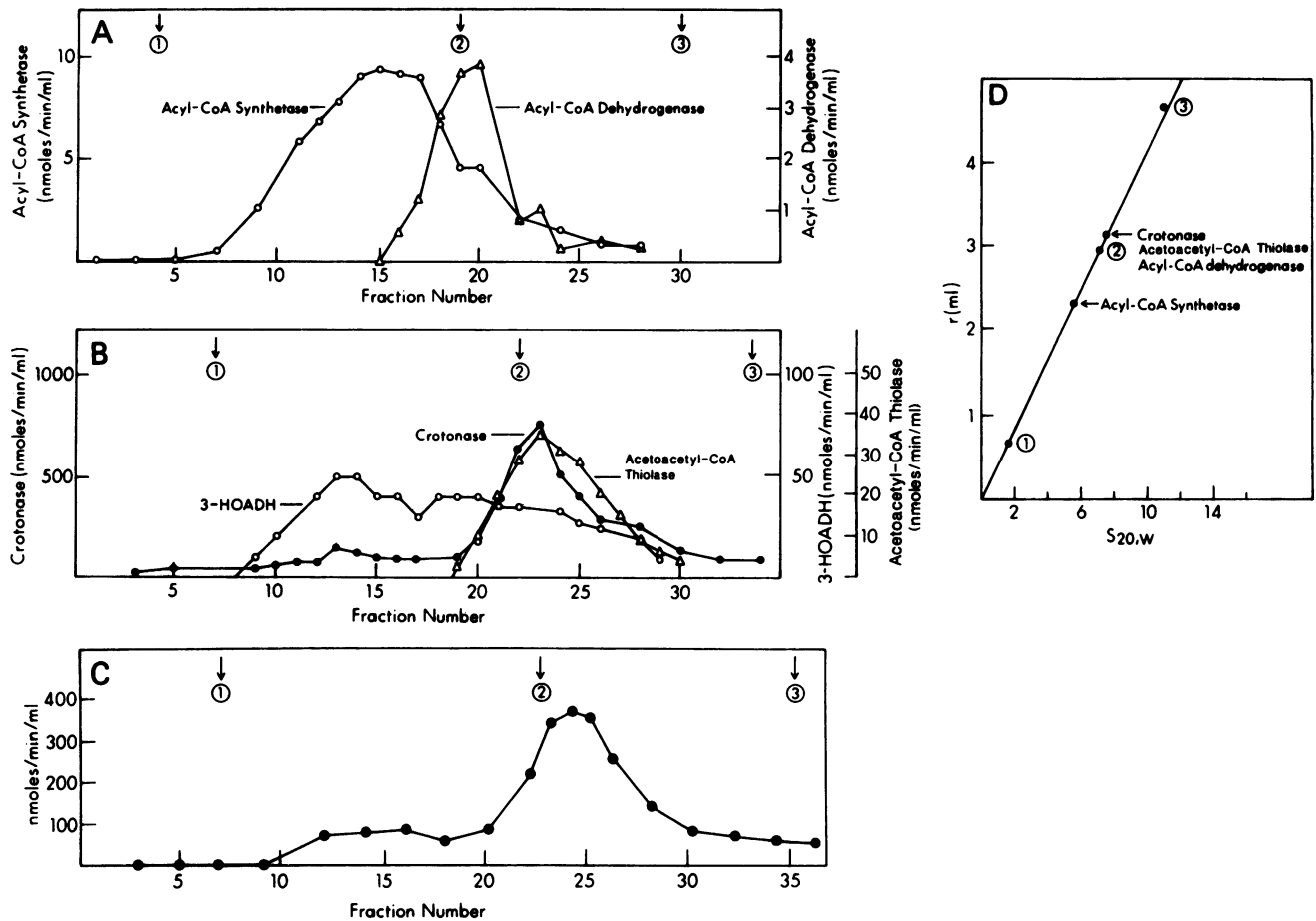
FIG. 6. Sucrose density gradient centrifugation of the fatty acid β-oxidation enzymes in crude cell extracts of *C. crescentus*. Cell extracts were prepared, and centrifugation was carried out in a 5 to 20% sucrose gradient at 45,000 rpm in a Spinco 50Ti rotor for 14 h as described in Materials and Methods. Panels A to C show the results from three separate gradients, each containing the indicated internal protein standards: cytochrome *c* (1), lactate dehydrogenase (2), and catalase (3). (C) Mono Q fraction of crotonase applied to a 5 to 20% sucrose gradient; D, calibration curve. 3-HOADH, 3-hydroxyacyl-CoA dehydrogenase.

approximately 7.5S. Increasing the salt concentration in the sucrose buffer from 10 to 100 mM KH_2PO_4 did not change the sedimentation rates of these three enzyme activities. If all three enzymes act as typical water-soluble globular proteins, then an $s_{20,w}$ of 7.5S should correspond to a Stokes radius of approximately 4.5 nm. As described earlier, crotonase exhibited a Stokes radius of 2.7 nm regardless of whether a crude extract or a partially purified crotonase fraction was subjected to Superose 6 chromatography. This discrepancy between the $s_{20,w}$ and Stokes radius suggests that the enzyme sediments as an aggregate during the sucrose gradient centrifugation and separates into subunits on the gel permeation column.

To determine whether partially purified crotonase can form a complex of identical subunits, the Mono Q-purified fraction of crotonase was centrifuged in a sucrose density gradient (Fig. 6C). The Mono Q fraction of the crotonase had been cleanly separated from the acetoacetyl-CoA thiolase (Fig. 5). The purified crotonase activity, like the crotonase activity in the crude extract (Fig. 6B), had a sedimentation coefficient of approximately 7.5S, suggesting that the crotonase eluted from the Superose 6 column (Fig. 4) could form aggregates.

The sedimentation behavior of both crotonase and acetoacetyl-CoA thiolase was independent of the state of purity

at which they were applied to a sucrose gradient, and their large sedimentation coefficient appears to result from the aggregation of homologous subunits. Other bacterial β-oxidation enzymes have been shown to form aggregates of homologous subunits; crotonase from *Clostridium acetobutylicum* forms a tetramer of four identical subunits (31), and acetoacetyl-CoA thiolase from *E. coli* is also composed of a tetramer of four identical subunits (5).

DISCUSSION

The *C. crescentus* β-oxidation enzymes, acyl-CoA synthetase, acyl-CoA dehydrogenase, crotonase, 3-hydroxyacyl-CoA dehydrogenase, and β-ketoacyl-CoA thiolase, have been identified, and their initial characterization has been described previously (22). The enzymes are constitutively expressed in *C. crescentus* and are not induced by oleic acid. The constitutive levels of expression in *C. crescentus* are significantly higher than the oleic-induced levels in *E. coli*. Enzyme activities are lowered in both organisms upon growth in the presence of glucose. However, the glucose effect is only 2- to 3-fold in *C. crescentus*, compared with 50-fold repression in *E. coli*. Addition of exogenous cyclic AMP to the growth medium reversed this catabolite repression in *C. crescentus*, as it does in *E. coli* (23).

In *E. coli*, the utilization of short-chain fatty acids includes three enzymes involved in long-chain fatty acid degradation (crotonase, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA dehydrogenase) (Fig. 1; 20, 21, 26) and also two enzymes that are specific for the degradation of β-keto short-chain fatty acids (acetyl-CoA transferase and ace-toacetyl-CoA thiolase) (Fig. 2; 6, 20). Both short-chain and long-chain fatty acids were used as substrates by *C. cres-centus* crude extracts. However, after heating of an extract to 63°C, the ability to use the long-chain fatty acid, β-keto-octanoyl-CoA, as a substrate was lost. The short-chain fatty acid activity, acetoacetyl-CoA thiolase, proved to be resistant to heat inactivation and was consequently purified to apparent homogeneity by the procedure described in Table 1. In *E. coli*, both β-ketoacyl-CoA thiolase and ace-toacetyl-CoA thiolase are relatively heat resistant, and this property was used in their purification (6).

The apparent subunit molecular mass of acetoacetyl-CoA thiolase derived from its electrophoretic mobility on SDS-polyacrylamide gel electrophoresis is 45,000 Da (Fig. 3). Although stable in crude extracts, the purified enzyme was very unstable; hence, its sedimentation coefficient in sucrose density gradients could not be determined directly by assay-ing for enzyme activity. Instead, the presence of the purified enzyme was detected in sucrose gradients by SDS-poly-acrylamide gel electrophoresis (Fig. 3). The same sedimen-tation coefficient, 7.5S, was obtained both for the purified enzyme and for the activity in crude extracts. This sedimen-tation coefficient is much larger than predicted from its mobility on SDS-polyacrylamide gels. One explanation is that since acetoacetyl-CoA thiolase sediments slightly ahead of lactate dehydrogenase, a tetrameric enzyme composed of identical 36,500-Da subunits, it is possible that acetoacetyl-CoA thiolase exists as a tetramer composed of 45,000-Da subunits. This is similar in size to acetoacetyl-CoA thiolase purified from *E. coli*, which is a tetrameric protein composed of subunits of 41,500 Da (5). It remains to be determined whether *C. crescentus* uses enzymes besides β-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase for the long- and short-chain fatty acid substrates. If more than one enzyme is present for different-chain-length fatty acids at specific steps of the β-oxidation pathway, it may explain our difficulty in obtaining mutants in the β-oxidation pathway (22). The substrate specificities of these enzymes might overlap and mask a lesion in a specific enzyme.

An attempt was made to purify the *C. crescentus* fatty acid β-oxidation proteins involved in the degradation of long-chain fatty acids by phosphocellulose chromatography at pH 6.5, as was successfully done in *E. coli* (1, 2, 6, 25). However, the β-oxidation enzymes in crude extracts of *C. crescentus* were not retained on phosphocellulose columns. The procedure developed for purification of the acetoacetyl-CoA thiolase (but omitting the heat inactivation step) yielded a partial purification for two of the β-oxidation enzymes: crotonase (100-fold) and 3-hydroxyacyl-CoA dehydrogenase (45-fold).

As was reported for *E. coli* (26), the *C. crescentus* enzymes were inactivated in a nonparallel fashion. Ace-toacetyl-CoA thiolase was resistant to heat inactivation but was unstable in purified fractions, and crotonase was heat sensitive but relatively stable in both the crude and purified forms. 3-Hydroxyacyl-CoA dehydrogenase was the least stable of the three enzymes; hence, its fold purification and yield were the lowest of the three. Like acetoacetyl-CoA thiolase, crotonase activity sedimented in sucrose gradients with an $s_{20,w}$ of 7.5S, yet its elution from a gel permeation

column, after acetoacetyl-CoA thiolase but before 3-hy-droxyacyl-CoA dehydrogenase, suggests a subunit size smaller than 45,000 Da. It is possible that the native croto-nase also forms a tetrameric noncovalent complex of homol-ogous subunits during sucrose gradient centrifugation. The partial purity of crotonase precluded its identification on SDS-polyacrylamide gels, and its subunit molecular weight could not be determined. However, SDS-polyacrylamide gel electrophoresis of the Mono Q fractions containing croto-nase, 3-hydroxyacyl-CoA dehydrogenase, and acetoacetyl-CoA thiolase activity showed relatively few protein bands, and none were as large as 78,000 in molecular weight.

3-hydroxyacyl-CoA dehydrogenase sedimented in a su-crose gradient at approximately 4.5S but with a significant trailing shoulder into the 7.5S range of the gradient (Fig. 6), indicating that this enzyme also has a propensity to form oligomers. Because of the partial purity of the enzyme, we were not able to identify the enzyme subunit by SDS-polyacrylamide gel electrophoresis.

Schulz and co-workers have purified a complex of β-oxidation enzymes that contains both 78,000- and 42,000-Da proteins (1, 25, 28). The complex appears to have at least five enzymatic activities associated with it, including crotonase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, β-hydroxyacyl-CoA epimerase, and thio-lase. The 42,000-Da subunit has β-ketoacyl-CoA thiolase activity (25). Spratt et al. have cloned the genes encoding several of the β-oxidation enzymes (28). The genes encoding 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydroge-nase, crotonase, epimerase, and isomerase form an operon. The gene encoding the 42,000-Da subunit is first, followed by the gene encoding a 78,000-Da protein (28). Pawar and Schulz (25) have suggested that a large, 78,000-Da multifunc-tional protein might harbor both the crotonase and 3-hy-droxyacyl-CoA dehydrogenase activities as well as epime-rase and isomerase. However, O'Brian and Frerman (21) have provided evidence that in *E. coli* a large complex containing crotonase, 3-hydroxyacyl-CoA dehydrogenase, and β-ketoacyl-CoA thiolase activity does exist, but con-trary to Pawar and Schulz (25), they found that this complex can be dissociated into separate functional proteins. In *C. crescentus*, acetoacetyl-CoA thiolase, crotonase, and 3-hydroxyacyl-CoA dehydrogenase appear to exist as separate proteins that form multimeric aggregates composed of ho-mologous subunits. Thus, *C. crescentus* resembles *Clostrid-ium acetobutylicum* and eucaryotic mitochondria in having separate proteins for different β-oxidation enzyme activities. It is possible that these separate proteins have evolved from a single multifunctional protein. On the basis of the presence of a multienzyme complex in *E. coli*, the β-oxidation en-zymes likely exist in a highly ordered arrangement and function in a coordinate manner. The parallel between the organization of the β-oxidation enzymes in *C. crescentus* and eucaryotic mitochondria suggests that studying enzyme organization and coordinate function in a genetically amen-able procaryote might contribute to our understanding of this complicated metabolic pathway in both bacteria and higher cells.

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