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Evidence for a Complex of Three Beta-Oxidation Enzymes in *Escherichia coli*: Induction and Localization

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The enzymes for β-oxidation of fatty acids in inducible and constitutive strains of *Escherichia coli* were assayed in soluble and membrane fractions of disrupted cells by using fatty acid and acyl-coenzyme A (CoA) substrates containing either 4 or 16 carbon atoms in the acyl moieties. Cell fractionation was monitored, using succinic dehydrogenase as a membrane marker and glucose 6-phosphate dehydrogenase as a soluble marker. Acyl-CoA synthetase activity was detected exclusively in the membrane fraction, whereas acyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase activities that utilized both C_4 and C_{16} acyl-CoA substrates were isolated from the soluble fraction. 3-Hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase activities assayed with both C_4 and C_{16} acyl-CoA substrates co-chromatographed on gel filtration and ion-exchange columns and cosedimented in glycerol gradients. The data show that these three enzyme activities of the *fad* regulon can be isolated as a multienzyme complex. This complex dissociates in very dilute preparations; however, in those preparations where the three activities are separated, the fractionated species retain activity with both C_4 and C_{16} acyl-CoA substrates.

Escherichia coli and a number of other bacteria are able to grow on a variety of fatty acids as the sole source of carbon and energy (24). Overath et al. (20) showed that the enzymes required for β-oxidation of fatty acids were inducible in *E. coli*. Klein et al. (13) and Weeks et al. (28) demonstrated that β-oxidation enzymes could be induced when *E. coli* was grown in a medium containing fatty acids with acyl chains of more than 10 carbon atoms; fatty acids containing 10 or less carbon atoms did not serve as inducers. Klein et al. (13) observed, however, that spontaneous mutants constitutive for the synthesis of β-oxidation enzymes could be selected by plating inducible strains on decanoate (C_{10}).

The structural genes for β-oxidation enzymes comprised a complex regulon, referred to as the fatty acid degradation regulon (*fad*) (19). It was proposed that the true inducers of the regulon were the fatty acyl-coenzyme A (CoA) derivatives of fatty acids, since mutants that lacked acyl-CoA synthetase activity were not induced when grown in a medium containing long-chain fatty acids. O'Brien and Frerman (18) have reported the isolation of a mutant with altered acyl-CoA synthetase substrate specificity that also possessed altered inducer specificity. The characterization of that mutant also implicated acyl-CoA synthetase as a regulator of the *fad* regulon.

Little is known about the structural or kinetic properties of the *fad* regulon enzymes. Samuel et al. (21) partially purified acyl-CoA synthetase from *E. coli*. Synthetase activities assayed with both long- and short-chain substrates copurified but differed in thermal stabilities. Feigenbaum and Schulz (9) purified the 3-ketoacyl-CoA thiolase and showed that a single enzyme catalyzed the CoA-dependent cleavage of 3-ketoacyl-CoA thiolesters containing 4 to 16 carbons in the acyl group. During the preparation of this paper, Binstock et al. (5) reported isolating an enzyme complex that contained three *fad* regulon enzyme activities. Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase activities with long-, medium-, and short-chain substrate specificities were isolated in the complex. It was concluded that β-oxidation in *E. coli* was catalyzed by enzymes with broad substrate specificity and not by series enzymes with limited substrate specificities.

This study presents evidence that four enzymes which catalyze the principal reactions of the cyclic oxidation of long-chain acyl-CoA to acetyl-CoA are active on both short-chain (C_4) and long-chain (C_{16}) acyl-CoA substrates. Our data confirm those of Binstock et al. (5) and show that the soluble complex of enoyl-CoA

hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase is a noncovalent complex, which, upon dissociation, can be resolved into three separate proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* ML308-225 was obtained from H. R. Kaback; strain K-12-YMel was obtained from U. Henning; strains *fad-5*, *fadB64*, and *fadA30* were obtained from P. Overath. Mutant D-1ML, constitutive for fatty acid degradation enzymes, (*fadR*), was isolated in this laboratory by selection on a medium containing 0.1% decanoate as the sole carbon source (19). Cells were routinely grown in medium A, the synthetic medium described by Davis and Mingioli (7), supplemented with the indicated carbon sources. Solid medium was prepared by adding agar (1.5%) to liquid medium. When fatty acids were used as a carbon source, a nonionic detergent, Triton X-100 (0.8%) or Brij 36T (0.4%), was added to the medium before autoclaving.

Enzyme preparations and cell fractionations. Cells were broken by sonic disruption before assaying total enzyme activities. The cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed twice by centrifugation from 0.05 M potassium phosphate buffer, pH 7.2. The washed cells were resuspended in the same buffer at a concentration of 0.1 g (wet weight)/ml of buffer. The suspension was sonically disrupted in 9 to 10 ml at 45 W with a Branson model W185 Sonifer for a total of 4 min. An ice-salt bath was used to maintain the temperature of the preparation between 4 and 10°C. The cells were broken with sonic bursts of 30 s with intermittent 30-s cooling periods. After disruption, unbroken cells were removed by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was used as the source of enzymes.

A French press was used to disrupt cells when the cellular localization of enzyme activities was determined. The cells were harvested and washed in potassium phosphate buffer, pH 7.2, as described above, and resuspended in the same buffer at a concentration of 0.1 g (wet weight) of cells per ml. The cells were broken in an Amicon French pressure cell at 10,000 lb/in^2. The French press lysate was made 2.5 mM in $MgCl_2$ and treated for 30 min at room temperature with 2 μg each of deoxyribonuclease and ribonuclease per ml. The nuclease-treated lysate was then centrifuged at 120,000 × *g* at 4°C for 90 min to remove membrane fragments and unbroken cells. The 120,000 × *g* supernatant was used as a source of soluble enzymes. The sediment from the 120,000 × *g* centrifugation was gently resuspended and diluted with 0.05 M potassium phosphate buffer, pH 7.2, in four times the volume in which the cells were broken. Unbroken cells were removed by centrifugation at 800 × *g* for 30 min at 4°C. The supernatant was then centrifuged at 120,000 × *g* for 90 min. The sediment from this centrifugation was resuspended by gentle homogenization and washed by centrifugation in twice the volume of the disrupted suspension. The washed membrane fraction was suspended in 0.05 M potassium phosphate buffer, pH 7.2, at a protein concentration of about 4 mg/ml. The membranes were stored at 4°C until used for enzyme assays.

Enzyme assays. The acyl-CoA synthetase was assayed in the presence of hydroxylamine as described by Kornberg and Pricer (14) and modified by Overath et al. (19). Acyl-CoA dehydrogenase activity was assayed by modification of the procedure used for other flavoprotein dehydrogenases (2). The incubation mixture contained 5 mM potassium phosphate buffer (pH 7.6)–43 μM oleyl-CoA or 0.2 mM butyryl-CoA–240 μM (3-4,5-dimethylthiazolyl-2-2,5-diphenyl)tetrazolium bromide (MTT)–1.7 mM phenazine methosulfate (PMS)–15 mM potassium cyanide. The reaction was initiated with acyl-CoA substrate, and reduction of MTT was followed by the increase in absorbance at 546 nm, using an extinction coefficient for reduced MTT of 22,800 cm^{-1} M^{-1} (29). The reaction was dependent on PMS concentration; velocities were linearly related to PMS concentration to 1.7 mM; above 1.7 mM PMS, the reduction of MTT was inhibited. Control incubations were performed in the absence of acyl-CoA substrate.

Enoyl-CoA hydratase activity was routinely assayed by following the decrease in absorbance at 263 nm caused by disappearance of the enoyl-CoA substrate (23). Incubation mixtures contained 83 mM potassium phosphate buffer (pH 8.0)–55 μM crotonyl-CoA or 25 μM *trans*-$\Delta^{2,3}$-hexadecanoyl-CoA–50 μg of bovine serum albumin in a final volume of 0.6 ml. Enoyl-CoA substrates were omitted in control incubation mixtures. The molar extinction coefficient of enoyl-CoA substrates at 263 nm was calculated to be 5,850 cm^{-1} M^{-1}, as described below. Short-chain enoyl-CoA dehydrogenase activity with crotonyl-CoA was also assayed by coupling 3-hydroxybutyryl-CoA formation with nicotinamide adenine dinucleotide (NAD) reduction in the presence of pig heart 3-hydroxyacyl-CoA dehydrogenase as described by Weeks et al. (28).

Short-chain 3-hydroxyacyl-CoA dehydrogenase was assayed with acetoacetyl-CoA as the substrate as described by Weeks et al. (28). The long-chain 3-hydroxyacyl-CoA dehydrogenase activity was assayed by the following procedure. The standard incubation mixture contained 62.5 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2)–40 μM DL-3-hydroxyhexadecanoyl-CoA–125 μM NAD–50 μg of bovine serum albumin. Control assays contained no DL-3-hydroxyhexadecanoyl-CoA. The reaction was initiated by adding NAD. The NAD-dependent formation of 3-ketohexadecanoyl-CoA was recorded by monitoring the increase in absorbance at 303 nm. An extinction coefficient of 3,600 cm^{-1} M^{-1} was used to calculate the amount of product formed (6).

Short-chain β-ketoacyl-CoA thiolase activity was determined with acetoacetyl-CoA as the substrate, as described by Clinkenbeard et al. (6). Long-chain thiolase activity was assayed, using endogenous long-chain thiolase 3-hydroxyacyl-CoA dehydrogenase to generate 3-ketohexadecanoyl-CoA as described above, except that the incubation mixture contained 7.5 mM $MgCl_2$. The reaction with the long-chain 3-ketoacyl-CoA substrate was initiated

by the addition of 150 μmol of CoA. The conversion of β-ketohexadecanoyl-CoA to acetyl-CoA and myristoyl-CoA was assayed spectrophotometrically by following the CoA-dependent decrease in absorbance at 303 nm caused by cleavage of the Mg^{2+} enolate of 3-ketohexadecanoyl-CoA with a molar extinction coefficient of 11,900 cm^{-1} M^{-1} (26).

Succinic dehydrogenase was assayed by the procedure of Arrigoni and Singer (2). Glucose 6-phosphate dehydrogenase was assayed as described by DeMoss (8).

Analytical methods. Protein was estimated by the method of Lowry et al. (15), as modified by Miller (17), with bovine serum albumin as the standard. The total bacterial and membrane protein was estimated by the same method after solubilizing the sample with 1% sodium dodecyl sulfate at 50°C for 10 min (10).

Lipid phosphorus was estimated by the method of Bartlett (4) after extraction of cell or protein samples with chloroform-methanol as described by Ames (1).

Synthesis of acyl-CoA substrates. *trans*-$\Delta^{2,3}$-Hexadecenoyl-CoA was synthesized from *trans*-$\Delta^{2,3}$-hexadecenoic acid and CoA as described by Schulz (5, 23). The *trans*-$\Delta^{2,3}$-hexadecenoyl-CoA was precipitated by acidifying to pH 1.0 with 1 M HCl. Unreacted hexadecenoic acid was extracted from the acidified solutions with diethyl ether. The ether phase was removed, and the aqueous phase and precipitate were washed three additional times with ether to remove the unreacted fatty acid. The aqueous phase, containing salts and other reactants that interfere with the enzyme assays, was removed from the precipitated product. The product, *trans*-$\Delta^{2,3}$-hexadecenoyl-CoA, was suspended in water, adjusted to pH 3.0 with HCl, and frozen at −20°C. The concentration of *trans*-$\Delta^{2,3}$-hexadecenoyl was determined by following the decrease in absorption at 263 nm caused by cleavage of the thioester bond in the presence of 3.0 M hydroxylamine at pH 7.0 (11). Crotonyl-CoA, obtained from P-L Biochemicals, and *trans*-$\Delta^{2,3}$-hexadecenoyl-CoA, synthesized by H. Schulz, were used as standards. The molar extinction coefficient of the thioester bond at 263 nm of *trans*-$\Delta^{2,3}$-enoyl-CoAs was determined to be 5,850 cm^{-1} M^{-1}.

The ethyl ester of DL-3-hydroxyhexadecanoic acid was synthesized by the Reformatsky reaction from 0.5 mol each of ethyl bromoacetate and tetradecanal in benzene (12). The ethyl ester of DL-3-hydroxyhexadecanoate was hydrolyzed with 1 M KOH in ethanol; the potassium salt was converted to a free acid by acidification to pH 1.0 with 6 N H_2SO_4, and the free acid was extracted into diethyl ether (27). Ether was removed under a stream of nitrogen leaving the DL-3-hydroxydecanoic acid as an oil. The CoA derivative of the free acid was formed by the reaction of the DL-3-hydroxydecanoic acid and CoA (23). The CoA esters were precipitated and washed as described above for long-chain enoyl-CoA. The product was suspended in distilled water and adjusted to pH 3.0 with 1 M HCl. The concentration of the DL-mixture of 3-hydroxyhexadecanoyl-CoA was determined by cleavage with neutral hydroxylamine (11), using an extinction coefficient of the thioester at 232 nm of 5,780 cm^{-1} M^{-1}. The 232-nm-to-260-nm ratio of absorbance, A_{232}/A_{260}, was 0.51.

Density-gradient centrifugation. Gradients were 10 to 50% glycerol in 0.05 M potassium phosphate buffer, pH 7.3 containing 5 mM mercaptoethanol and were centrifuged for 16 h at 40,000 rpm in an International B-60 centrifuge with an SB-283 rotor. Fractions were collected by pumping from the bottom of the gradient with a peristaltic pump (0.22-ml fractions from a 13.0-ml gradient).

Diethylaminoethyl (DEAE)-Sephadex chromatography. Columns were equilibrated in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.2, containing 10% glycerol–0.05 M KCl–5 mM mercaptoethanol. The columns (1.5 by 35 cm) were eluted with a 0.05 to 0.35 M KCl gradient (700 ml). The fraction size was 6.5 ml.

Materials. The following materials were obtained from the sources indicated: acetoacetyl-CoA, butyryl-CoA, oleyl-CoA, CoA, adenosine 5′-triphosphate, NAD, and reduced NAD were obtained from P-L Biochemicals, Milwaukee, Wis. Porcine heart 3-hydroxyacyl-CoA dehydrogenase was obtained from Calbiochem, La Jolla, Calif. *trans*-$\Delta^{2,3}$-Hexadecenoic acid was obtained from K & K Chemicals, Cleveland, Ohio. Tetradecanal, ethyl chloroformate, ethyl bromoacetate, and triethylamine were obtained from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals were obtained from commercial sources and were the best grades available.

RESULTS

Induction of β-oxidation enzymes in *E. coli*. *E. coli* ML308-225 contains an inducible *fad* regulon (*fadR*$^+$), whereas strain D-1ML, derived from ML308-225, was a constitutive strain (*fadR*). Cells were grown to midexponential phase in medium A containing 0.8% Triton X-100 and either acetate or oleate as sole source of carbon and energy. Total enzyme activities of five β-oxidation enzymes were assayed, and the results are summarized in Table 1.

The data show that the five enzyme activities required for the β-oxidation of fatty acids are inducible, as assayed both with long-chain (C_{16}) and short-chain (C_4) substrates when strain ML308-225 was grown on oleate (C_{18}). The amount of induction ranged from about 2-fold for short-chain acyl-CoA dehydrogenase activity to as high as 180-fold for short-chain enoyl-CoA hydratase activity.

The constitutive mutant, D-1ML, synthesized β-oxidation enzymes with both long-chain and short-chain activities when grown on acetate. The specific activities of the enzymes shown in Table 1 indicate that the levels are nearly the same in strain D-1ML grown on acetate and strain ML308-225 grown on oleate, as would be expected (19). Higher specific activ-

ities and coordinate increases in specific activities of all five enzymes were observed when strain D-1ML was grown on oleate. This observation was consistent with the assumption that enzymes with long-chain and short-chain acyl-CoA specificities are under the control of the same regulatory gene.

The β-oxidation enzyme activities were also assayed with both long- and short-chain substrates in several fatty acid degradation mutants isolated by Klein et al. (13). The data in Table 2 show induced levels of β-oxidation enzymes in the parental strain K-12-YMel and compare well with those reported by Overath et al. (19) when assayed with short-chain substrates. Specific activities with long-chain substrates are also shown. When K-12-YMel was grown in medium containing only acetate as the carbon source, only trace levels of the β-oxidation enzymes could be assayed, as reported by Overath et al. (19).

The mutant *fad-5*, previously shown by Klein et al. (13) to be deficient in short-chain acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, was also found to contain reduced amounts of these enzymes when assayed with long-chain-length substrates, suggesting that the long- and short-chain-length activities are coordintely controlled.

TABLE 1. *Specific activity of the β-oxidation enzymes in E. coli ML308-225 and D-1ML when grown on acetate or oleate*

Strain	Carbon source	Enzyme activity (nmol/min per mg)[a]								
		Acyl-CoA[b] synthetase	Acyl-CoA dehydrogenase		Enoyl-CoA hydratase		3-Hydroxyacyl-CoA dehydrogenase		3-Ketoacyl-CoA thiolase	
			LC[c]	SC[d]	LC	SC	LC	SC	LC	SC
ML308-225	Acetate	0.05	1.8	0.5	9	20	9	14	4	5
	Oleate	0.50	4.2	1.2	420	4,000	1,140	650	17	71
D-1ML	Acetate	0.56	4.2	1.3	310	4,900	710	570	8	38
	Oleate	1.10	8.1	2.4	530	9,900	1,600	1,200	17	82

[a] Measured in sonically disrupted preparations and assayed at 22°C.
[b] Assayed at 37°C.
[c] LC, Long-chain-length substrate with 16 carbon atoms.
[d] SC, Short-chain-length substrate with 4 carbon atoms.

TABLE 2. *Specific activities of β-oxidation enzymes in parental strain K-12-YMel and fatty acid degradation mutants*

Strain	Mutational defect	Carbon source	Enzyme activity (nmol/min per mg)							
			Acyl synthetase	Acyl-CoA dehydrogenase		Enoyl-CoA hydratase		3-Hydroxyacyl-CoA dehydrogenase		3-Ketoacyl-CoA thiolase
				LC[a]	SC[b]	LC	SC	LC	SC	LC	SC
K-12-YMel	None	0.1% Oleate + 0.4% acetate	2.4	9.0	2.4	84	1,400	1,800	720	17	79
fad-5	*fad* regulon	TS[c] + 0.1% oleate	1.9	0.1	—[d]	3	10	11	5	1	0.8
fad-5	*fad* regulon	TS + 0.4% acetate	1.1	—	—	2	12	16	7	1	0.5
fadb64	SC 3-hydroxyacyl-CoA dehydrogenase	TS + 0.1% oleate	2.0	7.8	2.0	78	1,320	590	36	5.4	33
fada30	SC 3-ketoacyl-CoA thiolase	0.4% Acetate + 0.1% oleate	2.2	8.1	2.2	75	1,300	1,000	430	7.3	2.3

[a] LC, Long-chain substrate containing 16 carbon atoms.
[b] SC, Short-chain substrate containing 4 carbon atoms.
[c] TS, Trypticase soy broth plus 0.1% oleate.
[d] —, No measurable activity.

Mutant *fadB64*, which was previously shown to be deficient in 3-hydroxyacyl-CoA dehydrogenase activity (19), showed a 95% decrease in the specific activity of 3-hydroxyacyl-CoA dehydrogenase activity relative to the parental strain grown on acetate plus oleate. However, only a 67% decrease in dehydrogenase activity was observed when C_{16} substrates were used to assay activity. Mutant *fadA30*, a 3-ketoacyl-CoA thiolase mutant isolated by Overath, showed a greater than 97% decrease in short-chain thiolase specific activity relative to K-12-YMel but only a 57% decrease in long-chain thiolase specific activity. These strains have been reported to β-oxidize [1-^{14}C]oleate at about 50% the rate observed in the wild type; however, CO_2 was released from [18-^{14}C]oleate at 14% or less of the wild-type rate. Both mutant strains were grown in medium supplemented with Trypticase soy to obtain reasonable yields of cells, as indicated by Overath et al. (19).

These observations suggest either that each step in the β-oxidation cycle is catalyzed by multiple enzymes with a limited range of substrate specificity or that these mutations have affected the structure of the enzymes with broad chain-length substrate specificity such that their activity on short-chain-length substrates was affected to a greater degree than with long-chain substrates.

Localization of the β-oxidation enzymes in membrane and soluble fractions of strain D-1ML. To determine whether β-oxidation enzymes with long-chain-length substrate specificity were membrane bound, cultures of D-1ML were grown, harvested, washed, disrupted, and fractionated as described in above. Succinic dehydrogenase was used as a membrane marker, and glucose 6-phosphate dehydrogenase was used as a marker of soluble enzymes. With the French press differential centrifugation method of cell fractionation, 92% of the succinic dehydrogenase activity was recovered in the membrane fraction, and 100% of the glucose 6-phosphate dehydrogenase activity was found in the soluble fraction.

The acyl-CoA synthetase was predominantly membrane associated; 83% of the recovered activity was found in the membrane fraction. This observation is consistent with those of both Overath et al. (19) and Samuel et al. (21). The remaining β-oxidation enzymes were found excusively in the cytoplasmic fraction when assayed with short-chain substrates. When these activities were assayed with long-chain substrates, a small but consistent amount of activity was found associated with a membranous fraction. The residual long-chain activities that were membrane associated did not appear to be due to contamination of the membrane fraction with small amounts of the soluble enzymes, since neither glucose 6-phosphate dehydrogenase nor β-oxidation activities with short-chain substrates could be found in the membrane fraction upon disruption of the membranes by sonic oscillation, Triton X-100, or Brij 36T.

Separation of soluble β-oxidation enzyme activities. Since the preceding experiments did not clearly distinguish between the possible existence of single β-oxidation enzymes with broad chain-length specificity and multiple enzymes with limited substrate specificity, the following experiments were performed to separate species of soluble β-oxidation enzymes. The supernatant of a French press lysate of acetate-grown D-1ML was subjected to gel filtration chromatography on Sephadex G-200.

The activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase assayed with both long-chain and short-chain acyl-CoA substrates co-chromatographed (Fig. 1). None of these activities was found in other column fractions. The ratio of short-chain-to-long-chain activities in the three fractions containing the most activity was virtually constant. The elution position from Sephadex G-200 of β-oxidation enzyme activities corresponded to a globular protein with a molecular weight of 245,000, as based on a comparison with known standards. The recovery of short-chain-length activities from the column was always greater than 60%, except for the activity of 3-ketoacyl-CoA thiolase where 20% of the initial activity was recovered. Approximately 20% of each activity measured with long-chain-length substrates was recovered. Subsequent stability studies indicated that 20% glycerol was required to obtain maximum stability with all the activities.

The cytoplasmic fraction (120,000 × *g* supernatant) was also subjected to velocity sedimentation in 10 to 50% glycerol gradients. Except for the acyl-CoA dehydrogenase activity, the long- and short-chain-length activities of the enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase were observed to cosediment at an $s_{20,w}$ value of about 9.7 S. As previously observed after gel filtration chromatography, ratios of long-chain-to-short-chain-length activities in the gradient were virtually constant (Fig. 2). Recovery of each activity from the glycerol gradient was about 60%, except for the thiolase activities, which were recovered in about 30% yields.

Fractionation by both gel filtration chromatography and density gradient centrifugation depend largely on Stokes radii and only indi-

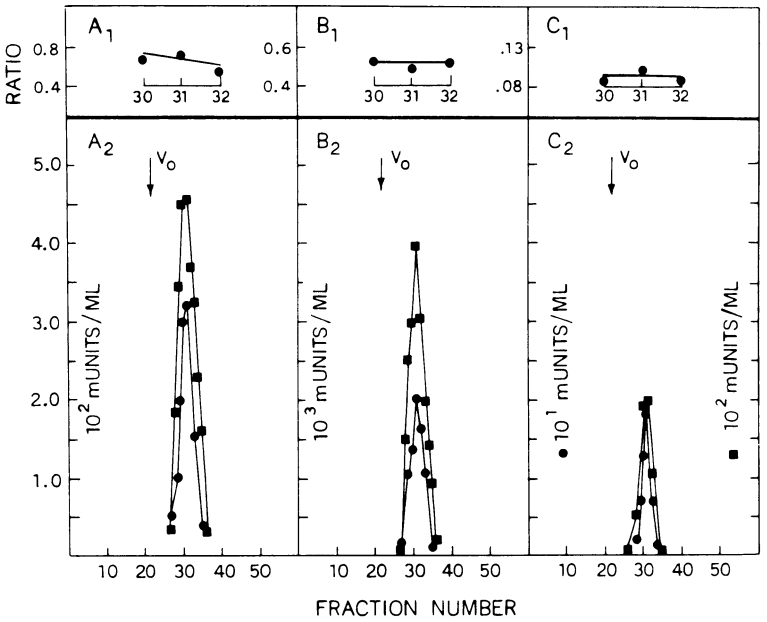

FIG. 1. *Chromatography of β-oxidation enzymes on Sephadex G-200. The 120,000 × g supernatant of a French press lysate of acetate-grown D-1ML was loaded on a Sephadex G-200 column and chromatographed in 0.05 M potassium buffer, pH 7.4, containing 2% glycerol and 5 mM mercaptoethanol.* v_0 *represents the void volume.* A_1*, Ratio of 3-hydroxyacyl-CoA dehydrogenase activity when assayed with long-chain-length substrate* (C_{16}) *to the activity when assayed with short-chain-length substrate* (C_4). B_1*, Ratio of long-chain-to-short-chain activity when enoyl hydratase was assayed.* C_1*, Ratio of long-chain-to-short-chain activity when 3-ketoacyl-CoA thiolase was assayed.* A_2, B_2, *and* C_2*, Elution patterns when* (●) *long-chain substrates and* (■) *short-chain substrates were used to assay 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase, respectively.*

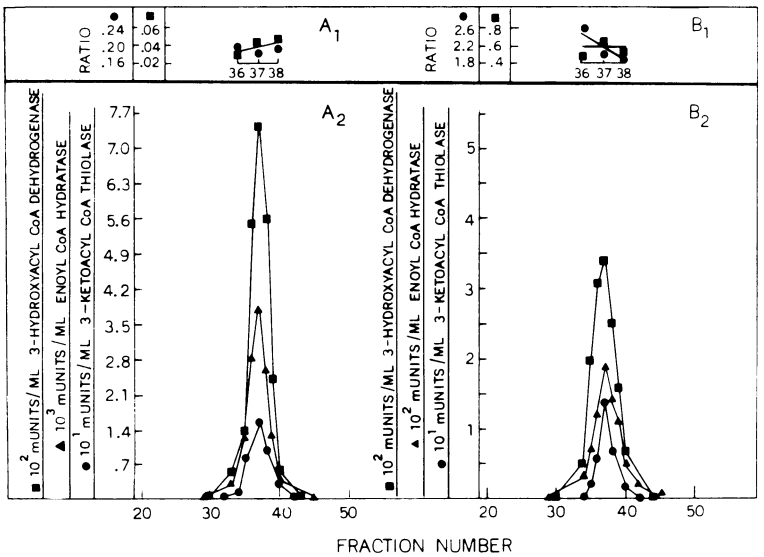

FIG. 2. *Density gradient profile of the β-oxidation enzyme activities. The 120,000 × g supernatant of a French press lysate of acetate-grown D-1ML was the source of enzymes layered on a 10 to 50% glycerol gradient of 0.05 M potassium phosphate buffer, pH 7.2, containing 5 mM mercaptoethanol.* A_2 *and* B_2*, The sedimentation of the long- and short-chain β-oxidation enzymes, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase.* A_1*, ratio of short-chain 3-hydroxyacyl-CoA dehydrogenase to enoyl-CoA hydratase activity* (●) *in fractions 36, 37, 38;* (■) *ratio of short-chain 3-ketoacyl-CoA thiolase to short-chain enoyl-CoA hydratase.* B_1*, Ratios of long-chain activities in these fractions.*

rectly on molecular weight. Therefore, in an effort to resolve the hydratase, dehydrogenase, and thiolase activities, the soluble fraction from a French press lysate was subjected to ion-exchange chromatography on DEAE-Sephadex. The column was eluted with a linear gradient of 0.05 to 0.35 M KCl. Again, the three activities were eluted coincidentally from the column, and the ratios of activities were relatively constant (Fig. 3). The recovery of activities ranged between 50 and 80% for the enzymes other than the thiolase. Thirty percent recovery of thiolase was achieved when assayed with both long- and short-chain substrates.

Crude lysates containing the soluble hydratase, dehydrogenase, and thiolase activities were subjected to preparative glycerol gradient centrifugation; the cosedimenting activities were then chromatographed on DEAE-Sephadex. Upon elution of the protein from the column with KCl, a majority of β-oxidation enzyme activity was found to elute in the same gradient position (Fig. 4). In addition to the coincident peak of long- and short-chain-length activities of hydratase, dehydrogenase, and thiolase, three clearly spearated peaks of individual enzyme activities were observed. Each peak contained long- and short-chain activity of the three respective enzymes. Recoveries of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase from an ion-exchange column were 48, 97, and 14% when assayed with short-chain substrates, and 16, 21, and 94% when assayed with the long-chain substrates.

Comparison of specific activities of the enzymes recovered from the fraction 59 column (Fig. 4) indicated that from the crude extract, a 30-fold purification of the hydroxyacyl-CoA dehydrogenase could be obtained by this procedure. Determination of lipid phosphorus in the chloroform-methanol extract of partially purified enzyme complex, obtained as described, indicated that these samples contained 66 nmol of lipid phosphorus per mg of protein. The amount of lipid phosphorus found in the crude, soluble *E. coli* lysate was 7 nmol/mg of protein.

DISCUSSION

Direct enzymatic assays show that growth of *E. coli* on long-chain fatty acids induces the biosynthesis of β-oxidation enzymes that utilize both long-chain (C_{16}) and short-chain (C_4) acyl-CoA substrates. A comparison of specific activities of the β-oxidation enzymes in induced cells indicates that fatty acid activation to the corresponding acyl-CoA thiolester is rate limiting in β-oxidation. This enzymatic reaction is also involved in translocation of fatty acids, as acyl-CoA thiolesters, into the cell (10, 13). The data also indicate that within any one cycle of β-oxidation, the reaction catalyzed by acyl-CoA dehydrogenase is rate limiting. Feigenbaum and Schulz (9) had proposed that the thiolase-catalyzed reaction was rate limiting.

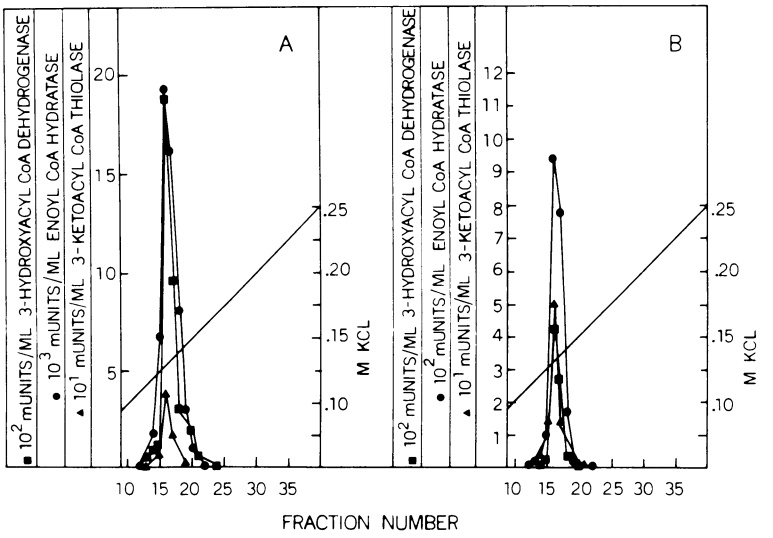

FIG. 3. *DEAE-Sephadex chromatography of β-oxidation enzymes of E. coli. The supernatant of a 120,000 × g centrifugation of a French press lysate of D-1ML grown on acetate was chromatographed on a column (42 by 1.5 cm) of DEAE-Sephadex. A, Activities of recovered enzymes as assayed with C_4 substrates; B, activities as assayed with C_{16} substrates.*

Another major finding of this study is that three of the soluble β-oxidation enzymes can be isolated as a noncovalent complex. While this paper was in preparation, Binstock et al. (5) reported isolating a complex of the same three enzymes from *E. coli* B. Our data confirm their findings and show that complexation of the three enzymes is a general phenomenon in *E. coli* and is not dependent on the allelic state of the *fadR* gene. The properties of that complex are virtually identical to those reported here. The complex isolated from *E. coli* B has a molecular weight of about 300,000 and, based on acrylamide gel electrophoresis in the presence of dodecyl sulfate, contains subunits with molecular weights of 78,000 and 42,000. The 42,000-molecular-weight subunit is undoubtedly derived from the tetrameric thiolase (16). Our observation that 3-hydroxyacyl-CoA dehydrogenase and hydratase activities could be separated when the complex dissociated on ion-exchange chromatography makes it unlikely that there is a single 78,000-dalton polypeptide that contains both dehydrogenase- and hydratase-active sites as proposed by Binstock et al. (5). The complex is nonclassical in the sense that its component enzymes dissociate or are inactivated in a nonparallel fashion (25). Binstock et al. also reported nonparallel losses of activities among components of the complex (5). In crude extracts, we found no evidence of lower-molecular-weight species of components of the complex after gradient centrifugation or gel filtration chromatography; Binstock et al. (5) did report a medium-chain-length enoyl-CoA hydratase activity that did not co-chromatograph with the complex. The complex may contain bound phospholipid. A complex of aminoacyl-transfer ribonucleic acid synthetases has been previously shown to be associated with lipid (3).

The complex of β-oxidation enzymes very likely plays an important role in regulation of acyl-CoA oxidation. Compartmentalization of substrates and products would facilitate control over the cyclic portion of the β-oxidation pathway by regulating substrate availability and product levels.

Several observations in the present study apparently contradict the hypothesis that single enzymes with broad chain-length specificity catalyze each step in the cyclic oxidation of acyl-CoA thiolesters in *E. coli*. First, in the mutants *fadA30* and *fadB64*, which are deficient in thiolase and 3-hydroxyacyl CoA dehydrogenase, respectively, there is a greater loss of short-chain activities compared to activities with long-chain substrates. The mutations in both *fadA30* and *fadB64* are leaky. The differential losses of activities with short-chain substrates compared to activities with long-chain substrates may simply reflect that the mutations affect the steric or charge properties of the active sites, resulting in differential binding of substrates or different rate-limiting steps in the catalytic pathways. A similar argument may be advanced to explain the differential loss of activities with short-chain and long-chain substrates during purification of the complex from the *fadR*, D1-ML. Second, whereas enzyme activities for short-chain substrates were found only in the soluble fractions of the cells, about 10% of the total activities with

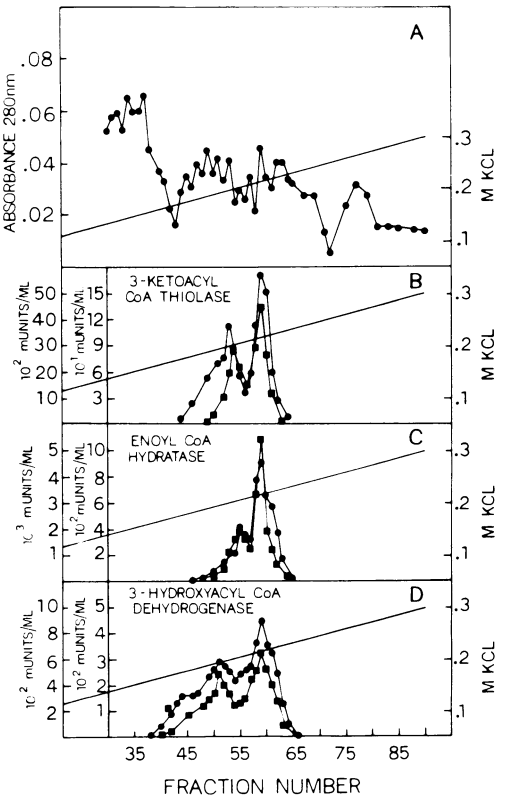

FIG. 4. *DEAE-Sephadex chromatography of β-oxidation enzyme complex obtained from a density gradient centrifugation. E. coli strain D-1ML was grown on acetate, harvested, and washed, then disrupted by the French press method. The complex of β-oxidation enzymes obtained from preparative glycerol gradients was then chromatographed on DEAE-Sephadex. A, 280-nm absorption profile of column eluant; B, C, and D, elution of the β-oxidation enzymes; B, 3-ketoacyl-CoA thiolase as assayed with C_4 substrate (●) and C_{16} substrate (■); C, enoyl-CoA hydratase as assayed with C_4 substrate (●) and C_{16} substrate (■); D, 3-hydroxyacyl-CoA dehydrogenase as assayed with C_4 substrate (●) and C_{16} substrate (■).*

long-chain acyl-CoA substrates was consistently membrane associated. This latter observation also applied to long-chain thiolase activity; it is well established that thiolase of the *fad* regulon catalyzes the thiolytic cleavage of both long-chain and short-chain 3-ketoacyl-CoA substrates. The finding of exclusively long-chain activities may represent artifacts in the assays of membrane-associated activities with short-chain acyl-CoA substrates. On the other hand, this observation may reflect differences in substrate specificity when these enzymes are associated with the membranes. Sartonelli et al. (22) showed that the mitochondrial guanosine 5'-triphosphate-dependent acyl-CoA synthetase has greatly increased activities with long-chain fatty acids when it is associated with the membrane. However, the co-chromatography on gel filtration and ion-exchange columns as well as the cosedimentation of hydratase, 3-hydroxyacyl-CoA dehydrogenase, and thiolase activities with both long-chain and short-chain specificity is compelling evidence for single enzymes with broad substrate specificities.

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