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Five Different Enzymatic Activities Are Associated with the Multienzyme Complex of Fatty Acid Oxidation from *Escherichia coli*

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The purified multienzyme complex of fatty acid oxidation from *Escherichia coli* was found to possess 3-hydroxyacyl-coenzyme A (CoA) epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities in addition to the previously identified enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoactyl-CoA thiolase activities. Evidence is presented in support of the proposed association of all five enzyme activities with one protein which apparently is composed of two types of subunits and which can exist in several aggregated forms. The five component enzymes of the complex were rapidly inactivated by tris(hydroxymethyl)aminomethane, whereas they remained active in the presence of potassium phosphate.

Fatty acid oxidation in *Escherichia coli* is catalyzed by an inducible enzyme system (13, 17). Acyl coenzyme A (acyl-CoA) synthetase (EC 6.2.1.3), at least two acyl-CoA dehydrogenases (EC 1.3.99.2 and 1.3.99.3), enoyl-CoA hydratase (EC 4.2.1.17), L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), 3-ketoacyl-CoA thiolase (EC 2.3.1.16), 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3) are induced when *E. coli* cells are grown on long-chain fatty acids as the sole carbon source (6, 12, 13, 17). The isolation and mapping of mutants of fatty acid oxidation led to the conclusion that the genes for the enzymes of fatty acid oxidation are located on three separate regions of the *E. coli* chromosome (6). The genes for acyl-CoA synthetase and for the acyl-CoA dehydrogenases were mapped on different locations of the chromosome unlinked to the genes of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, which apparently form an operon (6, 12).

In a previous publication we reported the isolation and purification of a multienzyme complex of fatty acid oxidation from *E. coli* B cells which exhibited enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase activities (2). In the present communication we report that the complex exhibits, additionally, 3-hydroxyacyl-CoA epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities, both of which are required for the degradation of unsaturated fatty acids. We also provide evidence for the association of these five enzyme activities with one protein which can exist in different polymeric forms.

MATERIALS AND METHODS

Materials. The preparation of several substrates and the sources of most materials have been presented recently (2, 3). Polyacrylamide gradient gels were obtained from Pharmacia Fine Chemicals. DL-3-Hydroxydodecanoic acid was synthesized by reduction of ethyl 3-ketododecanoate with $NaBH_4$, followed by alkaline hydrolysis. Ethyl 3-ketododecanoate was prepared by an established procedure (5). *cis*-$\Delta^{3,4}$-Octenoic acid was synthesized from 3-octyn-1-ol obtained from Pfaltz and Bauer by following the procedure of Stoffel and Ecker (16). The CoA derivatives of *cis*-$\Delta^{3,4}$-octenoic acid and DL-3-hydroxydodecanoic acid were prepared by the mixed anhydride method of Goldman and Vagelos (4). Pig heart 3-ketoacyl-CoA thiolase was purified as previously described (15). The conditions of growth of *E. coli* B cells (ATCC 11775) induced for the enzymes of β-oxidation and the purification of the multienzyme complex of fatty acid oxidation have been described previously (2, 3).

Protein and enzyme assays. Protein concentrations were determined by the method of Lowry et al. (8). Thiolase, L-3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase were assayed spectrophotometrically at 303, 340, 263 nm, respectively, as described in principal by Lynen and Ochoa (9) and as detailed previously (3, 14, 17). Acyl-CoA synthetase was assayed by the method of Kornberg and Pricer (7) as described by Overath et al. (12). Acyl-CoA dehydrogenase assays were performed as described by Beinert (1). 3-Hydroxyacyl-CoA epimerase activities were measured spectrophotometrically at 340 nm and at 30°C by an assay in which the epimerase-dependent formation of L-hydroxydodecanoyl-CoA was coupled

to its dehydrogenation and thiolytic cleavage, which were catalyzed by L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, respectively. The assay mixture contained 0.166 M potassium phosphate (pH 8), 0.3 mg of bovine serum albumin per ml, 0.5 mM oxidized nicotinamide adenine dinucleotide, 0.1 mM CoA-sulfhydryl, 60 μM DL-3-hydroxydodecanoyl-CoA, 2 μg of L-3-hydroxyacyl-CoA dehydrogenase per ml, and 1 μg of 3-ketoacyl-CoA thiolase per ml. The reaction was allowed to proceed until the L-isomer of 3-hydroxydodecanoyl-CoA was completely degraded. The epimerase assay was then initiated by the addition of the multienzyme complex of fatty acid oxidation. *cis*-Δ^3-*trans*-Δ^2-Enoyl-CoA epimerase activity was measured spectrophotometrically at 340 nm and at 30°C by an assay in which the isomerase-dependent formation of *trans*-Δ^2-enoyl-CoA was coupled to its hydration, dehydrogenation, and finally thiolytic cleavage, which were catalyzed by crotonase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, respectively. The assay mixture contained 0.166 M potassium phosphate (pH 8), 0.5 mM oxidized nicotinamide adenine dinucleotide, 0.1 mM CoA-sulfhydryl, 30 μM *cis*-Δ^3-octenoyl-CoA, 2.5 μg of 3-ketoacyl-CoA thiolase per ml, 2 μg of L-3-hydroxyacyl-CoA dehydrogenase per ml, and 7.5 μg of crotonase per ml. The reaction was started by the addition of the multienzyme complex of fatty acid oxidation. In homogenates, activities of L-3-hydroxyacyl-CoA dehydrogenase, epimerase, and isomerase were assayed after heating the preparations for 1 min at 70°C to destroy reduced nicotinamide adenine dinucleotide dehydrogenase. One unit of enzyme activity is defined as 1 μmol of substrate converted to product per min.

Gel electrophoresis. Electrophoresis experiments were performed with 4 to 30% polyacrylamide gradient gel slabs (7.6 by 7.5 cm). After application of the protein samples, the gels were subjected to electrophoresis for 5 h at 100 V in a Pharmacia GE-4 electrophoresis apparatus. The electrophoresis buffer was 0.09 M tris(hydroxymethyl)aminomethane (Tris)-boric acid (pH 8.35)-2.5 mM ethylenediaminetetraacetate. After completion of the electrophoresis, either the total slab or narrow slices cut vertically from each side of the slab were stained for 40 min with 1% Coomassie brilliant blue and destained for 1 h in 7% acetic acid, after which the protein bands were visible. In those cases where only two slices of the slab had been stained for protein, the remainder of the gel was cut horizontally into several segments so that each of the main protein bands was associated with a separate slice. All slices were extracted for 18 h in a minimal volume of 0.02 M potassium phosphate (pH 8) containing 10% glycerol, 1 mg of bovine serum albumin per ml, and 5 mM mercaptoethanol.

RESULTS AND DISCUSSION

Enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, three of the seven known enzyme activities required for the degradation of fatty acid to acetyl-CoA in *E. coli*, were found to be associated in a multienzyme complex (2, 10). The purified complex, after heat treatment and chromatography on phosphocellulose, was devoid of acyl-CoA synthetase and acyl-CoA dehydrogenase activities (2). Because the same observation was made when the heat treatment step was omitted, it is concluded that acyl-CoA synthetase and the acyl-CoA dehydrogenases are not component enzymes of the purified multienzyme complex of fatty acid oxidation. However, both 3-hydroxyacyl-CoA epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities were detected in purified preparations of the complex. To further investigate their possible association with the complex, the co-purification of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase was studied. Results (Table 1) show that all five enzymes were purified to the same extent (36- to 39-fold) when an *E. coli* homogenate was subjected to heat treatment and chromatography on a phosphocellulose column from which not only thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase (2), but also epimerase and isomerase, were eluted coincidently. These observations suggest that the epimerase and isomerase are possibly associated with the multienzyme complex of fatty acid oxidation.

When the homogeneity of the purified complex was evaluated by polyacrylamide gradient gel electrophoresis, two intense protein bands

TABLE 1. *Purification of the multienzyme complex of fatty acid oxidation from E. coli*[a]

Enzyme	Substrate	Sp act (μmol/min per mg)		Yield (%)	Purification (-fold)
		Homogenate	Purified protein		
Enoyl-CoA hydratase	Crotonyl-CoA	1.5	57	41	38
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	0.41	16	44	39
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA	0.05	1.9	42	38
3-Hydroxyacyl-CoA epimerase	D-3-Hydroxydodecanoyl-CoA	0.05	1.8	39	36
cis-Δ^3-*trans*-Δ^2-Enoyl-CoA isomerase	*cis*-Δ^3-Octenoyl-CoA	0.15	5.7	42	38

[a] For experimental details see text.

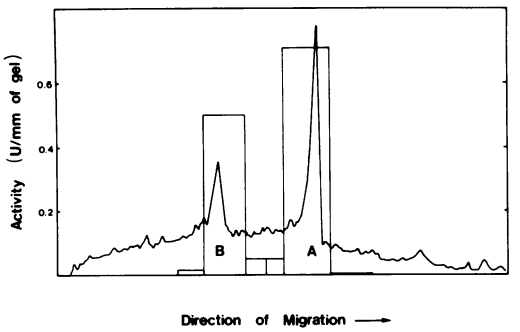

FIG. 1. *Electrophoresis of the purified complex of fatty acid oxidation (0.36 mg) on a 4 to 30% polyacrylamide gradient gel. The recorder tracing was obtained by scanning the absorbance at 500 nm of a gel slice stained for protein. The bars represent enoyl-CoA hydratase activity measured in the extracts of various gel segments. For details see text.*

were observed (Fig. 1). The molecular weights of the two corresponding proteins were estimated by comparison with proteins of known molecular weight to be 265,000 (peak A) and 580,000 (peak B) (Fig. 2). The relative amounts of the two proteins corresponding to peaks A and B differed from one preparation to the next, but the ratio of their molecular weights was consistently found to be approximately 1:2. Because the molecular weight of the multienzyme complex of fatty acid oxidation had been determined previously to be approximately 300,000 (2), peak A must correspond to the complex, whereas peak B was suspected to be due to a dimer of the complex. Preparations of the complex which contained a large amount of the putative dimeric form of the complex were resolved on polyacrylamide gradient gel electrophoresis into three bands (bands A through C), the slowest moving of which (band C) was apparently due to a trimeric form of the complex, with an estimated molecular weight of 820,000 (Fig. 2). Polyacrylamide gel electrophoresis of the purified complex in the presence of sodium dodecyl sulfate demonstrated the presence of only the 42,000- and 78,000-dalton polypeptides previously identified (2). Analysis by two-dimensional gel electrophoresis proved that the proteins corresponding to peaks A and B had identical subunit structures (Fig. 2). Definite proof for the close relationship between proteins A and B (Fig. 1) was obtained when the presence of the same enzymatic activities in these two proteins was established. All five enzymes listed in Table 1 were found to be associated with both protein A and protein B (Fig. 1 and Table 2). The specific and relative activities of the five enzymes observed in regions A and B of the gel are given in Table 2. In view of the low activities present in the gel extracts, the relative activities of the enzymes located in regions A and B of the gel agree reasonably well with those of the starting material (Table 2) except for thiolase, which under a variety of conditions was found to be inactivated more easily than the other enzymes. The activities of all five enzymes in the regions adjacent and between the peaks were either very low or undetectable, as in the case of enzymes with low specific activities. We therefore conclude that epimerase and isomerase, in addition to the three previously identified enzymes of β-oxidation, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, are component enzymes of the multienzyme complex of fatty acid oxidation and that protein B is an enzymatically active dimer of the multienzyme complex of fatty acid oxidation.

During our studies of *E. coli* thiolases we observed and reported (3) that 3-ketoacyl-CoA

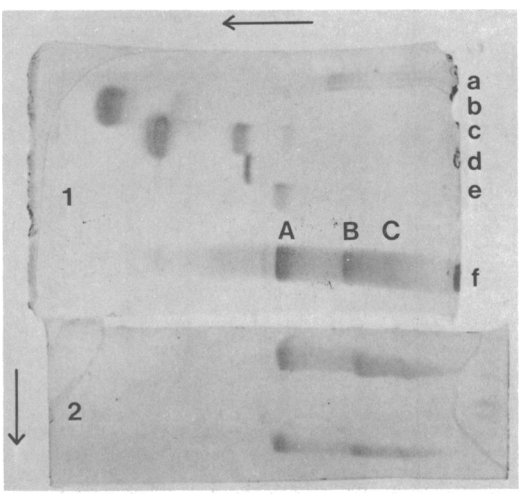

FIG. 2. *Two-dimensional polyacrylamide gel electrophoresis of the multienzyme complex of fatty acid oxidation from E. coli. In gel 1 the native complex and several protein standards were run on a 4% (top) to 30% polyacrylamide gel as described in the text. Before staining the gel, a slice identical to segment f, which contained only the multienzyme complex, was cut off. This slice was incubated with a buffer containing sodium dodecyl sulfate and mercaptoethanol, placed on top of a sodium dodecyl sulfate-polyacrylamide gel (10%, gel 2), and subjected to electrophoresis as described by O'Farrell (11). Arrows indicate directions of migration. Lane a, apoferritin; lane b, ovalbumin; lane c, bovine serum albumin; lane d, lactate dehydrogenase; lane e, catalase; lane f, multienzyme complex of fatty acid oxidation from E. coli. The positions of the monomeric, dimeric, and trimeric forms of the complex are marked as A, B, and C, respectively.*

TABLE 2. *Activities of the component enzymes of the monomer and dimer of the multienzyme complex of fatty acid oxidation from E. coli*[a]

Enzyme	Substrate	Sp act (μmol/min per mm of gel)[d]		Relative activity (%)		
		A[b]	B[b]	PC[c]	A[b]	B[b]
Enoyl-CoA hydratase	Crotonyl-CoA	0.71	0.5	100	100	100
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	0.24	0.21	29	34	42
3-Ketoacyl-CoA thiolase	3-Ketodecanoyl-CoA	0.2	0.15	69	28	30
3-Hydroxyacyl-CoA epimerase	D-3-Hydroxydodecanoyl-CoA	0.085	0.046	10	12	9.1
cis-Δ^3-*trans*-Δ^2-Enoyl-CoA isomerase	*cis*-Δ^3-Octenoyl-CoA	0.021	0.014	3.2	2.9	2.7

[a] Separation of the monomer and dimer of the complex and enzyme assays were performed as described in the text and in the legend to Fig. 1.

[b] A and B, Extracts from regions A and B of polyacrylamide gradient gel (Fig. 1).

[c] PC, Purified complex of fatty acid oxidation. Specific activities of this preparation are given in Table 1 except for thiolase activity with 3-ketodecanoyl-CoA, which was 39.3 U/mg.

[d] Because the gel was divided into unequal slices, the enzyme activities determined in the gel extracts were normalized with respect to the width of the gel slices.

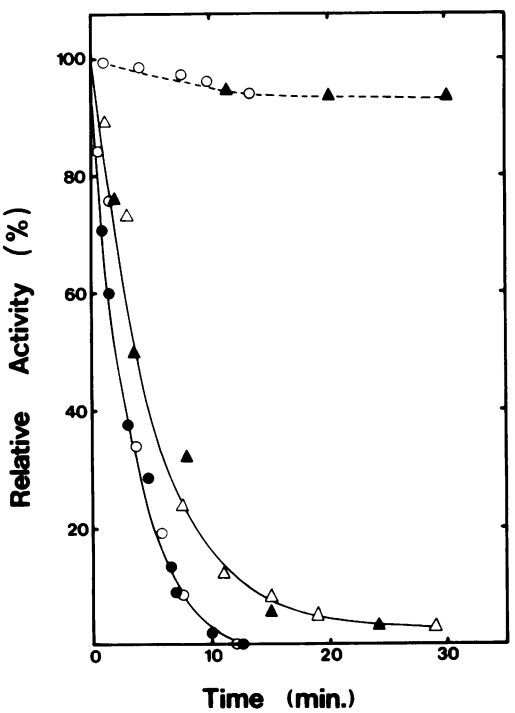

FIG. 3. *Effects of Tris-hydrochloride and potassium phosphate on the activities of the multienzyme complex of fatty acid oxidation. Purified complex (27 μg/ml) at 0°C in the presence of 1 M Tris-hydrochloride pH 8* (——) *or 0.2 M potassium phosphate, pH 8* (-----). *The activities of the five component enzymes were determined as a function of time. Symbols: ●, thiolase activity with acetoacetyl-CoA; ○, thiolase activity with 3-ketodecanoyl-CoA; ▲, L-3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA; △, L-3-hydroxyacyl-CoA dehydrogenase with 3-ketodecanoyl-CoA. The inactivation curves for enoyl-CoA hydratase with crotonyl-CoA, 3-hydroxyacyl-CoA epimerase, and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase were virtually identical to that of L-3-hydroxyacyl-CoA dehydrogenase.*

thiolase (thiolase I), which is a component enzyme of the fatty acid oxidation complex (2), was easily inactivated in dilute solution, although it was surprisingly heat stable. Further studies proved that this inactivation was caused by Tris-hydrochloride buffer, which was routinely used for diluting and assaying this enzyme. A systematic study of this phenomenon revealed that all component enzymes of the complex were inactivated by Tris-hydrochloride. The half-times for the inactivations were 2 min for thiolase and 4 to 4.5 min for the other four enzymes when the complex (at a concentration of 27 μg/ml) was kept in 1 M Tris-hydrochloride, pH 8.1 (Fig. 3). However, in 0.2 M potassium phosphate (pH 8) none of the enzymes was significantly inactivated (Fig. 3). The inactivation was slower and not complete when the concentration of the protein was higher or when that of Tris-hydrochloride was lower. Because the Tris-dependent inactivation of long-chain enoyl-CoA hydratase was slower than that of crotonase and because the ratio of short-chain to long-chain enoyl-CoA hydratase activities did not remain constant during the purification of the protein, the possibility exists that two enoyl-CoA hydratases are present in the complex. There is no evidence for the presence of more than one thiolase or one L-3-hydroxyacyl-CoA dehydrogenase in the complex.

We conclude that in *E. coli* the five enzymes enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hy-

droxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, whose genes are closely linked and possibly form an operon (12), are associated in a multienzyme complex of fatty acid oxidation. Further studies should establish the quaternary structure of the complex as well as the physiological significance of the arrangement of several, but not all, enzymes of β-oxidation in a multienzyme complex.

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