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Studies on fatty acid oxidation in rat brain and *Escherichia coli*

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City University of New York, 1991

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STUDIES ON FATTY ACID OXIDATION IN RAT BRAIN AND Escherichia coli

by

XUE-YING HE

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1991

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

STUDIES ON FATTY ACID OXIDATION IN RAT BRAIN AND Escherichia coli

by

XUE-YING HE

Adviser: Professor Horst Schulz

In an attempt to clarify why the brain oxidizes fatty acids poorly or not at all, the activities of β-oxidation enzymes present in rat brain and rat heart mitochondria were measured and compared with each other. Although the apparent K_m values and chain-length specificities of the brain and heart enzymes are similar, the specific activities of all but one brain enzyme are between 4% and 50% of those observed in heart mitochondria. The exception is 3-ketoacyl-CoA thiolase (EC 2.3.1.16) whose specific activity in brain mitochondria is 125-times lower than in heart mitochondria. The partially purified brain 3-ketoacyl-CoA thiolase was shown to be catalytically and immunologically identical with the heart enzyme. The low rate of fatty acid oxidation in brain mitochondria, estimated on the basis of palmitoylcarnitine-supported

respiration and [1-^{14}C]palmitoylcarnitine degradation to be less than 0.5 nmol/min/mg of protein, may be the consequence of the low activity of 3-ketoacyl-CoA thiolase. Inhibition of [1-^{14}C]palmitoylcarnitine oxidation by 4-bromocrotonic acid proves the observed oxidation of fatty acids in brain to be dependent on 3-ketoacyl-CoA thiolase and thus to occur via β-oxidation. Since the reactions catalyzed by carnitine palmitoyltransferase (EC 2.3.1.21) and acyl-CoA synthetase (EC 6.2.1.3) do not seem to restrict fatty acid oxidation in brain, it is concluded that the oxidation of fatty acids in rat brain is limited by the activity of the mitochondrial 3-ketoacyl-CoA thiolase.

In Escherichia coli, a multienzyme complex of fatty acid oxidation exhibiting five different enzymatic activities consists of two large α-subunits and two small β-subunits that are encoded by the fadBA operon. The operon's structure and organization were revealed by sequencing a 5.2-kb DNA (PstI-SalI) fragment located at 87 min on the E. coli chromosome. The direction of transcription of this operon was found to be from fadB to fadA. It was concluded that the orientation of the fadA and fadB genes was the reverse of what had been published previously [S.K. Spratt et al. (1984) J. Bacteriol. 158, 535-542]. The transcription start site of the fadBA operon was located 42 nucleotides upstream of the initiator codon of the fadB gene by primer extension analysis. Sequences of the -10 and -35 regions of the promoter responsible for interaction with RNA polymerase were found to be CACACT and TTTGCA, respectively, and the location of the promoter of the fadBA operon was thus defined. The α- and β-subunits of the E. coli fatty acid oxidation complex were separated by gel filtration in formic acid, and the sequences of the amino-terminal 10 amino acids of these two subunits determined by Edman degradation coincided with those deduced from the nucleotide sequence data. The coding regions of fadA and fadB genes were correctly identified, and the primary structures of both subunits of the multienzyme complex are reported. The 3-ketoacyl-CoA thiolase β-subunit encoded by the fadA gene, is composed of 387 amino acids residues and has a calculated molecular weight 40,876. Sequence comparisons suggest that Cys-91 of the E. coli enzyme is the active-site cysteine residue, and that the consensus sequence of the active sites of 3-ketoacyl-CoA thiolases is Asn-Arg-X_1-Cys-X_2-Ser-X_3-X_4-Gln. Although the quaternary structure of the E. coli 3-ketoacyl-CoA thiolase is different from that of other thiolases, the sequence is homologous with rat and human peroxisomal and rat mitochondrial 3-ketoacyl-CoA thiolases, to the extent of 42%, 41%, and 37% identify, respectively. Since the peroxisomal 3-ketoacyl-CoA thiolase is more homologous to the E. coli enzyme than to the corresponding mitochondrial enzyme, it is suggested that the primitive peroxisomal 3-ketoacyl-CoA thiolase gene evolved from a prokaryotic 3-ketoacyl-CoA thiolase gene. The large α-subunit, coded for the fadB gene, a multifunctional protein is composed of 729 amino acid residues and has a calculated M_r of 79,593. A putative NAD-binding $\beta\alpha\beta$-fold necessary for L-3-hydroxyacyl-CoA dehydrogenase function was found in the central region of the fadB gene product. Sequence analysis suggest that the functional domains of the multifunctional protein are arranged in the order: enoyl-CoA hydratase: L-3-hydroxyacyl-CoA dehydrogenase:

Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase. In addition, sequence comparisons strongly suggest that the genes of the E. coli multifunctional protein and rat peroxisomal trifunctional β-oxidation enzyme evolved from a common ancestral gene.

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I wish to express my sincere gratitude to Professor Horst Schulz for his advice, encouragement and guidance throughout the course of this work.

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ABBREVIATIONS

bp	base pair
CoASH	coenzyme A
DTT	dithiothreitol
EDTA	ethylenediamineteraacetate
FAD	flavin adenine dinucleotide (oxidized)
$FADH_2$	flavin adenine dinucleotide (reduced)
EGTA	[ethlenebis(oxyethylenenitrilo)]tetraacetic acid
FAO complex	fatty acid oxidation multienzyme complex
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Kb	kilobase(s)
M_r	relative molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

PART ONE

Fatty Acid Oxidation in Rat Brain Is Limited by the Low Activity of 3-Ketoacyl Coenzyme A Thiolase

INTRODUCTION

Fatty acids are an important energy source for many organisms. Fatty acids must be converted to fatty acyl-CoA thioesters before entering into their catabolic pathway. The activation of fatty acids is catalyzed by acyl-CoA synthetase (EC 6.2.1.3). Fatty acyl-CoA thioesters are degraded via a β-oxidation spiral [1]. The mitochondrial β-oxidation mechanism involves a sequence of four reactions as follows:

1) Dehydrogenation of acyl-CoA to 2-<u>trans</u>-enoyl-CoA catalyzed by a group of three acyl-CoA dehydrogenases which differ in their chain length specificities (EC 1.3.99.2 and EC 1.3.99.3),

$$\text{R-CH}_2\text{-CH}_2\text{-CO-SCoA} + \text{FAD} \longrightarrow \text{R-CH=CH-CO-SCoA} + \text{FADH}_2$$

The cooperation of short-chain acyl-CoA dehydrogenase, which acts on butyryl-CoA and hexanoyl-CoA, with the medium-chain dehydrogenase, which is highly active toward substrates from hexanoyl-CoA to dodecanoyl-CoA, and the long-chain dehydrogenase, which preferentially acts on octanoyl-CoA and longer chain substrates, assures high rates of dehydrogenation over the whole spectrum of normal fatty acids and their chain-shortened intermediates.

2) Hydration of 2-<u>trans</u>-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by enoyl-CoA hydratase or crotonase (EC 4.2.1.17),

$$R\text{-}CH{=}CH\text{-}CO\text{-}SCoA + H_2O \rightleftharpoons R\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}SCoA$$

Crotonase acts with decreasing efficiency on all substrates from crotonoyl-CoA to hexadecenoyl-CoA. A second enoyl-CoA hydratase, that has been partially purified from pig heart, is inactive toward crotonyl-CoA and most active with 2-octenoyl-CoA; it is referred to as long-chain enoyl-CoA hydratase (EC 4.2.1.74). It has been suggested that the long-chain hydratase complements crotonase to assure a high rate of hydration over the entire spectrum of enoyl-CoA intermediates.

3) Oxidation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35),

$$R\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}ScoA + NAD^+ \rightleftharpoons R\text{-}CO\text{-}CH_2\text{-}CO\text{-}SCoA + NADH + H^+$$

A second mitochondrial L-3-hydroxyacyl-CoA dehydrogenase is associated with the inner membrane and exhibits a preference for long-chain substrates.

4) Cleavage of 3-ketoacyl-CoA by 3-ketoacyl-CoA thiolase (EC 2.3.1.16) to acetyl-CoA and an acyl-CoA two carbons shorter in length. Since the equilibrium of this reaction is far to the side of the products, the thiolytic cleavage pulls β-oxidation to completion.

$$R\text{-}CO\text{-}CH_2\text{-}CO\text{-}SCoA + CoASH \rightleftharpoons R\text{-}CO\text{-}SCoA + CH_3\text{-}CO\text{-}SCoA$$

The resulting acyl-CoA compounds pass through the β-oxidation cycle again until they are completely degraded to acetyl-CoA.

It is well known that the brain utilizes primarily glucose and ketone bodies [2] while free fatty acids are the major fuel in heart and some other tissues [3]. The biochemical basis for this metabolic difference between heart and brain remains poorly understood. It was reported [4] that blood-borne fatty acids are not oxidized by the adult brain even though free fatty acids are readily oxidized by brain after ventricular administration. Since a number of reports [5-7] claimed that brain mitochondria can oxidize palmitate and other fatty acids at rates comparable to those observed with liver or heart mitochondria, it was believed that all enzymes necessary for fatty acid oxidation are present in brain at levels comparable to those of other tissues [8,9]. Considering that long-chain fatty acids are mostly bound to albumin in serum the inability of brain to oxidize fatty acids was attributed to their exclusion by the blood-brain barrier [8,9]. Because the endothelial cells of cerebral capillaries are joined by tight junctions, fatty acids must pass through two capillary cell membranes to reach extracellular fluid. However, Dhopeshwarkar et al. [10] observed that after the intraperitoneal injection of [1-^{14}C]palmitic acid into young rats, the ^{14}C-label persisted in the carboxyl carbon of palmitate of brain lipids even after a period of 2 months. More recently, isolated brain mitochondria were shown to oxidize fatty acids at low rates [11,12]. These observations are contrary to the hypothesis that the unavailability of fatty acids to brain is the cause for the inability of brain to oxidize fatty acids. Therefore, it is of great interest to determine what factor limits the ability of brain to use fatty acids as an energy source.

In this report, I show that the specific activities of the β-oxidation enzymes in rat brain mitochondria are about 4% to 50% of those observed in heart mitochondria except for 3-ketoacyl-CoA thiolase (EC 2.3.1.16) whose specificity activity in brain mitochondria is two orders of magnitude lower than that in heart mitochondria. It appears that the rate of fatty acid oxidation in rat brain mitochondria is actually limited by the low activity of 3-ketoacyl-CoA thiolase.

EXPERIMENTAL PROCEDURES

Materials: NAD^+, NADH, CoASH, n-butyryl-CoA, n-decanoyl-CoA and palmitoyl-CoA were purchased from P-L Biochemicals. Ethyl chloroformate, triethylamine, diketene, and trans-2-decenoic acid were obtained from Aldrich. [1-^{14}C]Palmitoyl-L-carnitine (45 mCi/nmol) was supplied by New England Nuclear. Scinti Verse II was obtained from Fisher. 4-Bromocrotonic acid [13], crotonyl-CoA [14], acetoacetyl-CoA [15], decenoyl-CoA [16] and 3-ketodecanoyl-CoA [17] were synthesized according to published procedures. The concentrations of CoA derivatives were determined by the method of Ellman [18] after cleaving the thioester bond with hydroxylamine at pH 7. Bovine liver crotonase [19], pig heart 3-ketoacyl-CoA thiolase [20], antibodies to pig heart 3-ketoacyl-CoA thiolase (thiolase I) and antibodies to E. coli acetoacetyl-CoA thiolase (thiolase II) [20] were prepared as previously described. Palmitoyl-L-carnitine was generously provided by Dr. K. Brendel, University of Arizona, College of Medicine. A suspension of Staphylococcus aureus cells (Cowan strain), pig heart 3-hydroxyacyl-CoA dehydrogenase, Ficoll (type 400-DL), L-malate, ADP and all other standard biochemicals were obtained from Sigma.

Isolation of Mitochondria and Oxygen Uptake Measurements: Nonsynaptic mitochondria from rat brain and heart mitochondria were isolated from male, Sprague-Dawley rats (240-260 g) by the procedures of Lai and Clark [21] and

Chappell and Hansford [22], respectively. Protein concentrations were determined by the biuret method [23]. For oxygen uptake measurements, brain mitochondria (3 mg protein/ml) were incubated at 25°C in 1.9 ml of a medium containing 0.1 M KCl, 5 mM KP_i, 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA. Heart mitochondria (0.5 mg protein/ml) were incubated in 1.9 ml of a medium containing 0.11 M KCl, 2 mM KP_i, 33 mM Tris-HCl (pH 7.4), and 0.1 mM EGTA. To the suspension were added bovine serum albumin (0.5 mg/ml), 2.5 mM L-malate and 0.5 mM ADP. Respiration was initiated by the addition of 30 μM palmitoylcarnitine or other substrates. When puruvate or oxoglutarate served as substrates, bovine serum albumin was omitted from the incubation medium. Rates of respiration were measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph.

Partial Purification of 3-Ketoacyl-CoA Thiolase and Acetoacetyl-CoA Thiolases from Rat Brain and Heart Mitochondria: Rat brain mitochondria (27.8 mg of protein) or rat heart mitochondria (19 mg of protein) suspended in 3 ml of 50 mM KP_i (pH 6.6) containing 10 mM mercaptoethanol were sonicated 6 times for 5 sec each. The resulting suspension was centrifuged at 120,000 x g for 30 min and the supernatant was applied to a phosphocellulose column (1.2 x 7 cm) which had been previously equilibrated with 50 mM KP_i (pH 6.6) containing 5% glycerol and 10 mM mercaptoethanol. The column was washed with the same buffer until materials absorbing light at 280 nm ceased to be eluted. The column was then developed with a gradient made up of 75 ml of 50 mM KP_i (pH 6.6) containing 10% glycerol, 10 mM mercaptoethanol and 75 ml of 0.5 M KP_i (pH 6.6) containing 10% glycerol, 10 mM mercaptoethanol. Fractions of 3 ml were collected and assayed for thiolase activities with acetoacetyl-CoA and 3-ketodecanoyl-CoA as substrates [24]. Fractions active with 3-ketodecanoyl-CoA and acetoacetyl-CoA as substrates (thiolase I) were pooled and concentrated in an Amicon concentrator fitted with a PM-10 membrane. Fractions active with acetoacetyl-CoA only (thiolase II) were also pooled and concentrated. All operations, except for enzyme assays, were performed at 4°C. The separation of the two thiolases was carried out twice with virtually the same result.

Immunotitration of Thiolase: The partially purified thiolases were incubated with varying amounts of antibodies for 1 min at 25°C, after which time 120 μl of assay buffer containing 0.1 M HEPES (pH 8.1), 25 mM $MgCl_2$, bovine serum albumin (0.2 mg/ml), 2 mM mercaptoethanol, and 5% (v/v) glycerol was added. This mixture was incubated together with 45 μl of a 10% cell suspension of Staphylococcus aureus (Cowan strain) for 1 min at 25°C, and then 830 μl H_2O and 120 μl of the assay buffer were added and well mixed. The resulting mixture was centrifuged on a Beckman Microfuge B for 2 min and 0.6 ml of the supernatant was assayed for thiolase I (3-ketoacyl-CoA thiolase) and thiolase II (acetoacetyl-CoA thiolase) activities with 3-ketodecanoyl-CoA and acetoacetyl-CoA, respectively. The thiolase reaction was always initiated by the addition of CoASH.

Oxidation of [1-^{14}C]Palmitoylcarnitine by Rat Brain Mitochondria: The reaction mixture contained in a final volume of 0.7 ml 60 mM Tris-HCl (pH 7.4), 120 mM KCl, 4 mM KP_i, 8 mM $MgCl_2$, 0.5 mM EDTA-K_2, fatty acid-free bovine serum albumin (0.1 mg/ml), 0.05 mM L-malate, 1 mM ADP, 40 μM [1-^{14}C]palmitoylcarnitine (160,000 cpm), and rat brain mitochondria which had been preincubated with or without 17.2 μM 4-bromocrotonic acid for 3 min at 37°C. Fatty acid oxidation was initiated by the addition of rat brain mitochondria (0.5 mg of protein), and the rate of [1-^{14}C]palmitoylcarnitine degradation was measured at 37°C under shaking in a 25 ml Erlenmeyer flask covered by a rubber septum to which a plastic center well was attached. At the end of the incubation period 0.3 ml of 1 M hyamine hydroxide was injected into the center well, and the reaction was stopped by the injection of 0.1 ml of 70% perchloric acid into the incubation medium. The flask was shaken for an additional 2 hrs to permit the complete absorption of $^{14}CO_2$ by hyamine hydroxide. The center well was then transferred to a scintillation vial, and the hyamine hydroxide was thoroughly mixed with 4 ml Scinti Verse II and the resulting solution was counted in a liquid scintillation counter. The acidified reaction mixture was centrifuged at 120,000 x g for 60 min at 4°C. Then, 0.1 ml of the supernatant was mixed with 4 ml of Scinti Verse II and the radioactivities of acid-soluble ^{14}C-labeled products were determined in a scintillation counter. Net radioactivity values were calculated by subtracting values obtained from control experiments in which mitochondria had been omitted.

Enzymes Assays: The activities of acyl-CoA dehydrogenase were measured as described by Davidson and Schulz [25], and those of enoyl-CoA hydratases by the indirect method of Fong and Schulz [26]. The L-3-hydroxyacyl-CoA dehydrogenase activities were determined in the forward direction with L-3-hydroxydecanoyl-CoA as a substrate [27] and also in the backward direction with acetoacetyl-CoA as a substrate [24]. The activities of 3-ketoacyl-CoA thiolases and acetoacetyl-CoA thiolases were assayed as described in principle by Lynen and Ochoa [28] and as detailed by Binstock and Schulz [24]. The thiolase reactions were initiated by the addition of CoASH. Extinction coefficients at 303 nm for acetoacetyl-CoA and 3-ketodecanoyl-CoA are 16,500 M^{-1} cm^{-1} and 9,500 M^{-1} $cm^{-1,}$ respectively. The enzyme assays were performed at 25°C on a Gilford recording spectrophotometer. A unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate to product per minute.

RESULTS

Specific Activities of β-Oxidation Enzymes in Rat Brain and Rat Heart Mitochondria: In an attempt to establish whether any single β-oxidation enzyme is responsible for the limited capacity of brain mitochondria to oxidize fatty acids, we determined the specific activities of these enzymes in rat brain and rat heart mitochondria and compared them with each other. For this purpose mitochondria from rat brain and rat heart were isolated, purified and used as sources of β-oxidation enzymes. Triton X-100 was used to disrupt the mitochondrial membrane. At the concentration used in these experiments (0.05%), Triton X-100 does not affect the activities of any of β-oxidation enzymes [29]. The specific activities measured with substrates of various chain lengths are listed in Table I. All enzymes could be detected and quantitated in brain mitochondria disrupted with Triton X-100 except for 3-ketoacyl-CoA thiolase for which activities with 3-ketoacyldecanoyl-CoA as substrate were virtually identical in the absence and presence of CoASH. This finding suggests that either 3-ketoacyl-CoA thiolase (thiolase I) is not present or its activity is very low, and furthermore that the thiolase activity detected with acetoacetyl-CoA as a substrate reflects mostly or exclusively the activity of acetoacetyl-CoA thiolase (thiolase II).

The activity levels of most β-oxidation enzymes in brain are approximately 4%

to 20% of the activities present in heart mitochondria. One exception is acyl-CoA dehydrogenase with decanoyl-CoA as a substrate, believed to reflect primarily the activity of medium-chain acyl-CoA dehydrogenase, which is present in brain at half the level observed in heart (see Table I). Since apparent K_m values and chain length specificities of enoyl-CoA hydratase(s) as well as 3-hydroxyacyl-CoA dehydrogenase of brain and heart mitochondria are very similar (see Table I and II), the brain and heart β-oxidation enzymes are most likely identical. If so, the lower activity levels of these enzymes in brain as compared to heart probably reflect differences between their intramitochondrial concentrations.

Separation and Partial Purification of 3-Ketoacyl-CoA Thiolases and Acetoacetyl-CoA Thiolases from Rat Brain and Heart Mitochondria: In order to establish whether or not 3-ketoacyl-CoA thiolase is present in rat brain mitochondria, a soluble mitochondrial extract was subjected to chromatography on phosphocellulose. When an extract from heart mitochondria was used to test the separation procedure, the thiolase activity was found to be separated into two fractions (see Fig. 1A). The fraction emerging first from the column (Peak I) acted on both acetoacetyl-CoA and 3-ketodecanoyl-CoA and thus was 3-ketoacyl-CoA thiolase (thiolase I), whereas the second fraction was active only with acetoacetyl-CoA as a substrate and therefore was assumed to be acetoacetyl-CoA thiolase (thiolase II) [30]. When an extract of brain mitochondria was subjected to the same separation procedure, again two peaks of activity were detected (see Fig. 1B). However, in contrast to heart, the first peak was very small but reflected 3-ketoacyl-CoA thiolase (thiolase I) activity as evidenced by the thiolytic cleavage of 3-ketodecanoyl-CoA which was CoA-dependent. The much larger second peak reflected acetoacetyl-CoA thiolase (thiolase II). Clearly, most of the acetoacetyl-CoA thiolase activity present in brain mitochondria is due to thiolase II. Estimates of the 3-ketoacyl-CoA thiolase activity in brain mitochondria yielded values of 1.3 and 3.9 mU/mg of mitochondrial protein for 3-ketoacyl-CoA thiolase with acetoacetyl-CoA and 3-ketodecanoyl-CoA as substrates, respectively (see Table I). These activity levels are less than 1% of those observed in heart mitochondria. In contrast to the extremely low 3-ketoacyl-CoA thiolase activity in brain, acetoacetyl-CoA thiolase, which is believed to function in ketone body degradation [20], is present in brain mitochondria at one-twelfth of the level characteristic of heart mitochondria.

Immunological and Catalytic Properties of 3-Ketoacyl-CoA Thiolase from Rat Brain and Rat Heart Mitochondria: The structural relationships between the mitochondrial thiolases of rat brain and rat heart were evaluated by immunotitration with antibodies raised against pig heart thiolase I and *E. coli* thiolase II. A bacterial suspension of *S. aureus*, which carries protein A on its surface, was used to facilitate the precipitation of the thiolase-antibody complexes by centrifugation [31] after which step the activity remaining in the supernatant was measured. The immunotitration curves thus obtained showed that 3-ketoacyl-CoA thiolase from either rat brain or heart, just as the homologous enzyme from pig heart, strongly reacted with antibodies raised against purified pig heart thiolase I. However, neither of the two antibodies used in this study cross-reacted with thiolase II from rat brain and heart (see Fig. 2). These observations suggest that rat brain 3-ketoacyl-CoA thiolase has antigenic sites, and consequently an overall structure, similar to those of the corresponding enzymes from pig heart and rat heart. However, the two thiolases present in rat brain are structurally different.

The functional similarity between rat brain and rat heart mitochondrial thiolases I was evaluated by studying their catalytic properties. The apparent K_m values of rat brain 3-ketoacyl-CoA thiolase were found to be 14.1 μM and 1.2 μM for acetoacetyl-CoA and 3-ketodecanoyl-CoA, respectively. These values are very close to the apparent K_m values of 15.4 μM and 1.1 μM, respectively, of the corresponding enzyme from rat heart (see Table II). The chain-length specificities of rat brain and rat heart 3-ketoacyl-CoA thiolases appear to be similar as evidenced by ratios of 3-ketodecanoyl-CoA to acetoacetyl-CoA activities of 3 for rat brain and 2.5 for rat heart.

Rates of Long-Chain Fatty Acid Oxidation in Rat Brain Mitochondria: Isolated nonsynaptic mitochondria from rat brain are metabolically active and well coupled. The oxygen uptake rates with pyruvate and oxoglutarate as substrates were determined 6 times each and found to be 111±23 and 75±5 nanoatoms oxygen/min/mg of protein at 25°C, respectively. However, the rates of long-chain fatty acid oxidation by the same mitochondria were found to be extremely low. For example, the addition of 30 μM palmitoylcarnitine to suspensions of mitochondria at 25°C stimulated the oxygen uptake by only 2.8 ± 0.7 nanoatoms of oxygen/min/mg of protein at state 3 respiration (see Fig. 3). This measurement proves the rate of fatty acid oxidation in rat brain mitochondria to be approximately 50 times lower than in heart mitochondria and 30 times lower than in liver mitochondria.

The observed rate of [1-^{14}C]palmitoylcarnitine degradation catalyzed by rat brain mitochondria at 37°C was 0.45 nmol/min/mg of protein (see Fig. 4). The preincubation of rat brain mitochondria with 4-bromocrotonic acid, which causes the inhibition of mitochondrial 3-ketoacyl-CoA thiolase [32], resulted in a 90% inhibition of [1-^{14}C]-palmitoylcarnitine oxidation together with a change of the ratio of acid-soluble products formed to CO_2 released from 5 or larger to 1 (see Fig. 4). The inhibitory effect of 4-bromocrotonic acid on the formation of CO_2 and acid-soluble products proves the degradation of [1-^{14}C]palmitoylcarnitine to occur by β-oxidation. Since 4-bromocrotonic acid must be activated and metabolized before causing the inactivation of thiolases, this experiment also provides evidence for the presence of a medium-chain acyl-CoA synthetase in rat brain mitochondria.

Since the complete oxidation of palmitoylcarnitine requires it to pass seven times through the β-oxidation spiral, 3-ketoacyl-CoA thiolase must act on seven intermediates formed during the breakdown of a single palmitate residue.

Consequently, the activity of thiolase must be seven times higher than the rate of palmitoylcarnitine degradation sustained by it. Taking this relationship between thiolase activity and palmitate degradation into account, it appears that the rate of β-oxidation in rat brain is limited by the activity of 3-ketoacyl-CoA thiolase.

DISCUSSION

Studies aimed at elucidating the rate-limiting step and regulation of fatty acid oxidation have primarily focused on liver and heart. For example, evidence suggests that the rate-limiting step in heart is catalyzed by 3-ketoacyl-CoA thiolase whose activity can be modulated by the acetyl-CoA/CoASH ratio [1]. In liver, fatty acid oxidation is believed to be controlled via the regulation of carnitine palmitoyltransferase I [33] which therefore catalyzes the rate-limiting step or one of the slow steps of the pathway. in contrast, the rate-limiting step of fatty acid oxidation in brain remains unknown.

Recently, Bird et al. reported [11] that the activities of carnitine palmitoyltransferase I and II in rat brain mitochondria are one-fourth and one-half, respectively, of the corresponding enzyme activities in liver mitochondria, and they concluded that the activity of neither of these enzymes is likely to be rate-limiting in fatty acid oxidation in brain.

This study was focused on the inherent capacity of brain mitochondria for fatty acid oxidation and specifically attempted to elucidate the rate-limiting step of β-oxidation in brain mitochondria. The activity levels of most enzymes are between 4% and 20% of the specific activities observed in heart mitochondria and therefore might sustain in brain a rate of fatty acid oxidation which would be significant even in comparison with heart. However, two of the brain enzymes, medium-chain acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, are present at surprising activity levels. The activity of the former enzyme is unusually high at half of the level characteristic of heart, whereas 3-ketoacyl-CoA thiolase is present in brain at a hardly detectable level of less than 1% of that observed in heart. The significance of the high activity level of medium-chain acyl-CoA dehydrogenase remains to be elucidated. However, the extremely low activity of 3-ketoacyl-CoA thiolase in brain mitochondria points to the thiolytic cleavage of 3-ketoacyl-CoA thioesters as the rate-limiting step in brain β-oxidation. If fact, a previous study of thiolases in various mammalian tissues seemed to indicate that 3-ketoacyl-CoA thiolase may be absent from brain [34]. However, the evidence gathered in this study clearly demonstrates the presence of this enzyme in brain mitochondria. The most convincing evidence for the existence of this enzyme in brain is its immunoprecipitation by antibodies to pig heart 3-ketoacyl-CoA thiolase. Also, the partial purification of a thiolase active with 3-ketodecanoyl-CoA and the elution of this enzyme from a phosphocellulose column at a position identical with that of heart 3-ketoacyl-CoA thiolase attest to the presence of this enzyme in brain.

In contrast to 3-ketoacyl-CoA thiolase, acetoacetyl-CoA thiolase is present in brain mitochondria at 8% of the level observed in heart. This relatively high activity level of short-chain specific thiolase is in accord with the significant capacity of brain to metabolize ketone bodies.

The thiolytic capacity of brain mitochondria is sufficient to sustain the degradation of approximately 0.6 nmol of palmitate/min/mg of mitochondrial protein. This value was calculated by dividing the specific activity of 3-ketoacyl-CoA thiolase of 3.9 mU/mg of mitochondrial protein by seven to account for the enzyme having to act on seven different metabolites formed during the breakdown of palmitate. Since the thiolase-catalyzed step is significantly slower than other β-oxidation reactions, 3-ketoacyl-CoA thiolase may be responsible for the extremely slow rate of fatty acid oxidation in brain mitochondria. In order to estimate the capacity of brain tissue to oxidize fatty acid, the respiration supported by palmitoylcarnitine and the degradation of [1-^{14}C]palmitoylcarnitine by brain mitochondria were measured. Since the oxidation of palmitate may be incomplete, the observed rate of palmitoylcarnitine degradation of 0.45 nmol/min/mg of protein may be greater than the maximal capacity of brain mitochondria to oxidize palmitoylcarnitine.

In view of this very low rate of fatty acid oxidation of brain it was necessary to prove that the observed degradation of palmitate had occurred by β-oxidation and not by α-oxidation or some other pathway. The availability of 4-bromocrotonic acid, an inhibitor of mitochondrial thiolases, provided the means to determine whether or not palmitoylcarnitine is β-oxidized in brain. 4-Bromocrotonic acid itself is metabolized by β-oxidation to yield 3-keto-4-bromobutyryl-CoA which irreversible inactivates both 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase [32]. The 90% inhibition of palmitate oxidation by 4-bromocrotonic acid agrees with the assumption that the observed fatty acid oxidation in brain mitochondria had occurred by β-oxidation. The simultaneous increase in the ratio of released CO_2 to acid-soluble products formed indicates that the tricarboxylic acid cycle is not affected by this treatment, whereas β-oxidation is strongly inhibited. In addition, the inhibition of palmitate oxidation by 4-bromocrotonic acid proves that medium-chain acyl-CoA synthetase is present in brain mitochondria or otherwise 4-bromocrotonic acid would not have been converted to its inhibitory metabolite.

Palmitate and other long-chain fatty acids can be activated in brain because of the presence of long-chain acyl-CoA synthetase in mitochondria, microsomes and the cytosol of rat brain [5]. Reddy et al. [35] reported activities of palmitoyl-CoA synthetase of 7.0 and 2.9 nmol/min/mg/ of protein in rat brain microsomes and mitochondria, respectively. Consequently, long-chain fatty acids can be rapidly converted to their CoA derivatives in rat brain.

The results presented here, when taken together with previously published data, prompt the suggestion that fatty acid oxidation in brain is limited by the acitvity of mitochondrial 3-ketoacyl-CoA thiolase [36]. However, the possibility must be considered that the transfer of fatty acids from plasma into brain cells through the blood-brain barrier is the slowest step in fatty acid metabolism in brain. In fact, Hawkins [9] seems to advocate such a model. Yet, some published data do not agree with his hypothesis. For example Pardridge and Mietus reported [37] that approximately 5% of plasma palmitate is unidirectionally cleared by brain on a single pass. Since the net uptake of free fatty acids by brain is zero [38,39], the unidirectional clearance of plasma palmitate is possible balanced by a simultaneous release of free fatty acids from the brain. Consequently, the metabolism of fatty acids by brain does not seem to be restricted by their uptake, but rather by the low capacity of brain to oxidize fatty acids appears to prevent their net uptake.

PART TWO

Nucleotide Sequence of the fadBA Operon and Primary Structures of the Multifunctional Fatty Acid Oxidation Protein and 3-Ketoacyl-CoA Thiolase from Escherichia coli

INTRODUCTION

The presence of an active pathway of fatty acid oxidation in E. coli was established in 1967 when Overath and coworkers [40] showed that growth of E. coli on long-chain fatty acids instead of glucose resulted in a 200-fold induction of the enzymes of fatty acid oxidation. The synthesis of the fatty acid degradation (fad) enzymes in E. coli is strongly repressed by the presence of glucose in the growth medium. Inducers such as oleate could not relieve this repression but addition of cAMP did cause derepression [41]. Like many other inducible enzyme systems, the expression of the genes coding for fad enzymes is negatively controlled by a repressor, the fadR gene product [42,43]. Since an E. coli mutant lacking acyl-CoA synthetase cannot be induced to synthesize the fad enzymes, Overath and coworkers [42] suggested that the fatty acyl-CoA derivative rather than fatty acids serve as inducers of the enzymes of fatty acid oxidation.

Purification of the β-oxidation enzymes from E. coli resulted in the isolation of a multienzyme complex which has an estimated molecular weight of 260,000 which is composed of two types of subunits with molecular weights of 78,000 and 42,000 [24]. The quaternary structure of the complex is $\alpha_2\beta_2$ where α and β denote the 78,000-Da and 42,000-Da subunits, respectively [44]. This multienzyme complex is encoded by the fadBA operon which was mapped at 87 min of the E. coli chromosome [45-48]. The fadA gene specifies 3-ketoacyl-CoA thiolase (thiolase I) (EC 2.3.1.16), the small β-subunit of the complex [44,45,47]. The fadB gene product, the large α-subunit of the complex, is a multifunctional polypeptide, exhibiting four different enzyme activities [45,46] as follows. Enoyl-CoA hydratase (crotonase) (EC 4.2.1.17) and L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) catalyze the second and third reactions of the β-oxidation spiral respectively, whereas Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) are auxiliary enzymes that function in the β-oxidation of polyunsaturated fatty acids [49].

The fatty acid oxidation complex was overproduced by expression of the fadBA operon located within a 5.2-kb (PstI-SalI) fragment of E. coli DNA and has been purified and characterized [46]. The important role of this multienzyme complex in fatty acid β-oxidation was intensively studied. The substrate chain-length specificities of the component β-oxidation enzymes: 3-ketoacyl-CoA thiolase, L-3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase were reported [24]. Based on a kinetic study, it was concluded that β-oxidation intermediates are directly transferred from the active site of enoyl-CoA hydratase (crotonase) to that of L-3-hydroxyacyl-CoA dehydrogenase on the large subunit of the multienzyme complex [49-51]. Because of intermediate channeling, the E. coli fatty acid oxidation complex is capable of directly β-oxidizing 2,4-dienoyl-CoAs, key intermediates in the β-oxidation of polyunsaturated fatty acids [49,51]. Furthermore, the degradation of 2-

trans, 4-cis-decadienoyl-CoA to hexanoyl-CoA by the E. coli fatty acid oxidation complex suggested that the epimerase-dependent pathway of unsaturated fatty acid oxidation [52] is operative in non-mitochondrial β-oxidation systems [49]. However, many unanswered questions have emerged as a result of these studies. Where on the single polypeptide chain do the four different enzymatic functions of the fadB gene product reside? Why does the fadA gene product have a broad substrate specificity distinct from that of acetoacetyl-CoA thiolase (thiolase II) (EC 2.3.1.9)? What is the structural basis for the channeling of β-oxidation intermediates on the large subunit of the multienzyme complex. How are the structural genes fadA and fadB expressed? Information about the fadBA promoter and the primary structures of the α and β subunits of the multienzyme complex is essential for seeking answers to such questions.

In this paper, I report the nucleotide sequence of the fadBA operon and the deduced primary structures of the E. coli 3-ketoacyl-CoA thiolase (β-subunit) and of the multifunctional protein (α-subunit). The transcription start site has been located at an adenine nucleotide 42-bp upstream of the fadB initiator codon by primer extension analysis, and the promoter region of the fadBA operon is defined. Based on sequence homologies, I propose the approximate locations of the enzymatic functional domains within the amino acid sequence of the E. coli multifunctional protein. Sequence comparisons suggest that the peroxisomal thiolase I gene evolved directly from the prokaryotic thiolase I gene, and that the E. coli multifunctional protein and the rat peroxisomal trifunctional β-oxidation enzyme have a common evolutionary origin.

EXPERIMENTAL PROCEDURES

Materials: E. coli strain MV1190 is a Δ(srl-recA)306::Tn10 transductant of JM101 [47]. E. coli strain LS6749 containing plasmid pK52 was described previously [46,47]. E. coli strain DH5αF' competent cells were purchased from Bethesda Research Laboratories. M13 mp19 and mp18 have been described by Yanisch-Perron et al. [53]. Restriction endonucleases, T4 polynucleotide kinase, and yeast tRNA were purchased from Boehringer Mannheim. M-MLV reverse transcriptase, RNasin, and RNase-free DNase were from Bethesda Research Laboratories. T4 polymerase and the Cyclone I Biosystem were obtained from International Biotechnologies, Inc. Sequenase Version 2.0 Kit, T4 ligase, and the Klenow fragment of DNA polymerase were purchased from United States Biochemical Corp. Taq DNA polymerase was from Perkin Elmer/Cetus. [α-^{35}S]dATP and [γ-^{32}P]dATP were obtained from Amersham Co. The fluorescent dye-labeled primers were supplied by Applied Biosystems, Inc.

Isolation of DNA: The 5.2-kb DNA fragment containing the fadBA operon was purified from a culture of strain LS6749 (pK52) as follows. The recombinant plasmid pK52 was amplified by culturing the cells in 500 ml minimal medium with chloramphenicol [54]. After the cells were resuspended in 70 ml of cold STET buffer

(8% sucrose, 10mM Tris-HCl at pH 8.0, 50mM EDTA, and 0.5% Triton X-100) containing 50 mg lysozyme, they were lysed by boiling. The plasmid DNA was isolated from the supernatant as described previously [55]. The plasmid pK52 was digested with Pst I and Sal I, and the resulting DNA fragments were separated by 1% agarose gel electrophoresis. A 5.2-kb DNA fragment was removed and purified from the gel according to a published procedure [56].

DNA Sequencing: The purified 5.2-kb DNA (PstI-SalI) was digested with Bgl II and/or Cla I. Various restriction fragments were cloned into M13 mp19 and mp18 vectors using MV1190 competent cells treated with TSS (Luria-Bertani broth with 10% polyethylene glycol, 5% dimethyl sulfoxide, and 50mM Mg^{2+} at pH 6.5) [57]. The orientation of an insert in a single BamH I site was ascertained by hybridization assay with single-stranded template DNA [58]. Overlapping deletions in single-stranded template DNA were generated with T4 polymerase according to the method of Dale et al. [59] but using DH5αF' competent cells as described by Hanahan [60]. The DNA sequencing strategy is shown in Fig. 5. The nucleotide sequence was determined by the dideoxy chain termination method [61,62] with 7-deaza-dGTP instead of dGTP [63], and base determination was automated by using an Applied Biosystems, Inc. 370A DNA sequencer [64]. The nucleotide sequence at regions containing compressions was verified manually by use of Sequenase Version 2.0 and [α-^{35}S]dATP, and the substitution of dITP for dGTP [65]. Computer assisted sequence analysis was accomplished using the PC/GENE (IntelliGenetics Co.)

programs [66].

Determination of N-terminal Amino Acid Sequences: The multienzyme complex of fatty acid oxidation was purified from strain LS6749 (pK52) after induction with oleic acid as a sole carbon source [46). Purified multienzyme complex (2.6mg/ml) was dissolved in 70% formic acid and injected onto a Pharmacia Superose 12HR 10/30 gel filtration column, which was developed with 70% formic acid at a flow rate of 0.5ml/min (see Fig. 6). The fractions collected under the peaks were concentrated about 20-fold, and a 30 µl aliquot of the concentrated sample was analyzed by automated Edman degradation [67] using an Applied Biosystems, Inc. 470A gas-phase sequencer.

Primer Extension Analysis: Total E. coli RNA was purified from strain LS6749 (pK52) growing exponentially in an M9 salts medium supplemented with 1% tryptic peptone and 0.1% (v/v) oleate dispensed in 0.4% (v/v) Triton X-100 by a procedure adapted from published methods [68,69]. A primer specific to fadB (5' CGGCAATGCCATCTTCCA 3') was synthesized with an Applied Biosystems, Inc. 380B DNA synthesizer [70], and was 5' end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase as described [71]. 20µg of RNA mixed with 400 f mol of [^{32}P]-labelled primer (5x10^4 cpm) in 20µl buffer (containing 250mM KCl, 5mM Tris-HCL and 0.5mM EDTA, pH 8.0) was denatured in a beating block set at 80°C for 2 min, and this sample was then transferred to a 46°C waterbath for hybridization at least

2 hrs. After the annealing reaction, the sample was mixed with 20 units RNasin and was diluted to 125µl of the final concentration:50mM Tris-HCL (pH 8.3), 3mM $MgCl_2$, 75mM KCL, 10mM DTT, and 2mM of each dATP, dGTP, dTTP, and dCTP. For each sample 2µl (400 units) M-MLV reverse transcriptase was added, and the extension reaction proceeded at 42°C for 60 min [72]. Two parallel experiments, one with 20µl of yeast tRNA as a substitute for the RNA samples and another without addition of the reverse transcriptase, were performed as controls. At the end of the primer extension, 4µl of 0.5M EDTA was added to stop the reaction. After the nucleic acid was recovered by ethanol precipitation, the pellet was resuspended in 6µl of formamide gel loading buffer. Then, primer extension products were separated by electrophoresis on a 8% polyacrylamide/8M urea gel alongside a dideoxy sequencing ladder [65], generated from a M13 mp19 clone of a 1.25-kb PstI-BglI fragment that includes the initiator codon and 5' flanking region of the fadB gene DNA using the above-mentioned, unlabelled oligonucleotide as primer.

RESULTS AND DISCUSSION

N-Terminal Amino Acid Sequences of the α- and β-subunits: The large and small subunits of the fatty acid oxidation complex were resolved by Fast Protein Liquid Chromatography gel filtration in 70% formic acid (see Fig. 2). Aliquots corresponding to the peaks were subjected to amino acid sequence analysis. The sequences obtained for the two subunits are presented in Table III.

DNA Sequence of the fadBA Operon: The approximate location of the fadA gene in the cloned 5.2-kb (Pst I - Sal I) DNA fragment was predicted by Spratt et al. [73]. For the purpose of sequencing the 3-ketoacyl-CoA thiolase gene, the nucleotide sequences of both strands around the Cla I restriction site on the 3.3-kb (Bgl II-Bgl II) DNA fragment were determined (see Fig. 5). Inspection of all six reading frames revealed that only one gave an open reading frame long enough to accommodate the 3-ketoacyl-CoA thiolase gene. The beginning of this open reading frame was located at 0.83-kb upstream from the Cla I site. The complete nucleotide sequence of fadA with 321-bp of 3' non-coding regions is shown in Fig. 7. A Shine-Dalgarno sequence is centered 9 nucleotides on the 5' side of the initiator codon ATG. Translation of the DNA sequence in this 1, 161-bp long open reading frame encodes a 387-amino acid polypeptide with a calculated M_r of 40,876. This value is in good agreement with the 42,000 molecular weight of the β-subunit of the E. coli fatty acid oxidation complex as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis [44]. The sequence of the first 10 residues of the β-subunit exactly matches the sequence of 3-ketoacyl-CoA thiolase deduced from the nucleotide sequence data (see Fig. 7). This result corroborates the deduced sequence, and indicates that the thiolase coding region was correctly identified. Subsequently, the nucleotide sequences of both strands at the 5' flanking region of the fadA gene were further determined for up to 2.6 kb (see Fig. 5). Inspection of all six reading frames revealed only one with a 2.2-kb open reading frame long enough to accommodate the multifunctional protein gene. The beginning of this open reading frame was located 150 bp upstream of a Bgl II site. A small 147 bp (Bgl II-Bgl II) DNA fragment was found to be lost in the previously published restriction map [73]. In fact, there is an additional Bgl II restriction site near the first Bgl II site (Fig. 5). The complete nucleotide sequence of the fadB gene with 381 base pairs of 5'-noncoding region and 12 base pairs in the 3'-noncoding region that links fadB to fadA is shown in Fig. 7. The fadA gene is located 9 nucleotides downstream of the stop codon of the fadB gene. A Shine-Dalgarno sequence is centered nine nucleotides on the 5'-side of the initiator codon of the fadB gene. The translation product of the DNA sequence in the 2,187-base pair-long open reading frame is a polypeptide made-up of 729 amino acid residues with a calculated molecular weight of 79,593. This value is in good agreement with the value of 78-kDa of the α-subunit of the E. coli fatty acid oxidation complex as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [44]. The sequence of the first 10 residues of the α-subunit determined by Edman degradation exactly matches the amino acid sequence of the multifunctional protein deduced from the nucleotide sequence data (see Fig. 7). These results indicate that the *fadB* coding region has been correctly identified. Several discrepancies between the data presented here and those of recent reports are apparent. Guanine residues are present at positions 120, 1933, and 2690 instead of cytosine residues, and there are cytosines rather than the guanine residues at positions 121, 1934, 2373, 2377 and 2691, as reported by DiRusso [74] and Nakahigashi and Inokuchi [75] . In addition, there is an adenine rather than a guanine residue at position 2936 as reported by Nakahigashi and Inokuchi [75], and a guanine residue was missing after nucleotide 3823 in that report [75]. Four of these nucleotide errors occurred in the *fadB* coding sequence, and resulted in changes of the protein sequence: (a) the residue 518, alanine, was replaced by arginine, and (b) a stretch of amino acid sequence LGFPPFH (residues 662-668) was changed to LGLPAFH. Therefore, the molecular weight of the *fadB* gene product, 79,678 Da, predicted previously by DiRusso [74] should be corrected. Moreover, residues 37 and 119 of 3-ketoacyl-CoA thiolase reported to be threonine and glycine [75] are actually serine and glutamic acid, respectively, and it is not clear how Nakahigashi and Inokuchi [75] could define the 5' ends of the structural genes *fadB* and *fadA* with confidence, since they do not report any studies of the amino-terminal sequences of the subunits of the *E. coli* fatty acid oxidation complex.

Structural Organization and Direction of Transcription of the *fadBA* Operon: Early genetic studies provided evidence for the close linkage of the *E. coli* 3-hydroxyacyl-CoA dehydrogenase gene (*fadB*) and the *fadA* gene [42]. More recently, it has been reported [46] that the *fadB* gene encodes 3-hydroxyacyl-CoA epimerase, Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase, and enoyl-CoA hydratase, in addition to 3-hydroxyacyl-CoA dehydrogenase; the product of *fadB* is apparently a multifunctional polypeptide. Based on the results of genetic and biochemical studies, Overath *et al.* [42] and Yang and Schulz [45] have suggested that *fadA* and *fadB* genes form a fatty acid oxidation operon in *E. coli*. Subsequently, Spratt *et al.* [73] attempted to determine the orientation of the *fadA* and *fadB* genes in this operon by subcloning and by Tn*5* mutagenesis, and concluded that the direction of transcription is from the *fadA* gene to the *fadB* gene, the latter being distal to the promotor. According to this model of operon organization, a 0.5-kb *Bgl* II - *Sal* I fragment would be upstream (5') of the *fadA* gene [43,73,76]. However, by inspection of the DNA sequence, it is clear that the location of this piece of DNA is about 0.3-kb downstream from the stop codon (TAA) of the *fadA* gene. Thus, only DNA *upstream* from the *fadA* gene is sufficiently long to accommodate the *fadB* gene. Furthermore, it was found that the stop codon (TAA) of the *fadB* gene, which lies in a 2.2-kb open reading frame, is located 9 nucleotides upstream of the *fadA* gene, and overlaps the putative ribosome-binding site of the *fadA* gene (Fig. 7). This revised organization of the operon, in which the transcription direction is from *fadB* to *fadA* as shown in Fig. 8, was first published by Yang *et al.* [47], and later confirmed by others [74,75].

Identification of Promoter of fadBA Operon: The transcription start site was determined by primer extension analysis. The primer used is complementary to the fadBA mRNA between nucleotides 35 and 52 downstream from the initiator codon of fadB coding sequence (Fig. 7). The results of a representative experiment are presented in Fig. 9. The size of the most prominent extended fragment indicates that the transcription initiates with an adenine residue (designated +1). The 5' end of fadBA mRNA was clearly mapped at 42 nucleotides upstream from the initiator codon of the fadB coding sequence. This result permits identification of the fadBA promoter region that interacts with RNA polymerase to form the initiator complex. The -10 region sequence CACACT, with a spacing of 7-bp upstream from the transcription start site, shows a reasonable homology to the consensus Pribnow sequence, and is very similar to the -10 region sequence of malPQ operon of E. coli [77]. Moreover, the -35 region sequence TTTGCA has 4 of 6 nucleotides that are identical with the consensus -35 hexamer. Homology to conserved base pairs around the -35 hexamer [78,79] was found from nucleotides -42 to -28 relative to the transcription start site, and the nucleotides sequence upstream of the -35 region is A/T rich. Based on the biochemical and genetic evidence, the promoter region of the fadBA operon is defined (see Fig. 7). The previously predicted promoter -10 region "TATTT" and -35 region "showing little homology to the consensus sequence" [74] are not confirmed by the experimental results, and appear to be incorrect.

When the E. coli RNA isolated before oleate induction was used as a template, little primer extension product was detected (data not shown). These results demonstrate that the efficiency of the fadBA promoter was apparently dramatically raised by the inducer. The identification of the promoter DNA sequence is important for elucidation of the induction mechanism, which is a key to the gene expression of the fad regulon [42,43]. Sequence analysis of the fadBA operon DNA in the region of the transcription start site revealed a 21-bp DNA fragment (from -2 to +19) that shows dyadic symmetry. This region is a presumed repressor binding site, at which the formation of a repressor-operator complex would block the synthesis of fadBA mRNA (see Fig. 7). In addtion, a 26-bp DNA fragment that extends from -10 to -35 exhibits partial twofold symmetry. If a repressor binds to this site, it would interfere with the formation of promoter-RNA polymerase initiation complex (Fig. 7). It was reported [41] that the expression of the genes encoding E. coli β-oxidation enzymes requires both cAMP and its receptor protein (CRP). Sequence analysis of the promoter region revealed that a 29-bp DNA fragment (from -48 to -76) has 8 of 12 nucleotides identical with the highly conserved base pairs of cAMP-CRP binding site [80]. The distance between the center of the putative cAMP-CRP binding site and the transcription start site is -61/62 (Fig. 7). Another CGTGA segment (from -81 to -85) may participate in the search of CRP for the primary binding site, but a secondary site is not essential for transcription activation [80].

Analysis of the Thiolase Sequence: 3-Ketoacyl-CoA thiolase is the only enzymatic activity associated with the small β-subunit of the E. coli fatty acid oxidation complex [45]. The sequence around Cys-91 of this subunit is similar to that around Cys-89 of acetoacetyl-CoA thiolase from the bacterium Zoogloea ramigera (see Fig. 10). The Cys-89 of the latter enzyme has recently been identified as the thiol nucleophile in the active site by sequencing of the [^{14}C]-acetyl-CoA-labeled peptide and by oligonucleotide site-directed mutagenesis [81]. Comparison of the active site peptide sequence of pig heart mitochondrial acetoacetyl-CoA thiolase [82] with the sequence of E. coli thiolase I also supports the suggestion that Cys-91 of the E. coli enzyme is the catalytically essential cysteine (see Fig. 10). This identification of the active site cysteine of E. coli thiolase I is valid for the following reasons: Both thiolase I and thiolase II act by a ping-pong mechanism, and form acyl-S-intermediates [81-85]. Furthermore, the rat liver mitochondrial thiolase II was found to be reversibly inhibited by 3-keto-4-bromoctanoyl-CoA [86], indicating that the active site of acetoacetyl-CoA thiolase has the capacity to bind medium-chain 3-ketoacyl-CoA derivatives as well.

As shown in Fig. 11, Cys-91 of E. coli 3-ketoacyl-CoA thiolase is equivalent to Cys-97 of peroxisomal and Cys-92 of mitochondrial 3-ketoacyl-CoA thiolase, and the sequences around these cysteine residues are homologous to each other. Based upon the locations that have identical amino acid residues, we propose the following consensus sequence for the active site of prokaryotic and eukaryotic 3-ketoacyl-CoA thiolases: Asn-Arg-X_1-Cys-X_2-Ser-X_3-X_4-Gln (see Fig. 11). We also suggest that the last residue (glutamine) is related to the broad substrate specificity of 3-ketoacyl-CoA thiolase because this position is occupied by a basic residue in all acetoacetyl-CoA thiolases [83,87-89].

The amino acid sequence from residue 260 to the carboxyl-terminus of E. coli thiolase I is more like the eukaryotic 3-ketoacyl-CoA thiolases than is the rest of the enzyme. Since this region has been conserved during evolution, it may have important functions common to all these thiolases [90]. An eleven amino acid residue deletion in the E. coli thiolase, between residues 159 and 160, is apparent when the sequence of this thiolase is aligned with those of eukaryotic thiolases I to achieve maximum homology (see Fig. 11), whereas another segment corresponding to residues 122-131 of E. coli thiolase I is absent from the rat and human peroxisomal 3-ketoacyl-CoA thiolases. These deletions or additions of sequences in the central portion of the proteins may account for the diversity of the quaternary structures of 3-ketoacyl-CoA thiolases. Comparison of the sequence of the E. coli thiolase I with those of rat and human peroxisomal and rat mitochondrial thiolases I [91-93], reveals that 42%, 41% and 37% of the amino acid residues are identical, respectively. However, rat liver peroxisomal and mitochondrial thiolases I show a 37% identity only [91,93]. The peroxisomal thiolase I is more homologous to the E. coli enzyme than to the corresponding mitochondrial enzyme (see Fig. 11). This result suggests that the peroxisomal 3-ketoacyl-CoA thiolase gene evolved directly from the prokaryotic 3-ketoacyl-CoA thiolase gene.

Analysis of the Multifunctional Protein Sequence: Since the activities of different mitochondrial β-oxidation enzymes are carried on separate polypeptide chains [1], a comparison of the sequence of the large α-subunit of the E. coli fatty acid oxidation complex with the sequence of the corresponding mitochondrial β-oxidation enzymes was carried out in an effort to locate the various enzymatic functions within the multifunctional protein. As shown in Fig. 12, the amino-terminal region (residues 4-253) is clearly homologous to rat mitochondrial enoyl-CoA hydratase [94]. The overall identity of the two peptides is 29%, and a region near the center (residues 108-148 in the E. coli multifunctional protein and residues 104-144 of rat mitochondrial enoyl-CoA hydratase) shows 54% identity. This region is quite well conserved throughout the "hydratase family" [94], and probably plays an important role in the catalytic function. Based upon this evidence, we propose that the amino-terminal region of the fadB gene product is an enoyl-CoA hydratase functional domain. The enoyl-CoA hydratase has a broad chain-length specificity [24]. The hydration of short-chain substrate (crotonyl-CoA) catalyzed by this enzyme is 2.5-fold faster than the hydration of the medium-chain substrate 2-trans-decenoyl-CoA [24]. Clearly, this hydratase is not specific for medium-chain substrates as claimed by DiRusso [74], who also seems to be unaware [74] that information about amino acid sequences of mitochondrial enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase are known.

A search for homology to pig heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase [95] revealed clear homology with a central region (residues 294-593) of the E. coli multifunctional protein. In the alignment shown in Fig. 12, there is a 36% identity between these two proteins. NAD^+ is a necessary cofactor for the dehydrogenase activity [45], and by searching the sequence for the "fingerprint" consensus sequence of an ADP-binding $\beta\alpha\beta$-fold structure, a predicted NAD-binding domain was identified in this region. Since a 29 amino acid residue fragment (residues 315-343) has 10 of 11 residues that match the "fingerprint" and contains a perfect "core-fingerprint" with glycine residues at positions 6, 8 and 11 and with an acidic residues (aspartic acid) at the end [96], it is clearly homologous with the NAD-binding $\beta\alpha\beta$-fold of the pig heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase (see Fig. 12). The conformation of the latter enzyme has been determined by X-ray diffraction [97]; it has a two-domain structure with a bilobal appearance. The larger amino-terminal lobe (residues 1-200) binds the NAD cofactor and displays considerable structural homology with the NAD-binding domains observed in other dehydrogenases. The L-3-hydroxyacyl-CoA substrate binds in the cleft between the lobes. The carboxyl-terminal smaller lobe bears little homology to other known dehydrogenases, and forms the subunit-subunit interface in the dimer [97]. As shown in Fig. 12, the sequence homology between mitochondrial and E. coli L-3-hydroxyacyl-CoA dehydrogenase extends over a range of about three hundred residues. A fragment of the E. coli multifunctional protein (residues 294-487) corresponding to the amino-terminal domain (residues 1-200) of the mitochondrial dehydrogenase contains the putative NAD-binding $\beta\alpha\beta$-fold, and must be responsible for binding the NAD cofactor. The next 106-residue fragment (residues 488-593) of the fadB gene product is very similar to the carboxyl-terminal smaller domain (residues 201-307) of the pig heart dehydrogenase. This finding also suggests that the gene of the latter enzyme probably evolved by partial duplication of an ancient multifunctional protein gene.

Δ^3-cis-Δ^2-trans-Enoyl-CoA isomerase has recently been purified from rat liver mitochondria [98]. However, the amino acid sequence of this enzyme has not yet been determined. It seems likely that Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase, is present in the carboxyl-terminal region (residues 594-729) of the multifunctional protein because the rest of the protein already harbors other enzymes (Fig. 12). Since the chemical modification of the multifunctional protein by N-ethylmaleimide only inhibited the isomerase and did not affect other enzyme activities [68], the functional domain of the isomerase is probably physically distinct from the others. The exact location and size of the isomerase functional domain, of course, need to be further confirmed by protein engineering, X-ray crystallography, or other techniques.

Besides the hydratase, dehydrogenase, and isomerase activities mentioned above, this multifunctional polypeptide also possesses the ability to epimerize D-3-hydroxyacyl-CoAs to their L-isomers [45.49]. Recent studies have revealed that the epimerization of D-3-hydroxacyl-CoAs in rat liver peroxisomes is accomplished by a two-step dehydration-hydration reaction sequence that is catalyzed by a novel hydratase and crotonase [99-101]. The epimerization of D-3-hydroxyacyl-CoAs in E. coli probably proceeds by the same mechanism, not through redox catalysis using NAD as a cofactor as does UDP-glucose 4- epimerase [102,103]. The following observations suggest that the E. coli L-3-hydroxyacyl-CoA dehydrogenase does not function as an epimerase: (a) while the 3-hydroxyacyl-CoA dehydrogenase activity was strongly inhibited by iodoacetamide labeling of the NAD binding site, the epimerase activity was only slightly affected by this reaction [45], and (b) a mutant of the E. coli fatty acid complex that exhibited a normal level of epimerase activity, lacked L-3-hydroxyacyl-CoA dehydrogenase activity [104]. The 3-hydroxyacyl-CoA epimerase activity is thus probably associated with the amino-terminal hydratase domain of the E. coli multifucntional protein. In summary, based on sequence homologies and other lines of evidence, it is proposed that the enzymatic activities associated with the multifunctional protein are located in the order: enoyl-CoA hydratases (3-hydroxyacyl-CoA epimerase and crotonase):L-3-hydroxyacyl-CoA dehydrogenase:Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase (see Fig. 13).

Sequence Similarity between Rat Peroxisomal Trifunctional β-Oxidation Enzyme and the E. coli Multifunctional Protein: Rat enoyl-CoA hydratase:L-3-hydroxyacyl-CoA dehydrogenase: Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase trifunctional enzyme catalyzes the second and third reactions of the peroxisomal β-oxidation system and additionally the isomerization of 3-enoyl-CoA thioesters [105]. It has been reported [94,106] that the amino-terminal region (residues 1-188 encoded by exon I-V) and the fragment from residue 296 to 527 of the trifunctional enzyme are homologous to mitochondrial enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, respectively. The similarity terminates abruptly at the end of exon V, and there is essentially no similarity between the polypeptide fragment encoded by exon VI (residues 189-302) and the corresponding regions of mitochondrial enzymes [94,106]. In contrast, the sequence similarity between the *E. coli* multifunctional protein and rat peroxisomal trifunctional enzyme is apparent over the full lengths of the polypeptides including the region encoded by exon VI of the gene for rat peroxisomal trifunctional enzyme (Fig. 14). As shown in Fig. 14, the carboxyl-terminal regions of these two proteins are definitely related, as judged by the criteria published by Dayhoff *et al*. [108]. The similarities of the homologous regions are illustrated by a segment of the polypeptide (residues 611-661 of rat peroxisomal trifunctional enzyme) where 21 out of 51 residues are identical to those in a counterpart of the *E. coli* multifunctional protein without the introduction of breaks or gaps in either sequence. The carboxyl-terminal region of rat peroxisomal trifunctional β-oxidation enzyme (residues 581-722) presumably has the same function as the corresponding region of the *E. coli* multifunctional protein, which is proposed to be the Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activity (see Fig. 13).

The rat peroxisomal trifunctional β-oxidation enzyme differs from the *E. coli* multifunctional protein in that it does not exhibit 3-hydroxyacyl-CoA epimerase activity [39,96]. However, the sequence of the dehydrogenase functional domain of the rat peroxisomal trifunctional enzyme (residues 278-580) shows greater homology to the corresponding dehydrogenase functional domain of the E. coli multifunctional protein (residues 294-593) (Fig. 14) than to the pig heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase [106]. Moreover, the sizes of the rat peroxisomal trifunctional enzyme (78 kDa) and of the E. coli multifunctional protein (79 kDa) are almost identical, and the hydratase and dehydrogenase in both proteins (Fig. 13) are capable of catalyzing the direct β-oxidation of 2, 4-dienoyl-CoAs formed during the β-oxidation of unsaturated fatty acids [49,50]. This metabolic feature may be due to juxtaposition of the functional domains of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogeanse. All together, the evidence suggests that the genes of the rat peroxisomal trifunctional β-oxidation enzyme and the E. coli multifunctional protein have a common ancestor. This is consistent with the proposal [47] that the peroxisomal 3-ketoacyl-CoA thiolase gene evolved directly from the prokaryotic 3-ketoacyl-CoA thiolase gene. The mammalian peroxisomal β-oxidation system thus appears to be evolutionarily more closely related to the prokaryotic β-oxidation system than to the mitochondrial β-oxidation system, and this provides evidence for the hypothesis [109] that peroxisomes are descendants of ancient endosymbionts.

TABLE I

Specific activities of β-oxdiation enzymes and acetoacetyl-CoA thiolase in rat brain mitochondria and rat heart mitochondria.

Enzyme	Substrate[*]	Specific Activity[°]		Activities of Brain Enzymes Relative to Those of Heart
		Brain Mitochondria	Heart Mitochondria	
		nmol/min/mg protein	nmol/min/mg protein	%
Acyl-CoA dehydrogenase	n-Butyryl-CoA[a]	1.9	50.8	4
	n-Decanoyl-CoA[b]	25.3	50.8	50
	Palmitoyl-CoA[b]	3	36.3	8
Enoyl-CoA hydratase	Crotonyl-CoA[a]	890	4,590	19
	Decenoyl-CoA[a]	430	2,490	17
L-3-Hydroxy-acyl-CoA dehydrogenase	Acetoacetyl-CoA[a]	140	2,120	7
	L-3-Hydroxydecanoyl-CoA[c]	46	500	9
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA[a]	1.3	200	0.7
	3-Ketodecanoyl-CoA[d]	3.9	500	0.8
Acetoacetyl-CoA thiolase	Acetoacetyl-CoA[e]	40	510	8

[*] Substrate concentrations: a, 30 μM; b, 50 μM; c, 20 μM; d, 10 μM; e, 14 μM.

[°] Data are averages of measurements with two mitochondrial preparations which were assayed at least three times each.

TABLE II

Apparent Michealis constant values of some β-oxdiation enzymes from rat brain mitochondria and rat heart mitochondria.

Enzyme	Substrate	Km[a] Brain Mitochondria	Km[a] Heart Mitochondria
		μM	μM
Enoyl-CoA hydratase	Crotonyl-CoA	74.1	62.5
	Decenoyl-CoA	14.9	21.6
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	8.7	6.2
	NADH	11.4	8.1
	L-3-Hydroxydecanoyl-CoA	1.7	2.0
	NAD^+	39.2	64.5
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA	14.1	15.4
	CoASH	18.8	58.8
	3-Ketodecanoyl-CoA	1.2	1.1
	CoASH	49.1	59.2

[a] The apparent Km values were obtained from the double reciprocal plots.

TABLE III

Amino terminal sequence analyses of the subunits of the Fatty Acid Oxidation Complex+

Step	phenylthiohydantoin-derivatives recovered			
	α-SUBUNIT	pmoles	β-SUBUNIT	pmoles
1	M	52	M	88
2	L	34	E	40
3	Y	21	Q	35
4	K	20	V	27
5	G	19	V	34
6	D	*	I	24
7	T	8	V	27
8	L	22	D	19
9	Y	13	A	33
10	L	18	I	12

+Standard one-letter amino acid abrreviations are used.

*Not identified in the run shown. When the fatty acid oxidation complex (containing both subunits) was sequenced, both isoleucine and aspartic acid were recovered at step 6.

Fig. 1. Separation of mitochondrial 3-ketoacyl-CoA thiolase (peak I) and acetoacetyl-CoA thiolase (Peak II) by chromatography on phosphocellulose. A, Rat heart; B, rat brain. For experimental details, see "Experimental Procedures". Thiolase activities were assayed with acetoacetyl-CoA (--X--) and 3-ketodecanoyl-CoA (--O--) as substrates.

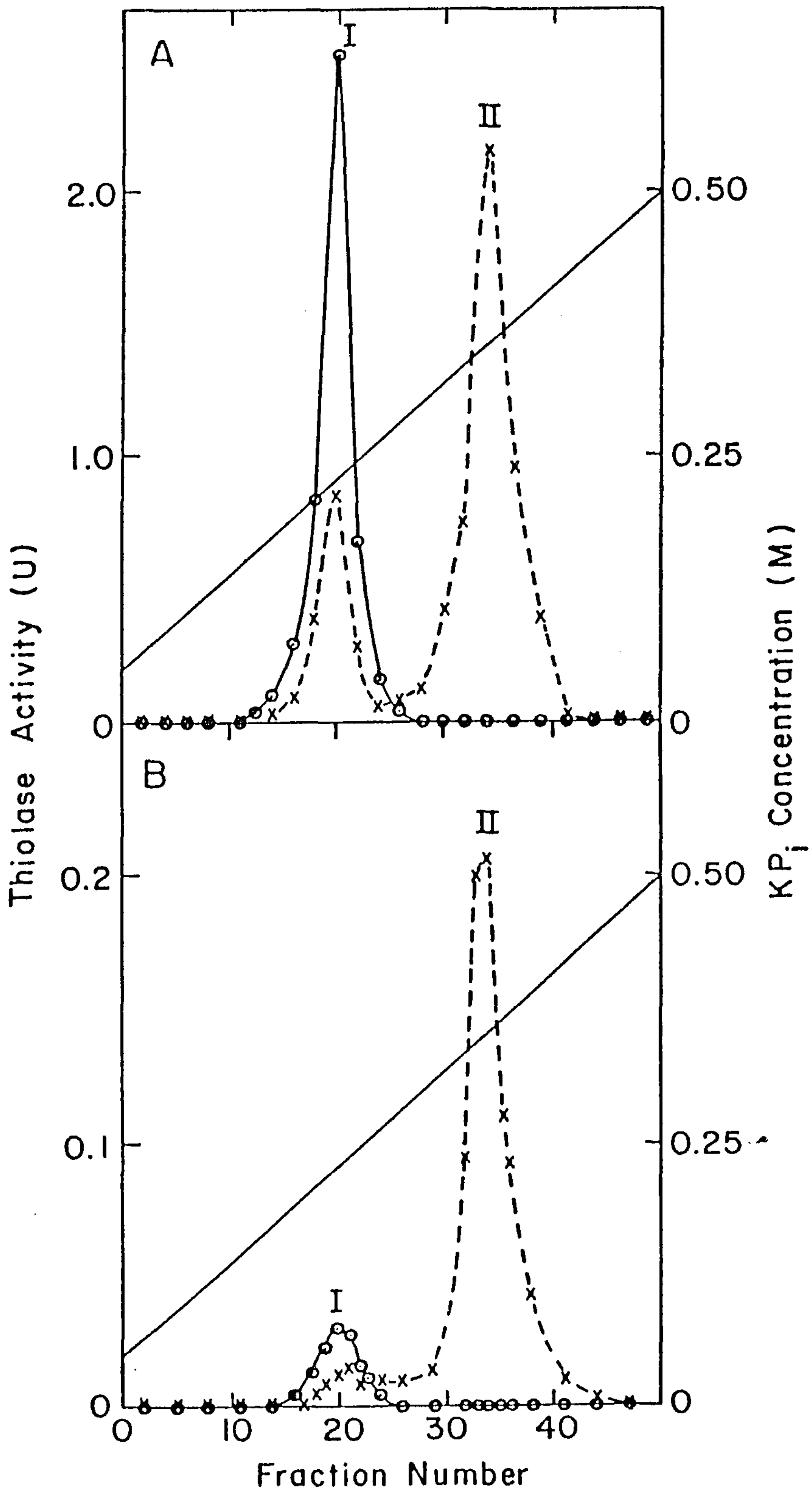
A
B
I
II
I
II
Thiolase Activity (U)
0
1.0
2.0
0
0.1
0.2
KP$_i$ Concentration (M)
0
0.25
0.50
0
0.25
0.50
0
10
20
30
40
Fraction Number

Fig. 2. Immunotitration of 3-ketoacyl-CoA thiolase (thiolase I) and acetoacetyl-CoA thiolase (thiolase II) from rat brain and rat heart mitochondria. The percent of remaining thiolase activity in the supernatant after precipitation of immune complexes was plotted as a function of the amount of added antibody (Ab). For experimental details, see "Experimental Procedures". The immunotitration curves shown are: O, rat brain thiolase I with antibodies to pig heart thiolase I; O, rat brain thiolase I with antibodies to *E. coli* thiolase II; ▾, rat brain thiolase II with antibodies to pig heart thiolase I; v, rat brain thiolase II with antibodies to *E. coli* thiolase II; ■, rat heart thiolase I with antibodies to pig heart thiolase I; ◆, rat heart thiolase II with antibodies to pig heart thiolase I, ▲, pig heart thiolase I with antibodies to pig heart thiolase I.

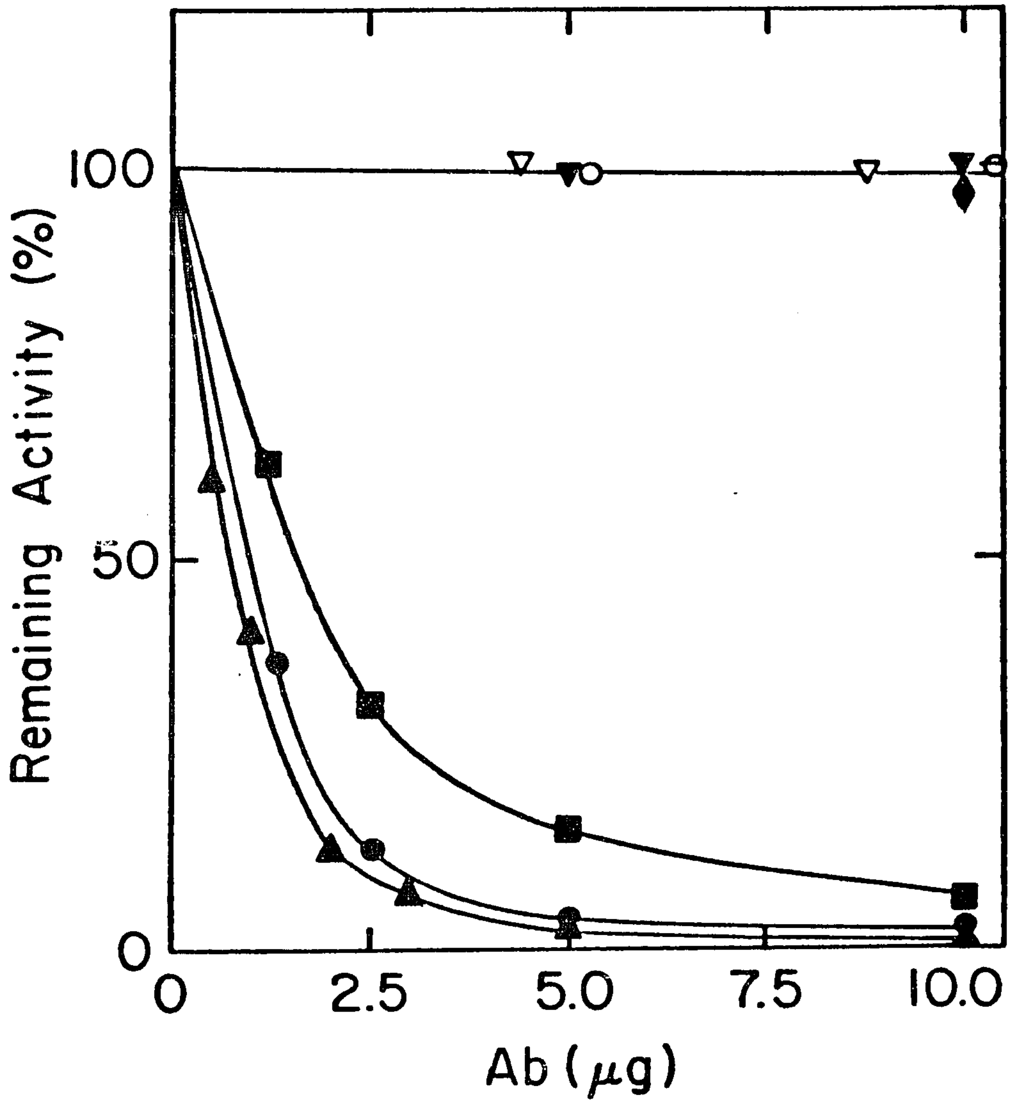
Remaining Activity (%)
100
50
0
0
2.5
5.0
7.5
10.0
Ab (μg)

Fig. 3. Respiration rates of coupled rat brain mitochondria (RBM) and coupled rat heart mitochondria (RHM) with palmitoylcarnitine as a substrate. For experimental details, see "Experimental Procedures". C_{16}-cn, palmitoylcarnitine (30 μM); Mal, L-malate (2.5 mM); and ADP (0.5 mM). The numbers represent the rates of respiration in nanoatoms of oxygen/min and mg of protein.

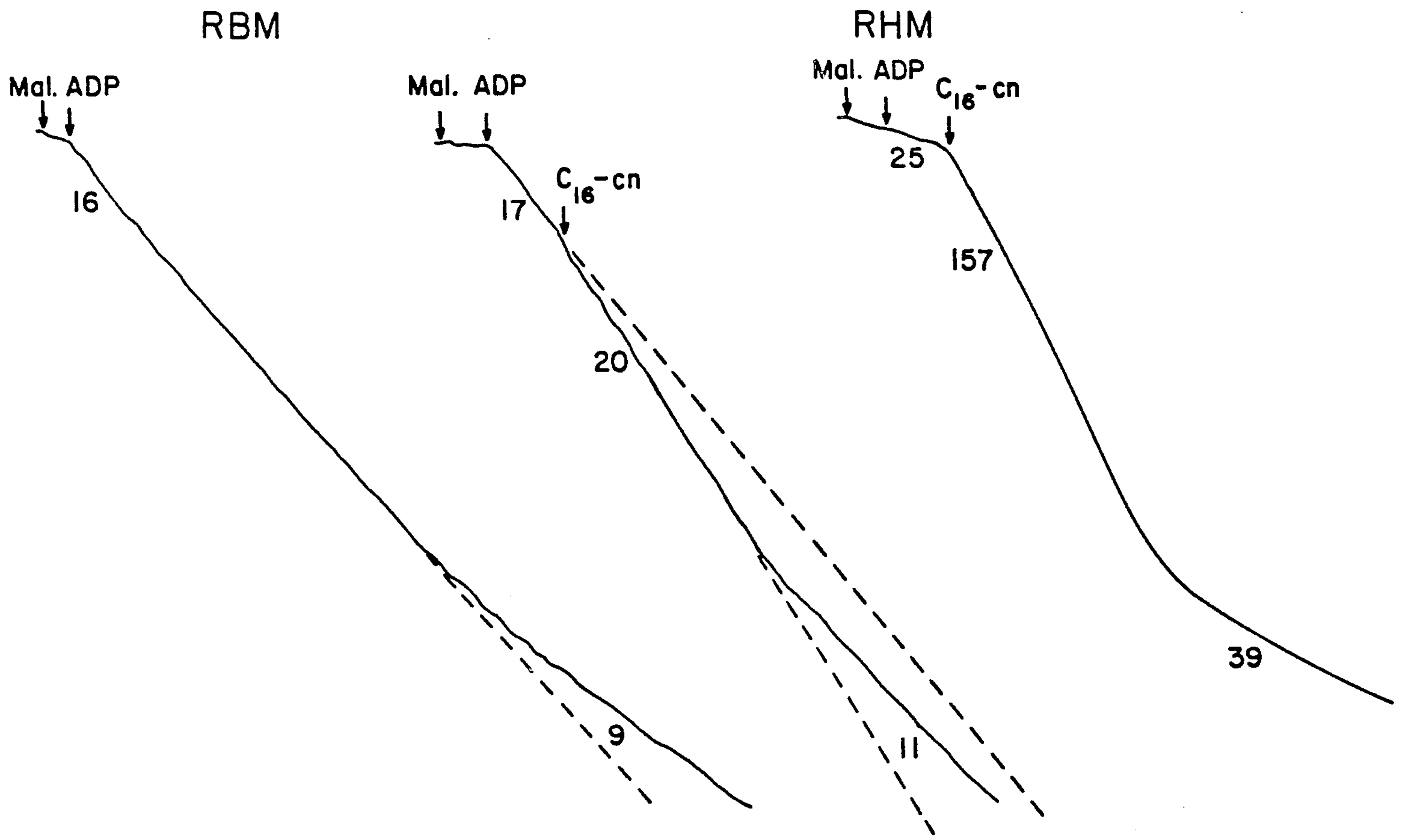
RBM
RHM
Mal. ADP
16
9
Mal. ADP
17
C_{16}-cn
20
11
Mal. ADP
C_{16}-cn
25
157
39

Fig. 4. Oxidation of [1-^{14}C]palmitoyl-carnitine by rat brain mitochondria as a function of time. For experimental details, see "Experimental Procedures". Symbols: circle, $^{14}CO_2$; square, ^{14}C-labeled acid-soluble products; triangle, sum of $^{14}CO_2$ and ^{14}C-labeled acid-soluble products. Data obtained with mitochondria that were preincubated for 3 min at 37°C with 4-bromocrotonic acid are marked with solid symbols. Data obtained with mitochondria preincubated without the inhibitor are marked by open symbols.

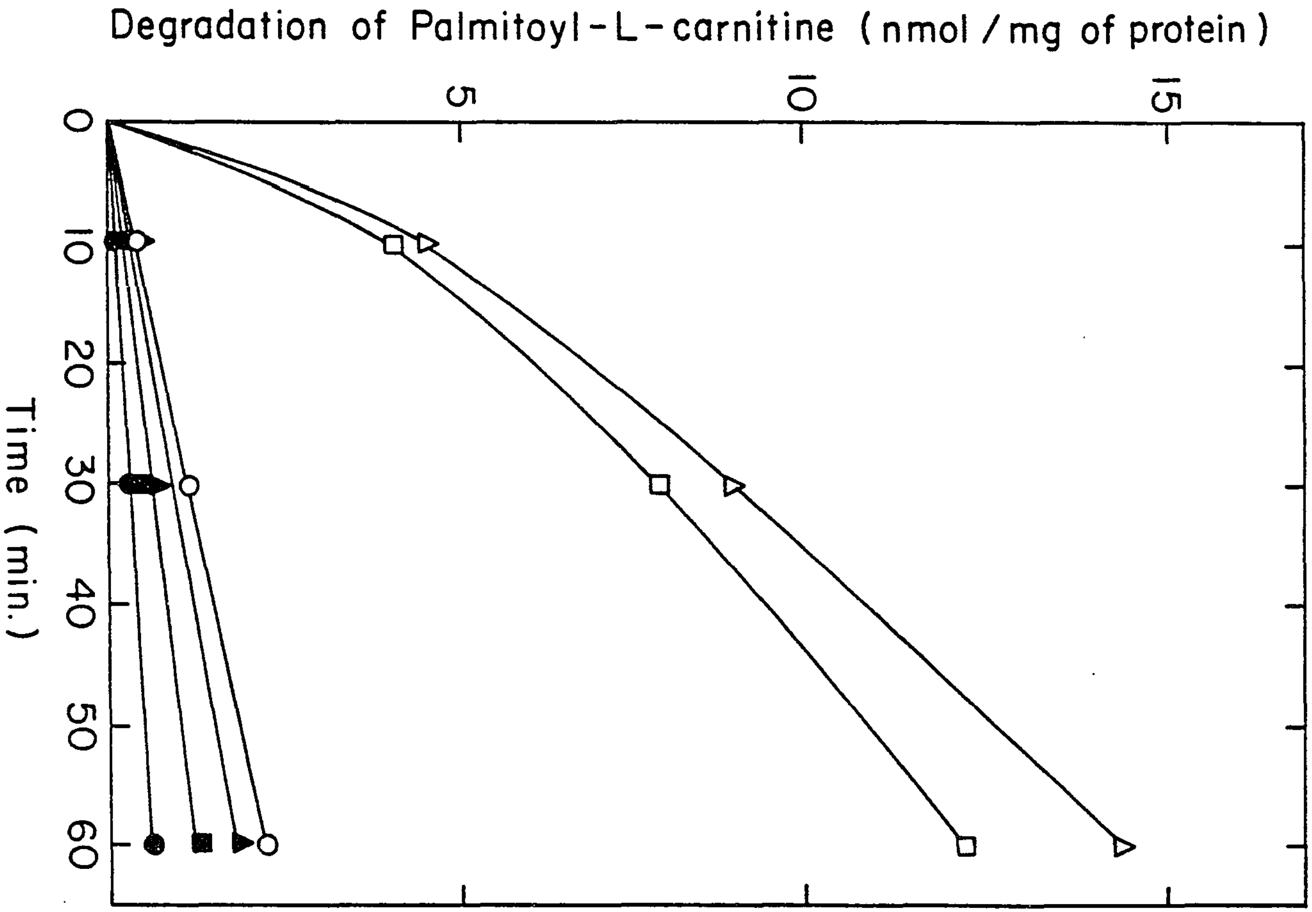
Degradation of Palmitoyl-L-carnitine (nmol / mg of protein)
5
10
15
0
10
20
30
40
50
60
Time (min.)

Fig. 5. DNA sequencing strategy. The coding regions for the multifunctional protein and 3-ketoacyl-CoA thiolase are indicated by the open and shaded boxes, respectively. The restriction sites from which subclones were made for sequencing are shown. The horizontal arrows under the DNA fragments indicate the directions and extents of sequencing.

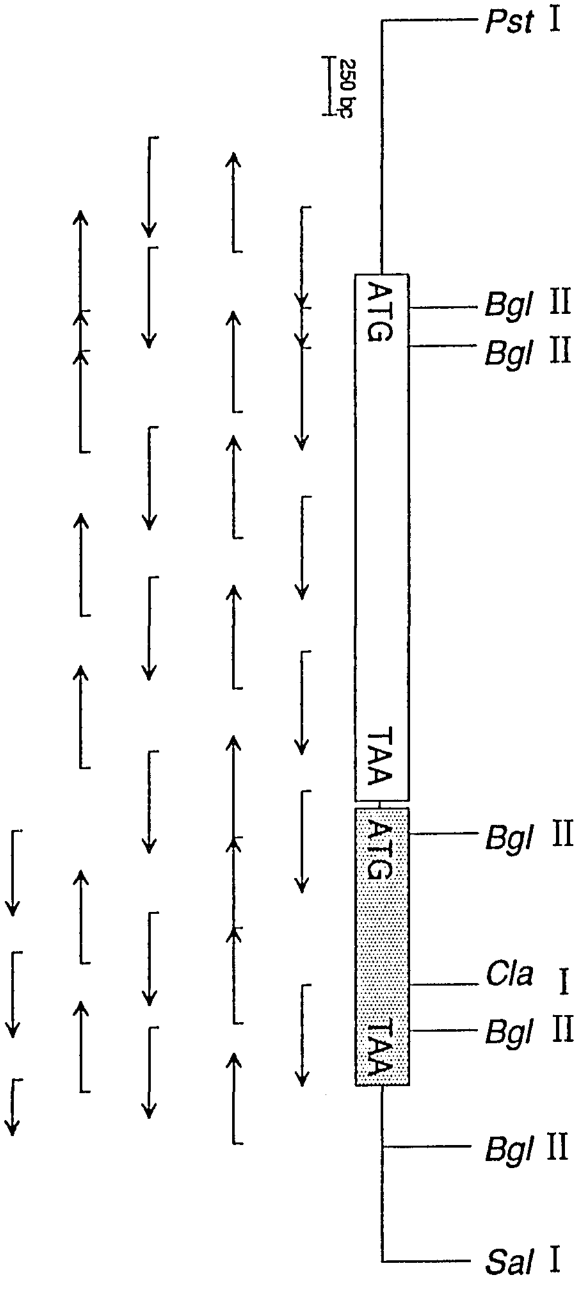
Pst I
250 bp
ATG
Bgl II
Bgl II
TAA
ATG
Bgl II
Cla I
TAA
Bgl II
Bgl II
Sal I

Fig. 6. Size exclusion chromatography of purified FAO complex. A 20 µl aliquot of a 2.6 µg/µl purified sample of the E. coli FAO complex was brought to 200 µl in 70% formic acid and injected onto a Pharmacia Superose 12 HR 10/30 gel filtration column, and monitored at 280 nm. An isocratic run was performed at 0.5 ml/min., room temperature in 70% formic acid. One ml. fractions were collected after a delay of 10.5 min. The fractions eluting at 14-18 min constituted the first peak (the α-subunit), and those eluting at 18-20 min the second peak (the β-subunit). The fractions were dried and reconstituted in 50 µl of 70% formic acid.

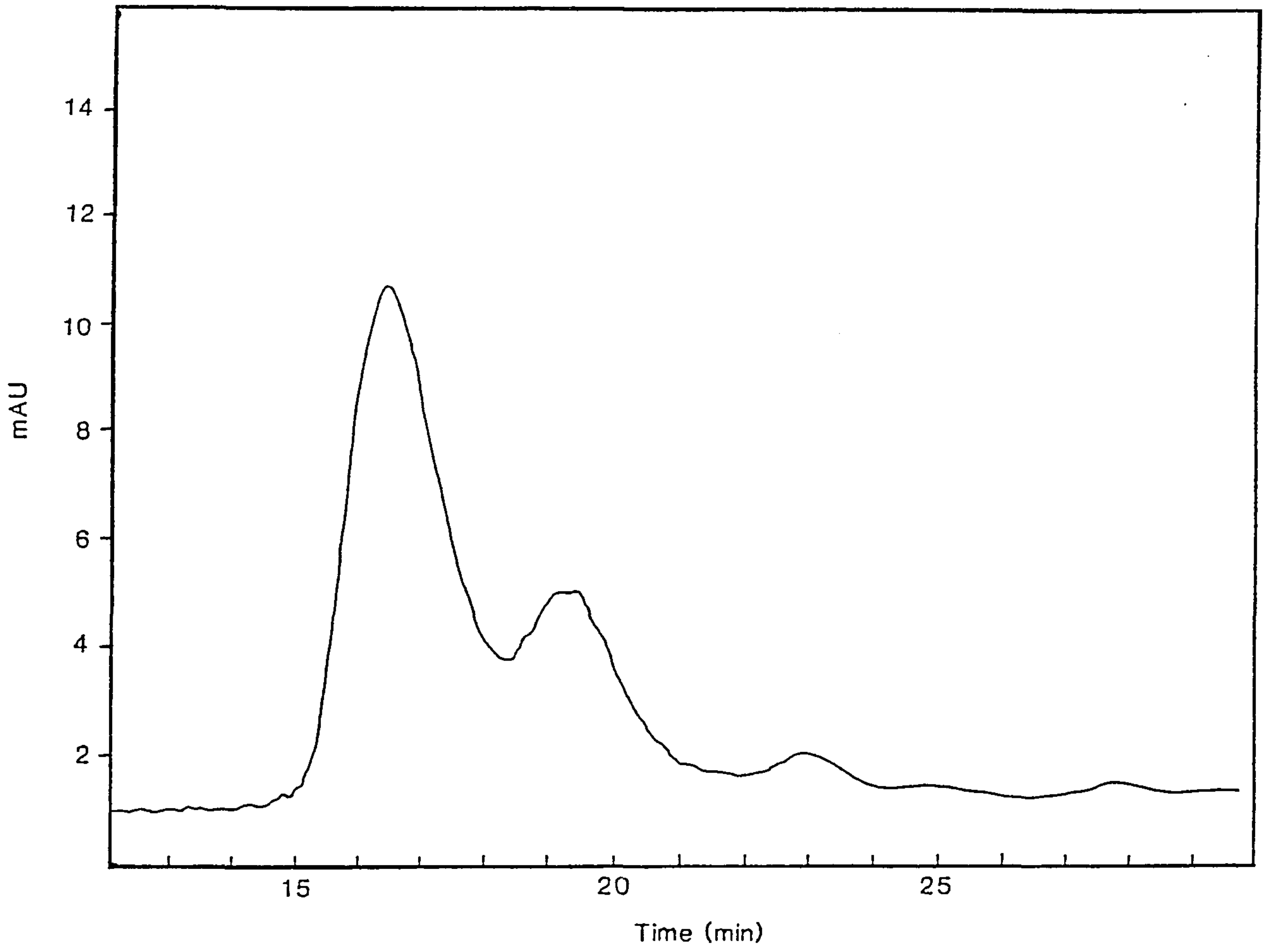
14
12
10
8
6
4
2
mAU
15
20
25
Time (min)

Fig. 7. Nucleotide sequence of the fadBA operon and the deduced primary structures of the multifunctional protein and 3-ketoacyl-CoA thiolase from E. coli. Nucleotides are numbered in the 5' to 3' direction. The transcription start site of the fadBA operon found at position 340 is marked as +1 above the sequence, and the nucleotides upstream of the transcription start site are given negative numbers for convenience in studying the promoter region. The promoter -10 and -35 regions are indicated by lines above the sequence. Regions showing dyadic symmetry within the putative repressor binding site are underlined. The nucleotide identical with very highly conserved nucleotides are marked by thick lines. Inverted repeats are delineated by arrows below the sequence. The potential Shine-Dalgarno sequence is underlined and marked with SD. Bgl II and Cla I restriction sites are indicated by dashed lines. The deduced amino acid sequences (with standard one-letter amino acid abbreviations) are shown below the nucleotide sequence. The amino-terminal amino acid sequences, which are boxed, were determined by Edman degradation [47]. The stop codon is represented by *.

```
CTGAGTTACCGGCACCCACGCTTTGAATTG CGGGTTCACTTTAAACGGATAGGGATGATC 60

GTCGAGAAAAACATTGAACAGCTCGCCGGA GTGAATAAGTAACGCATCCAGCTTGAAGCG 120

CGCCAGCGCATCGCGAGTCCGTTCTTGTAA GGTAGCTATATGATTTTTATAGAGCGAGGC 180

CAGTGATTCCATTTTTTACCCTTCTGTTTT TTTGACCTTAAGTCTCCGCATCTTAGCACA 240
       -92             -76             -62             -48
TCGTTCATCCAGAGCGTGATTTCTGCCGAG CGTGATCAGATCGGCATTTCTTTAATCTTT 300
   -35              -10           +1                  +20
TGTTTGCATATTTTTAACACAAAATACACA CTTCGACTCATCTGGTACGACCAGATCACC 360
                                     mRNA
TTGCGGATTCAGGAGACTGACATGCTTTAC AAAGGCGACACCCTGTACCTTGACTGGCTG 420
          SD            M  L  Y   K  G  D  T  L  Y  L  D  W  L 13

GAAGATGGCATTGCCGAACTGGTATTTGAT GCCCCAGGTTCAGTTAATAAACTCGACACT 480
 E  D  G  I  A  E  L  V  F  D   A  P  G  S  V  N  K  L  D  T 33
                                                   BglII
GCGACCGTCGCCAGCCTCGGCGAGGCCATC GGCGTGCTGGAACAGCAATCAGATCTAAAA 540
 A  T  V  A  S  L  G  E  A  I   G  V  L  E  Q  Q  S  D  L  K 53

GGGCTGCTGCTGCGTTCGAACAAAGCAGCC TTTATCGTCGGTGCTGATATCACCGAATTT 600
 G  L  L  L  R  S  N  K  A  A   F  I  V  G  A  D  I  T  E  F 73

TTGTCCCTGTTCCTCGTTCCTGAAGAACAG TTAAGTCAGTGGCTGCACTTTGCCAATAGC 660
 L  S  L  F  L  V  P  E  E  Q   L  S  Q  W  L  H  F  A  N  S 93
                BglII
GTGTTTAATCGCCTGGAAGATCTGCCGGTG CCGACCATTGCTGCCGTCAATGGCTATGCG 720
 V  F  N  R  L  E  D  L  P  V   P  T  I  A  A  V  N  G  Y  A 113

CTGGGCGGTGGCTGCGAATGCGTGCTGGCG ACCGATTATCGTCTGGCGACGCCGGATCTG 780
 L  G  G  G  C  E  C  V  L  A   T  D  Y  R  L  A  T  P  D  L 133

CGCATCGGTCTGCCGGAAACCAAACTGGGC ATCATGCCTGGCTTTGGCGGTTCTGTACGT 840
 R  I  G  L  P  E  T  K  L  G   I  M  P  G  F  G  G  S  V  R 153

ATGCCACGTATGCTGGGCGCTGACAGTGCG CTGGAAATCATTGCCGCCGGTAAAGATGTC 900
 M  P  R  M  L  G  A  D  S  A   L  E  I  I  A  A  G  K  D  V 173

GGCGCGGATCAGGCGCTGAAAATCGGTCTG GTGGATGGCGTAGTCAAAGCAGAAAAACTG 960
 G  A  D  Q  A  L  K  I  G  L   V  D  G  V  V  K  A  E  K  L 193

GTTGAAGGCGCAAAGGCGGTTTTACGCCAG GCCATTAACGGCGACCTCGACTGGAAAGCA 1020
 V  E  G  A  K  A  V  L  R  Q   A  I  N  G  D  L  D  W  K  A 213

AAACGTCAGCCGAAGCTGGAACCACTTAAA CTGAGCAAGATTGAAGCCACCATGAGCTTC 1080
 K  R  Q  P  K  L  E  P  L  K   L  S  K  I  E  A  T  M  S  F 233

ACCATCGCTAAAGGGATGGTCGCACAAACA GCGGGGAAACATTATCCGGCCCCCATCACC 1140
 T  I  A  K  G  M  V  A  Q  T   A  G  K  H  Y  P  A  P  I  T 253

GCAGTAAAAACCATTGAAGCTGCGGCCCGT TTTGGTCGTGAAGAAGCCTTAAACCTGGAA 1200
 A  V  K  T  I  E  A  A  A  R   F  G  R  E  E  A  L  N  L  E 273

AACAAAAGTTTTGTCCCGCTGGCGCATACC AACGAAGCCCGCGCACTGGTCGGCATTTTC 1260
 N  K  S  F  V  P  L  A  H  T   N  E  A  R  A  L  V  G  I  F 293

CTTAACGATCAATATGTAAAAGGCAAAGCG AAGAAACTCACCAAAGACGTTGAAACCCCG 1320
 L  N  D  Q  Y  V  K  G  K  A   K  K  L  T  K  D  V  E  T  P 313
```

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AAACAGGCCGCGGTGCTGGGTGCAGGCATT ATGGGCGGCGGCATCGCTTACCAGTCTGCG 1380
 K  Q  A  A  V  L  G  A  G  I   M  G  G  G  I  A  Y  Q  S  A 333

TGGAAAGGCGTGCCGGTTGTCATGAAAGAT ATCAACGACAAGTCGTTAACCCTCGGCATG 1440
 W  K  G  V  P  V  V  M  K  D   I  N  D  K  S  L  T  L  G  M 353

ACCGAAGCCGCGAAACTGCTGAACAAGCAG CTTGAGCGCGGCAAGATCGATGGTCTGAAA 1500
 T  E  A  A  K  L  L  N  K  Q   L  E  R  G  K  I  D  G  L  K 373

CTGGCTGGCGTGATCTCCACAATCCACCCA ACGCTCGACTACGCCGGATTTGACCGCGTG 1560
 L  A  G  V  I  S  T  I  H  P   T  L  D  Y  A  G  F  D  R  V 393

GATATTGTGGTAGAAGCGGTTGTTGAAAAC CCGAAAGTGAAAAAAGCCGTACTGGCAGAA 1620
 D  I  V  V  E  A  V  V  E  N   P  K  V  K  K  A  V  L  A  E 413

ACCGAACAAAAAGTACGCCAGGATACCGTG CTGGCGTCTAACACTTCAACCATTCCTATC 1680
 T  E  Q  K  V  R  Q  D  T  V   L  A  S  N  T  S  T  I  P  I 433

AGCGAACTGGCCAACGCGCTGGAACGCCCG GAAAACTTCTGCGGGATGCACTTCTTTAAC 1740
 S  E  L  A  N  A  L  E  R  P   E  N  F  C  G  M  H  F  F  N 453

CCGGTCCACCGAATGCCGTTGGTAGAAATT ATTCGCGGCGAGAAAAGCTCCGACGAAACC 1800
 P  V  H  R  M  P  L  V  E  I   I  R  G  E  K  S  S  D  E  T 473

ATCGCGAAAGTTGTCGCCTGGGCGAGCAAG ATGGGCAAGACGCCGATTGTGGTTAACGAC 1860
 I  A  K  V  V  A  W  A  S  K   M  G  K  T  P  I  V  V  N  D 493

TGCCCCGGCTTCTTTGTTAACCGCGTGCTG TTCCCGTATTTCGCCGGTTTCAGCCAGCTG 1920
 C  P  G  F  F  V  N  R  V  L   F  P  Y  F  A  G  F  S  Q  L 513

CTGCGCGACGGCGCGGATTTCCGCAAGATC GACAAAGTGATGGAAAAACAGTTTGGCTGG 1980
 L  R  D  G  A  D  F  R  K  I   D  K  V  M  E  K  Q  F  G  W 533

CCGATGGGCCCGGCATATCTGCTGGACGTT GTGGGCATTGATACCGCGCATCACGCTCAG 2040
 P  M  G  P  A  Y  L  L  D  V   V  G  I  D  T  A  H  H  A  Q 553

GCTGTCATGGCAGCAGGCTTCCCGCAGCGG ATGCAGAAAGATTACCGCGATGCCATCGAC 2100
 A  V  M  A  A  G  F  P  Q  R   M  Q  K  D  Y  R  D  A  I  D 573

GCGCTGTTTGATGCCAACCGCTTTGGTCAG AAGAACGGCCTCGGTTTCTGGCGTTATAAA 2160
 A  L  F  D  A  N  R  F  G  Q   K  N  G  L  G  F  W  R  Y  K 593

GAAGACAGCAAAGGTAAGCCGAAGAAAGAA GAAGACGCCGCCGTTGAAGACCTGCTGGCA 2220
 E  D  S  K  G  K  P  K  K  E   E  D  A  A  V  E  D  L  L  A 613

GAAGTGAGCCAGCCGAAGCGCGATTTCAGC GAAGAAGAGATTATCGCCCGCATGATGATC 2280
 E  V  S  Q  P  K  R  D  F  S   E  E  E  I  I  A  R  M  M  I 633

CCGATGGTCAACGAAGTGGTGCGCTGTCTG GAGGAAGGCATTATCGCCACTCCGGCGGAA 2340
 P  M  V  N  E  V  V  R  C  L   E  E  G  I  I  A  T  P  A  E 653

GCGGATATGGCGCTGGTCTACGGCCTGGGC TTCCCTCCGTTCCACGGCGGCGCGTTCCGC 2400
 A  D  M  A  L  V  Y  G  L  G   F  P  P  F  H  G  G  A  F  R 673

TGGCTGGACACCCTCGGTAGCGCAAAATAC CTCGATATGGCACAGCAATATCAGCACCTC 2460
 W  L  D  T  L  G  S  A  K  Y   L  D  M  A  Q  Q  Y  Q  H  L 693

GGCCCGCTGTATGAAGTGCCGGAAGGTCTG CGTAATAAAGCGCGTCATAACGAACCGTAC 2520
 G  P  L  Y  E  V  P  E  G  L   R  N  K  A  R  H  N  E  P  Y 713

TATCCTCCGGTTGAGCCAGCCCGTCCGGTT GGCGACCTGAAAACGGCTTAAGGAGTCACA 2580
 Y  P  P  V  E  P  A  R  P  V   G  D  L  K  T  A  *  SD      729
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```
ATGGAACAGGTTGTCATTGTCGATGCAATT CGCACCCCGATGGGCCGTTCGAAGGGCGGT 2640
 M  E  Q  V  V  I  V  D  A  I   R  T  P  M  G  R  S  K  G  G  20

                        Bgl II
GCTTTTCGTAACGTGCGTGCAGAAGATCTC TCCGCTCATTTAATGCGTAGCCTGCTGGCG 2700
 A  F  R  N  V  R  A  E  D  L   S  A  H  L  M  R  S  L  L  A  40

CGTAACCCGGCGCTGGAAGCGGCGGCCCTC GACGATATTTACTGGGGTTGTGTGCAGCAG 2760
 R  N  P  A  L  E  A  A  A  L   D  D  I  Y  W  G  C  V  Q  Q  60

ACGCTGGAGCAGGGTTTTAATATCGCCCGT AACGCGGCGCTGCTGGCAGAAGTACCACAC 2820
 T  L  E  Q  G  F  N  S  V  P   A  V  T  V  I  A  R  N  A  A  80

TCTGTCCCGGCGGTTACCGTTAATCGCTTG TGTGGTTCATCCATGCAGGCACTGCATGAC 2880
 L  L  A  E  V  P  H  N  R  L   C  G  S  S  M  Q  A  L  H  D  100

GCAGCACGAATGATCATGACTGGCGATGCG CAGGCATGTCTGGTTGGCGGCGTGGAGCAT 2940
 A  A  R  M  I  M  T  G  D  A   Q  A  C  L  V  G  G  V  E  H  120

ATGGGCCATGTGCCGATGAGTCACGGCGTC GATTTTCACCCCGGCCTGAGCCGCAATGTC 3000
 M  G  H  V  P  M  S  H  G  V   D  F  H  P  G  L  S  R  N  V  140

GCCAAAGCGGCGGGCATGATGGGCTTAACG GCAGAAATGCTGGCGCGTATGCACGGTATC 3060
 A  K  A  A  G  M  M  G  L  T   A  E  M  L  A  R  M  H  G  I  160

AGCCGTGAAATGCAGGATGCCTTTGCCGCG CGGTCACACGCCCGCGCCTGGGCCGCCACG 3120
 S  R  E  M  Q  D  A  F  A  A   R  S  H  A  R  A  W  A  A  T  180

CAGTCGGCCGCATTTAAAAATGAAATCATC CCGACCGGTGGTCACGATGCCGACGGCGTC 3180
 Q  S  A  A  F  K  N  E  I  I   P  T  G  G  H  D  A  D  G  V  200

CTGAAGCAGTTTAATTACGACGAAGTGATT CGCCCGGAAACCACCGTGGAAGCCCTCGCC 3240
 L  K  Q  F  N  Y  D  V  I  R   P  E  T  T  V  E  A  L  A  E  220

ACGCTGCGTCCGGCGTTTGATCCAGTAAAC GGTATGGTAACGGCGGGCACATCTTCTGCA 3300
 T  L  R  P  A  F  D  P  V  N   G  M  V  T  A  G  T  S  S  A  240

CTTTCCGATGGCGCAGCTGCCATGCTGGTG ATGAGTGAAAGCCGCGCCCATGAATTAGGT 3360
 L  S  D  G  A  A  A  M  L  V   M  S  E  S  R  A  H  E  L  G  260

                                                   Cla I
CTTAAGCCGCGCGCTCGTGTGCGTTCGATG GCGGTCGTTGGTTGTGACCCATCGATTATG 3420
 L  K  P  R  A  R  V  R  S  M   A  V  V  G  C  D  P  S  I  M  280

GGTTACGGCCCGGTTCCGGCCTCGAAACTG GCGCTGAAAAAAGCGGGGCTTTCTGCCAGC 3480
 G  Y  G  P  V  P  A  S  K  L   A  L  K  K  A  G  L  S  A  S  300

GATATCGGCGTGTTTGAAATGAACGAAGCC TTTGCCGCGCAGATCCTGCCATGTATTAAA 3540
 D  I  G  V  F  E  M  N  E  A   F  A  A  Q  I  L  P  C  I  K  320
Bgl II
GATCTGGGACTAATTGAGCAGATTGACGAG AAGATCAACCTCAACGGTGGCGCGATCGCG 3600
 D  L  G  L  I  E  Q  I  D  E   K  I  N  L  N  G  G  A  I  A  340

CTGGGTCATCCGCTGGGTTGTTCCGGTGCG CGTATCAGCACCACGCTGCTGAATCTGATG 3660
 L  G  H  P  L  G  C  S  G  A   R  I  S  T  T  L  L  N  L  M  360

GAACGCAAAGACGTTCAGTTTGGTCTGGCG ACGATGTGTATCGGTCTGGGTCAGGGTATT 3720
 E  R  K  D  V  Q  F  G  L  A   T  M  C  I  G  L  G  Q  G  I  380

GCGACGGTGTTTGAGCGGGTTTAAGCCACTT GCCGGATGCGGCGTGAACGCCTTATCCAC 3780
 A  T  V  F  E  R  V  *                 ----->  <---------    387

CGGTCTACCGATCCGGCAATGTAGGCCTGAT AAGACGCGCCAGCGTCGCATCAGGCATCA 3840

TGCACCAATTGCCGGATGCGGCGTAAACGCC TTATCCGGCCTACGGTTCGGACTATTTGT 3900

AGGCACGATAAGACGCGACAGCGTCGCATCA GGCATCATGCACCAATTGCCGGATGCGGC 3960

GTAAACGCCTTATCCGGCCTACGGTTCGGAC TATTTGTAGGCACGATAAGACGCGACAGC 4020

                                   Bgl II
GTCGCATCGGGCATGGTTTGCCAGATACGTC ATAGATCT 4059
```

Fig. 8. Structural organization and direction of transcription of the fadBA operon. The locations of fadA and fadB genes and the direction of transcription are indicated. The spheres depict the two protein subunits of the fatty acid oxidation multienzyme complex encoded by the fadBA operon. Restriction sites Pst I, Bgl II, Cla I and Sal I are indicated.

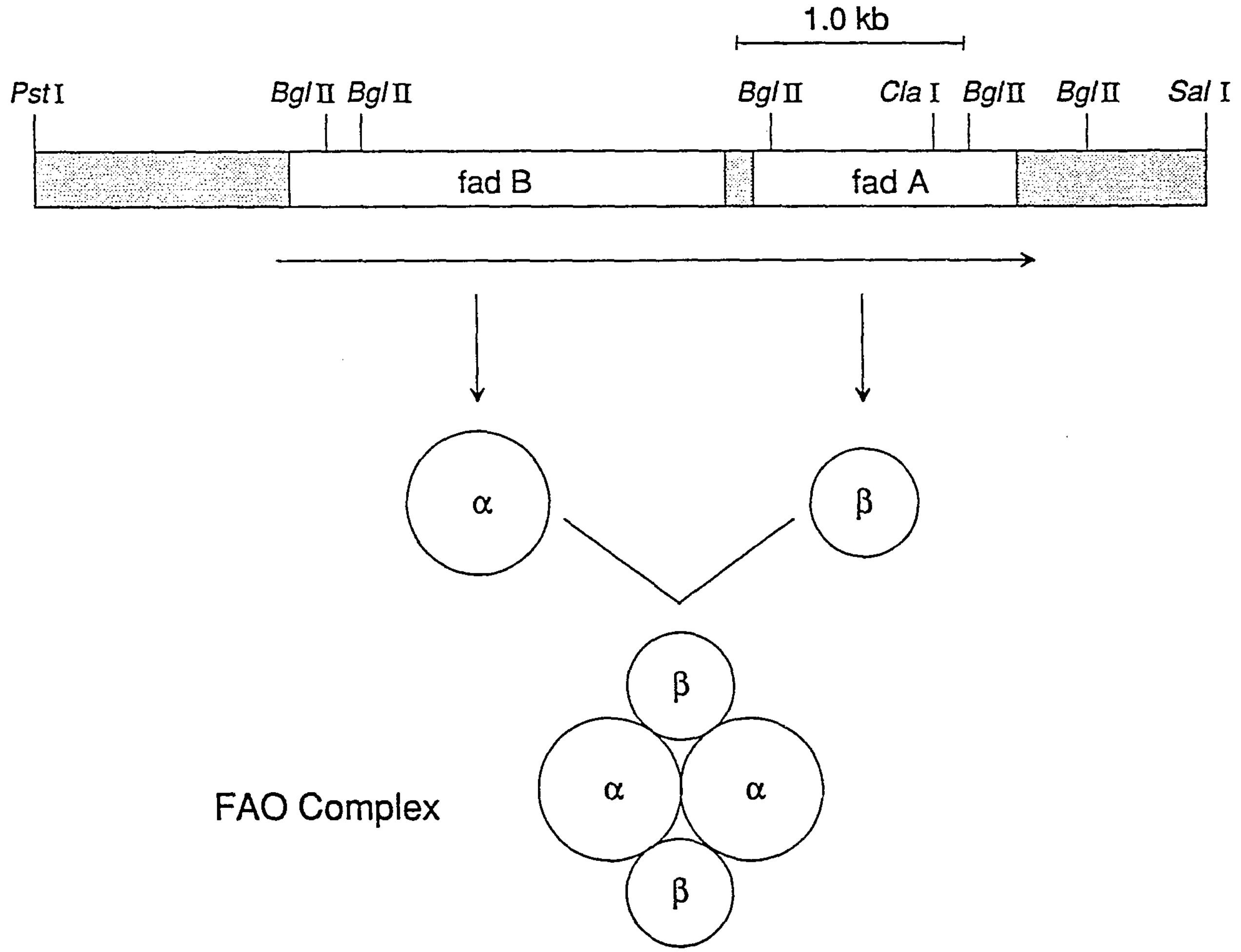
1.0 kb
Pst I
Bgl II
Bgl II
Bgl II
Cla I
Bgl II
Bgl II
Sal I
fad B
fad A
α
β
β
α
α
β
FAO Complex

Fig. 9. Determination of the transcription start site of the fadBA operon by primer extension analysis. An oligonucleotide complementary to the fadB sequence (from position 419 to 436, Fig. 2) was 5' end-labeled with ^{32}P, hybridized to RNA isolated from E. coli strain LS6749 containing plasmid pK52 after induction by oleate, and extended with reverse transcriptase. For details, see "Experimental Procedures". Lanes A,G,T,C, represent the dideoxy sequencing reaction products using the same oligonucleotide as primer and DNA of the fadB region as a template. In this experiment, samples were hybridized at 50°C (lane 1), 46°C (Lanes 2,4,5) and 42°C (Lane 3). Lane 4 is a control run without reverse transcriptase added in the primer extension reaction. Lane 5 is a control in which yeast tRNA was hybridized with the same primer, and the extension reaction was catalyzed by the addition of reverse transcriptase.

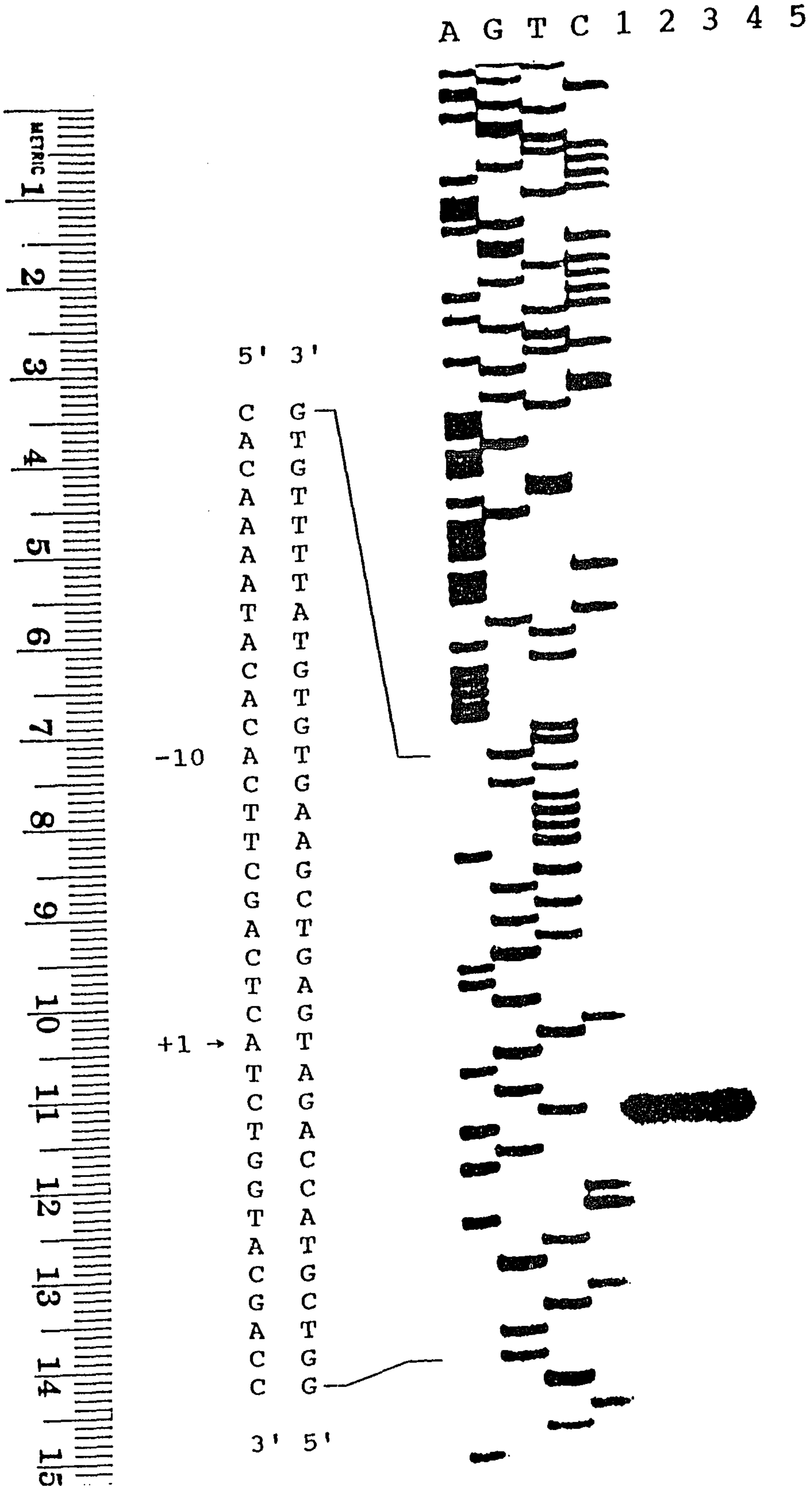
A G T C 1 2 3 4 5
METRIC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
5' 3'
C G
A T
C G
A T
A T
A T
A T
T A
A T
C G
A T
C G
-10 A T
C G
T A
T A
C G
G C
A T
C G
T A
C G
+1 → A T
T A
C G
T A
G C
G C
T A
A T
C G
G C
A T
C G
C G
3' 5'

Fig. 10. Amino acid sequence around the putative active-site cysteine residue of 3-ketoacyl-CoA thiolases from E. coli. The abbreviations used are ET, E. coli 3-ketoacyl-CoA thiolase; ZT, acetoacetyl-CoA thiolase of Zoogloea ramigera; HT, pig heart mitochondrial acetoacetyl-CoA thiolase. The portion of active site sequence of HT where only the amino acid composition has been determined is in parentheses. The cysteine residues indicated by the bold letter C in ZT and HT are directly involved in the formation of acyl-S-enzyme intermediates, and the counterpart in ET is indicated by an asterisk.

		*
ET	69-96	ARNAALLAEVPHSVPAVTVNRLCGSSMQ
ZT	67-94	ARQAAMKAGVPQEATAWGMNQLCGSGLR
HT		GAVLGAGLP(C,I_2,N,P,S,T_3)KVCASGMK

Fig. 11. Comparison of amino acid sequence of E. coli 3-ketoacyl-CoA thiolase (ET) with those of rat liver peroxisomal 3-ketoacyl-CoA thiolase precursor (PT), human fetal liver peroxisomal 3-ketoacyl-CoA thiolase (FT), and rat liver mitochondrial 3-ketoacyl-CoA thiolase (MT). Standard one-letter amino acid abbreviations are used. Gaps (-) are introduced to achieve maximum homology. Amino acid residues are numbered beginning at the initial methionine of the E. coli and mitochondrial thiolases, and at the amino terminal residue of the mature peroxisomal enzyme. The pre-sequence of peroxisomal 3-ketoacyl-CoA thiolase precursor is indicated by negative numbers. The cysteine residue indicated by an asterisk is the putative active-site cysteine.

```
ET    1                                    MEQVVIVDAIRTPMGRSKG-GAFRNV
PT  -26  MHRLQVVLGHLAGRSESSSALQAAPCSAGFPQASASDVVVVHGQRTPIGRA-GRGGFKDT
FT  -26  MQRLQVVLGHLRGPADSGWMPQAAPCLSGAPQASAADVVVVHGRRTAICRA-GRGGFKDT
MT    1                                 MALLRGVFIVAAKRTPFG-AYG-GLLKDF

ET   26  RAEDL--SAHLMRSLLARNPALEAAALDDIYWGCVQQTLEQGFNIARNAALLAEVP-HSV
PT   34  TPDELL-SAVLTAVLQDVKPKPE--CLGDISVGNVLQPGA-GAAMARIAQFLSGIP-ETV
FT   34  TPDELL-SAVMTAVLKDVHLRPE--QLGDICVGNVLQPGA-GAIMARIAQFLSDIP-ETV
MT   28  TATDLTEFAAR-AALSAGKVPPET-IDSVI-VGNVMQSSSDAAYLARIVGLRVGVPTETG

                 *
ET   83  PAVTVNRLCGSSMQALHDAARMIMTGDAQACLVGGVEHMGHVPMS-HGVDF--HPGLSRN
PT   89  PLSAVNRQCSSGLQAVANIAGGIRNGSYDIGMACGVESM-----------TLSERGNPGN
FT   89  PLSTVNRQCSSGLQAVASIAGGIRNGSYDIGMACGVESM-----------SLADRGNPGN
MT   85  -ALTLNRLCGSGFQSIVSGCQEICSKDAEVVLCGGTESMSQSPYSVRNVRFGTKFGLDLK

ET  140  VAKAAGMMGLTAEML-ARMHG-----------ISREMQDAFAARSHARAWAATQSA-AFK
PT  140  ISSRLLENEKARDCL-IPM-GITSENVAERFGISRQKQDAFALASQQKA-ASAQSKGCFR
FT  140  ITSRLMEKEKARDCL-IPM-GITSENVAERFGISREKQDTFALASQQKA-ARAQSKGCFQ
MT  144  LEDTLWA-GLTDQHVKLPM-GMTAENLAAKYNISREDCDRYALQSQQR-WKAANEAGYF-

ET  187  NEI-IP-TGG-HDADGVLK-QFNY--DEVIRPETTVEALATLRPAF--DPVNGMVTAGTS
PT  195  AEI-VPVTTTVLDDKGDRKTITVSQ-DEGVRPSTTMEGLAKLKPAF-KD--GGSTTAGNS
FT  195  AEI-VPVTTTVHDDKGTKRSITVTQ-DEGIRPSTTMEGLAKLKPAFKKD---GSTTAGNS
MT  200  NEEMAPIEVKT--KKG--K-QTM-QVDEHARPQTTLEQLQNLPPVFKKE---GTVTAGNA

ET  239  SALSDGAAAMLVMSESRAHE-LGLKPRARV-RSMAVVGCDPSIMGYGPVPASKLALKKAG
PT  250  SQVSDGAAAVLLARRSKAEE-LGL-PILGVLRSYAVVGVPPDIMGIGPAYAIPAALQKAG
FT  250  SQVSDGAAAILLARRSKAEE-LGL-PILGVLRSYAVVGVPPDIMGIGPAYAIPVALQKAG
MT  251  SGMSDGAGVVIIASED-AVKKHNFTPLARVV-GYFVSGCDPAIMGIGPVPAITGALKKAG

ET  297  LSASDIGVFEMNEAFAAQILPCIKDLGLIEQIDEKIN-LNGGAIALGHPLGCSGARISTT
PT  308  LTVNDIDIFEINEAFASQALYCVEKLG-IPA--EKVNPL-GGAIALGHPLGCTGARQVVT
FT  308  LTVSDVDIFEINEAFASQAAYCVEKLRL-PP--EKVNPL-GGAVALGHPLGCTGARQVIT
MT  309  LSLKDMDLIDVNEAFAPQFLAVQKSLDLDPS---KTNVS-GGAIALGHPLGGSGSRITAH

ET  356  LLN-LMERKDVQFGLASMCIGLGQGIATVFERV
PT  364  LLNELKRRGRRAYGVVSMCIGTGMGAAAVFEYPGN
FT  364  LLNELKRRGKRAYGVVSMCIGTGMGAAAVFEYPGN
MT  365  LVHELRRRGGK-YAVGSACIGGGQGISLIIQNTA
```

Fig. 12. Comparison of the amino acid sequence of the E. coli multifunctional protein (MP) with those of rat mitochondrial enoyl-CoA hydratase (EH) and pig heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase (HD). Standard one-letter amino acid abbreviations are used. Pairs of sequences are aligned with gaps (-) inserted to obtain maximal similarities. Residues identical in two sequences are shown by asterisks. Solid triangles indicate the positions of the "core-fingerprint" and the numbers below the sequence indicate the positions important to the formation of a $\beta\alpha\beta$-fold, which has been shown to bind the ADP moiety of NAD [96]. The sequences of rat mitochondrial enoyl-CoA hydratase and pig heart mitochondrial L-3-hydroxyacyl-CoA dehydrogenase are taken from Ref. [94] and [95], respectively.

```
MP   1   MLYKGDTLYLDWLEDGIAELVFDAPGSVNKLDTATVASLGE-AIGVLEQQ-SD
                 *      *    *        *   *    * *     **    *
EH   1      GANFQYIITEKKGKNSSVGLIQLNRPKALNALCNGLIEELNQALETFEED

MP  52   LKG--LLLRSNKAAFIVGADITEFLSLFLVPEEQ---LSQWLHFANSVFNRLE
                *     **  **** *              ** * *
EH  51   PAVGAIVLTGGEKAFAAGADIKEMQNRTFQDCYSGKFLSHWDHITRIKK----

MP 100   DLPVPTIAAVNGYALGGGCECVLAT--DYRLATPDLRIGLPETKLGIMPGFGG
           **  **************  **   *   *      * **  **  ** **
EH 100   --PV--IAAVNGYALGGGCE--LAMMCDIIYAGEKAQFGQPEILLGTIPGAGG

MP 151   SVRMPRMLGADSALEIIAAGKDVGADQALKIGLVDGVVKAEKLVEGAKAVLRQ
           *  *  *   * *    *    *  *   ***      * *** *
EH 147   TQRLTRAVGKSLAMEMVLTGDRISAQDAKQAGLVSKIFPVETLVEEAIQCAEK

MP 204   AINGDLDWKAKRQPKL---------EPLKLSKIEATMSF-TIAK--GMVAQTA
           *      *               *  ** *      * *     ** *
EH 200   IANNSKIIVAMAKESVNAAFEMTLTEGNKLEKKLFYSTFATDDRREGMSAFVE

MP 245   GKHYPAPITAVKTIEAAARFGREEALNLENKSFVPLAHTNEARALVGIF

EH 253   KRKANFKDH

                                    ▲ ▲  ▲                  ▲
MP 294   LNDQYVKGKAKK-LTKDVETPKQAAVLGAGIMGGGIAYQSAWK-GVPVVMKD-
                 **** * *         * * * ** *** * *   *  **  *
HD   1   SSSATAAAKAKKILVK------HVTVIGGGLMGAGIA-QVAAATGHTVVLVDQ

MP 344   ---INDKSLTLGMTEAAKLLN-KQLERGKIDGLKLAGVISTIHPTLDYAGFDR
            *  **         *    *  *  *          * *    * *
HD  47   TEDILAKSKKGIEESLRKVAKKKFAENPKAGDEFVEKTLSSISTSTDAAAVVH

MP 393   V-DIVVEAVVENPKVKKAVLAE-TEQKVRQD------TVLASNTSTIPISELA
           * **** **  **    *    *   * *      *  *****   *  **
HD 100   STDLVVEAIVEQLKVVGENLKVKSELFKRLDKFAAEHTIFASNTSSLQITSLA

MP 438   NALERPENFCGMHFFNPVHRMPLVEIIRGEKSSDETIAKVVAWASKM-GKTPI
         **  *   * * ** * *  * ***       *  *    *   **  ** *
HD 153   NATTRQDRFAGLHF-N-VPLMKLVEVVKTPMTSQKTLESLVDF-SKTLGKHPV

MP 490   VVNDCPGFFVNRVLFPYFAGFSQLLRDGADFRK--IDKVMEKQFGWPMGPAYL
            *  ** *** * **      *   * *  *  **  *    * ****  *
HD 203   SCKDTPGFIVNRLLVPYLIEAVRLYERG-DASKEDIDTAMKLGAGYPMGPFEL

MP 541   LDVVGIDTAHHAQAVMAAGFPQRMQKDYRDAIDALFDANRFGQKNGLGFWRYK
         ** ** **             *        *   *   * ** * * **  **
HD 255   LDYVGLDTTKFIIDGWHEMDSQNPLFQPSPAMNKLVAENKFGKKTGEGFYKYK

MP 594   EDSKGKPKKEEDAAVEDLLAEVSQPKRDFSEEEIIARMMIPMVNEVVRCLEEG

MP 647   IIATPAEADMALVYGLGFPPFHGGAFRWLDTLGSAKYLDMAQQYQHLGPLYEV

MP 700   PEGLRNKARHNEPYYPPVEPARPVGDLKTA
```

Fig. 13. Locations of functional domains in E. coli multifunctional protein (MP) and rat peroxisomal trifunctional β-oxidation enzyme (TE). The amino-terminal and the central regions of these two proteins are homologous to mitochondrial enoyl-CoA hydratase (EH) and L-3-hydroxyacyl-CoA dehydrogenase (HD), respectively. The rat peroxisomal trifunctional β-oxidation enzyme does not exhibit 3-hydroxyacyl-CoA epimerase activity, and we propose that the conserved carboxyl-terminal domains of these two proteins harbor Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase. The epimerase activity of the E. coli multifunctional protein is presumably associated with the enoyl-CoA hydratase functional domains.

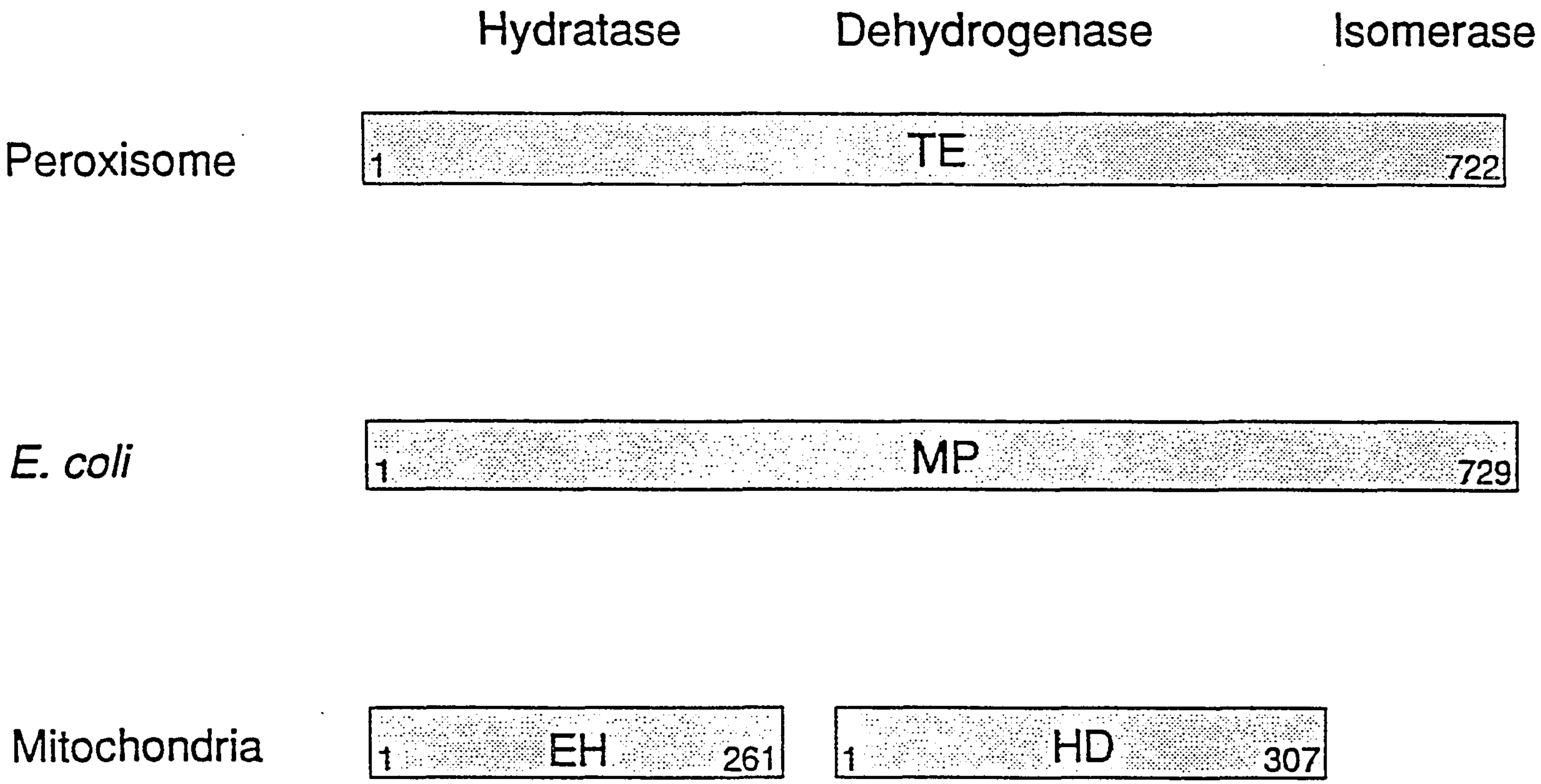
Hydratase
Dehydrogenase
Isomerase
Peroxisome
1
TE
722
E. coli
1
MP
729
Mitochondria
1
EH
261
1
HD
307

Fig. 14. Comparison between the amino acid sequences of rat peroxisomal trifunctional β-oxidation enzyme (TE) and E. coli multifunctional protein (MP). Standard one-letter amino acid abbreviations are used. The two sequences are aligned with appropriate gaps (-) to obtain maximum homology. Residues identical in both sequences are shown by asterisks. The rat peroxisomal trifunctional β-oxidation enzyme gene is divided into seven exons by six introns. Downward triangles indicate the positions of intron insertion. The sequence of rat peroxisomal trifunctional β-oxidation enzyme is taken from Ref. [107].

```
                                         ▼
BP   1        MAEYLRLPHSLAMIRLCNPP--VNAV-SPTVIREV-RNGLQKAGSDHT
                 **              *  **     **       *
MP   1   MLYKGDTLYLDWLEDGIAELVFDAPGSVNKLDTATVASLGEAIGVLEQQSD--

                        ▼
BP  45   VKAIVICGANGNFCAGADIHGF-SAFTPGLALGSLVDEIQR------YQKPVL
          *          *  ****  * * *       *                **
MP  52   LKGLLLRSNKAAFIVGADITEFLSLFLVPEEQLSQWLHFANSVFNRLEDLPVP

                                    ▼
BP  91   --AAIQGVALGGGLELALGCHYRIANAKARVGLPEVTLGILPGARGTQLLPRV
           **  * ***** *  *   ** *    * ****  *** **  *    **
MP 105   TIAAVNGYALGGGCECVLATDYRLATPDLRIGLPETKLGIMPGFGGSVRMPRM

                     ▼                                     ▼
BP 142   VGVPVALDLITSGKYLSADEALRLGILDAVVKSD-PVEEAIKFA--QKI-IDK
          *   **  *  **   ** **  *  * ***    ** * *    * *  *
MP 158   LGADSALEIIAAGKDVGADQALKIGLVDGVVKAEKLVEGA-KAVLRQAINGDL

BP 191   PIEPRRIFNKPVPSLPNMDSVFAEAIAK-VRKQYPGVL--APETCVRSIQASV
              *        *          ***    *  *    ** * *  * * *
MP 210   DWKAKRQPKLEPLKLSKIEATMSFTIAKGMVAQTAGKHYPAPITAVKTIEAAA

BP 241   KHPYEVGIKEEEKLFMYLRASGQAKALQYAFFAEKSAQKWSTPSGASWK-TAS
             *     * * *  *     * **   *             *   * *
MP 263   RFGREEALNLENKSFVPLAHTNEARALVGIFL------NDQYVKGKAKKLTKD

                   ▼
BP 293   AQPVSSVGVLGLGTMGRGIAI-SFARVGISVVAVESDPKQLDAAKKIITFTLE
                 *** * ** ***  * *  *  **      * *         *
MP 310   VETPKQAAVLGAGIMGGGIAYQS-AWKGVPVVMKDINDKSLT-----LGMTEA

BP 345   KEASRAHQNGQASAKPKLRFSSSTKELST-------VDLVVEAVFEDMNLK-K
                         **    **            ** ***** *  * * *
MP 357   AKLLNKQLERGKIDGLKLAGVISTIHPTLDYAGFDRVDIVVEAVVE--NPKVK

BP 390   K-VFAELSALCKPGAFLCTNTSALNVDDIASSTDRPQLVIGTHFFSPAHVMRL
         * * **          *  ***       *    **    * *** * * * *
MP 408   KAVLAETEQKVRQDTVLASNTSTIPISELANALERPENFCGMHFFNPVHRMPL

BP 442   LEVIPSRYSSPTTIATVMSL-SKKIGKIGVVVGN-CYGF-VGNRMLAPYYNQG
          * *    **  *** *    **  **   ** * * ** * ** * **   *
MP 461   VEIIRGEKSSDETIAKVVAWASKM-GKTPIVV-NDCPGFFV-NRVLFPYFA-G

BP 492   FF-LLEEGSKPEDVDGVLEE-FGFKMGPFRVSDLAGLDVGWKIRKGQGLTGPS
         *  **  *      * * *  **  ***    *  * *
MP 510   FSQLLRDGADFRKIDKVMEKQFGWPMGPAYLLDVVGIDTAHHAQAVMAA----

BP 543   LPPGTPVRKRGNSRYSPLGDMLCEAGRFGQKTGKG-W-YQYD-KPLGRIHKP-D
            * * *      *    * *  * ***** * * * *  * *  *   *  *
MP 559   ---GFPQRMQKD--YRDAIDALFDANRFGQKNGLGFWRYKEDSK--GKPKKEED

BP 593   PWLSTFLSQYREVHHIEQRTISKEEILERCLYSLINEAFRILEEGMAARPEHID
               *    **     *  * ***  *      **  * ****  * *   *
MP 606   AAVEDLL---AEVSQP-KRDFSEEEIIARMMIPMVNEVVRCLEEGIIATPAEAD

BP 647   VIYLHGYGWPRHKGGPMFYAASV-GLPTVLEKLQKYYRQNPDIPQLEPSDYLRR
              * * *   **  *      *    *   * *    *     *      *
MP 656   MALVYGLGFPPFHGGA-FRWLDTLGSAKYLDMAQQYQHLGPLYEVPEGLRNKAR

BP 700   LVAQGSPPLKEWQSLAGPHGSKL
               **  *     *
MP 709   HNEPYYPPV-EPARPVGDLKTA
```

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