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STRUCTURE AND KINETICS OF THE MULTIENZYME COMPLEX OF FATTY ACID OXIDATION FROM *Escherichia coli*

by

SONG-YU YANG

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.

1984

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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9-11-1984
Date

Executive Officer

Supervisory Committee

ABSTRACT

STRUCTURE AND KINETICS OF THE MULTIENZYME COMPLEX
OF FATTY ACID OXIDATION FROM Escherichia coli

by

SONG-YU YANG

Adviser: Professor Horst Schulz

The subunit locations of the five enzymes associated with the fatty acid oxidation complex from E. coli were studied by immunotitration and chemical modification. Antibodies raised against the purified complex caused the parallel inhibitions of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, while slightly stimulating 3-ketoacyl-CoA thiolase. All five component enzymes of the complex were inactivated by treatment with iodoacetamide. The inactivation of 3-ketoacyl-CoA thiolase was rapid, whereas the four other enzymes were inactivated at much slower, but almost equal rates. All enzymes except for 3-ketoacyl-CoA thiolase were protected against this inactivation by either NADH or crotonyl-CoA. The reaction of [1-^{14}C]iodoacetamide with the complex in the presence and absence of NADH resulted in the differential labeling of the large subunit only. These observations together with published results [Pawar, S. and H. Schulz (1981) J. Biol. Chem. 256, 3894-3899] lead to the suggestion that enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase are located on the 78,000-dalton subunit, whereas 3-ketoacyl-CoA thiolase is associated with the 42,000-dalton subunit. Additionally, this study provides further evidence for the existence of a fatty acid

oxidation (fad AB) operon that codes for the multienzyme complex of fatty acid oxidation and that is located at 85 min on the E. coli chromosome.

The kinetic properties of the fatty acid oxidation complex from E. coli were studied with the aim of elucidating the functional consequence of having enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase associated with a multifunctional polypeptide. The kinetic parameters of individual enzymes were determined and used in model calculations based on a published theory [Storer, A. C. and Cornish-Bowden, A. (1974) Biochem. J. 141, 205-209] to predict the kinetic behavior of a system of functionally unlinked enzymes. The validity of the theory for making these calculations was proven by demonstrating a good agreement between the calculated and observed rates of intermediate and product formation for the conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by a mixture of bovine liver enoyl-CoA hydratase and pig heart L-3-hydroxyacyl-CoA dehydrogenase. The V_{max} value of the dehydrogenase is 33.5 U/mg and the K_m values for 3-hydroxydecanoyl-CoA and NAD^+ are 2.9 μM and 191 μM, respectively. The conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by the sequential action of the hydratase and dehydrogenase of the complex were determined by measuring the rate of NADH formation. Stopped flow measurements showed the rate of NADH formation to be linear without any lag period. When the initial velocity of the hydratase was 10.2 μM min^{-1} that of the overall reaction was 8.4 μM min^{-1}. In contrast, the results calculated by use of the Storer and Cornish-Bowden equation for a system of unlinked enzymes predicted the overall reaction to exhibit a lag time of 30 s

and to result in the accumulation of 2.1 μM 3-hydroxydecanoyl-CoA before reaching a velocity corresponding to 82.5% of that of the hydratase reaction. The high initial rate and the unusual kinetic properties of the overall reaction observed in the present study cannot be explained unless a channeling mechanism is assumed to exist on the large subunit of the fatty acid oxidation complex. When the apparent degree of channeling is corrected for the percentage of the dehydrogenase active sites saturated with NAD^+, more than 90% of intermediates appears to be transferred directly from the active site of enoyl-CoA hydratase to that of 3-hydroxyacyl-CoA dehydrogenase. This channeling mechanism permits a rapid regulation of the rate of fatty acid degradation and prevents the accumulation of β-oxidation intermediates within cells.

ACKNOWLEDGMENTS

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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INTRODUCTION

Fatty acids are an important source of energy for many organisms. The catabolic pathway of fatty acids began to be unraveled in 1904 by the classical work of Knoop (1). Subsequent investigations were done by Lynen, Green, Ochoa and others with mammalian tissues. These studies led to the formulation of a universal pathway by which two carbon atoms are removed from a fatty acyl-CoA (2). Free fatty acids must be converted to fatty acyl-CoA thioesters before entering into this β-oxidation pathway. The activation of fatty acids is catalyzed by acyl-CoA synthetase (EC 6.2.1.3).

The β-oxidation mechanism involves a sequence of four reactions as follows:

1) dehydrogenation of acyl-CoA to 2-trans-enoyl-CoA catalyzed by acyl-CoA dehydrogenase (EC 1.3.99.3);
2) hydration of 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by enoyl-CoA hydratase (EC 4.2.1.17);
3) oxidation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); and
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.

The resulting acyl-CoA compounds pass through the β-oxidation cycle again as shown in Fig. 1, until they are completely degraded to acetyl-CoA.

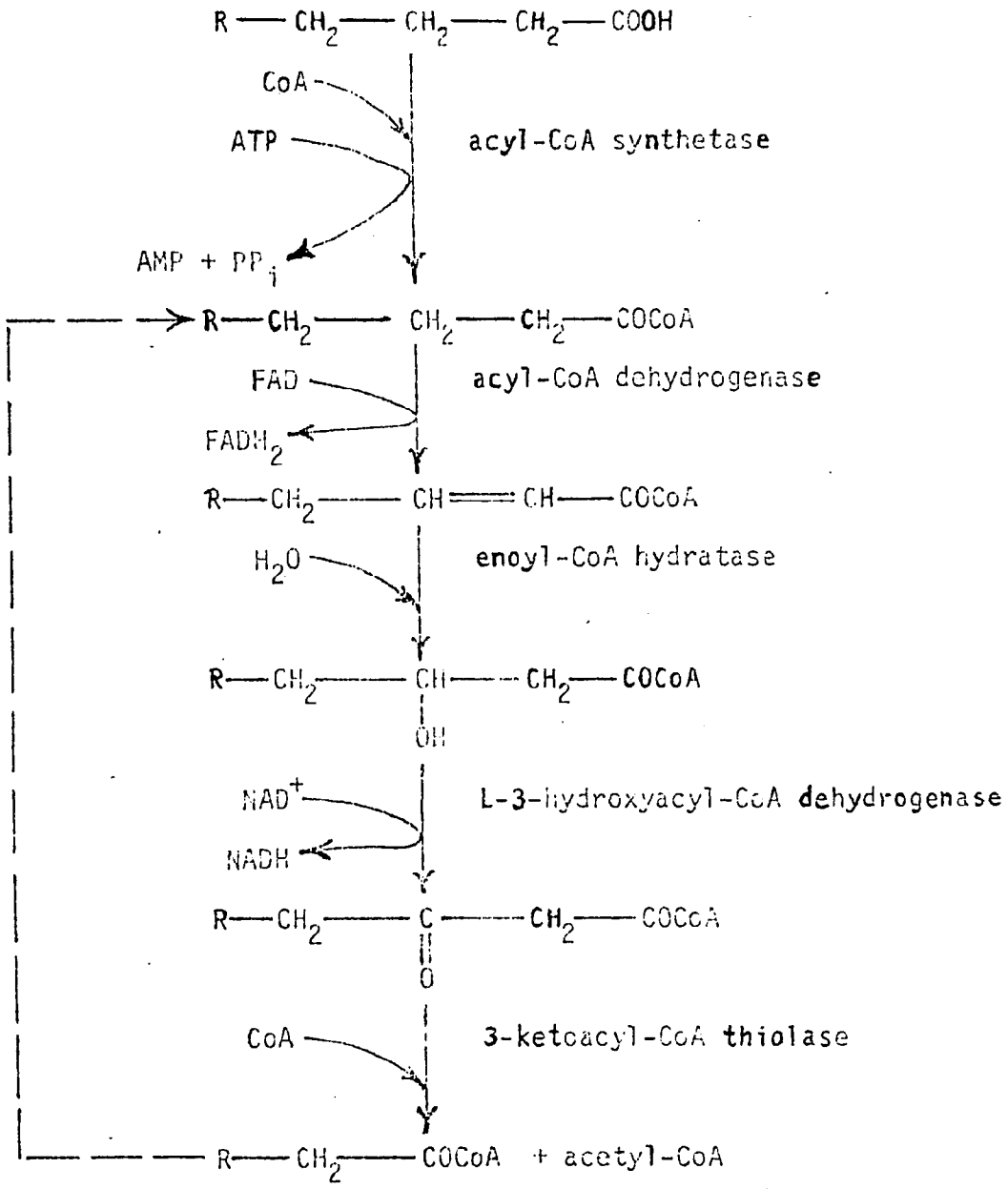

Figure 1. Pathway of Fatty Acid Oxidation.

The degradation of unsaturated fatty acids requires cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and NADPH dependent 2,4-dienoyl-CoA reductase (EC 1.3.1.-) in addition to the regular enzymes of β-oxidation (3,4). The isomerase acts on 3-cis-enoyl-CoA derived from unsaturated fatty acids containing cis double bonds extending from odd-numbered carbons. The products of the isomerization reaction are 2-trans-enoyl-CoA (5) that can be degraded by β-oxidation. Unsaturated fatty acids with cis double bonds extending from even-numbered carbons are chain-shortened by β-oxidation to 2-trans,4-cis-enoyl-CoA which can be reduced to 3-trans-enoyl-CoA by 2,4-dienyol-CoA reductase. The products of this reduction reaction are then isomerized by the isomerase to 2-trans-enoyl-CoA which can be further degraded by the β-oxidation pathway (3,4) (see Fig. 2). 3-Hydroxyacyl-CoA epimerase (EC 5.1.2.3), which catalyzes the isomerization of D-3-hydroxyacyl-CoA to its L-isomer, was previously proposed to be involved in the degradation of unsaturated fatty acids with cis double bonds extending from even-numbered carbons (6). However, it was recently found that 2-trans, 4-cis-decadienoyl-CoA cannot be converted to 2-cis-octenoyl-CoA, a precursor of D-3-hydroxyacyl-CoA, via the β-oxidation pathway (4). Thus, the physiological function of 3-hydroxyacyl-CoA epimerase remains to be established.

The association of β-oxidation with mitochondria has been known for a long time (2). Recently, some types of microbodies such as peroxisomes and glyoxisomes were also shown to be able to degrade fatty acids (7). The fatty acid β-oxidation pathways in microbodies and mitochondria

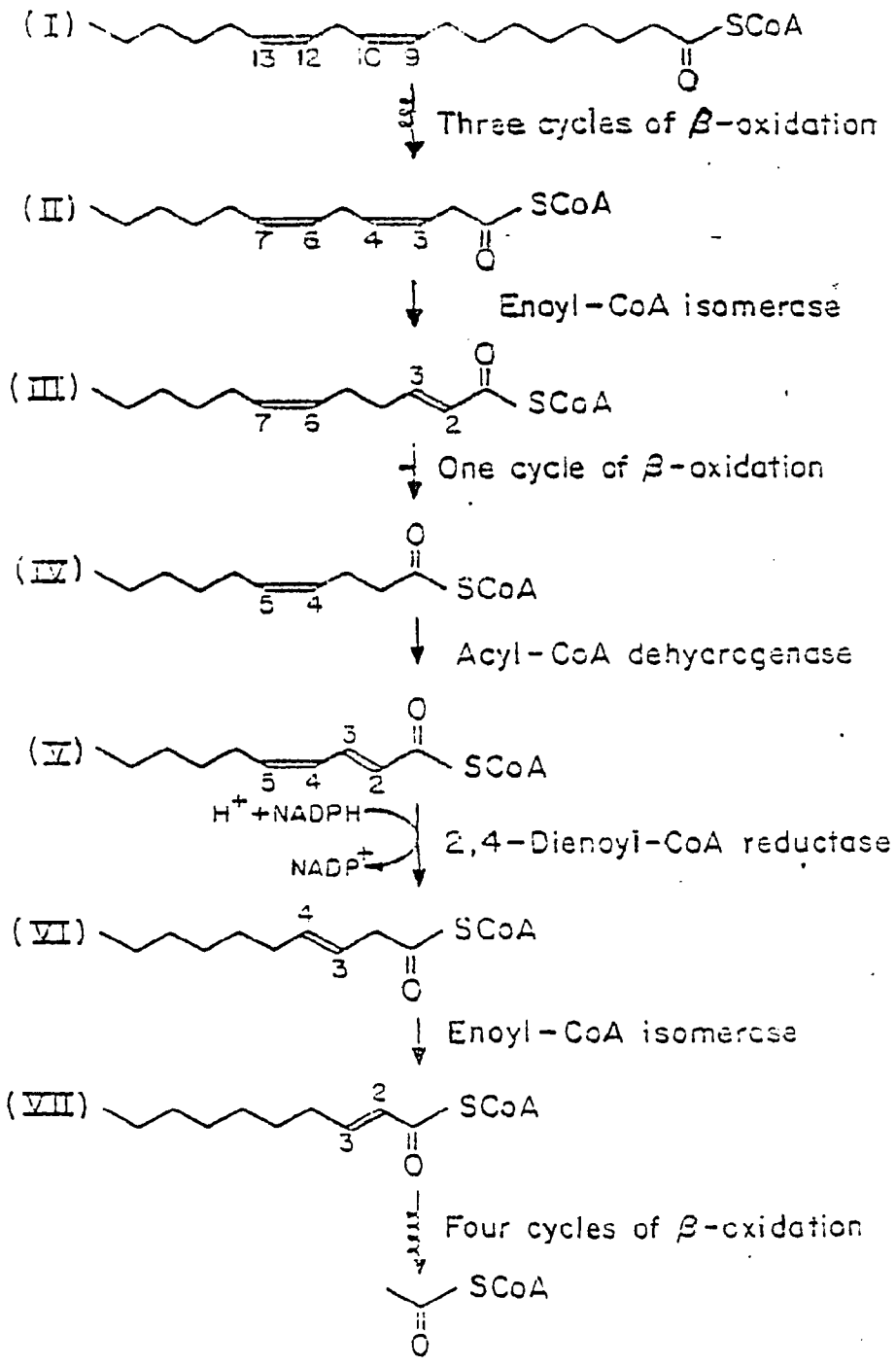

Figure 2. β-Oxidation of linoleoyl-CoA

are different in many ways. For example, β-oxidation in microbodies is carnitine independent, whereas mitochondrial β-oxidation of long-chain fatty acids is carnitine dependent (8,9). Instead of the FAD-linked acyl-CoA dehydrogenase functioning in mitochondria, peroxisomes in protozoa (10), liver (11) and yeast (12) as well as seed glyoxisomes (13) contain a FAD-linked acyl-CoA oxidase that transfer electrons to O_2 to form H_2O_2, which is rapidly decomposed by catalase. It appears that hepatic peroxisomal β-oxidation is specific for long-chain fatty acyl-CoAs, whereas mitochondria can degrade both long-chain and short-chain fatty acids. Interestingly, the end products of peroxisomal β-oxidation are mediun-chain acyl-carnitines and acetylcarnitine. These carnitine derivatives can passively diffuse out of the peroxisomes into the cytoplasm (8,9). Thus, peroxisomal β-oxidation may provide acetyl units for other cellular synthetic purpose and chain-shortened acylcarnitines for further oxidation in mitochondria. In contrast to rat liver peroxisomes, glyoxisomes of germinating seeds can degrade fatty acids completely to acetyl-CoA, which is utilized in the same glyoxysomes by the glyoxylate cycle to form succinate (14).

In 1951 it was first suggested by Silliker and Rittenberg (15) that the enzymes of fatty acid oxidation can be induced in E. coli cells. However, subsequent studies (16,17) failed to demonstrate the presence of β-oxidation enzymes in the extracts of E. coli or other microorganisms. The presence of an active pathway of fatty acid oxidation in E. coli was not established until 1967 when Overath and coworkers (18) 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. Overath et al. (18,19), Weeks et al. (20) and Klein et al. (21) have demonstrated that acyl-CoA synthetase, at least two acyl-CoA dehydrogenases, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase are induced when E. coli cells are grown on long-chain fatty acids as the sole carbon source. The synthesis of the fatty acid degradation (fad) enzymes in E. coli is strongly repressed by the presence of glucose in the growth medium (18). Inducers like oleate could not relieve this repression but addition of cAMP did cause derepression (22). This observation might explain why the presence of the β-oxidation enzymes in E. coli extract could not be demonstrated in previous studies (16,17). The synthesis of the fad enzymes is most likely regulated by a mechanism similar to that of the lac operon first postulated by Jacob and Monod (23). Like many other inducible enzyme systems the expression of the fad enzymes is subject to catabolite repression (21,22).

The isolation of E. coli mutants unable to grow on long-chain fatty acids permitted more detailed genetic studies of the fatty acid oxidation pathway (21,24,25). Genes essential for fatty acid oxidation have been mapped at five different locations on the E. coli chromosome as shown in Fig. 3. Since an E. coli mutant lacking acyl-CoA synthetase cannot be induced to synthesize the fad enzymes, Overath and coworkers (19) suggested that the fatty acyl-CoA derivatives rather than fatty acids serve as inducers

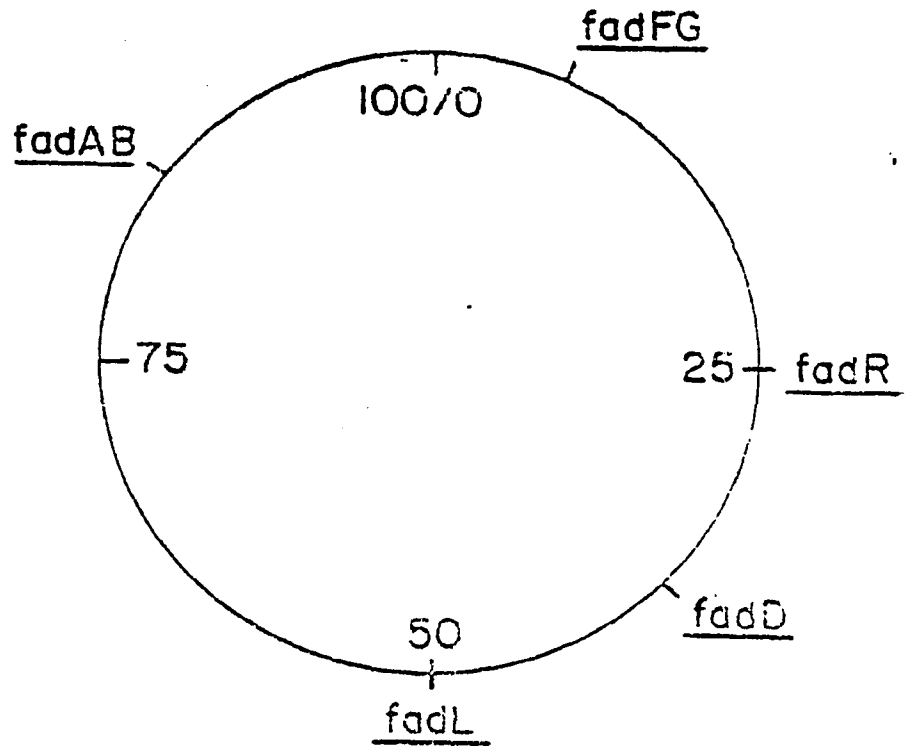

Figure 3. *E. coli* chromosome map with locations of genes required for fatty acid degradation (*fad*).

of the enzymes of fatty acid oxidation. The ideal coordinate induction was observed only for 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, while acyl-CoA synthetase and acyl-CoA dehydrogenase showed slightly lower rates of induction (18,20). On the basis of the above results, Overath and coworkers hypothesized that genes for 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase form a unit of expression, "an operon", while the genes of acyl-CoA synthetase and acyl-CoA dehydrogenase are not controlled by this operon and possibly have their own operators (18). Several fatty acid degradation mutants were isolated after mutagenesis with N-methyl-N'-nitro-N-nitroso-guanidine by plating first on minimal glucose plates and replica plating on minimal oleate medium (18,19). Mutants fadA and fadB have no detectable 3-ketoacyl-CoA thiolase activity and L-3-hydroxy-acyl-CoA dehydrogenase activity, respectively. Mutant fad5 is devoid of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase, but has inducible wild type levels of acyl-CoA synthetase and acyl-CoA dehydrogenase (18,19). Mapping data suggested that these three mutations are closely linked and are located between the loci metE and rha (19) (see position fadAB in Fig. 2).

Acyl-CoA synthetase is coded for by gene fadD. The region designated fadFG is believed to harbor the closely linked genes for at least one short-chain acyl-CoA dehydrogenase and one long-chain acyl-CoA dehydrogenase. Position fadL is that of a structural gene

for a membrane-bound protein that facilitates the entry of long-chain fatty acids through the cytoplasmic membrane (24,26). The product of the fadR gene appears to be a repressor protein involved in the expression of other fad genes (27,28). All fad genes form a fad regulon which codes for the enzymes of fatty acid oxidation and is controlled by the regulator gene fadR (19,27,28). Our knowledge about the structural and kinetic properties of enzymes of the fad regulon is still limited. Kameda and Nunn (29) have purified acyl-CoA synthetase from E. coli. This enzyme is partially membrane bound like the mammalian acyl-CoA synthetase (30).

Purification of the β-oxidation enzymes from E. coli resulted in the isolation of a multienzyme complex which exhibits enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase activities (31,32). The chain length specificities of 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase of the E. coli complex are similar to those of the mammalian enzymes. The complex has an estimated molecular weight of 260,000 and is composed of two types of subunits with molecular weights of 78,000 and 42,000. 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 (33). In addition, the purified complex contains the phospholipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (33). Immunological studies suggest that the total 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and short-chain enoyl-CoA hydratase activities present in E. coli extracts are associated with the complex.

However, a long-chain enoyl-CoA hydratase is apparently not part of the complex (33). The existence of a partially membrane-bound long-chain enoyl-CoA hydratase in E. coli was previously reported by Beadle et al.(34).

This investigation of the structural and kinetic properties of the fatty acid oxidation complex from E. coli is of great importance to the understanding of the fatty acid oxidation mechanism. The present thesis presents the results of such an investigation. The subunit locations of the component enzymes were successfully determined by immunotitration and chemical modification. Therefore, the large subunit of the fatty acid oxidation complex proves to be a multifunctional polypeptide (35). Moreover, a functional linkage between enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase is demonstrated by kinetic studies. The channeling of β-oxidation intermediates possibly permits the rapid regulation of the rate of fatty acid oxidation.

SECTION ONE

The Large Subunit of the Fatty Acid Oxidation Complex from Escherichia coli Is a Multifunctional Polypeptide.

The purified multienzyme complex of fatty acid oxidation from E. coli was found to possess enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), 3-ketoacyl-CoA thiolase (EC 2.3.1.16), cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) and 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) activities (31,32). By specifically labeling 3-ketoacyl-CoA thiolase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase with N-[2-^{14}C]ethylmaleimide, it was shown that 3-ketoacyl-CoA thiolase is a component enzyme of the β-subunit, whereas cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase is most likely associated with the larger α-subunit. Attempts were made to dissociate the complex in order to determine the subunit locations of the different enzymes. Unfortunately, these attempts resulted in a total loss of activities because of the tight association of the subunits in the complex and the instability of the subunits after their dissociation (36).

Evidence for the association of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase, and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase with the 78,000-Da α-subunit and thus proof that it is a multifunctional polypeptide is provided in this section. Since enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase, and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase are specified by the fadB gene which previously was believed to code for 3-hydroxyacyl-CoA dehydrogenase only (19), this study also provides further evidence for the existence of a fatty acid oxidation operon (fadAB)

that codes for the multienzyme complex of fatty acid oxidation and that is located at 85 min on the *E. coli* chromosome.

EXPERIMENTAL PROCEDURES

Materials - NADH, CoASH and crotonyl-CoA were purchased from P-L Biochemicals, Inc. Aldrich was the source of diketene and 2-decenoic acid. 3-Octyn-1-ol was bought from ICN Pharmaceuticals. [1-^{14}C]Iodoacetamide was supplied by New England Nuclear. NCS tissue solubilizer was purchased from Amersham Co. Iodoacetamide, L-3-hydroxyacyl-CoA dehydrogenase and all other standard biochemicals were obtained from Sigma Chemical Co. cis-3-Octenoic acid was synthesized from 3-octyn-1-ol by the procedure of Stoffel and Ecker (37). DL-3-Hydroxydodecanoic acid was prepared by reduction of ethyl 3-ketododecanoate with $NaBH_4$, followed by alkaline hydrolysis. Ethyl-3-ketododecanoate was synthesized by an established procedure (38). Beef liver crotonase (39), pig heart 3-ketoacyl thiolase (40) and rabbit antibodies against the fatty acid oxidation complex (33) were prepared as described.

Preparation of substrates - Acetoacetyl-CoA was synthesized by the method of Seubert (41). The CoA derivatives of cis-3-octenoic acid and DL-3-hydroxydodecanoic acid were prepared by the mixed anhydride method as detailed by Goldman and Vagelos (42). The concentrations of CoA derivatives were determined by the method of Ellman (43) after cleaving the thioester bond with hydroxylamine at pH 7.

Protein and Enzyme Assays - Protein concentrations were determined by the method of Lowry (44). Enoyl-CoA hydratase was assayed by following the decrease in absorbance at 263 nm due to the hydration of the $\Delta^{2,3}$-double bond of the substrate, as described in principle by Lynen

and Ochoa (45) and as detailed by Binstock and Schulz (46). L-3-Hydroxyacyl-CoA dehydrogenase was routinely assayed by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH, as detailed by Binstock and Schulz (46). 3-Ketoacyl-CoA thiolase was assayed by measuring the decrease in absorbance at 303 nm due to the disappearance of the Mg^{2+}-enolate complex of the substrate, as described by Binstock and Schulz (46). 3-Hydroxyacyl-CoA epimerase and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase were assayed spectrophotometrically at 340 nm as described (32,46).

Purification of the Fatty Acid Oxidation Complex - E. coli B (ATCC 11775) cells were grown in M 9 mineral salts medium with oleate (0.1%; v/v) as the sole carbon source. Induction of the enzymes of fatty acid oxidation was performed as described by Overath et al. (19) except that Triton X-100 (0.4%, v/v) was used instead of Brij 35 to disperse oleate. E. coli B cells (18 g) were homogenized by sonication for 4 min at 0°C in 36 ml of 10 mM potassium phosphate (pH 7.0) containing 10 mM 2-mercaptoethanol, 25% (v/v) glycerol and 2 mM PMSF.[1] Purification of the complex to homogeneity from the crude homogenate was achieved by chromatography on a phosphocellulose column (5x44 cm) as described in detail by Binstock and Schulz (46).

Immunotitration of the Fatty Acid Oxidation Complex - Fatty acid oxidation complex (1.4 µg) in 0.1 ml of the appropriate assay buffer was incubated at 25°C with antibodies raised against the fatty acid oxidation complex. The weight ratio of antibodies to complex was varied from 2 to 72. After incubation for 5 min enoyl-CoA hydratase,

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[1]PMSF: phenylmethanesulfonyl fluoride.

3-hydroxyacyl-CoA dehydrogenase, and thiolase were assayed in the incubation mixture.

Chemical Modification of the Fatty Acid Oxidation Complex - Fatty acid oxidation complex (27 μg/ml) in 0.2 M potassium phosphate (pH 8.0) was incubated with 20 mM iodoacetamide at 0°C in the absence or presence of either 1 mM NADH, 1 mM crotonyl-CoA or 1 mM acetoacetyl-CoA. 3-Hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and thiolase activities were determined as a function of the incubation time.

Fatty acid oxidation complex (3.6 mg/ml) was separated from 2-mercaptoethanol by centrifugation-filtration through Sephadex G-50 as described by Penefsky (47). The filtrate containing the complex in 0.2 M potassium phosphate (pH 8.0) was reacted with 80 mM iodoacetamide at 0°C for 90 min in the absence and presence of either 2 mM NADH or 2 mM crotonyl-CoA or both of them. The remaining activities of 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, thiolase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase of the fatty acid oxidation complex were determined as a function of the incubation time. In order to avoid an interference with the spectrophotometric measurement of thiolase activity, NADH was removed from the complex by the centrifugation-gel filtration procedure.

Labeling of the Fatty Acid Oxidation Complex with [1-^{14}C]-Iodoacetamide in the Presence and Absence of NADH - Fatty acid oxidation complex, which had been separated from 2-mercaptoethanol by the centrifugation-gel filtration procedure, was preincubated with 80 mM iodoacetamide at 0°C for 90 min in the presence of 2 mM NADH plus 2 mM crotonyl-CoA. The complex was separated from the incubation

mixture by the same centrifugation-gel filtration procedure. Half of the filtrate (at a protein concentration of 2.8 mg/ml) was reacted with 20 mM [1-^{14}C]iodoacetamide (15 μCi/ mol) at 0°C for 60 min in the presence of 1 mM NADH. The other half of the filtrate was reacted with [1-^{14}C]iodoacetamide under the same conditions but in the absence of NADH. The remaining activities of the five component enzymes of the fatty acid oxidation complex were determined during the labeling process. The fatty acid oxidation complex (30 μg) labeled with [1-^{14}C]iodoacetamide was subjected to polyacrylamide disc gel electrophoresis in the presence of SDS[1] at pH 8.3 (48). Gels were stained with Coomassie blue and destained in 7% acetic acid. Records of the gels were obtained by scanning their absorbance at 600 nm. The gels were then sliced into 2 mm wide segments each of which was incubated overnight with a mixture (1 ml) of NCS tissue solubilizer and water (9:1) at 50°C and thereafter combined with 10 ml of a toluene-based counting solution.

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[1]SDS: sodium dodecyl sulfate.

RESULTS

Immunological Study - Antibodies raised against the pure multienzyme complex of fatty acid oxidation from E. coli were used to study the responses of three component enzymes to the interaction of antibodies with the complex. Shown in Fig. 4 are the results obtained when increasing amounts of antibodies were added to a fixed amount of fatty acid oxidation complex which after incubation for 5 min at 25°C was assayed for 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. The activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase decreased upon the addition of increasing amounts of antibodies. It is interesting to note that the activities of these two component enzymes were affected by the antibodies in a nearly identical fashion. Most important was the finding that the activity of 3-ketoacyl-CoA thiolase was not inhibited by the binding of antibodies to the complex, but instead was increased approximately 25% at the highest antibody concentration used in this study. The measured 3-ketoacyl-CoA thiolase activity in the presence of antibodies was that of an antibody-antigen complex because it was completely precipitated together with the other two enzymes when the incubation mixture was centrifuged (33). Thus, the binding of antibodies at one or several sites of the fatty acid oxidation complex either interferes with the access of substrates to enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase but not to 3-ketoacyl-CoA thiolase or the antibody-antigen interaction affects the conformation of the hydratase and dehydrogenase adversely while having a beneficial effect on that of thiolase.

Fig. 4. Immunotitration of the fatty acid oxidation complex with antibodies raised against the purified complex. For experimental details, see under "Experimental Procedures". (▲) 3-Ketoacyl-CoA thiolase assayed with acetoacetyl-CoA as a substrate. (●) 3-Hydroxyacyl-CoA dehydrogenase assayed with acetoacetyl-CoA as a substrate. (■) Enoyl-CoA hydratase assayed with crotonyl-CoA as a substrate.

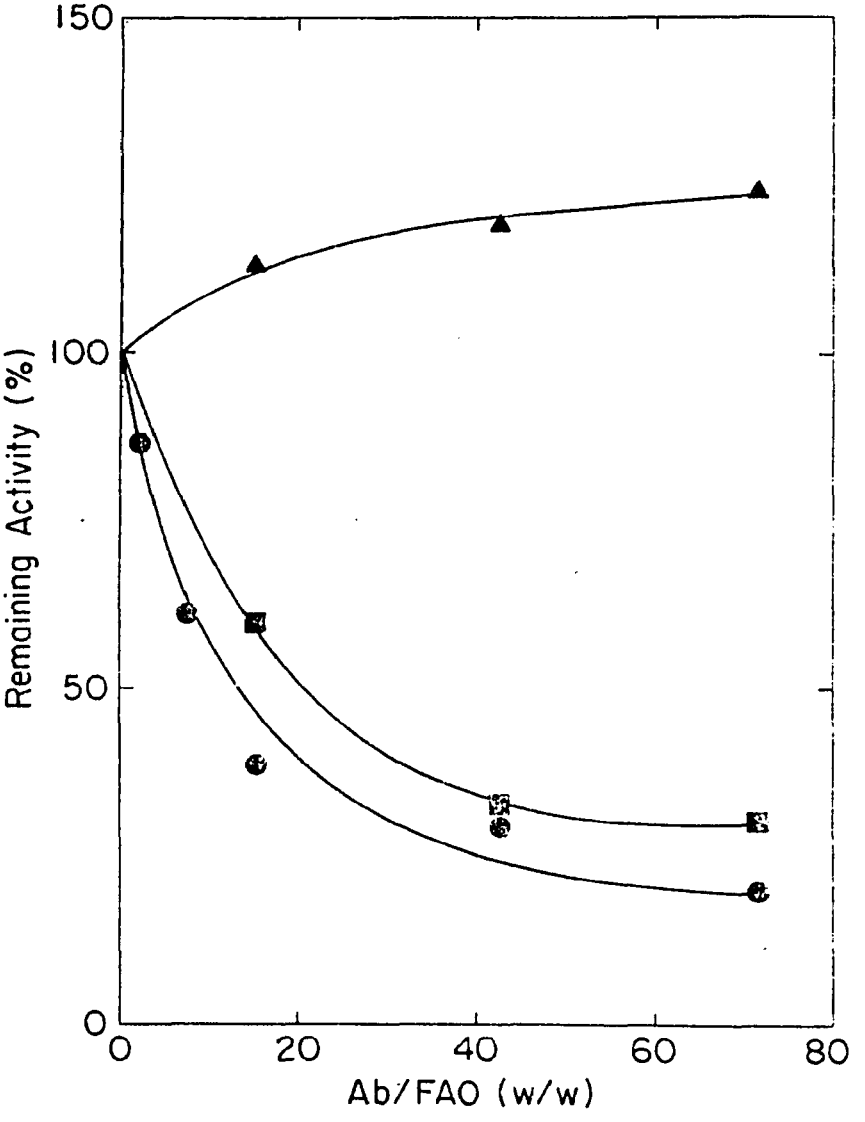


Figure 4

Inactivation of the Fatty Acid Oxidation Complex by Iodoacetamide and Protection Against this Inactivation - Incubation of the fatty acid oxidation complex with iodoacetamide at 0°C resulted in the inactivation of all its component enzymes. The enzymes were not reactivated when iodoacetamide was removed by gel filtration. The inactivations of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase as a function of time were studied as was the protection against this inactivation by several substrates. Inactivation of the complex (27 μg/ml) with 20 mM iodoacetamide caused the rapid inactivation of 3-ketoacyl-CoA thiolase with a half time of less than 30 sec, whereas enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were inactivated more slowly ($t_{\frac{1}{2}}$ = 30 min), but at nearly identical rates (see Fig. 5). NADH at a concentration of 1 mM completely protected enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, but not 3-ketoacyl-CoA thiolase against this inactivation (see Fig. 5). The protection of enoyl-CoA hydratase was surprising because the enzyme is not known to bind NADH. The effect of 1 mM crotonyl-CoA, a substrate of enoyl-CoA hydratase, on the inactivation of the complex by 20 mM iodoacetamide is shown in Fig. 6. As expected, 3-ketoacyl-CoA thiolase was not protected whereas enoyl-CoA hydratase was. The protection of 3-hydroxyacyl-CoA dehydrogenase was not anticipated, although crotonyl-CoA was partially converted to 3-hydroxybutyryl-CoA which is a substrate of the dehydrogenase and thus may bind to it and protect it. The protection provided by acetoacetyl-CoA is illustrated in Fig. 7. 3-Ketoacyl-CoA thiolase is completely protected by its substrate acetoacetyl-CoA. This result was expected because

Fig. 5. Effect of iodoacetamide on the enzyme activities of the fatty acid oxidation complex in the absence and presence of 1 mM NADH. The fatty acid oxidation complex (27 μg/ml) was incubated with 20 mM iodoacetamide at 0°C in 0.2 M potassium phosphate (pH 8.0). Enzyme activities were determined as a function of time. 3-Hydroxyacyl-CoA dehydrogenase activity in the presence of NADH (●) and in the absence of NADH (○). Enoyl-CoA hydratase activity in the presence of NADH (■) and in the absence of NADH (□). 3-Ketoacyl-CoA thiolase activity in the presence of NADH (▲) and in the absence of NADH (△).

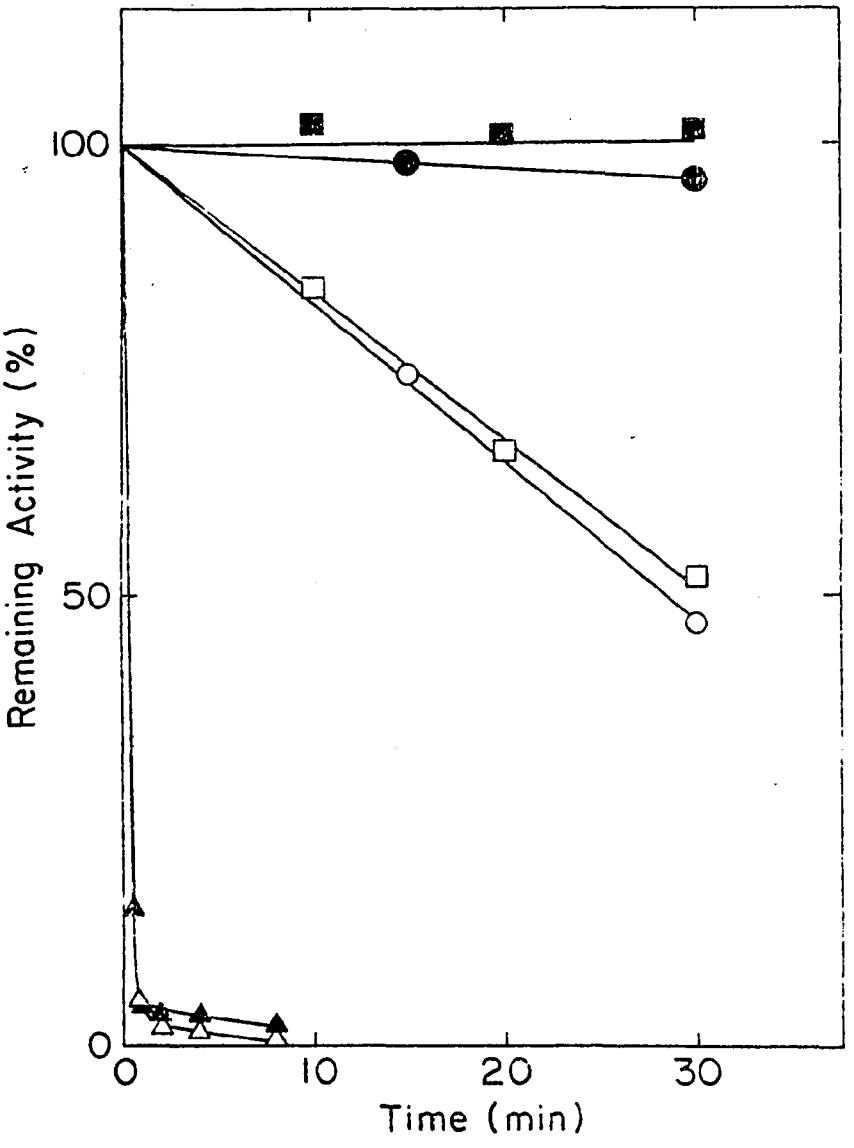


Figure 5

Fig. 6. Effect of iodoacetamide on the enzyme activities of the fatty acid oxidation complex in the absence and presence of 1 mM crotonyl-CoA. The fatty acid oxidation complex (27 μg/ml) was incubated with 20 mM iodoacetamide at 0°C in 0.2 M potassium phosphate (pH 8.0). Enzyme activities were determined as a function of time. 3-Hydroxyacyl-CoA dehydrogenase activity in the presence of crotonyl-CoA (●) and in the absence of crotonyl-CoA (○). Enoyl-CoA hydratase activity in the presence of crotonyl-CoA (■) and in the absence of crotonyl-CoA (□). 3-Ketoacyl-CoA thiolase activity in the presence of crotonyl-CoA (▲) and in the absence of crotonyl-CoA (△).

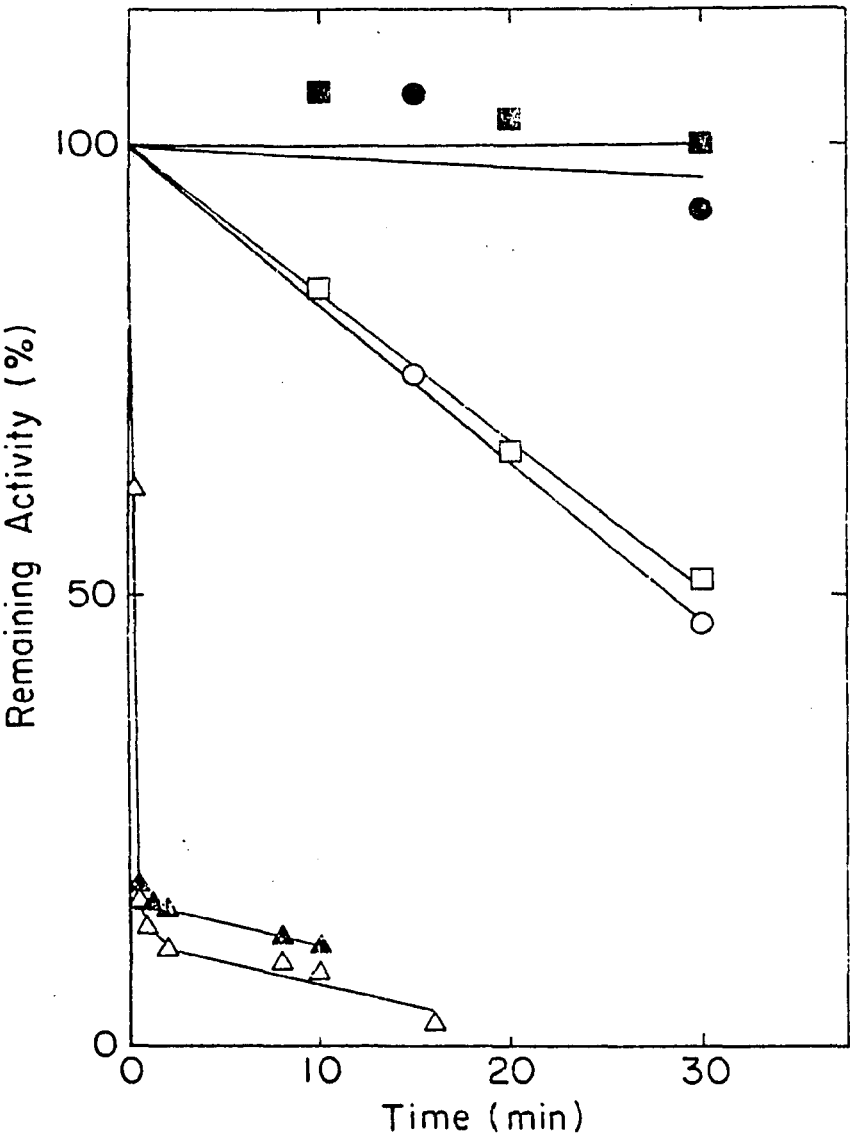


Figure 6

Fig. 7. Effect of iodoacetamide on the enzyme activities of the fatty acid oxidation complex in the absence and presence of 1 mM acetoacetyl-CoA. The fatty acid oxidation complex (27 µg/ml) was incubated with 20 mM iodoacetamide at 0°C in 0.2 M potassium phosphate (pH 8.0). Enzyme activities were determined as a function of time. 3-Hydroxyacyl-CoA dehydrogenase activity in the presence of acetoacetyl-CoA (●) and in the absence of acetoacetyl-CoA(○). Enoyl-CoA hydratase activity in the presence of acetoacetyl-CoA (■) and in the absence of acetoacetyl-CoA (□). 3-Ketoacyl-CoA thiolase activty in the presence of acetoacetyl-CoA (▲) and in the absence of acetoacetyl-CoA (△).

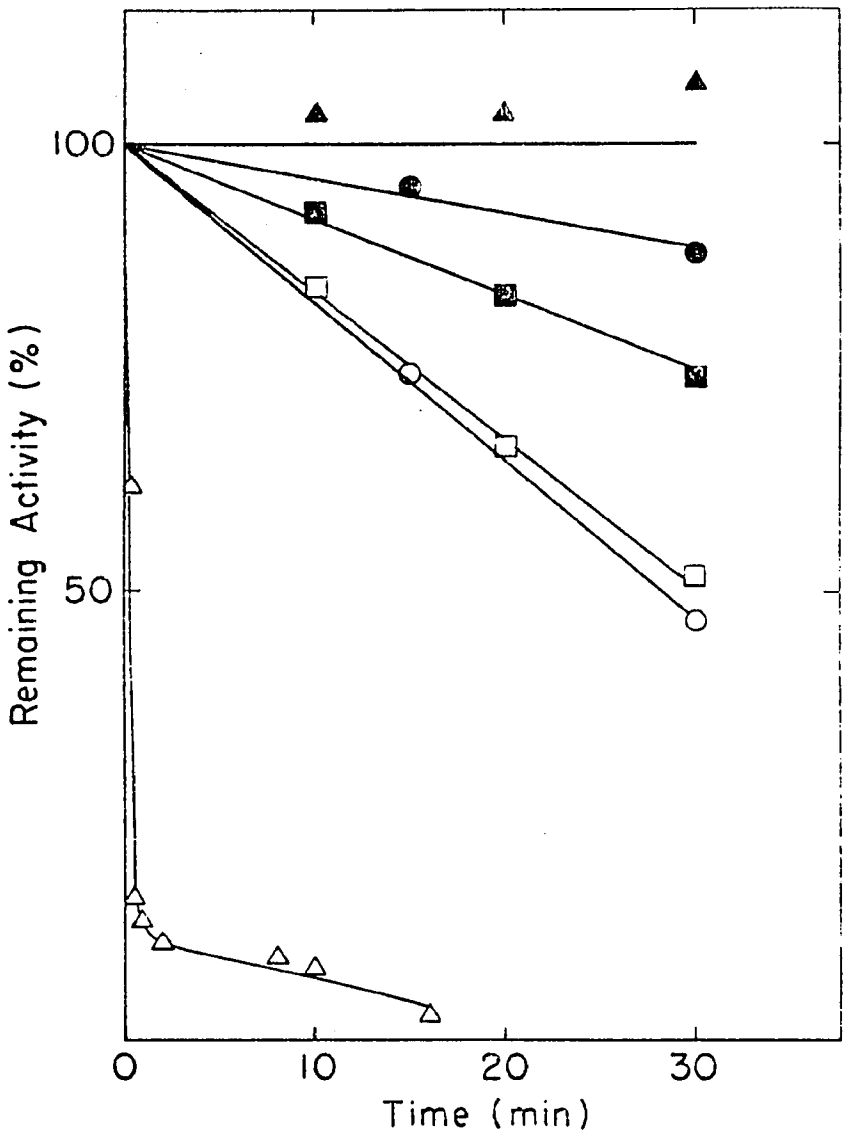


Figure 7

acetoacetyl-CoA has been shown to protect the essential sulfhydryl group of this thiolase against modification by N-ethylmaleimide (33). Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were partially protected by acetoacetyl-CoA, which is a substrate of the dehydrogenase and may even bind to enoyl-CoA hydratase in its enolate form (49).

We have additionally studied the inactivation of the fatty acid oxidation complex at a concentration of 3.6 mg/ml by 80 mM iodoacetamide and the protection provided against this inactivation by either NADH, or crotonyl-CoA or both of them. Included in this study were all five known component enzymes of the complex. The results are presented in Table I. As expected, an increase in the concentration of iodoacetamide from 20 mM to 80 mM resulted in a more rapid inactvation of the enzyme. Most interesting was the observation that all enzymes with the exception of 3-ketoacyl-CoA thiolase were inactivated at nearly identical rates and were protected by both NADH and crotonyl-CoA. However, the protection provided by NADH, even at concentrations as high as 12 mM was not complete. Similarly, crotonyl-CoA did not prevent the partial inactivation of the dehydrogenase, hydratase, isomerase and epimerase. However, in the presence of both 2 mM NADH and 2 mM crotonyl-CoA these four enzymes remained fully active or only lost a small fraction of their activities. Clearly, the protections provided by NADH and crotonyl-CoA are additive. A review of the data presented in Table I leads to the conclusion that the component enzymes of the fatty acid oxidation complex with the exception of 3-ketoacyl-CoA thiolase respond in a nearly identical fashion to the chemical modification by iodoacetamide and to the protection provided by NADH and crotonyl-CoA which are substrates of

TABLE I

Inactivation of the fatty acid oxidation complex by iodoacetamide and protection against this inactivation.

The complex (3.6 mg/ml) was reacted with 80 mM iodoacetamide in the absence or presence of NADH or/and crotonyl-CoA. For experimental details, see "Experimental Procedures."

Enzyme	Remaining Activity				
	No addition[a]		+12 mM NADH	+2 mM Crotonyl-CoA	+2 mM NADH +2 mM Crotonyl-CoA
	30 min	90 min	90 min	90 min	90 min
	%	%	%	%	%
3-Hydroxyacyl-CoA dehydrogenase	28	8	56	72	102
Enoyl-CoA hydratase	30	12	40	57	97
cis-Δ^3-trans-Δ^2-Enoyl-CoA isomerase	37	21	56[b]	76	89
3-Hydroxyacyl-CoA epimerase	39	14	61[b]	56	83
3-Ketoacyl-CoA thiolase	0	0	0	0	0

[a] The half-times for the inactivation of 3-hydroxyacyl-CoA dehydrogenase by 80 mM and 20 mM iodoacetamide under otherwise identical experimental conditions were 23 min and 54 min, respectively.

[b] In the presence of 24 mM NADH.

3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, respectively.

Labeling of the Fatty Acid Oxidation Complex with [1-^{14}C]-Iodoacetamide - It was planned to identify the subunit location of the functional groups that are modified by iodoacetamide in the absence but not in the presence of NADH and which are responsible for the inactivation of four of the five component enzymes of the fatty acid oxidation complex. In order to decrease the nonspecific background labeling, the complex was pretreated with 80 mM iodoacetamide in the presence of 2 mM NADH plus 2 mM crotonyl-CoA. Under these conditions the component enzymes of the complex with the exception of 3-ketoacyl-CoA thiolase remained fully active or nearly so (see Table I). Excess reagent and the protective agents were removed by gel filtration. One half of the sample was then reacted with 20 mM [1-^{14}C]iodoacetamide in the presence of 1 mM of NADH, while the other half was reacted with 20 mM [1-^{14}C]-iodoacetamide in the absence of NADH. The sample modified in the presence of NADH remained fully active, whereas in the absence of NADH the four active component enzymes were partially inactivated (see Table II).

Both samples were filtered through Sephadex G-50 and subjected to SDS gel electrophoresis. The protein and radioactive labeling patterns thus obtained are shown in Fig. 8. Both subunits of the complex were radioactively labeled. However, the degree of labeling of the small subunit (see Fig. 8, peak II) was unaffected by the presence of NADH. In contrast, the incorporation of [1-^{14}C]iodoacetamide into the large subunit was reduced by 50% when the labeling was performed in the presence of NADH (see Fig. 8, peak I). The third protein band shown

TABLE II

Inactivation of the fatty acid oxidation complex from *E. coli* by iodoacetamide.

The complex (2.8 mg/ml) was reacted with 20 mM [1-^{14}C]iodoacetamide for 60 min in the absence and presence of 1 mM NADH. For experimental details, see "Experimental Procedures."

Enzyme	Remaining Activity	
	+NADH	-NADH
	%	%
3-Hydroxyacyl-CoA dehydrogenase	98	50
Enoyl-CoA hydratase	94	60
cis-Δ^3-*trans*-Δ^2-Enoyl-CoA isomerase	96	76
3-Hydroxyacyl-CoA epimerase	105	79
3-Ketoacyl-CoA thiolase	0	0

Fig. 8. SDS-disc gel electrophoresis of the fatty acid oxidation complex labeled with [1-^{14}C]iodoacetamide. For experimental details, see under "Experimental Procedures". The fatty acid oxidation complex (2.8 mg/ml) was reacted for 60 min with 20 mM [1-^{14}C]iodoacetamide in the presence of 1 mM NADH (O) and in the absence of NADH (●). ———, protein; ------ and ....., radioactivity.

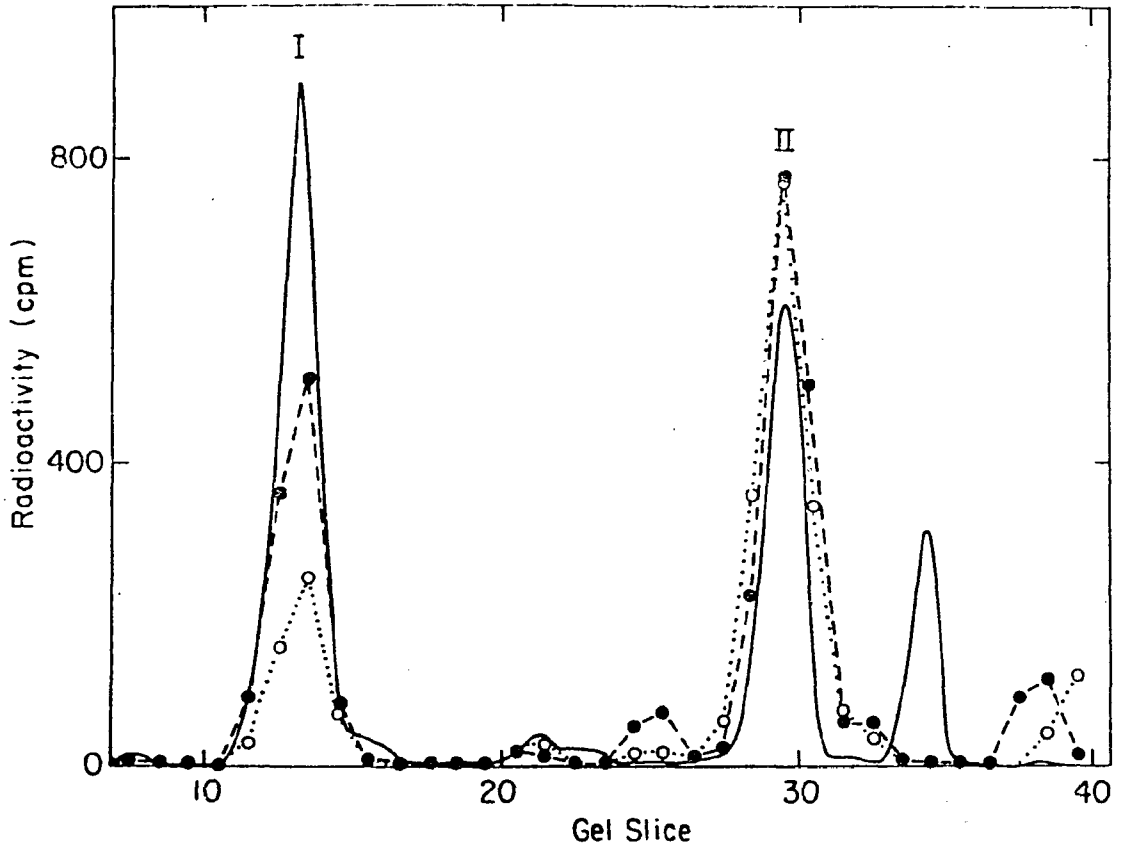


Figure 8

in Fig. 8 has previously been proven to be a contaminant or degradation product of the complex (33). These results strongly suggest that NADH prevents some groups on the large subunit of the complex from reacting with iodoacetamide and that the modification of these groups is the cause for the activity losses observed for enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase.

DISCUSSION

The isolation of a fatty acid oxidation complex from E. coli that exhibits five enzymatic activities and has an $\alpha_2\beta_2$ structure (31-33) prompted this investigation into the subunit location of the five component enzymes. In a previous publication from this laboratory evidence was presented for the association of 3-ketoacyl-CoA thiolase with the 42,000-dalton β-subunit and the possibly location of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase on the 78,000-dalton α-subunit (33). This conclusion was based on results obtained with N-ethylmaleimide as a labeling agent which caused the rapid inactivation of 3-ketoacyl-CoA thiolase and the slower inactivation of cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase, while it had no effect on the activities of the other three enzymes (33). It has previously been reported that the inactivation of all component enzymes of the complex by 1 M Tris-HCl (pH 8) (32). Interestingly, 3-ketoacyl-CoA thiolase was inactivated with a halftime of 2 min, whereas the halftimes for the other four enzymes were all between 4 and 5 min (32). This observation led to the formulation of the hypothesis that the four component enzymes, which were inactivated by Tris-HCl at equal rates, may be located on the complex in close proximity, possibly on the same subunit. This hypothesis is also supported by the observed parallel inactivations of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in response to the binding of antibodies to the complex. The bound antibodies may interfere with the access of substrates to the active sites of both component enzymes or adversely affect their conformations. Both enzymes possibly are affected in a similar fashion, because they are located on the complex in close proximity to each other and to the

antibody binding site(s). 3-Ketoacyl-CoA thiolase was not adversely affected by the bound antibodies, presumably because it is located on a region of the complex remote from the antibody binding site(s) and hence from the active sites of the two other enzymes.

The inactivation of the component enzymes of the complex by iodoacetamide permitted us to continue our investigation of their subunit locations. All enzymes except for 3-ketoacyl-CoA thiolase were inactivated at nearly equal rates which were approximately 60-times slower than the rate at which 3-ketoacyl-CoA thiolase was inactivated. The inactivation of thiolase was most likely a consequence of the modification of its essential sulfhydryl group which has been shown to be rapidly labeled by N-ethylmaleimide (33) and which was protected against both modifications by acetoacetyl-CoA. Since enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase were not affected by treatment with N-ethylmaleimide (33), it is likely that their inactivations by iodoacetamide were not due to the modification of a sulfhydryl residue, but involved another group, as for example an imidazole residue (50). Most important were two observations: (a) The four component enzymes, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase, were inactivated by iodoacetamide at almost equal rates and (b) all four enzymes were protected against this inactivation by either NADH or crotonyl-CoA which are substrates of 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, respectively. These observations lead us to suggest that the inactivations of these four enzymes is possible due to the modification of a single residue located at the

active sites of the hydratase and dehydrogenase, which appear to be close to the active sites of the isomerase and epimerase. Thus, these observations support our original hypothesis that these four component enzymes of the complex are located in close proximity to each other, most likely on the same subunit.

In order to identify the subunit that carries 3-hydroxyacyl-CoA dehydrogenase, the complex was labeled with [1-^{14}C]iodoacetamide in the absence and presence of NADH. Prelabeling with non-radioactive iodoacetamide in the presence of NADH plus crotonyl-CoA greatly reduced the non-specific labeling of the complex. Since the presence of NADH reduced the labeling of the large subunit only, 3-hydroxyacyl-CoA dehydrogenase is associated with the 78,000-dalton α-subunit. This fact together with conclusions presented above lead us to suggest that enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase are component enzymes of the α-subunit, whereas 3-ketoacyl-CoA thiolase is the only enzyme activity associated with the β-subunit. The 42,000-dalton β-subunit has the molecular weight characteristic of thiolases (40,51), whereas the 78,000-dalton α-subunit is similar in size to the 80,000-dalton peroxisomal and 75,000-dalton glyoxisomal bifunctional enzymes which exhibit enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (52,53). We have assayed the bifunctional enzyme from rat liver peroxisomes (kindly provided by Dr. Hashimoto, Shinshu University, Japan) for cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase but found it to be devoid of both enzyme activities (54). Shown in Fig. 9 are the reactions of β-oxidation catalyzed by the fatty

Fig. 9. Pathway of fatty acid degradation and the organization of the β-oxidation enzymes in E. coli. Shown is the association of the five component enzymes of the fatty acid oxidation complex with the large subunit (α) and the small subunit (β).

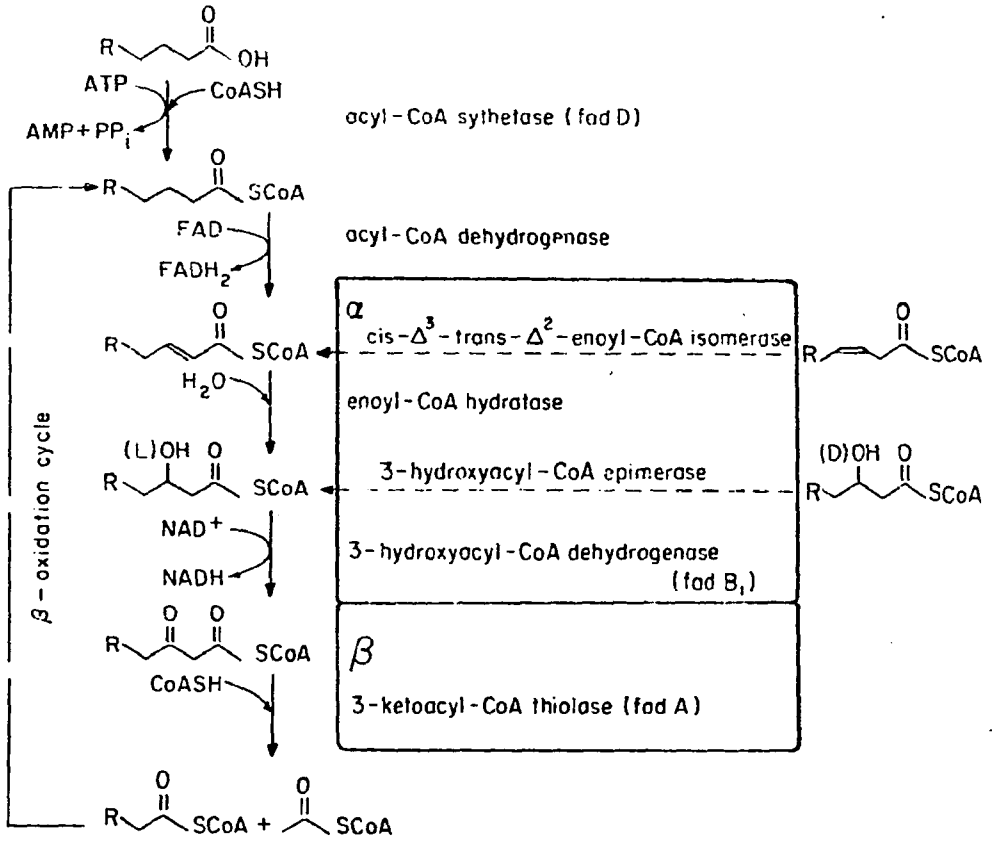

R
OH
ATP
CoASH
AMP+PP$_i$
acyl-CoA sythetase (fad D)
SCoA
FAD
FADH$_2$
acyl-CoA dehydrogenase
α
cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase
H$_2$O
enoyl-CoA hydratase
(L)OH
(D)OH
3-hydroxyacyl-CoA epimerase
NAD$^+$
NADH
3-hydroxyacyl-CoA dehydrogenase (fad B$_1$)
β
CoASH
3-ketoacyl-CoA thiolase (fad A)
β-oxidation cycle

Figure 9

acid oxidation complex from E. coli and the subunit locations of the five component enzymes of the complex. For simplicity only one α-subunit and β-subunit each are presented.

The results of this study additionally provide insight into the organization of the genes for the β-oxidation enzymes in E. coli. Overath et al. (18,19) have suggested that the genes for 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and possibly those for cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase behave as a single operon which was mapped between the loci met E and rha on the E. coli chromosome. This suggestion is based on the highly coordinate induction of the first three enzymes (18,20) and on the genetic mapping of the fad 5 mutant, deficient in all five enzymes, the fad A mutant, deficient in 3-ketoacyl-CoA thiolase, and the fad B mutant deficient in 3-hydroxyacyl-CoA dehydrogenase. These three mutations were found to be closely linked and the fad 5 behaved as a "polar mutation" found in many operons. However, the fad 5 mutant was obtained by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, a mutagen which causes with high probability mutations at more than one site on the chromosome (55). Since no mutants deficient in either enoyl-CoA hydratase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase or 3-hydroxyacyl-CoA epimerase have been obtained, the locations of their genes on the E. coli chromosome have not been determined. However, Overath et al. demonstrated that the fad 5 mutant still harbors the intact genetic information for 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase at the 85 min region of the chromosome (19). It thus appears that the expression of these two closely linked genes was

abolished by an upstream mutation. The results of the present study together with those of Overath and coworkers (18,19) provide evidence for the existence of a fatty acid oxidation (fad) operon in E. coli. We propose to name this operon fad AB, where A and B denote the genes for the 42,000-dalton β-subunit and the 78,000-dalton α-subunit of the complex, respectively. Since 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase are part of the same polypeptide, it follows that these four enzymes are specified by the fad B gene. Consequently the fad AB operon contains the genetic information for the five component enzymes of the fatty acid oxidation complex. The fatty acid oxidation complex from mutant fad B64 was found to have a defective 3-hydroxyacyl-CoA dehydrogenase whereas its gross structure was indistinguishable from the wild-type complex (56). This mutation in the fad B gene affected only one of the four catalytic functions of the fad B gene product. We refer to this mutation as fad B_1 (see Fig. 9) where B refers to the gene of the large subunit and the subscript 1 indicates the activity of the B gene product affected by this mutation.

SECTION TWO

Channeling of a β-Oxidation Intermediate on the Large Subunit of the Fatty Acid Oxidation Complex from Escherichia coli.

It was recently established that enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3) and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) are located on the large subunit, possibly in close proximity to each other (35). Since enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase catalyze two sequential reactions of β-oxidation, the functional consequence of having both enzymes associated with a multifunctional polypeptide was investigated.

The β-oxidation reactions catalyzed by enoyl-CoA hydratase (EH) and 3-hydroxyacyl-CoA dehydrogenase (HAD) of the large subunit of the fatty acid oxidation complex from E. coli are shown below.

$$R\text{-}CH_2\text{-}CH{=}CH\text{-}\underset{\underset{O}{\|}}{C}\text{-}SCoA + H_2O \xrightleftharpoons{EH} (L)R\text{-}CH_2\text{-}\underset{\underset{OH}{|}}{CH}\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}SCoA$$

$$NAD^+ + (L)R\text{-}CH_2\text{-}\underset{\underset{OH}{|}}{CH}\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}SCoA \xrightleftharpoons{HAD} R\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}SCoA + NADH + H^+$$

The association with a single polypeptide of two enzymes acting sequentially may or may not result in an increased rate of the overall reaction due to the channeling of intermediates. A number of multifunctional proteins show this behavior (57-59), whereas chorismate mutase-prephenate dehydratase and some other enzymes have component enzymes that operate independently (60-62).

In this section, it is shown that more than 90% of the intermediate L-3-hydroxydecanoyl-CoA is directly transferred from the active site of enoyl-CoA hydratase to that of 3-hydroxyacyl-CoA dehydrogenase without equilibrating with the bulk solvent. The channeling mechanism prevents the accumulation of intermediates in the medium and may be essential for the efficient regulation of β-oxidation.

EXPERIMENTAL PROCEDURES

Materials - NAD^+, NADH and CoASH were purchased from P-L Biochemicals. Ethyl chloroformate, triethylamine, diketene and trans-2-decenoic acid were obtained from Aldrich Chemical Company. Tetrahydrofuran, ethyl chloroformate and diketene were distilled before use. 3-Ketoacyl-CoA thiolase was isolated from pig heart according to the procedure of Staack et al. (40). Bovine liver enoyl-CoA hydratase was purified according to Steinman and Hill (39). L-3-Hydroxyacyl-CoA dehydrogenase and all other standard biochemicals were obtained from Sigma.

Preparation of Substrates - Crotonyl-CoA (63) and acetoacetyl-CoA (41) were synthesized according to published procedures. The CoA derivative of 2-decenoic acid was prepared by the mixed anhydride method as detailed by Goldman and Vagelos (42). The concentrations of CoA derivatives were determined by the method of Ellman (43) after cleaving the thioester bond with hydroxylamine at pH 7.

Purification of the Fatty Acid Oxidation Complex - *E. coli* B (ATCC 11775) cells were grown in M9 mineral salts medium with oleate (0.1%, V/V) as the sole carbon source. Induction of the enzymes of fatty acid oxidation was performed as described by Overath et al. (19) except that Triton X-100 (0.4%, V/V) was used instead of Brij 35 to disperse oleate. Cells of *E. coli* B (18 g, wet weight) were homogenized by sonication for 4 min at 0°C in 36 ml of 10 mM potassium phosphate (pH 7.0) containing 10 mM 2-mercaptoethanol, 25% (V/V) glycerol, and 2 mM phenylmethylsulfonyl fluoride. Purification of the complex to homogeneity from the crude homogenate was achieved by chromatography on a phosphocellulose column (5 x 44 cm) as described in detail by Binstock and Schulz (46).

Protein and Enzyme Assays - Protein concentrations were determined by the method of Lowry et al. (44). Enoyl-CoA hydratase was assayed spectrophotometrically at 340 nm by the indirect method, or at 263 nm by the direct method, as described in principle by Fong and Schulz (64). Indirect method: A standard assay mixture contained 0.2 M KP_i (pH 8), bovine serum albumin (0.2 mg/ml), 1.8 mM NAD^+, 0.25 mM CoASH, 4.7 µM 2-decenoyl-CoA, pig heart 3-hydroxyacyl-CoA dehydrogenase (0.95 U), pig heart 3-ketoacyl-CoA thiolase (12.5 mU) and enoyl-CoA hydratase to give an absorbance change of 0.08 A/min at 340 nm. The molar extinction coefficient used for calculating rates is 6220 M^{-1} cm^{-1}. Direct method: A standard assay mixture contained 0.2 M KP_i (pH 8), bovine serum albumin (0.2 mg/ml), 4.7 µM 2-decenoyl-CoA and enoyl-CoA hydratase to give an absorbance change of 0.09 A/min at 263 nm. The molar extinction coefficient used for calculating rates is 6700 M^{-1} cm^{-1}. The ratio of L-3-hydroxydecanoyl-CoA to 2-decenoyl-CoA at equilibrium is 2.23. L-3-Hydroxyacyl-CoA dehydrogenase was assayed in the forward direction spectrophotometrically at 340 nm as described in principle by Binstock and Schulz (46). First the substrate L-3-hydroxydecanoyl-CoA was generated enzymatically by reacting 4.7 µM 2-decenoyl-CoA in 0.2 M KP_i (pH 8) containing bovine serum albumin (0.2 mg/ml) with bovine liver enoyl-CoA hydratase (76 mU). At equilibrium the assay mixture contained 3.24 M L-3-hydroxydecanoyl-CoA; then 0.25 mM CoASH, 1.8 mM NAD^+ and pig heart 3-ketoacyl-CoA thiolase (12.5 mU) were added. The assay was started by the addition of 3-hydroxyacyl-CoA dehydrogenase to give an absorbance change of 0.08 A/min at 340 nm. The overall reaction, the conversion of 2-decenoyl-CoA to 3-hydroxydecanoyl-CoA, was

assayed spectrophotometrically by measuring the increase in absorbance at 340 nm. A standard assay mixture contained 0.2 M KP_i (pH 8), bovine serum albumin (0.2 mg/ml), 1.8 mM NAD^+, 0.25 mM CoASH, 4.7 μM 2-decenoyl-CoA and pig heart 3-ketoacyl-CoA thiolase (12.5 mU), and the assay was started by the addition of 0.38 μg of E. coli fatty acid oxidation complex. The conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by bovine liver enoyl-CoA hydratase (5.9 mU with 2-decenoyl-CoA as a substrate) and pig heart 3-hydroxyacyl-CoA dehydrogenase (6.2 mU with L-3-hydroxydecanoyl-CoA as a substrate) was measured by use of the same assay procedure except that the reaction was started by the addition of bovine liver enoyl-CoA hydratase. The molar extinction coefficient used for calculating the rate of NADH formation is 6220 M^{-1} cm^{-1}. Since NADH and 3-ketodecanoyl-CoA are found in equimolar amounts their rates of formation are identical. 3-Ketodecanoyl-CoA was instantly cleaved by pig heart 3-ketoacyl-CoA thiolase to prevent the inhibition of L-3-hydroxyacyl-CoA dehydrogenase by 3-ketodecanoyl-CoA. The assays were performed at 25°C on a Gilford recording spectrophotometer (Model 250) as well as a Durrum stopped-flow spectrophotometer (Model D-130) to determine the time course and the initial rate of the reactions. Enzyme activities of 3-ketoacyl-CoA thiolase, L-3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, that are listed above without a reference to a substrate, were measured with either acetoacetyl-CoA or crotonyl-CoA as substrates as described in principle by Lynen and Ochoa (45) and as detailed by Binstock and Schulz (46). A unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute.

Chemical Modification of the Fatty Acid Oxidation Complex - Fatty acid oxidation complex (38 µg/ml) in 0.2 M KP_i (pH 8) was incubated with 5 mM acetic anhydride at 0°C in the absence or presence of either 1 mM NADH or 1 mM crotonyl-CoA or both of them. Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were determined as a function of the incubation time.

Calculations - The kinetic parameters of the individual enzymes were obtained from Lineweaver-Burk plots (65). The theory of Storer and Cornish-Bowden was applied to simulate the kinetics of a system consisting of two enzymes that catalyze consecutive reactions, but operate independently (66). If the coenzyme NAD^+ is present at a saturating concentraton, or if the apparent K_m value of 3-hydroxyacyl-CoA dehydrogenase (HAD) for 3-hydroxydecanoyl-CoA (B) is equal to the real K_m value (K_{mB}), the concentration of intermediate ([B]) can be calculated from the value for K_{mB}, the maximal velocity of 3-hydroxyacyl-CoA dehydrogenase (V_{HAD}) and the rate of the overall reaction (v_{HAD}).

$$[B] = \frac{K_{mB} \quad v_{HAD}}{V_{HAD} - v_{HAD}} \qquad (1)$$

If the rate of the first reaction (v_{EH}) catalyzed by enoyl-CoA hydratase (EH) is constant, the time course of the overall reaction is defined by the following equation:

$$t = \frac{V_{HAD} \cdot K_{mB}}{(V_{HAD} - v_{EH})^2} \cdot \ln \left[\frac{v_{EH} (V_{HAD} - v_{HAD})}{V_{HAD} (v_{EH} - v_{HAD})} \right] - \frac{K_{mB} \cdot v_{HAD}}{(V_{HAD} - v_{HAD})(V_{HAD} - v_{EH})} \qquad (2)$$

The lag period expected for the formation of the final product and the accumulation of intermediate as a function of time can be calculated from both equations (2) and (1).

The above equations are derived as follows. Equation (1) is obtained by rearrangement of the Michaelis-Menten equation. This equation can be differentiated with respect to v_{HAD} to give:

$$\frac{d[B]}{dv_{HAD}} = \frac{V_{HAD} \cdot K_{mB}}{(V_{HAD} - v_{HAD})^2} \qquad (3)$$

and so:

$$\frac{d[B]}{dt} = \frac{V_{HAD} \cdot K_{mB}}{(V_{HAD} - v_{HAD})} \cdot \frac{dv_{HAD}}{dt} \qquad (4)$$

Since the intermediate B is generated in the first reaction at a velocity of v_{EH} which is assumed to be constant, and consumed in the second reaction at a velocity v_{HAD}, the rate of change of [B] is given by:

$$\frac{d[B]}{dt} = v_{EH} - V_{HAD} \qquad (5)$$

After substituting eqn. (4) into eqn. (5) and rearranging, eqn. (6) is obtained :

$$\int \frac{V_{HAD} \cdot K_{mB} \cdot dv_{HAD}}{[(v_{EH}-v_{HAD})(V_{HAD}-v_{HAD})^2]} = \int dt \qquad (6)$$

which may be integrated to give:

$$\frac{-V_{HAD} \cdot K_{mB}}{V_{HAD}-v_{EH}} \left[\frac{1}{V_{HAD}-v_{HAD}} + \frac{1}{V_{HAD}-v_{EH}} \right] \cdot \ln \left(\frac{v_{EH}-v_{HAD}}{V_{HAD}-v_{HAD}} \right) = t + \alpha \qquad (7)$$

where α is a constant of integration. The boundary condition $v_{HAD} = 0$ when $t = 0$ give the value of α.

$$\alpha = \frac{-V_{HAD} \cdot K_{mB}}{V_{HAD}-v_{EH}} \left[\frac{1}{V_{HAD}} + \frac{1}{V_{HAD}-v_{EH}} \right] \cdot \ln \frac{v_{EH}}{V_{HAD}} \qquad (8)$$

If $t = 0$, after substituting eqn. (8) into eqn. (7) and rearranging, eqn. (2) will be obtained. This equation shows, for any values of v_{EH}, K_{mB} and V_{HAD}, the time required for v_{HAD} (the rate of the overall reaction) to reach any value.

RESULTS

Kinetics of the Individual Enzymes - The kinetic parameters of bovine liver enoyl-CoA hydratase, pig heart 3-hydroxyacyl-CoA dehydrogenase and of the two corresponding enzymes present in the multienzyme complex of fatty acid oxidation from E. coli were determined according to Lineweaver and Burk (66) and are listed in Table III. The K_m values for 2-decenoyl-CoA obtained with the short-chain enoyl-CoA hydratase from bovine liver and E. coli are similar. However, the enoyl-CoA hydratase of the fatty acid oxidation complex, in contrast to the bovine liver enzyme, is inhibited at substrate concentrations higher than 25 μM (see Fig. 10). Since the K_m value for NAD^+ with the E. coli 3-hydroxyacyl-CoA dehydrogenase is unusually high, the value of the apparent V_{max} determined at 1.8 mM NAD^+ was used instead of V_{max} in all calculations. It was noted that the K_m value for L-3-hydroxydecanoyl-CoA with the E. coli 3-hydroxyacyl-CoA dehydrogenase is independent of the NAD^+ concentrations, and that the reactions catalyzed by the 3-hydroxyacyl-CoA dehydrogenase from either E. coli or pig heart obey the Michaelis-Menten equation at fixed concentrations of NAD^+ (see Fig. 11).

Kinetics of the Coupled Enzyme Reactions Catalyzed by Bovine Liver Enoyl-CoA Hydratase and Pig Heart 3-Hydroxyacyl-CoA Dehydrogenase - Progress curves for substrate consumption and product formation during the conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by bovine liver enoyl-CoA hydratase and pig heart 3-hydroxyacyl-CoA dehydrogenase are shown in Fig. 12A. The system contains 4.7 μM 2-decenoyl-CoA, 5.9 mU of enoyl-CoA hydratase determined with 2-decenoyl-CoA as a substrate and 6.2 mU of 3-hydroxyacyl-CoA dehydrogenase determined with

TABLE III

Kinetic parameters of bovine liver enoyl-CoA hydratase, pig heart 3-hydroxyacyl-CoA dehydrogenase, the corresponding enzymes associated with the α-subunit of the fatty acid oxidation (FAO) complex from E. coli.

	Enoyl-CoA Hydratase[a]				3-Hydroxyacyl-CoA Dehydrogenase			
	Bovine Liver		FAO Complex		Pig Heart		FAO Complex	
Substrate	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	μM	U/mg	μM	U/mg	μM	U/mg	μM	U/mg
2-Decenoyl-CoA	3.1	156	3.9	27.4				
3-Hydroxydecanoyl-CoA					2.9	101	2.9	33.5
NAD^+					10.7	101	191	33.5

[a] Enzyme activities were determined by the indirect method. For experimental details see under Experimental Procedures.

Fig. 10. Activities of short-chain enoyl-CoA hydratase from bovine liver and from E. coli as a function of 2-decenoyl-CoA (Δ^2C_{10}-CoA) concentration. Enzyme activities of enoyl-CoA hydratase from bovine liver (O) and of enoyl-CoA hydratase of the fatty acid oxidation complex from E. coli (●) were determined by the direct method. For experimental details, see under Experimental Procedures.

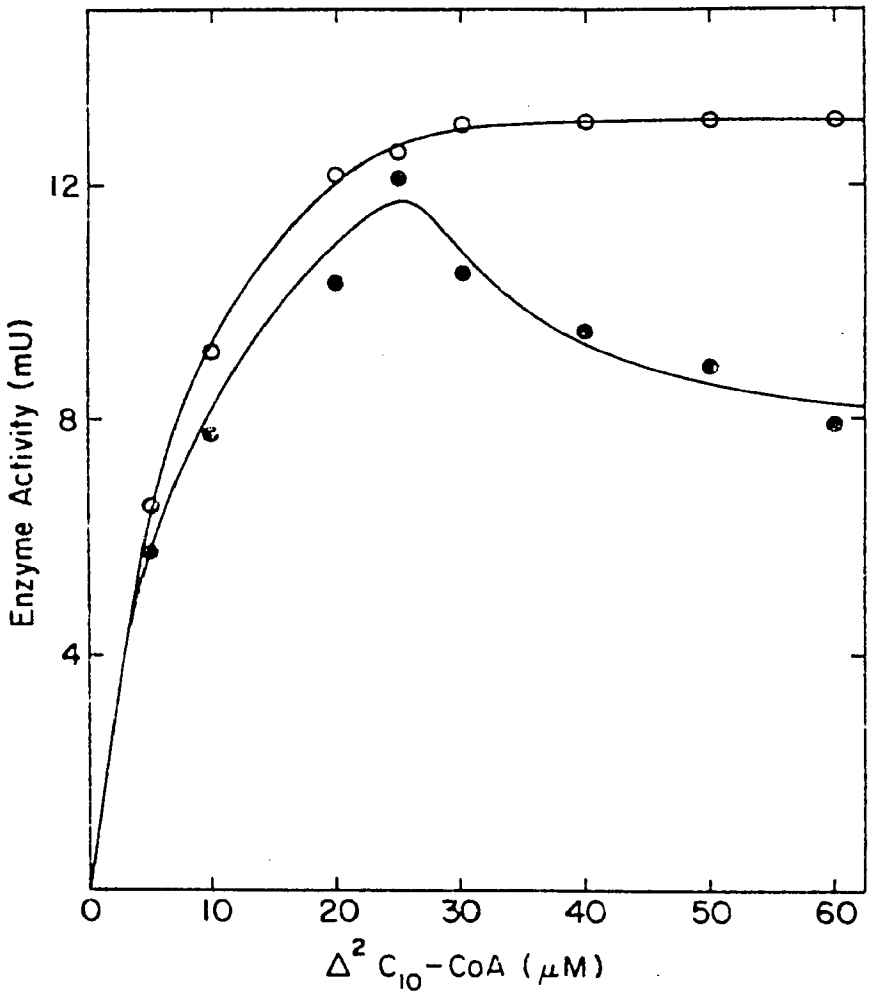


Figure 10

Fig. 11. Rates of the dehydrogenation of 3-hydroxydecanoyl-CoA catalyzed by 3-hydroxyacyl-CoA dehydrogenase of the fatty acid oxidation complex from E. coli (Panel A) and by the pig heart 3-hydroxyacyl-CoA dehydrogenase (Panel B) as a function of the substrate concentrations. The concentration of 3-hydroxydecanoyl-CoA ($3HOC_{10}$-CoA) was varied from 1.7 μM to 21.5 μM at three fixed concentrations of NAD^+: Panel A, (■) 0.26 mM, (▲) 0.52 mM, and (●) 1.80 mM; Panel B, (■) 0.024 mM, (▲) 0.048 mM, and (●) 1.80 mM. The insert shows replots of Slopes (▲) and y-Intercepts (●) versus $1/NAD^+$.

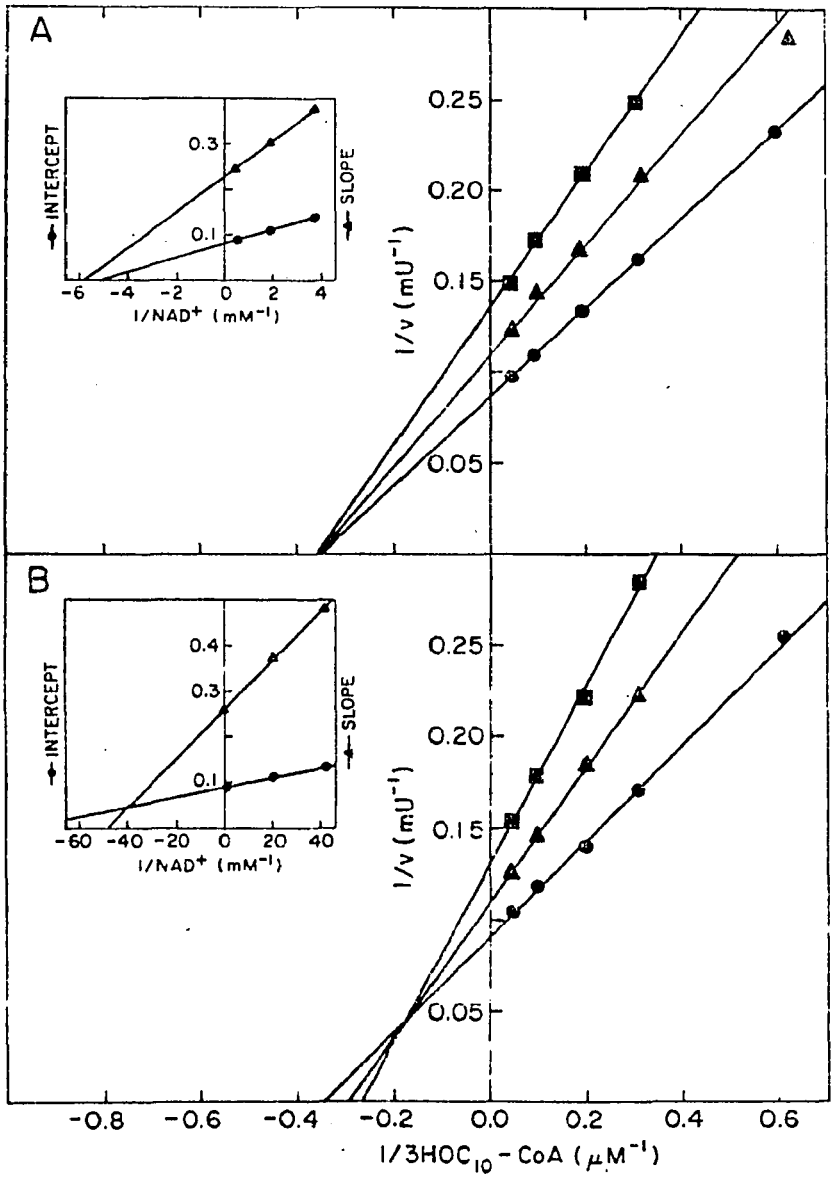

A
B
INTERCEPT
SLOPE
0.3
0.1
0.4
-6 -4 -2 0 2 4
-60 -40 -20 0 20 40
1/NAD+ (mM-1)
1/v (mU-1)
0.25
0.20
0.15
0.05
-0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6
1/3HOC10 - CoA (μM-1)

Figure 11

L-3-hydroxydecanoyl-CoA as a substrate. The concentration of 2-decenoyl-CoA and the enzyme activities used in this system are identical with those used in a corresponding kinetic study of the fatty acid oxidation complex from E. coli. When the experimental results are compared with the data calculated according to Storer and Cornish-Bowden by use of the kinetic parameters given in Table III, a near perfect agreement of data points was observed. This result shows that the theory of Storer and Cornish-Bowden is valid for predicting the kinetics of coupled reactions catalyzed by separate enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase.

The time course of NADH formation showed a significant lag (see Fig. 12A) characteristic of coupled enzyme system in which the amount of the second enzyme is suboptimal. The overall rate of coupled reactions catalyzed by two independent enzymes is a function of intermediate concentration in the bulk medium. Since these two individual enzymes function independently, the rate of NADH formation is expected to be initially zero. This prediction is shown to be correct by stopped flow measurements (see Fig. 13A).

Kinetics of the Overall Reaction Catalyzed by the Fatty Acid Oxidation Complex - The progress curve for the accumulation of intermediate and for the formation of product were computed by use of the kinetic parameters given in Table III and by assuming that there does not exist any functional linkage between the two component enzymes of the fatty acid oxidation complex. The dashed lines marked NADH (calc) and $3HOC_{10}$-CoA (calc) in Fig. 12B reflect the calculated formation of product and intermediate, respectively. The results resemble the kinetics of the coupled reactions catalyzed by the separate enzymes in that there is

Fig. 12. Progress curves for substrate consumption and product formation during the conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by bovine liver enoyl-CoA hydratase plus pig heart 3-hydroxyacyl-CoA dehydrogenase (Panel A) and catalyzed by the fatty acid oxidation complex from E. coli (Panel B). The concentrations of 2-decenoyl-CoA and NAD^+ were 4.7 μM and 1.8 mM, respectively. For experimental details, see under Experimental Procedures. The solid line labeled $-\Delta^2C_{10}$-CoA (obs) reflects the disappearance of 2-decenoyl-CoA and the production of equimolar amounts of L-3-hydroxydecanoyl-CoA. The conversion of L-3-hydroxydecanoyl-CoA to 3-ketodecanoyl-CoA and the equimolar formation of NADH is reflected by the solid line labeled NADH (obs). The difference between these two solid lines reflects the accumulation of the intermediate 3-hydroxydecanoyl-CoA which is labeled $3HOC_{10}$-CoA (obs). Assuming that enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase function independently, the progress curves for the accumulation of the intermediate were calculated according to Storer and Cornish-Bowden (66) by use of the kinetic parameters given in Table III. Calculated values for the accumulation of L-3-hydroxydecanoyl-CoA, $3HOC_{10}$-CoA (calc), are indicated by open circles. The difference between the consumption of 2-decenoyl-CoA and the calculated accumulation of L-3-hydroxydecanoyl-CoA reflects the predicted progress curve for the formation of NADH which is indicated by solid circles and labeled NADH (calc) in Panel B.

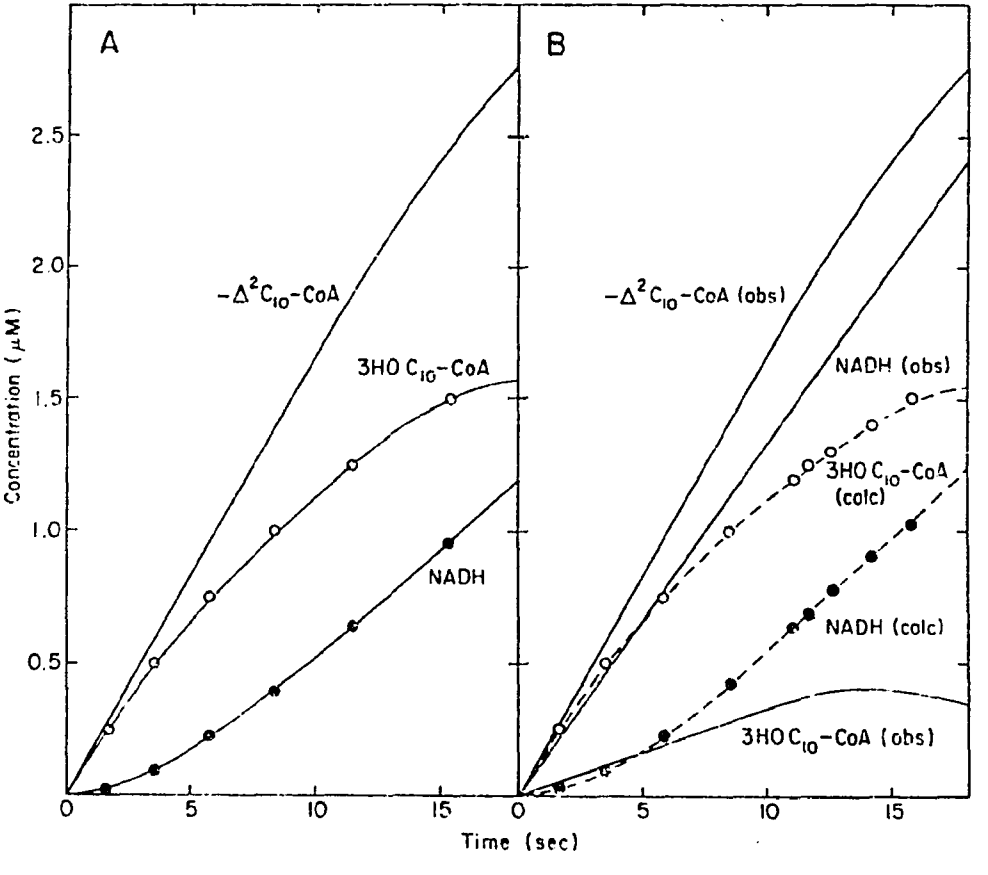
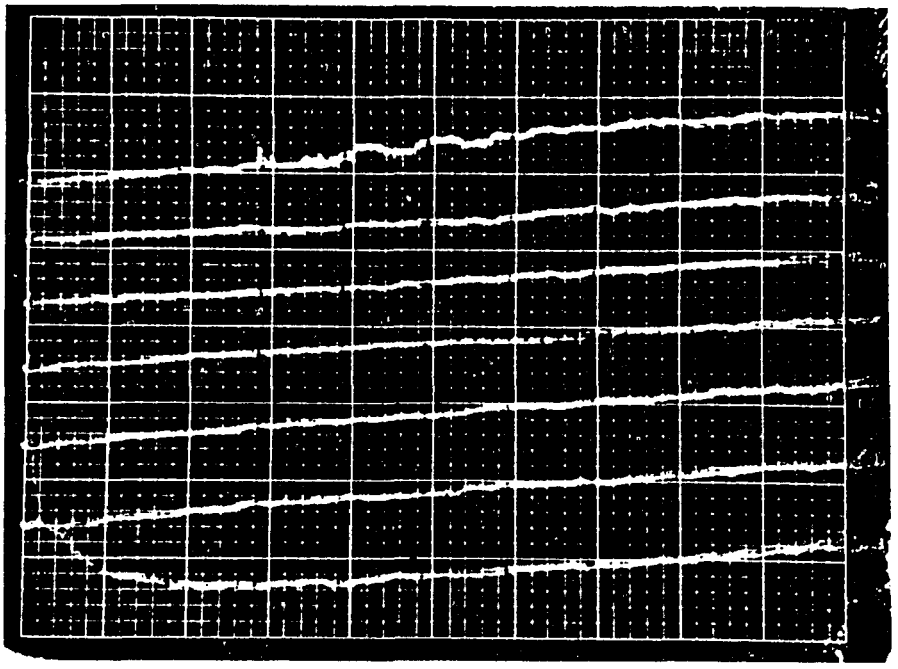

A
B
2.5
2.0
1.5
1.0
0.5
Concentration (μM)
$-\Delta^2 C_{10}$-CoA
3HO C_{10}-CoA
NADH
$-\Delta^2 C_{10}$-CoA (obs)
NADH (obs)
3HO C_{10}-CoA (calc)
NADH (calc)
3HO C_{10}-CoA (obs)
0
5
10
15
Time (sec)

Figure 12

Fig. 13. Stopped flow measurements of NADH formation during the conversion of 2-decenoyl-CoA to 3-ketodecanoyl-CoA catalyzed by enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. The vertical axis is 10 mV/large division; initial signal, 1.6 V. A. Photograph of the NADH formation in the coupled reaction catalyzed by bovine liver enoyl-CoA hydratase and pig heart 3-hydroxyacyl-CoA dehydrogenase. The horizontal axis is 0.5 s/large division. B. Photograph of the progress curve of NADH formation in the overall reaction catalyzed by the fatty acid oxidation complex from E. coli. The horizontal axis is 1 s/large division. Experimental conditions were the same as in Fig. 12.

A



B

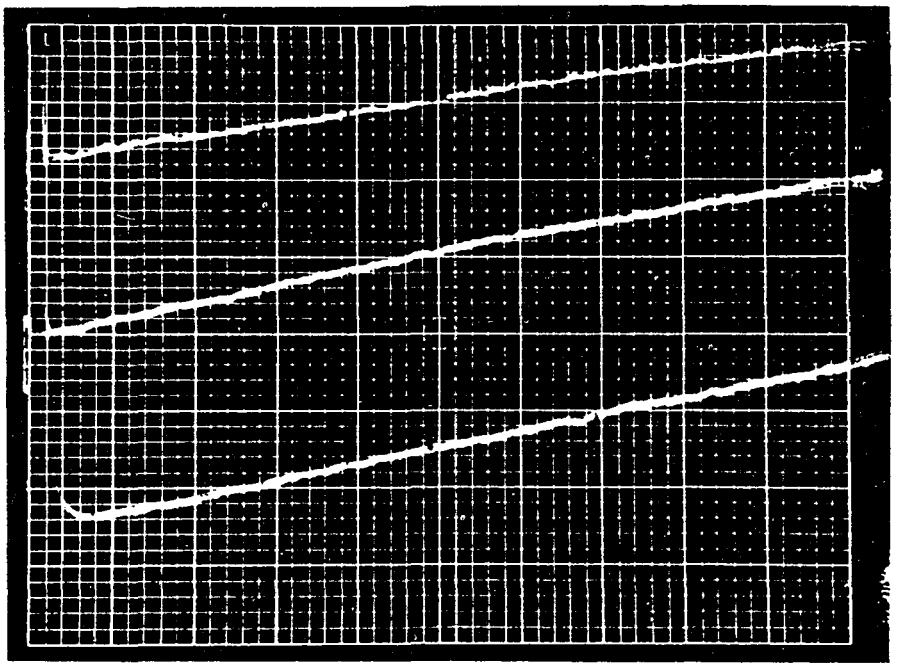


Figure 13

a distinct lag in the formation of NADH. The time required for the rate of the overall reaction to reach 50.9% (3.01 mU) of that of the enoyl-CoA hydratase reaction would be 8.5 s if the rate of the hydratase reaction remains constant (Fig. 12B). However, the calculated progress curves are different from the experimentally obtained progress curves shown in Fig. 12B as solid lines marked NADH (obs) and $3HOC_{10}$-CoA (obs), respectively. Thus, the assumption that no functional linkage exists between enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase of the fatty acid oxidation complex is incorrect.

The rate of the overall reaction (4.87 mU) catalyzed by the fatty acid oxidation complex is remarkably high and does not show any lag (see Fig. 13B). Since the sum of the concentrations of substrate, intermediate and product is constant, the accumulation of intermediate, $3HOC_{10}$-CoA, is extremely low (see Fig. 12B). The discrepancy between the high rate of 3-hydroxyacyl-CoA dehydrogenase and the low concentration of L-3-hydroxydecanoyl-CoA cannot be explained without assuming the occurrence of a direct transfer of the intermediate from the active site of enoyl-CoA hydratase to that of 3-hydroxyacyl-CoA dehydrogenase on the fatty acid oxidation complex. Thus, the existence of a channeling mechanism between these two β-oxidation enzymes appears likely in view of the conclusion that most of product of enoyl-CoA hydratase did not equilibrate with the bulk solvent prior to its dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase.

Estimation of the Degree of Channeling - The operational channeling can be calculated from the amount of substrate yielding the final product relative to the total consumption of substrate, or from the ratio

of the initial rate of the overall reaction to that of the enoyl-CoA hydratase reaction.

Although the rate of the hydratase reaction was not affected by the concentration of NAD^+ used in these measurements, the rate of the overall reaction changed significantly as a function of the NAD^+ concentration (see Fig. 14A). As a result, the apparent degree of channeling varied with the concentration of NAD^+. Since the dehydrogenation of L-3-hydroxydecanoyl-CoA can only be catalyzed by those active sites of 3-hydroxyacyl-CoA dehydrogenase to which NAD^+ is bound, the apparent degree of channeling should be corrected by the percentage of 3-hydroxyacyl-CoA dehydrogenase active sites saturated with NAD^+. If this is done, the degree of channeling is approximately 90% at any tested concentration of NAD^+. It is concluded that more than 90% of L-3-hydroxydecanoyl-CoA formed at the active site of enoyl-CoA hydratase is directly transferred to a linked active site of 3-hydroxyacyl-CoA dehydrogenase.

Inactivation of the Fatty Acid Oxidation Complex by Acetic Anhydride and Protection Against this Inactivation - Incubation of the fatty acid oxidation complex with acetic anhydride at 0°C resulted in the rapid inactivation of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase as shown in Fig. 15. Only enoyl-CoA hydratase was significantly protected against this inactivation in the presence of 1 mM crotonyl-CoA (54). However, neither enoyl-CoA hydratase nor 3-hydroxyacyl-CoA dehydrogenase was prevented from this inactivation by 1 mM NADH. It is important to note that no protection of 3-hydroxyacyl-CoA dehydrogenase was observed in the presence of both 2 mM crotonyl-CoA and 2 mM NADH (see Fig. 15). These observations suggest that the active sites of

Fig. 14. Effect of the NAD^+ concentration on the rates of the individual reactions and on the degree of channeling. Reactions were performed under conditions identical to those given in Fig. 12 except that the concentration of NAD^+ was varied. A. Rates of enoyl-CoA hydratase (●) and of the overall reaction (O) were determined as a function of the NAD^+ concentration. B. Comparison of the apparent degree of channeling (O) with the percentage of 3-hydroxyacyl-CoA dehydrogenase active sites saturated with NAD^+ (●).

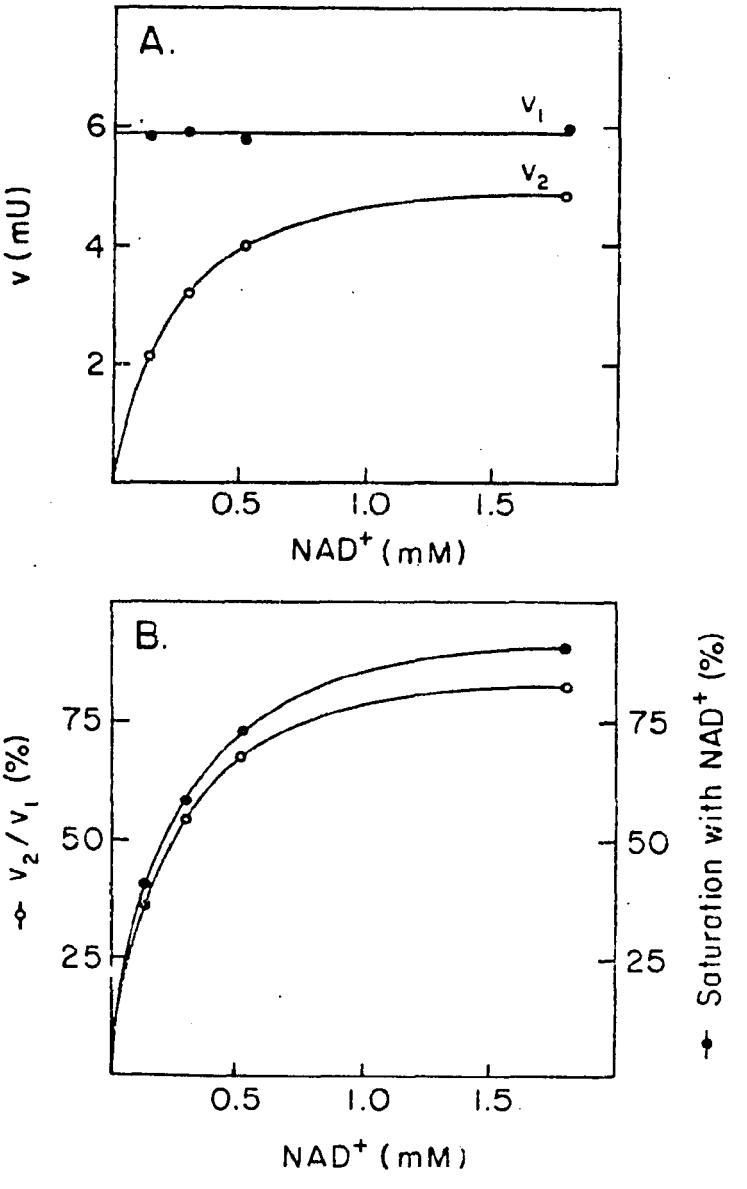

A.
V_1
V_2
6
4
2
v (mU)
0.5
1.0
1.5
NAD^+ (mM)
B.
75
50
25
V_2/V_1 (%)
Saturation with NAD^+ (%)
0.5
1.0
1.5
NAD^+ (mM)

Figure 14

Fig. 15. Effect of acetic anhydride on the enzyme activities of the fatty acid oxidation complex in the absence or presence of crotonyl-CoA and/or NADH. The fatty acid oxidation complex (38 µg/ml) was incubated with 5 mM acetic anhydride at 0°C in 0.2 M potassium phosphate (pH 8.0). Enzyme activities were determined as a function of time. A. Enoyl-CoA hydratase activity in the presence of 1 mM crotonyl-CoA (Δ), in the absence of crotonyl-CoA (O), and in the presence of 1 mM NADH (X). B. 3-Hydroxyacyl-CoA dehydrogenase activity in the presence of 1 mM NADH (X), in the absence of NADH (O), and in the presence of 2 mM crotonyl-CoA and 2 mM NADH (Δ).

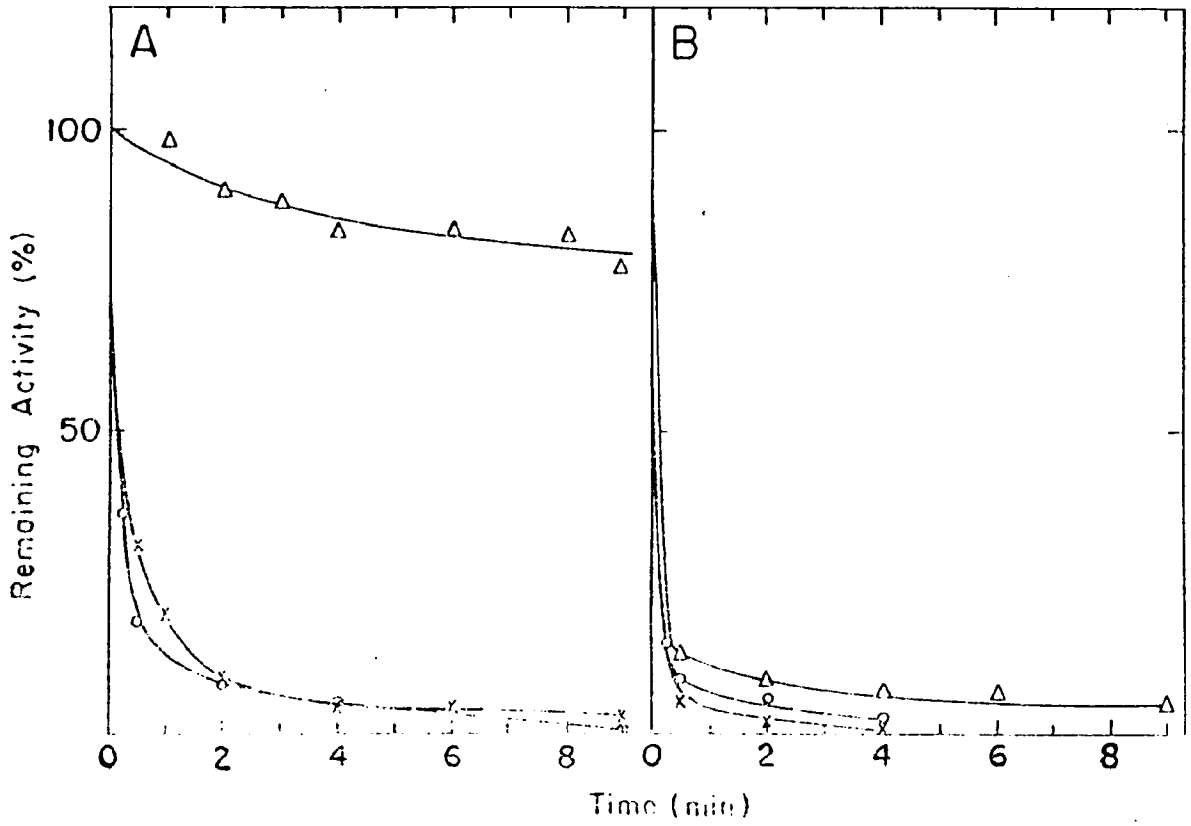


Figure 15

enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase have different essential groups that undergo reaction with acetic anhydride.

DISCUSSION

The channeling of L-3-hydroxyacyl-CoA between the active site of enoyl-CoA hydratase and that of 3-hydroxyacyl-CoA dehydrogenase on the fatty acid oxidation complex of E. coli was studied with L-3-hydroxydecanoyl-CoA because the specific activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase are very similar when acyl-CoA derivatives with 10 carbon atoms are used as substrates (46). In addition, the accumulation of 3-ketodecanoyl-CoA could be five times less than the accumulation of L-3-hydroxydecanoyl-CoA if the three β-oxidation enzymes of the fatty acid oxidation complex operated independently. A low substrate concentration of 4.7 μM was chosen for these studies to avoid the substrate inhibition that was observed with enoyl-CoA hydratase of the fatty acid oxidation complex at high substrate concentration (see Fig. 10). The substrate inhibition of enoyl-CoA hydratase, which catalyzes an Uni Uni reaction, may be due to a half-of-sites mechanism (65).

The K_m value of pig heart 3-hydroxyacyl-CoA dehydrogenase for 3-hydroxydecanoyl-CoA was determined to be 2.9 μM and thus is very close to the reported value of 3 μM (67). However, the K_m value of bovine liver enoyl-CoA hydratase for 2-decenoyl-CoA is much lower than the 300 μM reported previously (49) and is actually similar to that of E. coli enoyl-CoA hydratase. It should be noted that the K_m values of the mammalian and E. coli 3-hydroxyacyl-CoA dehydrogenase for 3-hydroxydecanoyl-CoA are identical, whereas the K_m values of these enzymes for NAD^+ differ dramatically (Table III). Calculations were made easier by the fact that at a NAD^+ concentration of 1.8 mM the apparent and true K_m values for 3-hydroxy-

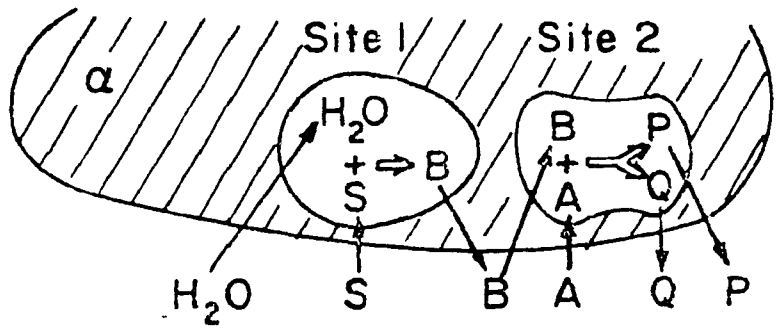
decanoyl-CoA are identical for the 3-hydroxyacyl-CoA dehydrogenase from both E. coli and pig heart (see Fig. 11). A system of two enzymes acting independently on sequential reactions was reconstituted by combining bovine liver enoyl-CoA hydratase and pig heart 3-hydroxyacyl-CoA dehydrogenase. This approach was chosen because attempts to isolate the component enzymes of the multienzyme complex had failed due to the irreversible inactivation of the fatty acid oxidation complex.

The theory of Storer and Cornish-Bowden was applied in this investigation of a possible channeling mechanism. The advantage of this theory is that it applies to coupled enzyme reactions without assuming that the second reaction follows first-order kinetics (66). Since the data obtained by calculations according to Storer and Cornish-Bowden agree perfectly with the experimental data observed for the coupled reactions catalyzed by separate enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (Fig. 12A), the validity of this theory for predicting the kinetics of coupled enzyme reactions was again confirmed. Since the β-oxidation reactions studied by us remained linear for a few seconds only, stopped flow measurements were performed to determine the time course of these reactions at less than four seconds after which time regular spectrophotometric measurements yielded optimal results.

A schematic model for the absence of intermediate channeling is shown in Fig. 16A. The diagram indicates that the intermediate must equilibrate with the bulk solvent before finding the second active site by free diffusion. The kinetics of such a system are described by the Storer and Cornish-Bowden theory. In contrast, a model of intermediate

Fig. 16. Schematic presentations of two kinetic models. A. No channeling of the intermediate is assumed to occur. B. Channeling of the intermediate is assumed to occur. Site 1 and site 2 represent the active sites of enoyl-CoA hydratase and of 3-hydroxyacyl-CoA dehydrogenase on the α-subunit of the fatty acid oxidation complex, respectively. The solid arrow (⟶) depicts the movement of compounds, whereas the open arrow (⟹) indicates the direction of the reaction. Symbols: S, trans-2-decenoyl-CoA; B, L-3-hydroxydecanoyl-CoA; A, NAD^+; P, 3-ketodecanoyl-CoA; Q, NADH.

A. No Channeling of Intermediate



B. Channeling of intermediate

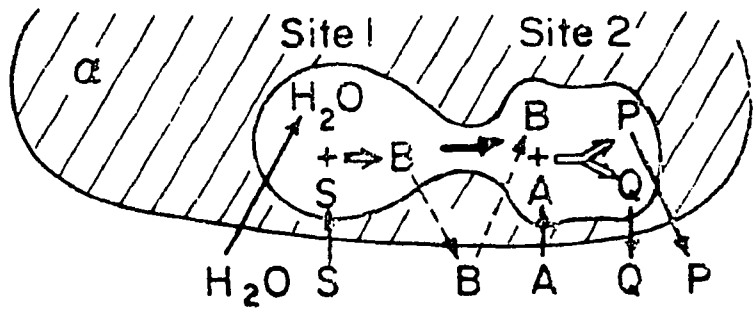


Figure 16

channeling should allow the partial or complete direct transfer of intermediate between active sites 1 and 2 (see Fig. 16B). Thus, the existence of a channeling mechanism results in unusual kinetic properties which are absolutely incompatible with the predictions made according to the theory of Storer and Cornish-Bowden. The experimental data (Fig. 12B) show the accumulation of little intermediate in the bulk medium and a high overall reaction rate from the start of the reaction. These observations suggest that channeling of intermediates occurs on the fatty acid oxidation complex.

The degree of operational channeling can be directly determined from the ratio of the initial rate of the overall reaction to that of the enoyl-CoA hydratase reaction. After a correction was made for the fraction of active site 2 saturated with NAD^+, more than 90% of intermediate molecules were found to be transferred directly from active site 1 to active site 2.

It was reported that either NADH or crotonyl-CoA can prevent the inactivation of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase by iodoacetamide. The protective effects of these two substrates are additive (35). It appears that a common group, probably an imidazole residue, is essential for the activities of both component enzymes of the fatty acid oxidation complex (35). However, the inactivation of 3-hydroxyacyl-CoA dehydrogenase by acetic anhydride was neither protected by NADH or crotonyl-CoA nor by both of them, whereas crotonyl-CoA but not NADH protected the enoyl-CoA hydratase against inactivation caused by acetic anhydride (Fig. 15). These findings suggest that the two enzymes differ with respect to some group essential for their activities (50).

The observations of this kinetic study together with results from chemical modifications suggest that the active sites of these two enzymes are in close proximity and possibly share some residues, but are not identical as depicted in Fig. 16B. This conclusion is also supported by the isolation and characterization of an E. coli mutant complex which has a defective 3-hydroxyacyl-CoA dehydrogenase but a fully active enoyl-CoA hydratase (56). A potent functional linkage between enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase on the fatty acid oxidation complex reduced the time required to reach the same rate of the overall conversion by 30 s. The catalytic efficiency is significantly enhanced due to the direct transfer of intermediate molecules without their equilibrating with the bulk medium (68,69). This situation permits a more rapid regulation of the fatty acid degradation flux and prevents the accumulation of β-oxidation intermediates in the cell.

The apparent absence of β-oxidation intermediates in mitochondria has prompted the proposal that mitochondrial enzymes of β-oxidation may exist as a multienzyme complex to which β-oxidation intermediates are tightly bound similar to intermediates bound to the mammalian fatty acid synthase. It was suggested that no intermediates whatsoever are released from such hypothetical "β-oxidation complex" (70-72). However, this hypothesis does not agree with the observations that saturated intermediates, acyl-CoA thioesters, accumulate in very low, but measurable amounts during β-oxidation in mitochondria (73,74). Therefore, a "leaky-hosepipe" model was proposed to account for the observed kinetics of β-oxidation of fatty acids in mitochondria. According to

this model the observed intermediates in the bulk phase arise by a constant "leakage" from the metabolic pathway. However, the underlying mechanism of such a model is still unclear (73).

The channeling of a β-oxidation intermediate on the E. coli fatty acid oxidation complex, to which intermediates are bound non-covalently, has been demonstrated. Such channeling mechanism can explain the kinetics of fatty acid β-oxidation in mitochondria observed by Stanley and Tubbs in 1975 (73) if the β-oxidation enzymes in mitochondria are organized in a complex. The results of the present study combined with previous observations made by Stanley and Tubbs (73) support the following hypothesis: the enzymes of β-oxidation in mitochondria exist as a labile multienzyme complex, which facilitates the perfect channeling of three β-oxidation intermediates, namely 2-enoyl-CoA, 3-hydroxyacyl-CoA and 3-ketoacyl-CoA. This complex, however, has a lower degree of channeling between the active sites of 3-ketoacyl-CoA thiolase and acyl-CoA dehydrogenase. This situation allows acyl-CoA thioesters to leak from the complex into the bulk medium. The differences between the levels of accumulation of acyl-CoA thioesters with various chain lengths may be due to the presence of three different acyl-CoA dehydrogenases and their dissimilar spatial relationships with regard to 3-ketoacyl-CoA thiolase. This proposal is also supported by the finding that some β-oxidation enzymes, namely 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, apparently bind in a specific manner to the inner mitochondrial membrane (75). It should be noted that the hypothetical fatty acid oxidation complex in mitochondria, in contrast to the E. coli complex, would be labile and would dissociate when mitochondria are disrupted.

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