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

EVIDENCE FOR THE EXISTENCE OF A FATTY ACID OXIDATION OPERON (fad AB) IN *ESCHERICHIA COLI*

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The subunit locations of the five enzymes associated with the fatty acid oxidation complex from *Escherichia 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 inhibited 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 iodo[1-14C]acetamide 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 Schulz, H. (1981) *J. Biol. Chem.* 256, 3894–3899) lead to the suggestion that enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ^2-trans-Δ^2-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase are located on the 78,000-Da subunit, whereas 3-ketoacyl-CoA thiolase is associated with the 42,000-Da subunit. Additionally, this study provides further evidence for the existence of a fatty acid oxidation (fad AB) operon that codes for the multifunctional enzyme of fatty acid oxidation and that is located at 85 min on the *E. coli* chromosome.

The synthesis of the fatty acid oxidation enzymes in *Escherichia coli* is highly induced when the bacterium is grown on long chain fatty acids as the sole carbon source (1, 2). Genetic studies have led Overath and coworkers to suggest that the genes for 3-ketoacyl-CoA thiolase (EC 2.3.1.16), 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, *cis*-Δ^2-trans-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3), and 3-hydroxyacyl-CoA epimerase (EC 5.1.3.5) are located on the 78,000-Da subunit, whereas 3-ketoacyl-CoA thiolase is associated with the 42,000-Da subunit. Additionally, this study provides further evidence for the existence of a fatty acid oxidation (fad AB) operon that codes for the multifunctional enzyme of fatty acid oxidation and that is located at 85 min on the *E. coli* chromosome.

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Fatty Acid Oxidation Complex from E. coli

RESULTS

Immunological Study—Antibodies raised against the pure multi-enzyme 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. 1 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 interaction. Shown in Fig. 2 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 0 °C was assayed for 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase, 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 (17). 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 or 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-Δ²-trans-Δ¹-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.
hydroxacyl-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 (19).

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 to 80 mM resulted in a more rapid inactivation 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

![Graphs showing the effect of iodoacetamide on enzyme activities](http://www.jbc.org/)

**Fig. 2 (left).** 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 (H) and in the absence of NADH (D). 3-Ketoacyl-CoA thiolase activity in the presence of NADH (Δ) and in the absence of NADH (□).

**Fig. 3 (center).** 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 (H) and in the absence of crotonyl-CoA (O). Enoyl-CoA hydratase activity in the presence of crotonyl-CoA (□) and in the absence of crotonyl-CoA (Δ).

**Fig. 4 (right).** 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 (H) and in the absence of acetoacetyl-CoA (O). Enoyl-CoA hydratase activity in the presence of acetoacetyl-CoA (□) and in the absence of acetoacetyl-CoA (Δ).

**Table I**

<table>
<thead>
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<th>Enzyme</th>
<th>Remaining activity (%)</th>
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<tr>
<td>3-Hydroxyacyl-CoA dehydrogenase</td>
<td>28</td>
</tr>
<tr>
<td>Enoyl-CoA hydratase</td>
<td>30</td>
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<tr>
<td>acr-Δ/-trans-Δ'-Enoyl-CoA isomerase</td>
<td>37</td>
</tr>
<tr>
<td>3-Hydroxyacyl-CoA epimerase</td>
<td>39</td>
</tr>
<tr>
<td>3-Ketoacyl-CoA thiolase</td>
<td>0</td>
</tr>
</tbody>
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*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.

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

![Graph showing the effect of iodoacetamide on enzyme activities](http://www.jbc.org/)

**Fig. 2 (left).** 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 (H) and in the absence of NADH (D). 3-Ketoacyl-CoA thiolase activity in the presence of NADH (Δ) and in the absence of NADH (□).

**Fig. 3 (center).** 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 (H) and in the absence of crotonyl-CoA (O). Enoyl-CoA hydratase activity in the presence of crotonyl-CoA (□) and in the absence of crotonyl-CoA (Δ).

**Fig. 4 (right).** 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 (H) and in the absence of acetoacetyl-CoA (O). Enoyl-CoA hydratase activity in the presence of acetoacetyl-CoA (□) and in the absence of acetoacetyl-CoA (Δ).
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 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, respectively.

LabeIing of the Fatty Acid Oxidation Complex with Iodo[1-14C]acetamide—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 iodo[1-14C]acetamide in the presence of 1 mM of NADH, while the other half was reacted with 20 mM iodo[1-14C]acetamide 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. 5. Both subunits of the complex were radioactively labeled.

| Table II |

<table>
<thead>
<tr>
<th>Enzyme</th>
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<td></td>
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<tr>
<td>3-Hydroxyacyl-CoA dehydrogenase</td>
<td>98</td>
</tr>
<tr>
<td>Enoyl-CoA hydratase</td>
<td>94</td>
</tr>
<tr>
<td>cis-Δ-trans-Δ'-Enoyl-CoA isomerase</td>
<td>96</td>
</tr>
<tr>
<td>3-Hydroxyacyl-CoA epimerase</td>
<td>105</td>
</tr>
<tr>
<td>3-Ketoacyl-CoA thiolase</td>
<td>0</td>
</tr>
</tbody>
</table>

The isolation of a fatty acid oxidation complex from E. coli that exhibits five enzymatic activities and has an αβδ structure (4–6) 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 49,000-Da β-subunit and the possible location of cis-Δ-trans-Δ'-enoyl-CoA isomerase on the 78,000-Da α-subunit (6). 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-trans-Δ'-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase.

Discussion

The inactivation of the fatty acid oxidation 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 (6) and which was protected against both modifications by acetocetyl-CoA. Since enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase were not affected by treatment with N-ethylmaleimide (6), it is likely that their inactivations by iodoacetamide were not due to the modification of a sulfhydryl residue, but involved another group, for example an imidazole residue (20). Most important were two observations: (a) the four component enzymes, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ-trans-Δ'-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 are 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 iodo[1-14C]acetamide in the absence and presence of NADH. Prelabeling with nonradioactive iodoacetamide in the presence of NADH plus crotonyl-CoA greatly reduced the nonspecific 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-Da α-subunit. The fact together with conclusions presented above lead us to suggest that enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, cis-Δ3-trans-Δ2-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-Da β-subunit has the molecular weight characteristic of thiolas (10, 21), whereas the 78,000-Da α-subunit is similar in size to the 80,000-Da peroxisomal and 75,000-Da glyoxisomal bifunctional enzymes which exhibit enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (22, 23). We have assayed the bifunctional enzyme from rat liver peroxisomes (kindly provided by Dr. Hashimoto, Shinnshu University, Japan) for cis-Δ3-trans-Δ2-enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase but found it to be devoid of both enzyme activities.2 Shown in Fig. 6 are the reactions of β-oxidation catalyzed by the fatty 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. (1, 3) have suggested that the genes for 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and possibly those for cis-Δ3-trans-Δ2-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 (1, 2) 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 (24). Since no mutants deficient in either enoyl-CoA hydratase, cis-Δ3-trans-Δ2-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 (3). 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 (1, 3) 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-Da β-subunit and the 78,000-Da α-subunit of the complex, respectively. Since 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, cis-Δ3-trans-Δ2-enoyl-CoA isomerase, and 3-hydroxyacyl-CoA epimerase are

2 A. Pramanik and H. Schulz, unpublished observation.
part of the same polypeptide, it follows that these four enzymes are specified by the \textit{fad B} gene. Consequently the \textit{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 \textit{fad B64} was found to have a defective 3-hydroxyacyl-CoA dehydrogenase whereas its gross structure was undistinguishable from the wild type complex (25). This mutation in the \textit{fad B} gene affected only one of the four catalytic functions of the \textit{fad B} gene product. We refer to this mutation as \textit{fad B1} (see Fig. 6) where \textit{B} refers to the gene of the large subunit and the subscript 1 indicates the activity of the \textit{B} gene product affected by this mutation.

REFERENCES
The large subunit of the fatty acid oxidation complex from Escherichia coli is a multifunctional polypeptide. Evidence for the existence of a fatty acid oxidation operon (fad AB) in Escherichia coli.

S Y Yang and H Schulz


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