An Alternative Pathway of Oleate \(\beta\)-Oxidation in *Escherichia coli* Involving the Hydrolysis of a Dead-end Intermediate by a Thioesterase*

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Running title: \(\beta\)-Oxidation of Oleic Acid in *E. coli*

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SUMMARY

The degradation of 2-trans,5-cis-tetradecadienoyl-CoA, a metabolite of oleic acid, by the purified complex of fatty acid oxidation from E. coli was studied to determine how much of the metabolite is converted to 3,5-cis-tetradecadienoyl-CoA and thereby diverted from the classical, isomerase-dependent pathway of oleate β-oxidation. Approximately 10% of the 2,5-intermediate was converted to the 3,5-isomer. When the latter compound was allowed to accumulate, it strongly inhibited the flux through the main pathway. Since Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase was not detected in E. coli cells grown on oleate, the 3,5-intermediate cannot be metabolized via the reductase-dependent pathway. However, it was hydrolyzed by a thioesterase, which was most active with 3,5-cis-tetradecadienoyl-CoA as substrate and which was induced by growth of E. coli on oleate. An analysis of fatty acids present in the medium after growth of E. coli on oleate revealed the presence of 3,5-tetradecadienoate, which was not detected after cells were grown on palmitate or glucose. Altogether, this data prompts the conclusion that oleate is mostly degraded via the classical, isomerase-dependent pathway in E. coli, but that a small amount of 2-trans,5-cis-tetradecadienoyl-CoA is diverted from the pathway via conversion to 3,5-cis-tetradecadienoyl-CoA by Δ^{3,Δ^{2-enoyl-CoA isomerase}. The 3,5-intermediate, which would strongly inhibit β-oxidation if allowed to accumulate, is hydrolyzed and the resultant 3,5-tetradecadienoate is excreted into the growth medium. This study provides evidence for the novel function of a thioesterase in β-oxidation.
\(\beta\)-Oxidation of oleic acid in mammalian mitochondria proceeds by two pathways. One is the classical or isomerase-dependent pathway that involves only one auxiliary enzyme, \(\Delta^3,\Delta^2\)-enoyl-CoA isomerase (enoyl-CoA isomerase)\(^1\) (EC 5.3.3.8), in addition to the enzymes required for the degradation of saturated fatty acids (for a review see reference 1). The other is the alternative or reductase-dependent pathway that requires three auxiliary enzymes, namely enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase (EC 1.3.1.34), and \(\Delta^{3,5},\Delta^{2,4}\)-dienoyl-CoA isomerase (dienoyl-CoA isomerase), to reductively remove the preexisting double bond of oleic acid \(^2,3\).

A recent study of the two pathways reached the conclusion that in rat heart mitochondria more than 80% of oleate is degraded via the classical pathway while the alternative pathway accounts for the remainder of oleate \(\beta\)-oxidation \(^4\). That study relied on the use of a mitochondrial extract that permitted the analysis of intermediates but did not maintain the supramolecular organization of enzymes as they exist in intact mitochondria. Since the organization of these enzymes may affect the flux through one pathway relative to the other, the use of an organized \(\beta\)-oxidation system uncompromised by its isolation would be advantageous. Such system is present in \textit{E. coli} where a multienzyme complex of fatty acid oxidation (FAO complex) is highly expressed when cells are grown on long-chain fatty acids as the sole carbon source \(^5\). The purified complex contains the cellular activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, and enoyl-CoA isomerase \(^6\). A study of fatty acid oxidation in \textit{E. coli} would also reveal whether the alternative pathway of oleate \(\beta\)-oxidation, which requires dienoyl-CoA isomerase, exists in prokaryotes. If yes, it may be a ubiquitous process that is operative in all organisms capable of oxidizing fatty acids. These considerations prompted the following study of oleate \(\beta\)-oxidation in \textit{E. coli}.
EXPERIMENTAL PROCEDURES

Materials - CoASH, NAD⁺, NADH, NADPH, stearoyl-CoA, palmitoyl-CoA, tetradecanoyl-CoA, dodecanoyl-CoA, decanoyl-CoA, octanoyl-CoA, butyryl-CoA, and acetyl-CoA were purchased from Life Science Resources, Milwaukee, WI. 5-cis-Tetradecenoic acid was synthesized by Cayman Chemical, Ann Arbor, MI. Oleic acid was obtained from Matreya, Inc., Pleasant Gap, PA. BCl₃-methanol (12%, w/w) was purchased from Supelco, Bellefonte, PA. Burdick & Jackson, Muskegon, MI was the source of ethyl ether while hexane was from Fisher Scientific. Sep-Pak C₁₈ cartridges used for concentrating acyl-CoAs and µBondapak C₁₈ columns (30 cm × 3.9 mm) were purchased from Waters Associates. Acyl-CoA oxidase from Arthrobacter species and most of the standard biochemicals were obtained from Sigma. Bovine liver enoyl-CoA hydratase (crotonase) (7), recombinant pig liver L-3-hydroxyacyl-CoA dehydrogenase (8), pig heart 3-ketoacyl-CoA thiolase (9), recombinant human peroxisomal enoyl-CoA isomerase (10), rat liver enoyl-CoA isomerase (11), recombinant rat liver dienoyl-CoA isomerase (12) and E. coli FAO complex (13) were purified by published procedures.

Syntheses of Substrates and Metabolites - 2-trans-Dodecenoic acid and 2-trans-tetradecenoic acid were synthesized by reacting malonic acid with n-decanal and n-dodecanal, respectively, as described in principle by Linestead et al. (14). Oleoyl-CoA, 5-cis-tetradecenoyl-CoA, 2-trans-tetradecenoyl-CoA, and 2-trans-dodecenoyl-CoA were synthesized from oleic acid, 5-cis-tetradecenoic acid, 2-trans-tetradecenoic acid, and 2-trans-dodecenoic acid, respectively, by the mixed anhydride method as described by Fong and Schulz (15). 2-trans-Tetradecenoyl-CoA was partially converted to L-3-hydroxytetradecanoyl-CoA by hydration in the presence of crotonase in 0.1 M KP₁ (pH 8.0). The resultant L-3-hydroxytetradecanoyl-CoA was purified by HPLC. 3-cis-Tetradecenoyl-CoA (16) and 3-ketohexadecanoyl-CoA (17) were synthesized as
described. 2-trans-5-cis-Tetradecadienoyl-CoA, L-3-hydroxy-5-cis-tetradecenoyl-CoA, 3-keto-5-cis-tetradecenoyl-CoA, L-3-hydroxydodecanoyl-CoA, 3-ketododecanoyl-CoA and 2-trans,4-trans-tetradecadienoyl-CoA were prepared as published (4). 3,5-cis-Tetradecadienoyl-CoA was synthesized by incubating 5 µmol of 5-cis-tetradecadienoyl-CoA in 15 ml of 0.1 M KP1 (pH 8.0) with 12 units of acyl-CoA oxidase at room temperature. The progress of the reaction was monitored by HPLC. After completion of the reaction, NAD+, CoASH, 0.2 unit of enoyl-CoA hydratase, 0.4 unit of 3-hydroxyacyl-CoA dehydrogenase, and 0.4 unit of 3-ketoacyl-CoA thiolase were added to remove traces of 2-trans-5-cis-tetradecadienoyl-CoA by converting it to 3-cis-dodecenoyl-CoA and acetyl-CoA, which were removed by HPLC. All products were purified by HPLC. The pH values of the acyl-CoA preparations were adjusted to approximately 3 to 4 and the thioester concentrations of these solutions were determined spectrophotometrically by quantification of CoASH with Ellman’s reagent (18) after quantitatively cleaving the thioester bond with NH2OH at pH 7.0 (15). The concentrations of 2-trans-5-cis-tetradecadienoyl-CoA and 3,5-cis-tetradecadienoyl-CoA were also determined by converting them enzymatically to 2-trans,4-trans-tetradecadienoyl-CoA (4), and calculating the concentration of the latter compound based on its absorbance at 300 nm by using an extinction coefficient of 28,000 M⁻¹ cm⁻¹ (19).

**Bacterial Growth Conditions -** E. coli cells (strain B) were grown on LB medium from single colonies. The initial culture was diluted 5-fold into M9 minimal medium containing 1% (w/v) trypton, 2 mM MgSO4, 10 µM CaCl2, 1 µM FeCl3 and additionally either glucose (0.2%-0.5%,w/v), oleic acid (0.1%-0.2%, v/v), or palmitic acid (0.1%) in the presence of 0.4% Triton X-100. The cultures were grown at 37 °C in a shaker-incubator to stationary phase when they were diluted 20-times into the same growth medium but without trypton. The final culture was
harvested at stationary phase by centrifugation at 3,500 x g for 30 min at 4 °C. Cell pellets were washed twice with M9 minimal medium and stored at –80 °C.

**Preparation and Fractionation of Bacterial Extracts** – Seven g of *E. coli* cell paste were suspended in 14 ml of 0.1 M KP, (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 10% glycerol, sonicated for altogether 2 min (10 sec x 12) at 0 °C and centrifuged at 100,000 x g for 1hr at 4°C. The resultant supernatant was collected for enzyme assays and protein purification. The precipitate was resuspended in 0.1 M KP, (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10% glycerol, and 1% Triton X-100 or Tween 40, or Tween 80, or β-D-glucopyranoside and incubated for 30 min on ice. The mixture was centrifuged at 100,000 x g for 1 hr at 4 °C and the supernatant was used for enzyme assays. The soluble *E. coli* extract was dialyzed overnight against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol and 1 mM benzamidine. The dialyzed supernatant was applied to a DEAE-cellulose column (13.5 x 2.5 cm) equilibrated with dialysis buffer. The column was then developed with a linear gradient made up of 500 ml each of 0.02 M Tris-HCl (pH 7.8) containing either 50 mM NaCl or 500 mM NaCl. Fractions were assayed for thioesterase activity and those with high activities were pooled and stored at –80 °C.

**Metabolic and Enzyme Assays** - Rates of degradation of 2-trans-5-cis-tetradecadienoyl-CoA via the isomerase-dependent pathway were determined by incubating various amounts of 2-trans-5-cis-tetradecadienoyl-CoA in 0.2 M KP, (pH 8.0) with 0.6 µg of *E. coli* FAO complex in the presence of bovine serum albumin (0.1 mg/ml), 1 mM NAD⁺ plus 0.3 mM CoASH and measuring the rate of NADH formation spectrophotometrically at 340 nm. An extinction coefficient of 6,220 M⁻¹ cm⁻¹ was used to calculate rates. The conversion of 2-trans-5-cis-tetradecadienoyl-CoA to 2,4-tetradecadienoyl-CoA was measured by incubating the substrate in
0.2 M KP$_1$ (pH 8.0) with 0.6 µg *E. coli* FAO complex in the presence of 0.04 unit of dienoyl-CoA isomerase. The absorbance change at 300 nm was recorded and an extinction coefficient of 28,000 M$^{-1}$cm$^{-1}$ was used to calculate rates. When the time-dependent formation of metabolites was studied, 20 µM 2-*trans*-5-*cis*-tetradecadienoyl-CoA was incubated in 0.2 M KP$_1$ (pH 8.0) with 0.6 µg *E. coli* FAO complex, 1 mM NAD$^+$, 0.3 mM CoASH, and in the presence or absence of dienoyl-CoA isomerase (0.04 unit/ml). Reactions were terminated by adjusting the pH to 1.5 with 6 N HCl. The pH was readjusted to 4.5 with 4 N KOH before the reaction mixtures were clarified by filtration through 0.22 µm pore size membranes and analyzed by HPLC.

Metabolites were quantified by the following procedure. Areas under the peaks were obtained by integration with Millenium software from Waters Corporation. Peak areas were normalized by use of extinction coefficients of 15,000, 19,650, and 28,800 M$^{-1}$cm$^{-1}$ that had been determined for acyl-CoA thioesters with a saturated $\alpha$ carbon, one double bond and two double bonds in conjugation with the thioester function, respectively at 254 nm (4). The sum of all normalized peak areas remained fairly constant throughout the experiment. Hence, the sum of all metabolites was 20 µM, the concentration of the substrate that was added to the incubation mixture. The normalized area of one peak relative to the sum of all normalized areas gives the percentage of substrate converted to the indicated metabolite. These values are plotted in Fig. 3 and are labelled “Metabolites (%).” Activities of dienoyl-CoA isomerase were determined by incubating 20 µM 3,5-tetradecadienoyl-CoA in 0.2 M KP$_1$ (pH 8.0) with various amounts of soluble cell extract or membranes solubilized with detergents (see under *Preparation and Fractionation of Bacterial Extracts*) from oleate grown or glucose grown cells. After recording the absorbance at 300 nm for 2 min, mammalian dienoyl-CoA isomerase (18 milliunits) was added to the assay to determine whether or not the substrate was still present. An extinction
coefficient of 28,000 M$^{-1}$ cm$^{-1}$ was used to calculate rates. Thioesterase was assayed by measuring the release of CoASH from acyl-CoAs with Ellman’s reagent (18). A standard assay mixture contained 0.175 M KPi (pH 8), 0.2 mM 5,5’-dithiobis (2-nitrobenzoic acid) (Ellman’s reagent), and 20 µM acyl-CoA. The progress of the reaction was determined spectrophotometrically at 412 nm and rates were calculated using an extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$.

*Isolation and Analysis of Fatty Acids Present in the Growth Medium - E.coli* cells were grown to early exponential or stationary phase in M9 medium containing oleate (0.1%) and Triton X-100 (0.4%) or were grown to stationary phase on M9 medium containing glucose (0.5%, w/v) plus Triton X-100 or palmitate (0.1%, w/v) plus Triton X-100. Cells were separated from the growth medium by centrifugation at 2,300 x g for 30 min at 4°C. The supernatant was acidified (pH 1-2) with 2 N H$_2$SO$_4$ and then extracted 4-times with 100 ml ether each. The organic phase was extracted with aqueous sodium bicarbonate. After acidifying the aqueous phase with 2 N H$_2$SO$_4$, it was extracted 3-times with 8 ml ether each. The combined ether extracts were dried over anhydrous sodium sulfate and the residual material, after removal of drying agent by filtration and ether by evaporation under a stream of N$_2$, was methylated by reacting it with 2 ml of BCl$_3$-methanol, 12% w/w, for 10 min at 60°C. After allowing the reaction mixture to cool down, 1 ml of H$_2$O and 1 ml of hexane were added. The organic layer was carefully removed and dried over anhydrous sodium sulfate. The residue after removal of sodium sulfate and evaporation of ether was dissolved in a minimal volume of anhydrous ethanol. This fraction, which contained the methyl esters of fatty acids that were present in the growth medium, was analyzed by gas chromatography in combination with mass spectrometry (GC/MS). For the purpose of identifying methyl 3,5-tetradecadienoate, a sample containing 20
nmol of 3,5-tetradecadienoyl-CoA and 20 nmol of n-pentadecanoyl-CoA (internal standard) was
hydrolyzed by reacting it with 4 N KOH at 25 °C for 1 hr. The reaction mixture was acidified
(pH 1-2) with 2 N H₂SO₄ and extracted 3-times with 8 ml of ether each. The extracted fatty
acids were converted to their methyl esters as described above. Aliquots of 1 µl of the fatty acid
methyl esters were injected at 250 °C into a GC/MS instrument (Shimadzu Scientific
Instruments) consisting of a gas chromatograph (model GC-17A) interphased with a mass
spectrometer (QP-5000) and equipped with a capillary column (30 m, ID: 0.25 mm, film
thickness: 0.25 µm; EC-5, Alltech Associates Inc., Deerfield, IL). The oven temperature was
raised from 100 °C to 230 °C at 5 °C/min, to 300 °C at 20 °C/min and then held constant for 6
min. The mass spectrometer served as a detector and was operated at 280 °C.

Purification and Analyses of Acyl-CoA Thioesters by HPLC - Acyl-CoA thioesters were purified
and metabolites were analyzed by reverse-phase HPLC on a Waters µBondapak C₁₈ column (30 cm x
3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the eluate was monitored at
254 nm. Separation of substrates and metabolites was achieved by washing the µBondapak C₁₈
column with 50 mM ammonium phosphate (pH 5.5) containing 40% of acetonitrile/water (9:1, v/v) for
20 min and then eluting acyl-CoAs by linearly increasing the organic phase from 40 to 70% in 20 min
at a flow rate of 2 ml/min. All samples were cleared of particulate matter by passing them through a
0.22-µm pore size membrane before they were injected into the HPLC system. Diluted samples were
concentrated by passing them through Sep-Pak C₁₈ cartridges and eluting them with small amounts of
methanol, which subsequently were removed by evaporation under reduced pressure.

RESULTS
Degradation of 2-trans,5-cis-Tetradecadienoyl-CoA by the E. coli Fatty Acid Oxidation Complex - 2-trans,5-cis-Tetradecadienoyl-CoA (compound III in Scheme 1)\(^2\) is an intermediate of oleate \(\beta\)-oxidation, which can be metabolized either by continuing on its path through the \(\beta\)-oxidation cycle (Scheme 1, pathway A) or by isomerization to 3,5-tetradecadienoyl-CoA (XI) (see Scheme 1). The enzymes necessary for these two divergent metabolic processes are enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, enoyl-CoA isomerase, all of which are associated with the \(E.\ coli\) fatty acid oxidation complex (FAO complex) that can be isolated and purified as an intact entity. The availability of this purified multienzyme complex provides the opportunity to study \textit{in vitro} the entry of 2-trans,5-cis-tetradecadienoyl-CoA into the classical and alternative pathways of \(\beta\)-oxidation because the key reactions of both pathways, the isomerization of the 2,5 to the 3,5 isomer (III to XI) and completion of the \(\beta\)-oxidation cycle (III to VI), are practically irreversible (4). Rates of the flux through the classical, isomerase-dependent pathway (III to X) were determined by incubating 2-trans,5-cis-tetradecadienoyl-CoA with NAD\(^+\) and CoASH in the presence of purified FAO complex (13) and measuring the formation of NADH spectrophotometrically at 340 nm. Rates of the entry into the alternative pathway were obtained by measuring at 300 nm the formation of 2,4-tetradecadienoyl-CoA, which was generated from 3,5-tetradecadienoyl-CoA (XI) by added dienoyl-CoA isomerase. Since the conversion of 2-trans,5-cis-tetradecadienoyl-CoA to 3,5-tetradecadienoyl-CoA is practically irreversible (4), the rate of this reaction is a measure of the flux through the alternate pathway. The results demonstrate that approximately 90% of 2-trans,5-cis-tetradecadienoyl-CoA was degraded via the classical pathway (Fig. 1, curve 1) while a small but significant amount (approximately 10%) of this intermediate was converted to 3,5-tetradecadienoyl-CoA as measured by conversion to 2,4-tetradecadienoyl-CoA (Fig. 1, curve 2).
and thereby diverted from the main pathway. The fraction of 2-trans,5-cis-tetradecadienoyl-CoA entering either of the two pathways was relatively constant over a wide concentration range of this compound. Hence subsequent studies were carried out at a fixed concentration of 20 µM 2-trans,5-cis-tetradecadienoyl-CoA.

The time course for the degradation of 2-trans,5-cis-tetradecadienoyl-CoA by the FAO complex in the presence of NAD⁺ and CoASH was determined by analyzing and quantifying metabolites by HPLC. Since 3,5-cis-tetradecadienoyl-CoA was coeluted with its 2,5 isomer, dienoyl-CoA isomerase was added to the incubation mixture to convert the 3,5 isomer to the 2,4 isomer, which is well separated from the starting material. Shown in Fig. 2 is the spectrum of metabolites that were formed during the first minute of the incubation period. All expected metabolites of 2-trans,5-cis-tetradecadienoyl-CoA (see Scheme 1) were detected and identified by use of authentic compounds that were synthesized by chemical and enzymatic reactions. Changes of metabolite concentrations as a function of the incubation time are shown in Fig. 3A. The rapid hydration of the starting material, 2-trans,5-cis-tetradecadienoyl-CoA (III), led to a built-up of 3-hydroxy-5-cis-tetradecenoyl-CoA (IV) because the latter compound seemed to be more slowly dehydrogenated than formed. Since the product of the dehydrogenation, 3-keto-5-cis-tetradecenoyl-CoA (V), only accumulated to a limited extent (see Fig. 2), it seems that initially the dehydrogenation step limited the flux through the pathway. The subsequent built-up of 3-dodecenoyl-CoA (VI) at 1 min followed by a more pronounced accumulation of 2-dodecenoyl-CoA (VII) at 2 min suggests that a competition between these compounds for the hydratase/isomerase active site (20) may restrict the flux through the pathway during the later part of the incubation period. The time course for the degradation of 2-trans,5-cis-tetradecadienoyl-CoA (III) by the FAO complex was quite different when dienoyl-CoA
isomerase was omitted from the incubation mixture (see Fig. 3B). Most dramatic was the much slower progress of the degradation process as illustrated by the conversion of approximately 10% of the starting material to decanoyl-CoA (X) during the first five minutes of the incubation, whereas close to 80% of the substrate was converted to decanoyl-CoA (X) during the same time period when dienoyl-CoA isomerase was present (compare Fig. 3A & 3B). All metabolites were more slowly formed and degraded in the absence of dienoyl-CoA isomerase. This observation prompts the suggestion that 3,5-tetradecadienoyl-CoA inhibits the FAO complex and hence may interfere with the efficient β-oxidation of oleic acid in intact *E. coli* cells unless this intermediate is further metabolized.

*Search for Dienoyl-CoA Isomerase in Extracts of E. coli Cells* – An extract of soluble proteins from *E. coli* cells grown on oleate as the sole carbon source was assayed for dienoyl-CoA isomerase. As shown in Table I, no activity was detected in the extract prepared from cells that express the enzymes of β-oxidation at high levels. Dienoyl-CoA isomerase activity was also not observed in an extract of membrane-bound proteins that were solubilized with Triton X-100, Tween 40, Tween 80, or β-D-glucopyranoside. As expected, cell extracts prepared from glucose-grown cells also were devoid of dienoyl-CoA isomerase activity. The lower limit of detecting the activity of this enzyme in a soluble cell extract was 0.2 milliunits per mg of protein under conditions used in this study. Since the specific activities of other β-oxidation enzymes are 2-3 orders of magnitude higher (5,6), it is unlikely that dienoyl-CoA isomerase is expressed in *E. coli* at a functional level. This conclusion raises the question as to how 3,5-tetradecadienoyl-CoA is metabolized in the absence of dienoyl-CoA isomerase. A clue to an answer was the observation that the apparent activity of mammalian dienoyl-CoA isomerase was lower than expected when it was added to the assay mixture 2 min after the addition of the *E.*
coli cell extract (see Table I). Furthermore, the observed activity of dienoyl-CoA isomerase was lower as the amount of extract was increased (see Table I) or the preincubation time was extended (data not shown). Together these observations prompted the idea that the substrate of dienoyl-CoA isomerase, 3,5-
\textit{cis}-tetradecadienoyl-CoA, was used up in another reaction catalyzed by the \textit{E. coli} extract. The most likely reaction that would cause the disappearance of 3,5-
\textit{cis}-tetradecadienoyl-CoA is its hydrolysis catalyzed by a thioesterase, which might be present in the \textit{E. coli} extract.

\textit{Identification of an E. coli Thioesterase Activity with 3,5-Tetradecadienoyl-CoA} – The following experiments were prompted by the idea that a thioesterase may be expressed in \textit{E. coli} cells for the purpose of hydrolyzing metabolites of β-oxidation when an accumulation of such intermediates would block the flux through the pathway. If such thioesterase exists, it should be highly expressed in oleate-grown \textit{E. coli} cells that have a high capacity to oxidize fatty acids but should not be expressed or poorly expressed in glucose-grown cells with a repressed β-oxidation system. The search for such thioesterase was initiated by preparing extracts from oleate-grown and glucose-grown \textit{E. coli} cells and by fractionating them on DEAE-cellulose. Fractions were assayed for thioesterase with tetradecanoyl-CoA as the substrate instead of 3,5-tetradecadienoyl-CoA because the latter compound is difficult to synthesize. This experiment revealed the presence of at least two thioesterases that correspond to peaks I and II (see Fig. 4 A&B). The same two enzymes may be present in either extract because similar elution patterns were obtained with both extracts. However, an important difference between the two extracts is the 6-times higher specific activity of thioesterase II in oleate-grown cells as compared to glucose-grown cells (compare peaks II of Fig. A & B). In contrast, the specific activities of thioesterase I were nearly the same in both extracts. Thus, growth on oleate seems to induce the expression of
thioesterase II but not that of the type I enzyme. An evaluation of substrate specificities revealed thioesterase I to be most active with saturated long-chain fatty acyl-CoAs like palmitoyl-CoA, myristoyl-CoA, and stearoyl-CoA (see Fig 5A). The enzyme exhibited significant activity with 3,5-\textit{cis}-tetradecadienoyl-CoA but was less active with other long-chain intermediates of \( \beta \)-oxidation. Thioesterase II also had a preference for long-chain fatty acyl-CoAs. Its highest activity, however, was observed with 3,5-\textit{cis}-tetradecadienoyl-CoA as substrate. Noteworthy is that thioesterase II effectively hydrolyzed a number of long-chain \( \beta \)-oxidation intermediates besides 3,5-\textit{cis}-tetradecadienoyl-CoA. Good substrates of this enzyme were \( \beta \)-oxidation intermediates that have no double bond at the \( \alpha \) carbon, like fatty acyl-CoAs, 3-enoyl-CoA, 3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. Thus, it seems that thioesterase II would be well suited to remove a block in \( \beta \)-oxidation by hydrolyzing one or several intermediates that might reduce the flux through the pathway because they would either inhibit certain reactions of \( \beta \)-oxidation, tie up free CoA, or do both.

\textit{Identification of 3,5-Tetradecadienoic Acid in the Growth Medium of E. coli Cells Grown on Oleate} – If 3,5-tetradecadienoyl-CoA is a metabolite of oleate in \textit{E. coli} and is hydrolyzed to CoASH and 3,5-tetradecadienoic acid, the latter compound is expected to exit from cells and accumulate in the growth medium. To test for the presence of 3,5-tetradecadienoic acid in the growth medium, cells were separated from the medium by centrifugation and the medium, after acidification, was extracted with ether. Neutral material present in the ether extract was removed by extraction with ether under alkaline conditions. The remaining acidic compounds were converted to methyl esters and analyzed by GC/MS. The analysis of this material by GC (see Fig. 6A) demonstrated the presence of many acidic compounds in the medium after growth of \textit{E. coli} cells on oleate. Fortunately, the region of the chromatogram between 17.5 min and 19.5
min, which is important for the identification of methyl tetradecanoate, methyl tetradecenoate and methyl tetradecadienoate, was relatively uncongested. Shown in Fig. 6B is an expanded view of the region between 18 min to 19.5 min where approximately ten peaks are visible. Mass spectra corresponding to these peaks were analyzed for ions with mass-to-charge (m/z) ratios of 238 that might be due to the molecular ion of methyl 3,5-tetradecadienoate. Spectra related to four of the peaks that are marked as 1 through 4 in Fig. 6B were similar to each other and had apparent molecular ions at m/z= 238. Hence these peaks may correspond to methyl tetradecadienoates. This view was supported by the absence of these peaks from chromatograms that were obtained with extracts of control cultures grown on either glucose or palmitate to stationary phase or grown on oleate to early exponential phase. For the purpose of identifying the materials that gave rise to the four peaks, methyl 3,5-tetradecadienoate was prepared by hydrolyzing 3,5-cis-tetradecadienoyl-CoA and converting the resultant acid to the methyl ester. The material obtained by this procedure was analyzed by GC/MS. The gas chromatogram showed six significant peaks in the region between 18 min and 19.5 min (see Fig. 6C). The mass spectra corresponding to four of the six peaks (see Fig. 6C) had apparent molecular ions at m/z=238. Moreover, the positions of these four peaks were virtually identical with the positions of the four peaks tentatively attributed to methyl 3,5-tetradecadienoate in the chromatogram of the material extracted from the medium after the growth of E. coli cells on oleate (compare panels B & C of Fig. 6). In an effort to identify the compounds that gave rise to peaks 1 through 4 in panel B of Fig. 6, their mass spectra were compared with the mass spectra of methyl 3,5-tetradecadienoates corresponding to peaks 1 through 4 in Fig. 6C. Since the mass spectrum related to the major peak (peak #3) in chromatogram B of Fig. 6 was virtually identical with the spectrum corresponding to peak 3 in Fig. C (shown in Fig. 6D is the mass spectrum of the
material corresponding to peak #3 in Fig. 6C), the compound that was isolated from the medium after growth of *E. coli* cells on oleate was most likely 3,5-tetradecadienoate. The stereochemistry of this 3,5-tetradecadienoate is not certain but it seems reasonable to assume that it may have a 5-cis double bond because the authentic methyl 3,5-tetradecadienoate was prepared from 3,5-cis-tetradecadienoyl-CoA. The spectra corresponding to peaks #1 and #4 in chromatograms B & C were very similar and only the spectra corresponding to peaks #2 of chromatograms B & C showed significant differences. Contaminations may account for some differences between mass spectra. The presence of different stereoisomers of methyl 3,5-tetradecadienoate may cause spectral variations, which additionally may reflect the existence of positional isomers. Overall, the results of these experiments suggest that growth on oleate gives rise to 3,5-tetradecadienoate in the growth medium.

**DISCUSSION**

The observation that a double bond at position 5 of an unsaturated fatty acids can be reduced by rat mitochondria in the presence of NADPH (21) led to an investigation that resulted in the characterization of an alternate pathway of β-oxidation for unsaturated fatty acids with odd-numbered double bonds (2). The degradation of oleic acid via the classical or isomerase-dependent pathway is outlined in Scheme 1A. The alternate pathway diverts from the classical pathway because of the conversion of 2-trans,5-cis-tetradecadienoyl-CoA (compound III in Scheme 1) to 3,5-cis-tetradecadienoyl-CoA (compound XI) catalyzed by enoyl-CoA isomerase. In rat, the 3,5-intermediate is converted by an auxiliary enzyme, named Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase (3), to 2-trans,4-trans-tetradecadienoyl-CoA that is reduced in an NADPH-dependent reaction catalyzed by 2,4-dienoyl-CoA reductase to 3-trans-tetradecenoyl-CoA. The latter
intermediate is converted by enoyl-CoA isomerase to 2-trans-tetradecenoyl-CoA, which is a substrate of β-oxidation and is completely degraded by this process. The reduction of the double bond by 2,4-dienoyl-CoA reductase was the reason for naming the alternate pathway reductase-dependent pathway. The existence of two pathways for the β-oxidation of unsaturated fatty acids with odd-numbered double bonds raised the question as to the flux through each of the two branches. Experiments with extracts from rat mitochondria led to the conclusion that the isomerase-dependent pathway accounted for more than 80% of the total flux (4, 22). In contrast, an earlier study suggested that the reductase-dependent pathway might be the major pathway (23). Different experimental approaches and interpretations may account for the contradictory conclusions. A concern regarding the use of mitochondrial extracts was the loss of enzyme organization upon extracting or solubilizing mitochondria, a change that may affect the flux pattern. The use of the FAO complex from E. coli alleviates this problem because the enzymes, which catalyze the reactions that determine the entry of 2-trans,5-cis-tetradecadienoyl-CoA into the two pathways, remain associated during the isolation and purification of the complex. The demonstration that only a small amount of 2-trans,5-cis-tetradecadienoyl-CoA is diverted from the isomerase-dependent pathway in E. coli agrees with the hypothesis that the classical, isomerase-dependent pathway accommodates most of the flux through β-oxidation. It has been suspected that an accumulation of fatty acid metabolites like 3,5-tetradecadienoyl-CoA might cause an inhibition of β-oxidation because the pool of free CoA would be reduced and/or enzymes of β-oxidation would be inhibited by metabolites (4). This study proves this prediction to be correct. The kinetics of the degradation of 2-trans,5-cis-tetradecadienoyl-CoA by the FAO complex reveal a severe inhibition of the classical, isomerase-dependent pathway in the absence of dienoyl-CoA isomerase. This inhibition occurs even though free CoA is
available. Consequently, the inhibition of at least one enzyme of the FAO complex is most likely responsible for the reduced flux through β-oxidation. Since this inhibition is observed when dienoyl-CoA isomerase is omitted from the incubation mixture, 3,5-tetradecadienoyl-CoA accumulates and most likely inhibits the FAO complex. It is reasonable to suggest that this compound binds to the hydratase/isomerase active site of the FAO complex (20) and thereby inhibits hydration or isomerization of enoyl-CoA intermediates.

The conclusion that dienoyl-CoA isomerase is not present in *E. coli* posed the question as to how 3,5-tetradecadienoyl-CoA is metabolized to prevent a severe inhibition of oleate β-oxidation. The surprising answer was that 3,5-tetradecadienoyl-CoA is hydrolyzed by a thioesterase. This solution of a metabolic problem is simple and the cost to the organism only is the incomplete oxidation of a small percentage of oleate that is passing through β-oxidation. The thioesterase assumed to be responsible for the hydrolysis of 3,5-tetradecadienoyl-CoA was more active with 3,5-tetradecadienoyl-CoA than with any other acyl-CoA tested as substrate and the activity of this enzyme was induced when *E. coli* was grown on oleate. Separation of an *E. coli* extract by chromatography on DEAE-cellulose yielded two thioesterase fractions that were named thioesterase I and II according to the order of their elution from the column. Barnes et al. (24) introduced this nomenclature. Thioesterase I was purified by Barnes and Wakil (25) and later shown to be a periplasmic protein (26). This enzyme should not be able to hydrolyze fatty acyl-CoAs located in the cytoplasm. Thioesterase II also was purified (27) and could be the thioesterase highly active with 3,5-tetradecadienoyl-CoA as substrate. However, it remains to be determined if the thioesterase fraction used in this study, referred to as thioesterase II, contained only thioesterase II (27) or perhaps contained more than one thioesterase. Several attempts have been made to elucidate the function of thioesterase II (26, 28, 29). So far no specific function
has been assigned to this enzyme because the growth properties of E. coli cells seem to be unaffected when thioesterase II is overexpressed or its gene (tesB) is silenced (29). The assumption underlying most of these studies was that thioesterase II might have a function in controlling or editing fatty acid synthesis that takes place with the growing acyl chain esterified to acyl carrier protein (ACP). Since thioesterase II exhibits little or no activity with fatty acyl-ACPs (28), it is unlikely to function in fatty acid synthesis, but it could be involved in fatty acid oxidation because the substrates and intermediates of the latter process are fatty acyl-CoA thioesters.

The results presented here establish that in E. coli 3,5-tetradecadienoyl-CoA is not metabolized by the reductase-dependent pathway as in mammals but is hydrolyzed so that the resultant carboxylic acid can be excreted (see Scheme 1B). This is a simple but not energy-efficient solution for disposing of this metabolite. The emergence of this metabolic shortcut does not support the idea that an alternative pathway of β-oxidation is required to accommodate an increased flux through β-oxidation. It seems that in E. coli fatty acids with odd-numbered double bonds are efficiently degraded via the classical, isomerase-dependent pathway. It is likely that in mammals too the isomerase-dependent pathway alone assures a flux of fatty acids through β-oxidation that is sufficient to meet the energy needs of the organism. If this assumption is correct, the main function of the alternative pathway is the removal of the 3,5-intermediate. A unicellular organism like E. coli can easily dispose of fatty acyl-CoAs by hydrolyzing them and excreting the resultant fatty acids from the cell. In a mammal, in contrast, fatty acids that move out of cells enter the circulation where they bind to serum albumin and are retained as long as they are hydrophobic enough and not metabolized. Removal of fatty acids that are resistant to β-oxidation from an animal would require their partial degradation and
conversion to a water-soluble product that could be excreted in the urine. Since such conversion of fatty acids is a multi-step process that takes place inside of cells, the easiest disposal of a dead-end fatty acyl-CoA may be its complete β-oxidation even if an additional enzyme like dienoyl-CoA isomerase is required to facilitate degradation by an alternative pathway.

A comparison of oleate β-oxidation in *E. coli* and mammals prompts the idea that the classical pathway accommodates a sufficient flux through β-oxidation when unsaturated fatty acids with odd-numbered double bonds serve as substrates. The formation of 3,5-*cis*-tetradecadienoyl-CoA may be an unavoidable side reaction due to the presence of enoyl-CoA isomerase, which catalyzes the conversion of 2,5-tetradecadienoyl-CoA to the more stable 3,5-isomer. Since 3,5-*cis*-tetradecadienoyl-CoA is an effective inhibitor of β-oxidation, it must be removed. This is achieved in *E. coli* by hydrolysis of 3,5-*cis*-tetradecadienoyl-CoA and excretion of 3,5-tetradecadienoate, whereas in mammals and perhaps in all multicellular organisms a pathway has evolved for the β-oxidation of 3,5-*cis*-tetradecadienoyl-CoA as the most efficient way for its disposal.

The work described herein represents the first example of a thioesterase serving an essential role in fatty acid β-oxidation coupled to oxidative phosphorylation. The enzyme hydrolyzes an intermediate that would inhibit the pathway if allowed to accumulate. Such function of thioesterases has been discussed in the past but not yet demonstrated (30). It is likely that other acyl-CoA intermediates will be identified, which are formed during fatty acid β-oxidation in mitochondria and peroxisomes and which are hydrolyzed by thioesterases to maintain a rapid flux through the pathways.
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Footnotes

*This work was supported by U.S. Public Health Service Grant HL30847 from the National Heart, Lung, and Blood Institute and by Grant RR03060 to Research Centers of Minority Institutions. J.A. was supported by the Halfway to Careers in Medicine and Research Programs of The New York Community Trust.

1 Abbreviations used are: Enoyl-CoA isomerase, Δ3,Δ2-enoyl-CoA isomerase; dienoyl-CoA isomerase, Δ3,5,Δ2,4-dienoyl-CoA isomerase; HPLC, high performance liquid chromatography; FAO complex, fatty acid oxidation complex; GC, gas chromatography; MS, mass spectrometry; ACP, acyl carrier protein.

2 Roman numerals refer to the structures of oleate metabolites shown in Scheme 1.
### Table I

*Dienoyl-CoA isomerase activity in an extract of soluble proteins from *Escherichia coli* cells grown on oleate as the sole carbon source*

| Protein per assay | Dienoyl-CoA isomerase activity |  |
|-------------------|--------------------------------|---|
|                  | Original extract<sup>a</sup> | Original extract plus 18 mU of DI<sup>b</sup> |
| µg               | milliunits                    | milliunits |
| 22               | 0                              | 2.6         |
| 44               | 0                              | 1.8         |
| 88               | 0                              | 0.6         |

<sup>a</sup>The assay mixture contained 20 µM 3,5-cis-tetradecadienoyl-CoA in 0.2 M KP<sub>i</sub> (pH 8) and the indicated amount of protein extracted from *E. coli*.

<sup>b</sup>After allowing the reaction to proceed for 2 min in the presence of the indicated amount of *E. coli* protein, 18 milliunits (mU) of rat dienoyl-CoA isomerase (DI) were added and the assay was continued.
Legends to Figures

Scheme 1. β-Oxidation of oleoyl-CoA in *E. coli*. A, classical or isomerase-dependent pathway; B, alternative pathway. Abbreviations: AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ^3,Δ^2-enoyl-CoA isomerase; TE, thioesterase.

Fig. 1. Rates of the metabolism of 2-trans,5-cis-tetradecadienoyl-CoA catalyzed by the purified FAO complex from *E. coli*. (●) Rates of 2-trans,5-cis-tetradecadienoyl-CoA degradation via the classical pathway as a function of the substrate concentration. Rates were determined by measuring the formation of NADH in the presence of NAD⁺ and CoASH. (○) Rates of the conversion of 2-trans,5-cis-tetradecadienoyl-CoA to 3,5-cis-tetradecadienoyl-CoA as a function of the substrate concentration. Rates were determined by measuring spectrophotometrically at 300 nm the formation of 2,4-tetradecadienoyl-CoA in the presence of recombinant rat dienoyl-CoA isomerase but in the absence of cofactors. All rates are means of three or four measurements ± S.D. For experimental details see “Experimental Procedures”.

Fig. 2. HPLC analysis of metabolites formed from 2-trans,5-cis-tetradecadienoyl-CoA by the purified FAO complex from *E. coli* in the presence of NAD⁺, CoASH, and dienoyl-CoA reductase. Products formed 1 min after initiating the incubation. Peaks identified by use of authentic compounds (listed in the order of decreasing elution time): Δ^{2,4}-C14:2, 2-trans,4-trans-tetradecadienoyl-CoA; Δ^{2,5}-C14:2, 2-trans,5-cis-tetradecadienoyl-CoA; Δ^2-C12:1, 2-trans-decenoyl-CoA; 3-Keto-Δ^5-C14:1, 3-keto-5-cis-tetradecenoyl-CoA; Δ^3-C12:1, 3-decenoyl-CoA; 3-HO-Δ^5-C14:1, 3-hydroxy-5-cis-tetradecenoyl-CoA; 3-Keto-C12:0, 3-ketodecanoyl-CoA; C10:0, decanoyl-CoA; 3-
HO-C12:0, 3-hydroxydodecanoyl-CoA-CoA; (+) impurity in substrate. For details see “Experimental Procedures”.

Fig. 3. **Kinetics of 2-trans,5-cis-tetradecadienoyl-CoA utilization and metabolite formation and degradation by the FAO complex from *E. coli*:** A. In the presence of dienoyl-CoA isomerase (0.04 unit/ml). B. In the absence of dienoyl-CoA isomerase. (●) 2-trans,5-cis-Tetradecadienoyl-CoA (Δ^2,5^-C_{14}-CoA); (Ø) 3-hydroxy-5-cis-tetradecenoyl-CoA (3-OH-Δ^5^-C_{14}-CoA); (♦) 2-trans,4-trans-tetradecadienoyl-CoA (Δ^2,4^-C_{14}-CoA); (▼) 3-cis-dodecenoyl-CoA; (▽) 2-trans-dodecenoyl-CoA; (■) 3-hydroxydodecanoyl-CoA; (□) decanoyl-CoA (C_{10}-CoA). All values are means of three or four determinations. Standard deviations are displayed for the major metabolites.

Fig. 4. **Separation of *E. coli* thioesterases.** Extracts of soluble proteins from *E. coli* cells grown on either oleate (panel A) or glucose (panel B) as the sole carbon source were subjected to column chromatography on DEAE-cellulose. Fractions were assayed for thioesterase with tetradecanoyl-CoA (myristoyl-CoA) as substrate.

Fig. 5. **Substrate specificities of *E. coli* thioesterase fractions.** Activities of thioesterase fractions I and II were determined at acyl-CoA concentrations of 20 µM with: C2:0, acetyl-CoA; C4:0, butyryl-CoA; C8:0, octanoyl-CoA; C14:0, tetradecanoyl-CoA (myristoyl-CoA); 2t-C14:1, 2-trans-tetradecenoyl-CoA; 2,5-C14:2, 2-trans,5-cis-tetradecadienoyl-CoA; 3t-C14:1, 3-trans-tetradecenoyl-CoA; 3,5-C14:2, 3,5-cis-tetradecadienoyl-CoA; 3OH-C14:0, 3-hydroxytetradecanoyl-CoA; C16:0, hexadecanoyl-CoA (palmitoyl-CoA); 3Keto-C16:0, 3-ketohexadecanoyl-CoA; C18:0, octadecanoyl-
CoA (stearoyl-CoA); 9c-C18:1, 9-\textit{cis}-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of two measurements that differed by 10% or less.

Fig. 6. **Identification of 3,5-tetradecadienoic acid in the medium after growth of \textit{E. coli} on oleate as the sole carbon source.** A. Gas chromatogram of the methyl esters of the acidic fraction extracted from the growth medium. B. Region of the gas chromatogram where methyl 3,5-tetradecadienoate would be eluted. Peaks marked 1 through 4 have molecular ions with mass-to-charge ratios (m/z) of 238. C. Gas chromatogram of methyl 3,5-tetradecadienoate prepared from 3,5-\textit{cis}-tetradecadienoyl-CoA. Peaks 1 through 4 are due to 3,5-tetradecadienoates or isomers with molecular ions at m/z = 238. D. Mass spectrum of the material that gave rise to peak 3 of panel C.
Two cycles of beta oxidation

Scheme 1
Fig. 1

Specific Activity (unit/mg)

2-trans,5-cis-Tetradecadienoyl-CoA (μM)
Fig. 3
Fig. 4

A. grown on oleate

B. grown on glucose

Volume (ml) vs. Thioesterase Activity (unit/ml)
Fig. 5

Graph A shows the thioesterase activity (mU/ml) for different substrates, with Thioesterase I being the primary focus. Graph B, labeled Thioesterase II, also presents activity levels for various substrates. The substrates include different carbon chain lengths and modifications, as indicated by their respective positions on the x-axis.

The y-axes in both graphs represent the thioesterase activity levels, with Graph A having a range up to 400 mU/ml and Graph B up to 700 mU/ml.
An alternative pathway of oleate β-oxidation in Escherichia coli involving the hydrolysis of a dead-end intermediate by a tioesterase
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J. Biol. Chem. published online January 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310032200

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