Metabolic Functions of the Two Pathways of Oleate β-Oxidation
Double Bond Metabolism During the β-Oxidation of Oleic Acid in Rat Heart Mitochondria

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SUMMARY

Unsaturated fatty acids with odd-numbered double bonds, e. g. oleic acid, can be degraded by β-oxidation via the isomerase-dependent pathway or the reductase-dependent pathway that differ with respect to the metabolism of the double bond. In an attempt to elucidate the metabolic functions of the two pathways and to determine their contributions to the β-oxidation of unsaturated fatty acids, the degradation of 2-trans,5-cis-tetradecadienoyl-CoA, a metabolite of oleic acid, was studied with rat heart mitochondria. Kinetic measurements of metabolite and cofactor formation demonstrated that more than 80% of oleate β-oxidation occurs via the classical isomerase-dependent pathway while the more recently discovered reductase-dependent pathway is the minor pathway. However, the reductase-dependent pathway is indispensable for the degradation of 3,5-cis-tetradecadienoyl-CoA, which is formed from 2-trans,5-cis-tetradecadienoyl-CoA by Δ3,Δ2-enoyl-CoA isomerase, the auxiliary enzyme that is essential for the operation of the major pathway of oleate β-oxidation. The degradation of 3,5-cis-tetradecadienoyl-CoA is limited by the capacity of 2,4-dienoyl-CoA reductase to reduce 2-trans,4-trans-tetradecadienoyl-CoA, which is rapidly formed from its 3,5 isomer by Δ3,5,Δ2,4-dienoyl-CoA isomerase. It is concluded that both pathways are essential for the degradation of unsaturated fatty acids with odd-numbered double bonds inasmuch as the isomerase-dependent pathway facilitates the major flux through β-oxidation and the reductase-dependent pathway prevents the accumulation of an otherwise undegradable metabolite.
The degradation of unsaturated and polyunsaturated fatty acids by β-oxidation requires the involvement of auxiliary enzymes that act on preexisting double bonds. Even-numbered double bonds are reductively removed via two reactions that are catalyzed by the auxiliary enzymes 2,4-dienoyl-CoA reductase (EC 1.3.1.34) and Δ3,Δ2-enoyl-CoA isomerase (enoyl-CoA isomerase)1 (EC 5.3.3.8) (for a review see Reference 1). Odd-numbered double bonds like the 9-cis double bond, which is present in oleic acid and in many other dietary fatty acids, are either isomerized or reduced during β-oxidation (for a review see Ref. 2). As summarized in Scheme 1, oleoyl-CoA (I)2 is chain-shortened by two cycles of β-oxidation to 5-cis-tetradecenoyl-CoA (II). Dehydrogenation of the latter compound by long-chain acyl-CoA dehydrogenase (3) produces 2-trans,5-cis-tetradecadienoyl-CoA (III), which can complete its pass through the β-oxidation cycle. The resultant 3-cis-dodecenoyl-CoA (VI), after isomerization to 2-trans-dodecenoyl-CoA (VII), can be completely degraded via the β-oxidation spiral. This pathway is referred to as the isomerase-dependent pathway because it only requires enoyl-CoA isomerase as an auxiliary enzyme. Most textbooks only mention this pathway when they discuss the β-oxidation of oleate or linoleate. However, the reductive removal of the double bond of 5-cis-enoyl-CoAs in mitochondria has been observed (4) and was explained by a four-step reaction sequence (5) that would convert 2,5-tetradecadienoyl-CoA (III) to 2-tetradecenoyl-CoA (XIV). The latter intermediate can be completely degraded via the β-oxidation spiral. The auxiliary enzymes required for this pathway are a novel enzyme, Δ3,5,Δ2,4-dienoyl-CoA isomerase (dienoyl-CoA isomerase) (6), in addition to 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase. This pathway has been referred to as reductase-dependent pathway.

Attempts to determine the relative fluxes through the reductase-dependent and isomerase-dependent pathways have not yet produced satisfactory answers for the degradation of typical long-chain dietary fatty acids. An estimate of the degradation of 5-cis-tetradecenoyl-CoA, an intermediate of oleate β-oxidation, via the reductase-dependent pathway yielded values of 86% and 65% for liver and heart
mitochondria, respectively (7). However, that study relied on the quantification of fatty acid metabolites in intact mitochondria. These metabolites are not true intermediates of β-oxidation but rather products that have leaked from the pathway, especially when functionally compromised mitochondria are involved (8). Hence, it is very doubtful that these values are meaningful estimates of the flux through the reductase-dependent pathway. In fact, when the degradation of 2-trans,5-cis-octadienoyl-CoA, a medium-chain intermediate of linolenic acid metabolism, was studied with a soluble extract of rat liver mitochondria in the presence of NAD⁺, CoASH, and NADPH, 80% of the metabolite was observed to be degraded via the isomerase-dependent pathway (9). The uncertainty about the contributions of the two pathways to the β-oxidation of long-chain dietary fatty acids prompted this study of the degradation of the oleate metabolite 2-trans,5-cis-tetradecadienoyl-CoA (III) by solubilized rat heart mitochondria.

**Experimental Procedures**

*Materials* - CoASH, NAD⁺, NADH, NADPH, dodecanoyl-CoA, decanoyl-CoA, and acetyl-CoA were purchased from Life Science Resources, Milwaukee, WI. Acyl-CoA oxidase from *Arthrobacter* species was bought from Boehringer Mannheim. Sep-Pak C₁₈ cartridges used for concentrating acyl-CoAs and µBondapak C₁₈ columns (30 cm × 3.9 mm) were purchased from Waters. Sigma was the supplier of most standard biochemicals. Bovine liver enoyl-CoA hydratase (crotonase) (10), recombinant pig liver L-3-hydroxyacyl-CoA dehydrogenase (11), pig heart 3-ketoacyl-CoA thiolase (12), recombinant human peroxisomal enoyl-CoA isomerase (13), rat liver enoyl-CoA isomerase (14), and recombinant rat liver dienoyl-CoA isomerase (15) were purified by published procedures. 2-trans-Dodecenoic acid was synthesized from n-decanal and malonic acid as described in principle by Linestead *et al.* (16). 5-cis-Tetradecenoic acid was a kind gift from Dr. Howard Sprecher, Ohio State University.
Syntheses of Substrates and Metabolites - 5-cis-Tetradecenoyl-CoA and 2-trans-dodecenoyl-CoA were synthesized from 5-cis-tetradecenoic acid and 2-trans-dodecenoic acid, respectively, by the mixed anhydride method as described by Fong and Schulz (17). Both products were purified by HPLC. For the synthesis of 2-trans-5-cis-tetradecadienoyl-CoA, a solution of 5 µmol of 5-cis-tetradecenoyl-CoA in 30 ml of 0.1 M KP₁ (pH 9.0) was saturated with air for 30 min and dehydrogenated by acyl-CoA oxidase at room temperature. The near complete conversion was achieved by the addition of 10 to 20 units of acyl-CoA oxidase in several aliquots over a period of 45 min. The progress of the conversion was monitored by HPLC. When a maximal conversion was achieved as indicated by the disappearance of 5-cis-tetradecaenoyl-CoA, the pH of the solution was adjusted to 1.5 with 6 N HCl to terminate the reaction. Precipitated protein was removed by filtering the solution through a 0.22-µm pore size membrane. After adjusting the pH to 4 with 4 N KOH, the solution was concentrated by passing it through a Sep-Pak C₁₈ cartridge and eluting it with a small volume of methanol, which subsequently was evaporated under reduced pressure. The resultant 2-trans-5-cis-tetradecadienoyl-CoA was purified by HPLC. Fractions containing 2-trans-5-cis-tetradecadienoyl-CoA were combined, concentrated as described above, and finally dissolved in deionized water. The pH of the final preparation was adjusted to 3 ~ 4 and the thioester concentration of this solution was determined spectrophotometrically by quantification of CoASH with Ellman’s reagent (18) after cleaving the thioester bond with NH₂OH at pH 7.0 (17). The concentration of 2-trans,5-cis-tetradecadienoyl-CoA was calculated by subtracting the concentration of 3,5-cis-tetradecadienoyl-CoA from that of 2-trans,5-cis-tetradecadienoyl-CoA plus 3,5-cis-tetradecadienoyl-CoA. The concentrations of 2-trans,5-cis-tetradecadienoyl-CoA plus 3,5-cis-tetradecadienoyl-CoA and of 3,5-cis-tetradecadienoyl-CoA were determined by measuring the absorbance changes at 300 nm due to their conversions in 0.1 M KP₁ (pH 8.0) to 2,4-tetradecadienoyl-CoA upon additions of 0.1 unit of dienoyl-CoA isomerase plus 0.05 unit of enoyl-CoA isomerase and of
0.1 unit of dienoyl-CoA isomerase, respectively. Concentrations of 2,4-dienoyl-CoA were calculated using an extinction coefficient of 28,000 M⁻¹ cm⁻¹ (19).

For the synthesis of 3,5-cis-tetradecadienoyl-CoA, 5 µmol of 2-trans-5-cis-tetradecadienoyl-CoA were incubated with 10 units of human peroxisomal enoyl-CoA isomerase in 30 ml of 0.1 M KP₄ (pH 8.0) at room temperature. The conversion was monitored by HPLC. After completion of the reaction, NAD⁺, CoASH, 0.2 unit of enoyl-CoA hydratase, 0.4 unit of 3-hydroxyacy-CoA dehydrogenase, and 0.4 unit of 3-ketoacyl-CoA thiolase were added to remove traces of 2-trans-5-cis-tetradecadienoyl-CoA. L-3-Hydroxy-5-cis-tetradecenoyl-CoA was prepared by incubating 2 µmol of 2-trans,5-cis-tetradecadienoyl-CoA in 10 ml of 0.1M KP₄ buffer (pH 8.0) with 9 units of enoyl-CoA hydratase at room temperature and separating the product from the substrate by HPLC. For the synthesis of 3-keto-5-cis-tetradecenoyl-CoA, 1.5 µmol of L-3-hydroxy-5-cis-tetradecenoyl-CoA in 10ml of 20 mM KP₄ buffer (pH 8.0) was incubated with 0.5 mM pyruvate and 0.5 mM NAD⁺ in the presence of 5 units of L-3-hydroxyacyl-CoA dehydrogenase and 7 units of lactate dehydrogenase at room temperature. The desired product was purified by HPLC. L-3-Hydroxydodecanoyl-CoA and 3-ketododecanoyl-CoA were synthesized from 2-trans-dodecenoyl-CoA and L-3-hydroxydodecanoyl-CoA, respectively, as described above for the corresponding longer chain acyl-CoAs. 2-trans,4-trans-Tetradecadienoyl-CoA was prepared by incubating 3,5-cis-tetradecadienoyl-CoA with dienoyl-CoA isomerase and purifying the product by HPLC.

Preparation of a Solubilized Extract from Rat Heart Mitochondria - Rat heart mitochondria were isolated as described by Chappell and Hansford (20) and stored at –70°C. The thawed rat heart mitochondria were suspended in 0.2 M KP₄ containing 0.5 mM ethylenediaminetetraacetate, 1 mM phenylmethylsulfonfyl fluoride, 1% Triton X-100, 10 mM benzamidin, and 5mM 2-mercaptoethanol
and incubated for 30 min on ice. The mixture was centrifuged at 100,000 x g at 4°C and the supernatant was used for metabolic assays.

*Metabolic Assays* - Rates of degradation of 2-trans-5-cis-tetradecadienoyl-CoA via the isomerase-dependent pathway were determined by incubating various amounts of the substrate in 0.2 M KPi (pH 8) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) in the presence of 1 mM NAD$^+$ plus 0.3 mM CoASH and measuring the rate of NADH formation spectrophotometrically at 360 nm. An extinction coefficient of 4,140 M$^{-1}$ cm$^{-1}$ was used to calculate rates. A concentration of 1 mM was chosen for NAD$^+$ because it is saturating (21,22) while 0.3 mM is the estimated concentration of free CoASH in mitochondria at state 3 respiration with palmitoyl-L-carnitine as substrate (23). The conversion of 2-trans-5-cis-tetradecadienoyl-CoA to 2,4-tetradecadienoyl-CoA was measured by incubating the substrate in 0.2 M KPi (pH 8) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) fortified with 0.1 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 or 20 µM 3,5-cis-tetradecadienoyl-CoA was incubated in 0.2 M KPi (pH 8.0) with an extract of solubilized rat heart mitochondria (0.1 mg/ml) in the presence of 1 mM NAD$^+$, 0.3 mM CoASH, and 0.5 mM NADPH. 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. Extinction coefficients of 15,000, 19,650, and 28,800 M$^{-1}$ cm$^{-1}$ were determined at 254 nm for acyl-CoA thioesters that have a saturated α carbon, one double bond and two double bonds in conjugation with the thioester function, respectively. These extinction coefficients were used to calculate concentrations of metabolites from the peak areas of HPLC chromatograms. This approach is made possible by the fact that the total concentration of all acyl-CoA metabolites, except for
newly formed acetyl-CoA, does not change during the course of the reaction and is equal to the starting concentration of the substrate, which was 20 µM. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate to product in 1 min.

Purification and Analyses of Acyl-CoA Thioesters by HPLC - Acyl-CoA substrates 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 30% of acetonitrile/water (9:1, v/v) for 20 min and then eluting acyl-CoAs by linearly increasing the organic phase from 30 to 60% 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

Kinetics of 2-trans,5-cis-Tetradecadienoyl-CoA Degradation - 2-trans,5-cis-Tetradecadienoyl-CoA (III) is an intermediate that is formed during the β-oxidation of oleate and that can be further metabolized by either the isomerase-dependent pathway or the reductase-dependent pathway (see Scheme 1). The kinetics of 2-trans,5-cis-tetradecadienoyl-CoA degradation via these two pathways were studied with rat heart mitochondria because of their minimal contamination with peroxisomes that are estimated to account for less than 3% of cardiac fatty acid β-oxidation (24). Treatment of rat heart mitochondria with 1% of Triton X-100 yielded a soluble extract that contained all β-oxidation enzymes required for the degradation of 2-trans,5-cis-tetradecadienoyl-
CoA (III) to decanoyl-CoA (X) and dodecanoyl-CoA (XVII). Since it had previously been
determined that Triton X-100 at the applied concentration did not affect the activities of the
enzymes of the β-oxidation spiral (25,26), it was only necessary to assess how Triton X-100
affects the activities of enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and dienoyl-CoA
isomerase. Such test revealed that none of these three auxiliary enzymes was negatively affected
by 1% of Triton X-100 (data not shown). When 2-trans,5-cis-tetradecadienoyl-CoA (III) was
incubated with an extract of rat heart mitochondria in the presence of 1 mM NAD+ and 0.3 mM
CoASH but in the absence of NADPH, it was possible to determine rates of β-oxidation via the
isomerase-dependent pathway without interference from the reductase-dependent pathway by
measuring spectrophotometrically the formation of NADH at 360 nm. The entry into the
reductase-dependent pathway was determined separately by measuring at 300 nm the
accumulation of 2,4-tetradecadienoyl-CoA (XII) in the absence of any cofactor. The results of
these experiments are shown in Fig. 1. Specific activities for the isomerase-dependent pathway
are based on initial velocity measurements that were linear during the first two minutes when, on
the average, 1.5 moles of NADH were produced per mole of degraded 2-trans,5-cis-
tetradecadienoyl-CoA (III). The conversion of 2-trans,5-cis-tetradecadienoyl-CoA (III) to 2,4-
tetradecadienoyl-CoA (XII) was measured 30 sec after initiation of the reaction when rates were
linear. When the flux of 2-trans,5-cis-tetradecadienoyl-CoA (III) through the isomerase-
dependent pathways is compared with its entry into the reductase-dependent pathway it is obvious
that the former pathway is the dominant one and that the ratio of rates for the two pathways does
not vary significantly over a considerable range of substrate concentrations (see Fig. 1).
Consequently, the results that were obtained by studying the degradation of 2-trans,5-cis-
tetradecadienoyl-CoA at one concentration may reflect the situation in intact mitochondria, for which the concentrations of true intermediates are unknown.

In subsequent experiments we analyzed the time-dependent formation of metabolites that accumulate when 20 µM 2-trans,5-cis-tetradecadienoyl-CoA (III) was incubated with an extract of solubilized rat heart mitochondria in the presence of 1mM NAD⁺, 0.3 mM CoASH, and 0.5 mM NADPH. Representative HPLC chromatograms are shown in Fig. 2. Product analysis five seconds after initiating the incubation revealed the rapid hydration of 2-trans,5-cis-tetradecadienoyl-CoA (III) to 3-hydroxy-5-cis-tetradecenoyl-CoA (IV) (see Fig. 2A). Since the hydration is freely reversible, both intermediates can enter either pathway. In addition, traces of 2-dodecenoyl-CoA (VII) and 2,4-tetradecadienoyl-CoA (XII) were detected. These two metabolites are committed to proceed through the isomerase-dependent pathway and reductase-dependent pathway, respectively. After one minute of incubation all intermediates of the isomerase-dependent pathway with the exception of 3-ketododecanoyl-CoA (IX) were present at detectable levels (see Fig. 2B). Decanoyl-CoA was the final product of this metabolic sequence due to the absence of cofactors that are necessary for its further degradation by β-oxidation. Entry into the reductase-dependent pathway had also continued as indicated by the formation of more 2,4-tetradecadienoyl-CoA (XII) while dodecanoyl-CoA (XVII) remained undetectable (see Fig. 2B).

Five minutes after initiating the incubation, dodecanoyl-CoA (XVII), the end product of the reductase-dependent pathway under the prevailing experimental conditions, was present together with its precursor, 2,4-tetradecadienoyl-CoA (XII) (see Fig. 2C). 3,5-Tetradecadienoyl-CoA was difficult to detect because it was insufficiently separated from 2-trans,5-cis-tetradecadienoyl-CoA. However, it is unlikely to accumulate because of the high activity of dienoyl-CoA isomerase in the mitochondrial extract. After a total reaction time of 5 minutes, 2-trans,5-cis-tetradecadienoyl-CoA had been completely metabolized and all intermediates of the isomerase-dependent pathway had been converted
to decanoyl-CoA (X). The small amount of material marked \( \Delta^2 \text{-C}_{12} \text{-CoA} \) was identified as a non-
metabolizable side product that seems to be formed from either 2,5-tetradecadienoyl-CoA (III) or 2,4-
tetradecadienoyl-CoA (XII) in a time-dependent manner and that was eluted from the reverse-phase
column together with \( \Delta^2 \text{-C}_{12} \text{-CoA} \).

The kinetics of 2-trans,5-cis-tetradecadienoyl-CoA degradation and metabolite formation are
shown in Fig. 3. Most dramatic was the rapid hydration of 2-trans,5-cis-tetradecadienoyl-CoA to
3-hydroxy-5-cis-tetradecenoyl-CoA. This reaction preceded the slower dehydrogenation of the 3-
hydroxy intermediate and the even slower appearance of the final product, decanoyl-CoA (C\text{10} 
CoA). It was noted that the utilizations of 2-trans,5-cis-tetradecadienoyl-CoA and 3-hydroxy-5-cis-
tetradecenoyl-CoA followed a similar time course and that the concentrations of 2-dodecenoyl-
CoA and 3-hydroxydodecanoyl-CoA changed almost in parallel (see Fig. 3). Thus, the two
hydration reactions seem to be at or near equilibrium. The fact that the levels of 3-ketoacyl-CoAs
and 3-cis-dodecenoyl-CoA were low or undetectable suggests that these intermediates are rapidly
degraded. Taken together, the observed kinetics of intermediate formation and degradation point
to the dehydrogenations of 3-hydroxyacyl-CoAs as the reactions that exert the greatest control
over the flux through the isomerase-dependent pathway. The entry of 2-trans,5-cis-
tetradecadienoyl-CoA into the reductase-dependent pathway was initially quite rapid as indicated
by the formation of 2,4-tetradienoyl-CoA but declined as the concentration of 2-trans,5-cis-
tetradecadienoyl-CoA decreased due to its hydration. However, dodecanoyl-CoA, the end product
of this pathway, was formed very slowly with the result that only a fraction of its precursor, 2,4-
tetradecadienoyl-CoA, was converted to the final product during the five-minute incubation
period, which was sufficient for the complete conversion of all intermediates of the isomerase-
dependent pathway to the final product decanoyl-CoA. Thus, it seems that the NADPH-dependent reduction of 2,4-tetradecadienoyl-CoA restricts the flux through the reductase-dependent pathway.

Degradation of 3,5-Tetradecadienoyl-CoA - 3,5-cis-Tetradecadienoyl-CoA is an assumed intermediate of oleate β-oxidation that we did not detect during the characterization of metabolites formed from 2-trans,5-cis-tetradecadienoyl-CoA because it was not separated from its precursor by HPLC. In addition we asked whether 3,5-cis-tetradecadienoyl-CoA could be metabolized via the isomerase-dependent pathway in addition to being degraded by the reductase-dependent pathway. To address these issues, 3,5-cis-tetradecadienoyl-CoA was incubated with an extract of rat heart mitochondria in the presence of NAD+ and CoASH and its metabolites were analyzed by HPLC. Since the absence of NADPH prevents degradation via the reductase-dependent pathway, the flux through the isomerase-dependent pathway can be evaluated. As shown in Fig. 4A, 3,5-cis-tetradecadienoyl-CoA was rapidly converted to its 2,4-isomer, but did not enter the isomerase-dependent pathway to a significant degree. After five minutes of incubation a trace of decanoyl-CoA was detected (data not shown), which could have been formed either via the isomerase-dependent pathway as outlined in Scheme 1 or more likely by direct β-oxidation of 2,4-tetradecadienoyl-CoA. The rapid degradation of 3,5-cis-tetradecadienoyl-CoA via the reductase-dependent pathway was demonstrated by incubating it with an extract of rat heart mitochondria in the presence of all required cofactors including NAD+, CoASH, and NADPH. As shown in Fig. 4B, 3,5-cis-tetradecadienoyl-CoA was rapidly converted to its 2,4 isomer, which was slowly reduced as indicated by the delayed appearance of dodecanoyl-CoA (C12-CoA) in the absence of significant amounts of downstream metabolites. This experiment demonstrates that 3,5-cis-tetradecadienoyl-CoA is only metabolized via the reductase-dependent pathway and additionally
confirms the conclusion reached during the first part of this study that the reduction of 2,4-
tetradecadienoyl-CoA is the rate-limiting reaction in the reductase-dependent pathway.

Effects of NADH and Acetyl-CoA on the β-Oxidation of 2-trans,5-cis-Tetradecadienoyl-CoA - The
metabolic studies described above were carried out with NAD⁺, CoASH, and NADPH as cofactors
but in the absence of NADH and acetyl-CoA that are present in mitochondria. Since NADH and
acetyl-CoA may inhibit β-oxidation enzymes and thereby the flux through the pathways, we
assessed their effects on the degradation of 2-trans,5-cis-tetradecadienoyl-CoA. For this purpose
we determined the formation of decanoyl-CoA (C₁₀-CoA) and dodecanoyl-CoA (C₁₂-CoA) plus
2,4-tetradecadienoyl-CoA (Δ₂,₄-C₁₄-CoA) as a function of the incubation time to measure fluxes
through the isomerase-dependent pathway and reductase-dependent pathway, respectively. Shown
in Fig. 5 are the results that were obtained when no NADH (Fig. 5A), 0.17 mM NADH (Fig. 5B),
or 0.5 mM NADH (Fig. 5C) was included in the incubation mixture in addition to the required
cofactors NAD⁺, CoASH, and NADPH. When the product formation during the first three
minutes was evaluated, the presence of NADH at the lower level resulted in slightly lower rates of
β-oxidation but did not affect the relative flux through the reductase-dependent pathway of
approximately 10%. At the higher NADH concentration, the rate of product formation was further
reduced while the relative flux through the reductase-dependent pathway was only slightly
increased to approximately 15% of the total. Thus, NADH inhibits β-oxidation without
significantly affecting the relative contributions of the two pathways to the degradation of 2-
trans,5-cis-tetradecadienoyl-CoA. The effect of acetyl-CoA on the operation of the two pathways
was also investigated. An increasing substitution of up to 80% of CoASH in the incubation
mixture by acetyl-CoA did not affect the rate of 2-trans,5-cis-tetradecadienoyl-CoA β-oxidation
nor did it change the contributions of the two pathways to this process (data not shown).
DISCUSSION

The demonstration that unsaturated fatty acids with odd-numbered double bonds can be degraded by two pathways, the isomerase-dependent pathway and the reductase-dependent pathway (5), prompted the questions as to how much each pathway may contribute to the total flux through \( \beta \)-oxidation and what specific functions the two pathways may have? The focus of this investigation was the \( \beta \)-oxidation of unsaturated dietary fatty acids in mitochondria. Given that oleic acid is abundantly present in the human diet and only contains a 9-\( cis \) double bond, it was selected as the fatty acid best suited for this study. The oleate metabolite, 2-\( trans \),5-\( cis \)-tetradecadienoyl-CoA, served as the substrate for the required flux measurements and product determinations. Mitochondria from rat heart were used because of their minimal contamination by peroxisomes, which contain a \( \beta \)-oxidation system different from the mitochondrial one. Mitochondria were solubilized with Triton X-100 to obtain a system that, in contrast to intact mitochondria, would permit rate measurements of the individual pathways. The concentration of CoASH was fixed at 0.3 mM because this is its estimated concentration in mitochondria that rapidly oxidize fatty acids (23). The concentrations of NAD\(^+\) and NADPH were set at 1 mM and 0.5 mM, respectively, because these are saturating concentrations even though they are lower than their estimated intramitochondrial concentrations. When rates of 2-\( trans \),5-\( cis \)-tetradecadienoyl-CoA degradation via the isomerase-dependent pathway were compared with rates of its entry into the reductase-dependent pathway, the former pathway was estimated to account for more than 85% of the \( \beta \)-oxidation of this metabolite of oleic acid. Similar results were obtained when the accumulation of products was determined. Decanoyl-CoA, which is formed via the isomerase-dependent pathway, accounted for 85% of the products formed from 2-\( trans \),5-\( cis \)-tetradecadienoyl-CoA. The ratio of
substrate utilization via the two pathways varied little over a significant range of 2-trans,5-cis-tetradecadienoyl-CoA concentrations and hence may reflect the relative contributions of the two pathways to β-oxidation under a variety of conditions including conditions that exist in intact mitochondria. However, intramitochondrial conditions may change during β-oxidation, especially as NAD⁺ is converted to NADH and CoASH to acetyl-CoA. Increased concentrations of NADH and acetyl-CoA may inhibit β-oxidation and thereby affect the relative contributions of the two pathways. This idea was tested by determining the effects that NADH and acetyl-CoA have on the formation of products via the two pathways. When 15% of the total NADH was in the reduced form, the relative contributions of the two pathways were unchanged even though the total flux through β-oxidation was reduced. An increase of NADH to one-third of the total coenzyme level further reduced the rate of oxidation but only slightly increased the relative contribution of the reductase-dependent pathway from 10% to 15%. Since only 5% of the total NAD⁺ is estimated to be in the reduced state during fatty acid β-oxidation in actively respiring mitochondria (23), it is unlikely that NADH would significantly change the contribution of the reductase-dependent pathway to oleate β-oxidation. The same conclusion was reached with regard to the effect of acetyl-CoA. This product of β-oxidation neither affected the rate of the process nor the contributions of the two pathways even when it comprised 80% of the total CoA content of the system. A major reason for the limited flux through the reductase-dependent pathway is the rapid and dramatic decrease in the concentration of 2-trans,5-cis-tetradecadienoyl-CoA due to its hydration. The consequence is a greatly reduced rate of its isomerization to 3,5-cis-tetradecadienoyl-CoA, the first metabolite of the reductase-dependent pathway. Together the results of this study lead to the conclusion that the reductase-dependent pathway only makes a minor contribution to the total β-oxidation of oleate.
If the reductase-dependent pathway contributes little to the β-oxidation of oleate, what is its metabolic function? In an attempt to answer this question, we studied the degradation of 3,5-cis-tetradecadienoyl-CoA, the first metabolite of oleate with two conjugated double bonds. Although it was assumed that this oleate intermediate could be metabolized via the reductase-dependent pathway, it was uncertain if it also could be degraded by way of the isomerase-dependent pathway. The results clearly demonstrate that 3,5-cis-tetradecadienoyl-CoA is rapidly converted to 2,4-tetradecadienoyl-CoA, which is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase before being degraded by β-oxidation to dodecanoyl-CoA. Since only a trace of decanoyl-CoA was detected, 3,5-cis-tetradecadienoyl-CoA is not a substrate of the isomerase-dependent pathway nor is its product, 2,4-tetradecadienoyl-CoA, effectively degraded by direct β-oxidation. The first observation agrees with the previous conclusion that 3,5-dienoyl-CoAs cannot be metabolized via the isomerase-dependent pathway (9). This is most likely due to the unfavorable energetics of the 3,5-dienoyl-CoA to 2,5-dienoy-CoA conversion. Surprising was the observation that 2-trans,4-trans-tetradecadienoyl-CoA, in contrast to the medium-chain metabolite 2-trans,4-trans-octadienoyl-CoA (9), was not directly degraded by β-oxidation. It should be noted that 2-trans,4-trans-decadienoyl-CoA but not its 2-trans,4-cis isomer is a substrate, albeit a poor one, of direct β-oxidation (19). The most likely reason for the different reactivities of 2,4-tetradecadienoyl-CoA and 2,4-octadienoyl-CoA is the involvement of two different sets of β-oxidation enzymes. 2,4-Octadienoyl-CoA is presumably hydrated by crotonase and the resultant 3-hydroxyoctanoyl-CoA is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase because both of these enzymes are more active with short-chain and medium-chain substrates than with long-chain ones (17, 21, 22). In contrast, 2,4-tetradecadienoyl-CoA is most likely acted upon by long-chain enoyl-CoA hydratase and long-chain 3-hydroxyacyl-CoA dehydrogenase of the trifunctional β-oxidation complex.
because crotonase and 3-hydroxyacyl-CoA dehydrogenase exhibit little activity toward substrates with acyl chains having 14 carbon atoms. (22). Since the equilibrium concentration of 3-hydroxy-4-enoyl-CoA formed by hydration of 2,4-dienoyl-CoAs is extremely low (equilibrium constant for the hydration is 0.003) (27), the 3-hydroxy intermediate will only be dehydrogenated at a measurable rate if the catalytic efficiency and concentration of the relevant 3-hydroxyacyl-CoA dehydrogenase are sufficiently high. These conditions seem to be met for the matrix 3-hydroxyacyl-CoA dehydrogenase acting on 3-hydroxyoct-4-enoyl-CoA but not for long-chain 3-hydroxyacyl-CoA dehydrogenase catalyzing the dehydrogenation of 3-hydroxy-tetradec-4-enoyl-CoA. The general conclusion is that once 3,5-\textit{cis}-tetradecadienoyl-CoA has been formed, it can be effectively degraded only via the reductase-dependent pathway. In the absence of this pathway, 3,5-dienoyl-CoAs would most likely accumulate and impair the oxidative function of mitochondria due to a decline of free CoA and possibly by inhibiting some of the enzymes of β-oxidation.

The reductase-dependent pathway is, however, the major pathway for the β-oxidation of unsaturated fatty acids with conjugated double bonds. Such fatty acids, specifically conjugated linoleic acid, are constituents of the human diet because they are formed in ruminants and during the partial hydrogenation of fats. The most common conjugated linoleic acid is 9-\textit{cis},11-\textit{trans}-octadecadienoic acid. β-Oxidation of this fatty acid is expected to produce 3-\textit{cis},5-\textit{trans}-dodecadienoyl-CoA as an intermediate, which can only be degraded by way of the reductase-dependent pathway. As previously pointed out (28), β-oxidation of 9-\textit{cis},11-\textit{trans}-octadecadienoic acid also yields 2-\textit{trans},5-\textit{cis},7-\textit{trans}-tetradecadienoyl-CoA as an intermediate. This metabolite might be degraded in part via the reductase-dependent pathway that requires the participation of $\Delta^{3,5,7},\Delta^{2,4,6}$-triencyl-CoA isomerase, which is an inherent activity of dienoyl-CoA isomerase (29).
Surprising and interesting was the observed accumulation of 2,4-tetradecadienoyl-CoA during the β-oxidation of either 2-trans,5-cis-tetradecadienoyl-CoA or 3,5-cis-tetradecadienoyl-CoA. This finding prompted the idea that the reaction catalyzed by 2,4-dienoyl-CoA reductase may limit the flux through the pathway even though the entry into this pathway is already restricted by competition with the dominant isomerase-dependent pathway. A previous evaluation of a possible control exerted by 2,4-dienoyl-CoA reductase over the β-oxidation of oleic acid and docosahexaenoic acid in cardiomyocytes came to the conclusion that an increase in the activity of 2,4-dienoyl-CoA reductase in response to the treatment of rats with growth hormone did not result in higher rates of β-oxidation (30). It is possible, however, that isolated and mostly quiescent cardiomyocytes are not suitable for such study because their low energy need severely restricts fatty acid oxidation with the possible result that none of the reactions of β-oxidation is limiting the rate of the energy production.

In summary, the reductase-dependent pathway only makes a minor contribution to the β-oxidation of oleic acid, which is mostly degraded via the classical isomerase-dependent pathway. However, the reductase-dependent pathway is essential for the degradation of 3,5-cis-tetradecadienoyl-CoA, which is formed from the oleate metabolite 2-trans,5-cis-tetradecadienoyl-CoA by enoyl-CoA isomerase that functions in the isomerase-dependent pathway. The reductase-dependent pathway is also essential for the β-oxidation of conjugated linoleic acid like 9-cis,10-trans-octadecadienoic acid.
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Footnotes

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1Abbreviations used are: Enoyl-CoA isomerase, $\Delta^3,\Delta^2$-enoyl-CoA isomerase; dienoyl-CoA isomerase, $\Delta^3,\Delta^2,\Delta^5,\Delta^4$-dienoyl-CoA isomerase; HPLC, high performance liquid chromatography.

2The roman numerals refer to the structures of oleate metabolites presented in Scheme 1.
Legends to Figures

Scheme 1. β-Oxidation of oleoyl-CoA in rat mitochondria. A, isomerase-dependent pathway; B, reductase-dependent pathway. Abbreviations: AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ³,Δ²-enoyl-CoA isomerase; DI, Δ³,Δ⁵,Δ²,4-dienoyl-CoA isomerase; DR, 2,4-dienoyl-CoA reductase.

Fig. 1. Rates of β-oxidation of 2-trans,5-cis-tetradecadienoyl-CoA by solubilized rat heart mitochondria. (●) Rates of 2-trans,5-cis-tetradecadienoyl-CoA degradation via the isomerase-dependent 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 entry of 2-trans,5-cis-tetradecadienoyl-CoA into the reductase-dependent pathway as a function of the substrate concentration. Rates were determined by measuring the formation of 2,4-tetradecadienoyl-CoA in the presence of 0.1 unit of recombinant rat dienoyl-CoA isomerase but in the absence of cofactors. All rates are means of three measurements ± standard deviations. (▲) Ratio of 2-trans,5-cis-tetradecadienoyl-CoA degradation via the isomerase-dependent pathway vs. its entry into the reductase-dependent pathway. For details see “Experimental Procedures”.

Fig. 2. HPLC analysis of metabolites formed from 2-trans,5-cis-tetradecadienoyl-CoA by solubilized rat heart mitochondria in the presence of NAD⁺, CoASH, and NADPH. Products formed (A) 5 s, (B) 1 min, and (C) 5 min after initiating the incubation. Peaks identified by use of authentic compounds: Δ²,5-C₁₄-CoA, 2-trans,5-cis-tetradecadienoyl-CoA; 3OHΔ⁵-C₁₄-CoA, 3-hydroxy-5-cis-tetradecenoyl-CoA; Δ²-C₁₂-CoA, 2-trans-decenoyl-CoA; Δ²,4-C₁₄-CoA, 2-trans,4-trans-tetradecadienoyl-CoA; 3OH-C₁₂-CoA, 3-hydroxydodecanoyl-CoA; C₁₀-CoA, decanoyl-CoA; C₁₂-CoA,
dodecanoyl-CoA; (x) 3-keto-5-cis-tetradecenoyl-CoA; (*) 3-decenoyl-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. (●) 2-trans,5-cis-Tetradecadienoyl-CoA (Δ2,5-C14-CoA); (○) 3-hydroxy-5-cis-tetradecenoyl-CoA; (□) 2-trans,4-trans-tetradecadienoyl-CoA; (▼) 2-trans-dodecenoyl-CoA; (▽) 3-hydroxydodecanoyl-CoA; (■) decanoyl-CoA; (◆) dodecanoyl-CoA.

Fig. 4. Metabolism of 3,5-cis-tetradecadienoyl-CoA by solubilized rat heart mitochondria. A, in the presence of NAD⁺ and CoASH; B, in the presence of NAD⁺, CoASH, and NADPH. (●) 3,5-cis-Tetradecadienoyl-CoA (Δ3,5-C14-CoA); (○) 2-trans,4-trans-tetradecadienoyl-CoA (Δ2,4-C14-CoA); (▼) dodecanoyl-CoA (C12-CoA). For details see “Experimental Procedures”.

Fig. 5. The effect of NADH on the degradation of 2-trans,5-cis-tetradecadienoyl-CoA via the isomerase-dependent pathway and the reductase-dependent pathway. 2-trans,5-cis-Tetradecadienoyl-CoA was incubated with solubilized rat heart mitochondria in the presence of 1 mM NAD⁺, 0.3 mM CoASH, 0.5 mM NADPH, and (A) no NADH; (B) 0.17 mM NADH; (C) 0.5 mM NADH. (●) Decanoyl-CoA (C10-CoA) and (○) dodecanoyl-CoA (C12-CoA) plus 2,4-tetradecanoyl-CoA (Δ2,4-C14-CoA) were quantified to evaluate β-oxidation via the isomerase-dependent pathway and reductase-dependent pathway, respectively. For details see “Experimental Procedures”.

23
Two cycles of beta oxidation

EH \rightarrow \text{H}_2\text{O}

AD \rightarrow SCoA

EI \rightarrow SCoA

DI \rightarrow SCoA

DR \rightarrow SCoA

EH \rightarrow \text{H}_2\text{O}

HD \rightarrow NAD^+

NADH + H^+

CoASH

CoASCOC\text{H}_2

CoASH

Complete beta oxidation

Scheme 1.
Fig 3.
Fig. 5

Metabolites (µM)

C₁₀-CoA

C₁₂-CoA + Δ²⁴-C₁₂-CoA

C₁₀-CoA + Δ²⁴-C₁₀-CoA

C₁₀-CoA + Δ²⁴-C₁₂-CoA

Time (min)
Ying Ren and Horst Schulz

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