Intermediate Channeling on the Trifunctional $\beta$-Oxidation Complex from Pig Heart Mitochondria*

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EXPERIMENTAL PROCEDURES

Materials—NAD$^+$, NADH, CoASH, acetyl-CoA, myristoyl-CoA, l-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) from pig heart, and enoyl-CoA hydratase (EC 4.2.1.17) or crotonase from bovine liver were purchased from Sigma. The trifunctional $\beta$-oxidation complex and 3-ketoacyl-CoA thiolase were purified from pig heart as described by Luo et al. (12) and Staack et al. (14), respectively.

Preparation of Substrates—2-trans-Hexadecenoic acid and 2-hexadecynoic acid were prepared by general methods developed for the synthesis of 2-trans-ene-3 acids (15) and 2-ynoic acid (16), respectively. The CoASH derivatives of the two acids were synthesized by the mixed


d " in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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anhydride method as detailed by Fong and Schulz (15). 2-Hexadecynoyl-CoA, after purification by hydrophobic chromatography on octyl-Sepharose (16), was converted to 3-ketohexadecanoyl-CoA by incubating 1 mM 2-hexadecynoyl-CoA in 20 mM HEPES buffer (pH 7) with crotonase (40 units/ml) for 1 h at 25 °C as described in principle by Thorpe (17). 3-Hydroxyhexadecanoyl-CoA was prepared by incubating 1 mM 3-hexadecynoyl-CoA and 3 μg of TOC. Long-chain enoyl-CoA hydratase of TOC was assayed by either the direct or indirect method. The indirect method is based on the decrease in absorbance at 280 nm due to the hydration of 2-enoyl-CoA. A standard assay mixture contained in 0.1 M KPi (pH 7.6), 0.2 mM CoASH, 20 μM 2-trans-hexadecynoyl-CoA, and 3 μg of TOC. Long-chain enoyl-CoA hydratase of TOC was assayed by either the direct or indirect method. The direct method is based on the change of approximately 0.02 A/min at 340 nm. Long-chain 3-ketoacyl-CoA dehydrogenase of TOC was assayed by measuring the formation of NADH spectrophotometrically at 340 nm. A standard assay mixture contained 0.1 mM KP, (pH 7.6) 20 μM 2-trans-hexadecynoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (0.1 unit/ml), and TOC to give an absorbance change of approximately 0.02 A/min. The molar extinction coefficient for calculating rates is 5,100 M-1 cm-1 (19). The indirect method is based on a coupled assay, in which 3-hydroxoyacyl-CoA formed by the hydration of 2-trans-enoyl-CoA is dehydrogenated and thiolated by the combined actions of 3-ketoacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in the presence of NAD+ plus CoASH. A standard assay mixture contained 0.1 mM KP, (pH 7.6) 1 mM NAD+, 0.2 mM CoASH, 20 μM 2-trans-hexadecynoyl-CoA, pig heart 3-ketoacyl-CoA dehydrogenase (2 units/ml), and pig heart 3-ketoacyl-CoA thiolase (0.1 unit/ml), and TOC to give an absorbance change of approximately 0.02 A/min. Long-chain 3-ketoacyl-CoA dehydrogenase of TOC was assayed by measuring the formation of NADH spectrophotometrically at 340 nm. A standard assay mixture contained 0.1 mM KP, (pH 7.6) 1 mM NAD+, 0.2 mM CoASH, 20 μM 3-hydroxyhexadecanoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (0.1 unit/ml), and TOC to give an absorbance change of approximately 0.02 A/min. Long-chain 3-ketoacyl-CoA thiolase of TOC was assayed by determining the concentration of acetyl-CoA or myristoyl-CoA by HPLC. A standard assay mixture contained in 1 ml of 0.1 M KP, (pH 7.6) 1 mM NAD+, 0.2 mM CoASH, 20 μM 2-trans-hexadecynoyl-CoA, and 3 μg of TOC. Long-chain enoyl-CoA hydratase of TOC was assayed by measuring its complete reduction by NADH at pH 7 in the presence of 3-hydroxoyacyl-CoA dehydrogenase. The concentration of purified 3-hydroxoyacyl-CoA was also calculated based on its absorbance at 259 nm and an extinction coefficient of 15,400 M-1 cm-1.

Enzyme Assays and Protein Determination—The three coupled reactions (overall reaction) catalyzed by TOC were assayed either by measuring spectrophotometrically the formation of NADH or by determining the concentration of acetyl-CoA or myristoyl-CoA by HPLC. A standard assay mixture contained in 1 ml of 0.1 M KP, (pH 7.6) 1 mM NAD+, 0.2 mM CoASH, 20 μM 2-trans-hexadecynoyl-CoA, and 3 μg of TOC. Kinetic parameters (Vmax, Km) of TOC were determined with long-chain substrates by measuring the formation of NADH spectrophotometrically at 340 nm. A standard assay mixture contained 0.1 mM KP, (pH 7.6) 20 μM 2-trans-hexadecynoyl-CoA and TOC to give an absorbance change of approximately 0.02 A/min. The kinetic parameters of the individual reactions catalyzed by long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase of TOC were determined. The results, shown in Table I, indicate that the dehydrogenase catalyzes the slowest reaction in the sequence and that the apparent Km values for 2-hexadecenoyl-CoA in the first and overall reactions are similar but lower than the Km values for the substrates of the second and third reactions. The Km value of 3.9 μM for 3-hydroxyhexadecanoyl-CoA in the dehydrogenase reaction was obtained by assuming that the substrate was in equilibrium with its dehydration product, whereas the Km value of 5.5 μM is based on the assumption that the substrate was not dehydrated.

Determination of Intermediates and Comparison of Actual and Calculated Reaction Rates—The substrate, intermediates, and products of the TOC-catalyzed overall reaction were separated by HPLC and quantified by use of standard curves established for each of the compounds with HPLC-purified acyl-CoA thioesters. As is apparent from Fig. 2A, only the first intermediate, 3-hydroxyhexadecanoyl-CoA (I1) was detected besides the substrate (ΔC16Δ) and the two products myristoyl-CoA (C14) and acetyl-CoA (C2). The second intermediate, 3-ketoxyhexadecanoyl-CoA (I2), would have been detected, because it can be separated from other compounds under the conditions used in this experiment (see Fig. 2B). The quantitative recovery of acyl-CoA thioesters was demonstrated by adding pentadecanoyl-CoA to the reaction mixture and determining its concentration by HPLC (data not shown). The concentration of intermediate I1, 3-hydroxyhexadecanoyl-CoA, was determined as a function of the incubation time. As can be seen from Fig. 3A, the concentration of I1 increased during the first minute of the reaction but declined thereafter. The measured concentration of I1 and the kinetic parameters of the dehydrogenase (see Table I) were used to calculate rates of NADH formation catalyzed by long-chain 3-hydroxyacyl-CoA dehydrogenase. The calculated formation of NADH, based on the concentration of free 3-hydroxyhexadecanoyl-CoA in the reaction mixture, is significantly lower than the observed formation of NADH (see Fig. 3B). Lines 1 and 2 in Fig. 3B represent the lower and upper limits, respectively, of the theoretical NADH formation based
on \( K_m \) values of 3.9 and 5.5 \( \mu M \). These \( K_m \) values reflect the lower and upper limits of the effective substrate concentrations in the assays of long-chain 3-hydroxyacyl-CoA dehydrogenase. Since the observed rate of NADH formation was higher than either of the calculated rates, the effective concentration of I1 must be higher than the concentration of I1 in the bulk phase. Such condition could be achieved if I1 were channeled from the active site of long-chain enoyl-CoA hydratase to that of long-chain 3-hydroxyacyl-CoA dehydrogenase. The presence of I1 in the bulk phase may be due to an excess capacity of the hydratase and a leaky channeling mechanism.

In contrast to the leaky channeling of I1, 3-ketoheaxadecanoyl-CoA (I2) was not detected at any time during the course of the overall reaction under the same conditions at which free I1 was formed. Since 0.5 \( \mu M \) I2 or less could have been detected, the observed formation of acetyl-CoA was compared with the calculated formation supported by 0.5 \( \mu M \) and 0.1 \( \mu M \) I2. As shown in Fig. 4, the observed rate is much higher than the expected rate in the presence of 0.5 \( \mu M \) I2 in the bulk phase.

This discrepancy between observed and predicted rates supports the hypothesis of intermediate channeling between the active sites of the dehydratase and thiolase of TOC.

**Effect of Acetyl-CoA on the Formation of Intermediates and Products of the TOC-catalyzed Reaction Sequence**—The product inhibition of the TOC-catalyzed overall reaction by acetyl-CoA was studied with the aim of evaluating the proposed intermediate channeling. Acetyl-CoA inhibited the formation of myristoyl-CoA from 2-hexadecenoyl-CoA by 50% when the acetyl-CoA concentration was raised from zero to 1 \( \mu M \) (see Fig. 5). However, the effect of this inhibitor on the concentration of intermediates was limited. Noteworthy is the accumulation of 0.5 \( \mu M \) I2 in the presence of 1 \( \mu M \) acetyl-CoA, whereas this intermediate was not detected in the absence of acetyl-CoA. A small amount of I2 also accumulated when the rate of the thiolase-catalyzed reaction was reduced by lowering the con-

| Enzyme | Substrate | \( K_m \) \( \mu M \) | \( V_{max} \) units/mg |
|--------|-----------|----------------|------------------|
| Overall reaction | 2-Hexadecenoyl-CoA | 2.7 | 1.0 |
| Hydratase | 2-Hexadecenoyl-CoA | 2.0 | 13 |
| Dehydrogenase | 3-Hydroxyhexadecanoyl-CoA | 5.5 (3.9) | 1.1 |
| Thiolase | 3-Ketoheaxadecanoyl-CoA | 8.4 | 2.0 |

*Overall reaction, the three coupled reactions catalyzed by TOC as measured by the formation of NADH; hydratase, long-chain enoyl-CoA hydratase; dehydrogenase, long-chain L-3-hydroxyacyl-CoA dehydrogenase; thiolase, long-chain 3-ketoacyl-CoA thiolase. For experimental details, see "Experimental Procedures."*  

*Apparent \( K_m \) and \( V_{max} \) values are means of two determinations, which differed by 12% or less.*  

*The \( K_m \) value of 5.5 \( \mu M \) was obtained by assuming that the substrate was not dehydrated, whereas the \( K_m \) value of 3.9 \( \mu M \) is based on the assumption that the equilibrium of the dehydratase/hydration was reached instantaneously.*
The concentration of CoASH from 0.2 mM to 0.05 mM (data not shown). However, the concentration of I₁ changed insignificantly even though the hydration of 2-hexadecenoyl-CoA had not reached the equilibrium. In fact, the observed concentration of I₁ was less than 50% of its equilibrium concentration. Acetyl-CoA, the product of the last of the three sequential reactions, was expected to inhibit thiolase. This assumption was proven to be correct by demonstrating that acetyl-CoA inhibited the thiolytic cleavage of 3-ketohexadecanoyl-CoA to myristoyl-CoA and acetyl-CoA (see Fig. 6A). Since 3-ketoacyl-CoAs, as for example acetocetyl-CoA, are known inhibitors of 3-hydroxyacyl-CoA dehydrogenase (22), the effect of 3-ketohexadecanoyl-CoA on the reduction of NAD⁺ in the overall reaction was determined. As shown in Fig. 6B, 3-ketohexadecanoyl-CoA at low micromolar concentrations inhibited the dehydrogenation of 3-hydroxyhexadecanoyl-CoA, the slowest reaction in the reaction sequence. This inhibition could explain the decreased formation of NADH by acetyl-CoA in the overall reaction, because the latter compound inhibits thiolase with the result that I₂ accumulates, which in turn inhibits the dehydrogenase-catalyzed formation of NADH. However, if intermediate channeling occurs, the concentration of bound intermediate would most likely be higher than reflected by the concentration of free intermediate. Hence the degree of inhibition might be higher than could be accounted for by the concentration of free intermediate. This seems to be the situation when the inhibition of the overall reaction by acetyl-CoA is analyzed. Although 1 mM acetyl-CoA caused an inhibition of the overall reaction by 50% (see Fig. 6A), the accumulation of 0.5 μM I₂ only explains a 30% decrease of the dehydrogenase-catalyzed reaction (see Fig. 6B), which is limiting the overall reaction. The differences between the observed and predicted degrees of inhibition are attributed to intermediate channeling.

DISCUSSION

The recent characterization of several long-chain specific β-oxidation enzymes (10–13) has made it necessary to modify the traditional view of how the enzymes of β-oxidation cooperate to completely degrade fatty acids. As schematically shown in Fig. 7, a set of four long-chain specific enzymes, located in the inner mitochondrial membrane and consisting of very long-chain acyl-CoA dehydrogenase, long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase, are presumed to catalyze the chain shortening of long-chain fatty acyl-CoAs. After one or
several rounds of β-oxidation, the soluble matrix enzymes, consisting of short-chain, medium-chain and perhaps long-chain acyl-CoA dehydrogenases in addition to enoyl-CoA hydratase (crotonase), 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoyl-CoA thiolase, take over to complete the degradation of acyl-CoAs. This revised hypothetical view of mitochondrial β-oxidation necessitates a reevaluation of the proposed control mechanism(s) of β-oxidation, especially of the energy-linked regulation in extra-hepatic tissues, e.g. in heart (23). Changes in the energy demand of a tissue oxidizing fatty acids were thought to cause changes in the concentrations of β-oxidation intermediates which in turn may control the activity of the pathway by regulating activities of key enzymes (23). However, the question of whether and if so, to which degree intermediates accumulate during β-oxidation in vivo has not been answered unambiguously.

The evidence presented here prompts the conclusion that long-chain intermediates of mitochondrial β-oxidation are channeled between the active sites of TOC. This conclusion does not exclude the possibility of intermediates dissociating from TOC under certain conditions and exiting from mitochondria and even cells. For example, 3-hydroxyacyl-CoAs have been reported to accumulate in isolated mitochondria and to exit from them as acylcarnitines, especially when the reoxidation of NADH is impaired (4–8) or when long-chain 3-hydroxyacyl-CoA dehydrogenase is deficient (24). In contrast, the accumulation of 3-ketoacyl-CoAs has not been observed. The observed accumulation of a relatively large quantity of 3-hydroxycarboxylic-CoA (CoA) in this study may be a consequence of the specific experimental set-up with 2-hexadecanoyl-CoA and with the activity of long-chain 3-hydroxyacyl-CoA dehydrogenase is deficient (24). In contrast, the accumulation of 3-ketoacyl-CoAs has not been observed. The observed accumulation of a relatively large quantity of 3-hydroxycarboxylic-CoA (CoA) in this study may be a consequence of the specific experimental set-up with 2-hexadecanoyl-CoA and with the activity of long-chain 3-hydroxyacyl-CoA dehydrogenase is deficient (24).

This study prompts the conclusion that the channelling of long-chain β-oxidation intermediates is most likely the underlying cause for their absence from the mitochondrial matrix or for their presence at very low levels. This situation also would explain why long-chain intermediates of β-oxidation were not detected in the extracellular fluid of fibroblasts unless an enzyme defect like long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency impaired the further metabolism of long-chain intermediates (9). If experiments with isolated mitochondria and whole cells yield different results with respect to the formation of β-oxidation intermediates, the results obtained with whole cells may be more relevant to the in vivo situation because mitochondria present in whole cells, in contrast to isolated ones, are most likely undamaged. However, when extracellular or extra-mitochondrial levels of intermediates are measured, the question arises whether they truly reflect the intramitochondrial concentrations of acyl-CoA. In fact the available evidence indicates that this may not be the case (7).

In considering the metabolic consequences of the non-accumulation of β-oxidation intermediates, the availability of more free coenzyme A in the mitochondrial matrix is perhaps most important. If each intermediate of β-oxidation were present in the matrix only at a low micromolar concentration, a substantial amount of the available CoASH would be tied up by the 27 intermediates that are formed along the pathway from palmitoyl-CoA to acetyl-CoA. Moreover, if β-oxidation intermediates accumulate, they might inhibit various mitochondrial enzymes, especially those with binding sites for acyl-CoAs. In fact, 3-ketoacyl-CoA intermediates at nanomolar concentra-

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