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Study of the Energy-dependent Regulation of Fatty Acid Oxidation in Rat Heart Mitochondria

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

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ABSTRACT

STUDY OF THE ENERGY-DEPENDENT REGULATION OF FATTY ACID OXIDATION IN RAT HEART MITOCHONDRIA

BY

AZFAR SYED ABBAS

ADVISOR: PROF. HORST SCHULZ

The subcellular location of cardiac carnitine acetyltransferase (CAT) was investigated by measuring the release of CAT and of marker enzymes from isolated rat myocytes permeabilized with digitonin. Additionally, the CAT activity exposed to the cytosolic compartment was quantified. The results indicate that soluble CAT is not present in the cytosol and that only 5% of the cellular CAT activity is positioned to catalyze the formation of cytosolic acetyl coenzyme A. This situation makes it unlikely that the energy-linked regulation of cardiac fatty acid oxidation proceeds by mechanisms which require the conversion of acetylcarnitine to acetyl coenzyme A in the cytosol.

The phosphorylation of trifunctional β-oxidation complex (TOC), a mitochondrial membrane-bound enzyme, was investigated to assess the possible role of such modification in the energy-linked regulation of fatty acid oxidation in heart. Phosphorylation was assayed with partially purified TOC, rat heart mitochondrial membrane vesicles (RHMM), and intact rat heart mitochondria (RHM). Results with partially purified TOC and RHMM in the presence and absence of cAMP-dependent protein

kinase and at [acetyl-CoA]\[CoASH] ratios characteristic of high and low energy utilizations did not reveal any phosphorylation of TOC. In addition, different metabolic conditions were generated in intact RHM to determine the phosphorylation of TOC. All these observations led to the conclusion that TOC is not phosphorylated and therefore, can not be regulated by it.

Vesicles prepared from RHMM were used as a system to quickly and efficiently assess the channeling of long-chain intermediates on the membrane bound β-oxidation system. When the membrane-bound β-oxidation system present in such vesicles was assayed with hexadecanoyl-CoA as a substrate, the accumulation of all possible intermediates was observed in the incubation mixture. Rates calculated based on the observed concentration of 2-hexadecenoyl-CoA and appropriate kinetic parameters, were higher than those observed with β-oxidation system A. These observations are indicative of a non-channeling situation, therefore, vesicles are not a suitable system to study of intermediate channeling despite the fact that evidence for channeling of long-chain intermediates has been obtained in whole cell (27).

Lastly, the effect of 5,6-*cis*-double bonds present in unsaturated acyl-CoAs on the activities of acyl-CoA dehydrogenases was determined. It was observed that very long-chain acyl-CoA dehydrogenase acts poorly on these unsaturated substrates compared to their saturated ones of the

same acyl chain length. In contrast, long-chain acyl-CoA dehydrogenase (LCAD) acted equally well with both saturated and unsaturated acyl-CoAs. This study provides evidence that LCAD might be essential for β-oxidation of long-chain unsaturated fatty acids having double bond at 5 position.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Carnitine acetyltransferase
CoA	Coenzyme A
CPT I	Carnitine palmitoyltransferase I
CPT II	Carnitine palmitoyltransferase II
DCPIP	2,6-dichlorophenol-indophenol
DEAE	Diethylaminoethyl
DPM	Disintegration per minute
EDTA	Ethylenediaminetetraacetic acid
EGTA	[Ethylene-bis (oxy-ethylenenitrilo)] tetraacetic acid
HPLC	High performance liquid chromatography
KCN	Potassium thiocyanide
kDa	Kilodalton
KPi	Potassium phosphate
LCAD	Long-chain acyl-CoA dehydrogenase

MCAD	Medium-chain acyl-CoA dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide (oxid)
NADH	Nicotinamide adenine dinucleotide (red)
NaF	Sodium fluoride
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PMSF	Phenylmethylsulfonyl Fluoride
PMS	Phenazine methosulfate
RCR	Respiratory control ratio
RHM	Rat heart mitochondria
RHMM	Rat heart mitochondrial membrane
SCAD	Short-chain acyl-CoA dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOC	The trifunctional β-oxidation complex
Tris	Tris-(hydroxymethyl)-aminomethane
VLCAD	Very long-chain acyl-CoA dehydrogenase

INTRODUCTION

Overview of β-Oxidation

Fatty acids are transported between organs either as unesterified fatty acids carried by serum albumin or in the form of triacylglycerols associated with lipoproteins. The mechanism by which free fatty acids enters cells remains poorly understood. Once fatty acids have entered the cell, they are activated to their CoA thioesters in mitochondria, peroxisome, and the endoplasmic reticulum by ATP-dependent acyl-CoA synthetases (1). These enzymes are classified as short-chain, medium-chain, long-chain and very long-chain acyl-CoA synthetases, based on their specificities for fatty acids of different chain lengths. Since the inner mitochondrial membrane is impermeable to CoA and its derivatives, therefore the acyl residue of acyl-CoA thioesters are carried across the inner mitochondrial membrane by L-carnitine. Their formation is catalyzed by carnitine palmitoyltransferase I (CPT I) located at the outer mitochondrial membrane (2). The resultant acylcarnitines cross the inner mitochondrial membrane by the carnitine:acylcarnitine translocase (3). In the mitochondrial matrix, acyl residues enter the β-oxidation spiral after being converted back to acyl-CoA thioesters by an inner mitochondrial membrane enzyme named carnitine palmitoyltransferase II (CPT II). The enzymes of β-oxidation specific for

long-chain and very long-chain acyl-CoAs are located at the inner mitochondrial membrane, whereas enzymes specific for medium-chain, and short-chain acyl-CoAs are in the mitochondrial matrix. In the first of four reactions that constitute one cycle of β-oxidation, acyl-CoA is dehydrogenated to 2-*trans*-enoyl-CoA. Four acyl-CoA dehydrogenases with different, but overlapping chain length specificities catalyze this reaction and they are named short-chain, medium-chain, long-chain, and very long-chain acyl-CoA dehydrogenases to indicate their chain length preferences. In the second step of β-oxidation, 2-trans-enoyl-CoA is reversibly hydrated by enoyl-CoA hydratase to L-3-hydroxacyl-CoA. Two enoyl-CoA hydratases have been identified in mitochondria (4), an enoyl-CoA hydratase or crotonase and long-chain enoyl-CoA hydratase which is a component enzyme of the membrane bound trifunctional β-oxidation complex (5). The third reaction in the β-oxidation cycle is the reversible dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA catalyzed by L-3-hydroxyacyl-CoA dehydrogenase (4). Three L-3-hydroxyacyl-CoA dehydrogenases have been identified in mitochondria. They include L-3-hydroxyacyl-CoA dehydrogenase, L-3-hydroxy-2-methylacyl-CoA dehydrogenase (soluble matrix), and long-chain L-3-hydroxyacyl-CoA dehydrogenase, associated with TOC (5). In the last reaction of the β-oxidation cycle, thiolase catalyzes the cleavage of 3-ketoacyl-CoA to acetyl-CoA and an acyl-CoA shortened by two carbon atoms. Mitochondria contain three classes of thiolases: acetoacetyl-CoA

thiolase, 3-ketoacyl-CoA thiolase, and long–chain 3-ketoacyl-CoA thiolase. The latter is a component enzyme of trifunctional β-oxidation complex (TOC).

The degradation of unsaturated fatty acids in mitochondria requires Δ^3,Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase as auxiliary enzymes in addition to the enzymes of the β-oxidation spiral.

Dehydrogenation of Unsaturated Long-chain Acyl-CoAs

The first step of β-oxidation for both saturated and unsaturated fatty acids is catalyzed by one of four distinct FAD-containing acyl-CoA dehydrogenases (4). They are short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD), and very long-chain acyl-CoA dehydrogenases (VCLAD). In the dehydrogenation reaction, one proton is removed from C-2 and a hydride ion from C-3 of the saturated acyl-CoA, resulting in the formation of *trans*-2-enoyl-CoA (6), which is chain shortened by the remaining three steps of β-oxidation. However the breakdown of unsaturated fatty acid requires additional enzymes that specifically function in the metabolism of pre-existing double bonds.

The chain-length specificities of all acyl-CoA dehydrogenases are well established. They seem to complement each other except for an overlap between LCAD and VLCAD. Similar substrate specificities of LCAD and VLCAD and little activity of VLCAD with unsaturated fatty acids that have a double bond at position 4,5 or 5,6 have prompted the idea that

LCAD may serve a specific function in β-oxidation of these fatty acids. The question addressed here is if long-chain unsaturated acyl-CoAs having double bond at position 5 are degraded by LCAD, VLCAD or both?

Energy-Linked Regulation of β-Oxidation

The rate of fatty acid β-oxidation in the heart is linked to the energy consumption by this organ. However, the regulatory mechanism by which β-oxidation is tuned to energy use is still under investigation. Experiments with perfused working hearts have revealed an inverse relationship between the work performed by the hearts and the intracellular concentration of acetyl-CoA at sufficiently high concentrations of fatty acids (0.6 to 1.2 mM) in the perfusate (7). Studies with isolated rat heart mitochondria have shown that β-oxidation under conditions of restricted energy dissipation (state 4 respiration) is associated with a high ratio of intramitochondrial [acetyl-CoA]/[CoASH] (8). Together these observations prompt the suggestion that the energy-linked β-oxidation of fatty acids in heart mitochondria is controlled by the intramitochondrial ratio of [acetyl-CoA]/[CoASH]. However, no consensus has been reached with respect to the site and mechanism of the energy-linked regulation of fatty acid oxidation in the heart.

It has been proposed that the activity of 3-ketoacyl-CoA thiolase (EC 2.3.1.16), and thereby the rate of β-oxidation, may be regulated by the [acetyl-CoA]/[CoASH] ratio (9). Other proposals are based on the

assumption that changes in the intramitochondrial concentrations of acetyl-CoA and CoASH result in corresponding changes in their cytosolic concentrations. For example, a change in the cytosolic CoASH concentration was proposed to result in corresponding activity changes of long-chain acyl-CoA synthetase (EC 6.2.1.3) which activates fatty acids for their β-oxidation (10).

Another proposal assumed that changes in the cytosolic concentration of acetyl-CoA would result in parallel changes of the malonyl-CoA level which may regulate carnitine palmitoyltransferase I (EC 2.3.1.21), thereby controlling the rate of β-oxidation (11).

However, all these proposals are based on the assumption that carnitine acetyltransferase (CAT) (EC 2.3.1.7) is present in the cytosol and can catalyze the conversion of acetylcarnitine to acetyl-CoA at a significant rate. This assumption has been questioned (12). This study was undertaken with the aim of determining whether or not CAT is present in the cytosol of heart cells.

Phosphorylation of Trifunctional β-Oxidation Complex

Protein phosphorylation is one of the principle ways by which intracellular events respond to external physiological stimuli. It is a general regulatory mechanism in metabolism, gene expression, cell growth and other cellular functions. Many protein kinases and phosphatases have been identified in different cellular compartments

including mitochondria. The presence of cAMP-dependent protein kinase in mammalian mitochondria is still a matter of controversy. As for mammalian mitochondria, fragmentary and contradictory reports have appeared regarding the cAMP-dependent (13) and cAMP-independent (14, 15) phosphorylation of proteins. For example, in yeast a cAMP-dependent protein kinase, loosely associated to the inner mitochondrial membrane, is responsible for the phosphorylation of a 40 kDa protein (16). In mammalian mitochondria, a cAMP-dependent protein kinase is apparently localized in the inner mitochondrial membrane/matrix space (17, 18). Recent studies (19) showed that in isolated bovine heart mitochondria, protein other than pyruvate dehydrogenase complex (20) and branched-chain alpha oxoacid dehydrogenase are phosphorylated by the cAMP-dependent and cAMP-independent kinase. Pyruvate dehydrogenase complex, a crucial enzyme in glucose metabolism, is inhibited via end product inhibition and by the activation of pyruvate dehydrogenase kinase, which in turn deactivates the pyruvate dehydrogenase complex by phosphorylation.

It has been proposed that β-oxidation in the heart is linked to energy consumption and is controlled by the intramitochondrial ratio of [acetyl-CoA]/[CoASH] at several possible sites of regulation. Thiolase, as mentioned earlier, is one possible site since its activity is affected by this ratio. Recently the effect of intramitochondrial [acetyl-CoA]/[CoASH] on the purified trifunctional β-oxidation complex (TOC) was evaluated (21)

and it was demonstrated that changing [acetyl-CoA]/[CoASH] alters TOC activity. TOC is a multienzyme complex, located in the inner mitochondrial membrane, consists of long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase, and functions in the chain shortening of long-chain acyl-CoAs. Here, it is proposed that TOC could be one of the possible sites for the regulation of β-oxidation. The [acetyl-CoA]/[CoASH] ratio, which is the signal of the energy-linked regulation, may cause both end product inhibition and covalent modification of TOC and therefore regulate it.

Channeling of Intermediates on the Long-chain Specific β-Oxidation System

The discovery of long-chain fatty acid-specific β-oxidation system consisting of very long-chain acyl-CoA dehydrogenase (VLCAD) and TOC in the inner mitochondrial membrane and the apparent absence of long-chain fatty acid intermediates in mitochondria raises questions about the proposed control mechanism(s) of β-oxidation. Specifically questioned is the energy-linked regulation in extrahepatic tissues that oxidize fatty acids and where changes in the concentrations of β-oxidation intermediates were thought to control the activity of the pathway by regulating the activities of key enzymes (22).

In vitro studies have shown that intermediates inhibit the activities of certain enzymes of β-oxidation. The available evidence suggests that the accumulation of intermediates would strongly inhibit the flux through the β-oxidation spiral. Such situations could be avoided by the absence of intermediates due to channeling. The apparent absence of intermediates of fatty acid oxidation from mitochondria (23) prompted the suggestion that the soluble matrix enzymes may exist as multi-enzyme complexes or as a dynamic aggregate of enzymes (21, 24-25). The existence of metabolite channeling by an enzyme complex or by a particular organization could have many catalytic advantages such as: "preventing or impeding the loss of intermediates by diffusion; decreasing the transient time required for an intermediate to reach the active site of the next enzyme; decreasing the transient time for the system to reach a new steady state; protecting chemically labile intermediates; circumventing unfavorable equilibria; or segregating the intermediates of competing chemical and enzymatic reactions" (26).

An increasing amount of compelling data supports the concept of channeling. In recent studies of β-oxidation with human fibroblasts, only saturated short-chain and medium-chain intermediates were detected in the incubation medium (27). Studies with radio-labeled fatty acids revealed the formation of low levels of chain-shortened acyl-CoAs in respiring mitochondria (28-32) and some investigators identified even smaller amounts of 2-enoyl-CoA and L-3-hydroxyacyl-CoA (29-31).

Thus, its seems that under normal conditions long-chain intermediates do not accumulate in fibroblasts, possibly because they are channeled between the active sites of enzymes catalyzing consecutive reactions. Such channeling most likely occurs if the enzymes of β-oxidation are organized as multi-enzyme complexes. This has been demonstrated for the purified pig heart trifunctional β-oxidation complex (TOC) and for the long-chain acyl-CoA-specific β-oxidation system (located at the inner mitochondrial membrane) in whole cell (21, 27).

Here I have used rat heart mitochondrial membrane vesicles as a system to study the channeling of intermediates on the long-chain acyl-CoA specific β-oxidation system. The long chain acyl-CoA dehydrogenase (LCAD) has a chain length preference for acyl-CoAs that is similar to that of very long-chain acyl-CoA dehydrogenase (VLCAD). The tendency of LCAD to stay associated with the mitochondrial membrane in whole cells or intact mitochondria could make it part of the membrane-bound long-chain specific β-oxidation system. If such situation exist, channeling between VLCAD and TOC might be difficult to study. Unlike whole cells and intact mitochondria, the mitochondrial membrane vesicles would not have this situation because its preparation would remove most of the matrix proteins and proteins loosely associated with it. Thus, channeling between VLCAD and TOC can be studied without the involvement of other β-oxidation enzymes like LCAD.

EXPERIMENTAL PROCEDURES

Materials

ADP, benzamidine hydrochloride, partially purified bovine heart cAMP-dependent protein kinase, bovine serum albumin, collagenase (type VIII), crude extract of porcine heart pyruvate dehydrogenase, cyclic AMP, 2,6-dichlorophenol-indophenol, digitonin, hyaluronidase (type II), , leupeptin, NADH, NAD^+, pepstatin A, sweet potato acid phosphatase (EC 3.1.3.2), rat brain protein kinase C (EC 2.7.1.37), rat liver casein kinase, Triton X-100, and other standard biochemicals were purchased from Sigma. Minimum essential medium (Joklik-modified) was obtained from Life Technologies (Gibco BRL). Dye regents for protein assay and SDS-PAGE mini gels (4-20%) were purchased from Biorad. The source of ferricenium hexafluorophosphate was Aldrich. Acetyl-CoA, CoASH, and other saturated CoA derivatives were obtained from Life Science Products. X-ray films (X-OMATAR) were ordered from Kodak. [^{3}H] acetyl-CoA was purchased from DuPont. [γ-^{32}P] ATP and radio-labeled inorganic phosphate were obtained from NEN Life Science Products. Male Sprague-Dawley rats (240-260 g) were procured from Taconic Farms, Germantown, New York.

Pig heart trifunctional β-oxidation complex, bovine liver medium-chain, long-chain, and rat liver very long-chain acyl-CoA dehydrogenases were purified as described by Luo et al. (33), Davidson et al. (34), Ikeda et al (35), and Izai et al. (36), respectively.

Methods

Synthesis of Substrates

2-*trans*-Hexadecenoic acid and 2-hexadecynoic acid were prepared by general methods developed for the synthesis of 2-*trans*-enoic (37) and 2-ynoic acid (38), respectively. The CoASH derivatives of these two acids were synthesized by the mixed anhydride method as described by Fong and Schulz (37). 2-Hexadecynoyl-CoA, after purification by hydrophobic chromatography on octyl-Sepharose (38), was converted to 3-ketohexadecanoyl-CoA by incubating 2 mM 2-hexadecanoyl-CoA in 20 mM HEPES buffer (pH 7.0) with crotonase (50 units/ml) for 1 hour at room temperature as described by Thorpe (39). L-3-Hydroxhexadecanoyl-CoA was prepared by incubating 2 mM 2-*trans*-hexadecenoyl-CoA in 0.1 M KPi (pH 7.6) with crotonase (30 units/ml) for 1 hour at room temperature. All substrates were purified by HPLC. Their concentrations were determined by measuring released CoASH according to Ellman (40) after quantitatively cleaving the thioester bonds with 1 M hydroxylamine at pH 7.0.

Isolation and Permeabilization of Cardiomyocytes

Adult rat cardiomyocytes were isolated by Langendorff perfusion of the rat heart with a calcium-free Joklik modified tissue culture medium containing collagenase (1 mg/ml), hyaluronidase (1 mg/ml), and 0.03%

bovine serum albumin followed by the gentle shaking of tissue segments in the perfusion medium as described by Frangakis et al (41). The viability of the isolated cells was 80% as judged by Trypan blue exclusion. Aliquots of the resultant cell suspension were incubated for 4 minutes at 37°C with 20 μM, 40μM, or no digitonin and thereafter centrifuged for 5 minutes at 13,000 x g. The supernatants, containing enzymes released from the cells, were assayed to determine the activities of lactate dehydrogenase (LDH), citrate synthase (CS), and CAT. The total cellular activities of these enzymes were measured with aliquots of the cell suspension after their solubilization with 0.2% Triton X-100. Aliquots of the cell suspension treated with digitonin were used as the enzyme source to measure the CAT activities present in the cytosol. When CAT activities present in the cytosol and cell supernatant were measured, aliquots of the cell suspension treated with digitonin were used as the enzyme source.

Spectrophotometric Enzyme Assays

LDH was assayed spectrophotometrically by measuring the pyruvate-dependent oxidation of NADH at 340 nm as described by Stolzenbach (42). CS was assayed by measuring spectrophotometrically at 412 nm the release of CoASH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Srere (43). The activity of CAT was measured as described by Fritz et al (44). The reaction mixture for the spectrophotometric assay

contained 0.2 M Tris-HCl (pH 8.0), 125 μM DTNB, 0.1 mM acetyl-CoA, 1.1 mM L-carnitine, and aliquots of the enzyme source to give an absorbance change at 412 nm of at least 0.02 per min.

Both long-chain and very long-chain acyl CoA dehydrogenases were assayed by recording the reduction of 2,6-dichlorophenolindophenol (DCPIP) with phenazine methosulfate (PMS) as an intermediate electron carrier at 600 nm (45). The assay mixture contained 0.1 M potassium phosphate (pH 7.6), 28 μM DCPIP, 0.2 mM N-ethylmaleimide, 0.45 mM KCN, 30 μM tetradecanoyl-CoA or 5-*cis*-tetradecenoyl-CoA and 3 μg of medium-chain acyl-CoA dehydrogenase (MCAD) or LCAD or VLCAD. The reaction was initiated by the addition of 1.6 mM PMS. Assays were performed at 25°C and an extinction coefficient of 21,300 M^{-1} was used to calculate rates, which were corrected for non-specific reactions.

Isolation of the Rat Heart Mitochondria and Mitochondrial Membrane

Rat heart mitochondria were isolated as described by Chappell and Hansford (46). For the isolation of rat heart mitochondrial membrane, 41 mg of frozen rat heart mitochondria were thawed at 4°C and suspended in 10 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 mM EGTA, 0.1 mM EDTA, 1 mM benzamidine, 2 mM merceptoethanol, leupeptin (1μg/ml), 10 μM antimycin (buffer-A). Next, this suspension was gently vortexed and subsequently centrifuged at 100,000x g for 30 minutes at 4°C to

obtain rat heart mitochondrial membrane (pellet). The resulting pellet was homogenized in buffer-A and centrifuged again for 30 minutes at 100,000x g to obtain washed mitochondrial membrane. The rat heart mitochondrial membrane was re-suspended in buffer-A, homogenized for 5 minutes and then sonicated at 4°C for four 15 second bursts with an ultrasonic sonifier (Model 385) equipped with a microtip. This preparation of the rat heart mitochondrial membrane vesicles was used for phosphorylation assays of TOC.

Radiometric Enzyme Assays

Carnitine Acetyltransferase Assay

The activity of CAT was also determined radiometrically. The reaction mixture of the radiometric assay contained in 0.2 ml: 33 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 2 mM $MgCl_2$, 0.1 M KCl, 0.1 mM EGTA, 20 nmol of [^{3}H]acetyl-CoA (8,000 dpm/nmol), 1.1 mM L-carnitine and an aliquot of the suspension of cardiomyocytes (39 μg of protein) treated with 0 μM, 20 μM, or 40 μM digitonin for 4 minutes. Reactions were terminated by acidification with HCl 0, 2, or 4 minutes after starting them. Assay mixtures were applied to a DEAE-cellulose column (0.9 x 3.5 cm) which was equilibrated with 5 mM HCl. Upon washing with 5 mM HCl, acetylcarnitine passed through the column. Fractions containing acetylcarnitine were collected and their radioactivity was determined by scintillation counting.

Phosphorylation Assay of Partially Purified Trifunctional β-Oxidation Complex

Partially purified pig heart TOC was assayed by incubating 40 μg of partially purified pig heart TOC in 25 mM Tris-HCl (pH 7.4) containing 10 mM $MgSO_4$, 1 mM EGTA, 1 mM EDTA, and 25 mM NaF with 100 μM [γ-^{32}P] ATP (0.45 μCi). To stop the reaction, 100 μl of the electrophoresis buffer, containing 60 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 10% glycerol (v/v), 5% merceptoethanol (v/v) and 0.1% bromophenol blue (w/v) (electrophoresis buffer), was added and boiled for 4 minutes. The resulting suspension was resolved on SDS-PAGE and phosphorylated proteins were visualized by autoradiography. In a control experiment, Mg^{+2} was omitted.

cAMP-dependent Protein Kinase Assay

Partially purified bovine heart cAMP-dependent protein kinase was assayed with partially purified pig heart TOC or rat heart mitochondrial membrane to identify endogenous substrate(s). For this purpose, 100 μl of 25 mM Tris-HCl (pH 7.4) containing 40 μg of partially purified pig heart TOC, 10 mM $MgSO_4$, 1 mM EGTA, 1 mM EDTA, 25 mM NaF, 1.5 mM $CaCl_2$, 50 μM cAMP, 14 μg cAMP-dependent protein kinase (1.4 picomolar/μg), and 0.3 mM [γ-^{32}P] ATP (2.2 μCi) was incubated for 30 minutes at 30°C under constant shaking. The reaction was terminated by adding 100 μl of electrophoresis buffer and heating the sample at

100°C for 4 minutes. Partially purified pig heart TOC was replaced by 0.2 mg of rat heart mitochondrial membrane and 1 mM isobutylmethylxanthine when cAMP-dependent protein kinase was assayed with rat heart mitochondrial membrane as a substrate. Pyruvate dehydrogenase (PDH) was used as a positive control. Phosphorylation of PDH was achieved when 17 μg of PDH was mixed with 100 μl of 25 mM Tris-HCl (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, 25 mM NaF, 10 mM $MgSO_4$, and 0.3 mM [γ-^{32}P] ATP (2.2 μCi) and incubated for 30 minutes at 30°C under constant shaking. The reaction was terminated by adding electrophoresis buffer and heating it to 100°C for 4 minutes. All samples were resolved on SDS-PAGE and phosphoproteins were visualized by autoradiography.

Effect of [Acetyl-CoA]/[CoASH] on the Kinase Activity of Rat Heart Mitochondrial Membranes

The effect of [acetyl-CoA]/[CoASH] on the phosphorylation of TOC in rat heart mitochondrial membranes was determined by autoradiography. One hundred microliters of a typical phosphorylation assay mixture contained 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM $MgSO_4$, 25 mM NaF, 0.3% Triton X-100, 0.19 mg rat heart mitochondrial membrane, and different concentrations of acetyl CoA and CoASH. The ratios are indicated in the legend of Figure 7. In each experiment, the total concentration of acetyl-CoA plus CoASH was 1.15 mM. Reactions

were started by adding 0.3 mM of ATP^{32} (2 μCi). The samples were incubated for 30 minutes at 30°C under constant shaking and the reactions were terminated by boiling the samples for 5 minutes after combining them with electrophoresis buffer. The resultant suspensions were centrifuged at 13000 x g for 10 minutes and the supernatants were subjected to SDS-PAGE to resolve proteins.

Polarographic Assays

Thirteen mg of rat heart mitochondria suspended in 2 ml of isolation buffer that contained 0.21 M mannitol, 70 mM sucrose, 5 mM Tris-HCl, and 1 mM EGTA, were incubated with 0.25 mCi (900-1100 μCi/mmole) inorganic phosphate for 10 minutes to label the mitochondrial ATP pool. For respiration measurements, rat heart mitochondria (0.375 mg/ml) were suspended in 1.9 ml of a basal isoosmotic medium containing 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 1 mM KPi, 2 mM $MgCl_2$, 0.1 mM EGTA, defatted BSA (0.5 mg/ml), and 0.5 mM malate. One minute later, 0.25 mM ADP and 15 μM palmitoyl-carnitine were added to this suspension to stimulate respiration. The respiratory control ratio was >5.

Oxidation of Palmitoyl-carnitine and Pyruvate by Labeled Rat Heart Mitochondria

The reaction mixture contained in a final volume of 19 ml, 0.11 M KCl, 3.3 mM Tris-HCl (pH 7.4), 1 mM KPi, 2 mM $MgCl_2$, 0.1 mM EGTA,

defatted BSA (0.1 mg/ml), 0.5 mM malate and 9.4 mg of pre-labeled rat heart mitochondria. After 2 minutes of incubation, 1 mg of rat heart mitochondria (blank) was withdrawn and immediately inactivated by lowering the pH to 1. Subsequently, 0.5 mM ADP and 15 μM of palmitoyl-carnitine were added to the remaining reaction mixture to stimulate respiration. After initiating respiration, samples of 1 mg were withdrawn at 0.5 minute (state-3 respiration), 1 minute (state-3½ respiration), 4 minute (state-4 respiration) and the immediately terminated by lowering the pH to 1. To the remaining reaction mixture were added 5 mM glucose and hexokinase (30 U) to change the respiration from state-4 to state-3. The last sample of this reaction mixture was withdrawn 2 minute after the reversal of state-4 respiration to state-3 respiration and then terminated by lowering the pH to 1. All samples were centrifuged at 13,000x g for 10 minutes to isolate mitochondria. The resulting pellets were washed twice and centrifuged with 30 mM Tris-HCl (pH 7.5) to remove soluble radioactive compounds and proteins. Finally, pellets were re-suspended in 300 μl of 30 mM Tris-HCl (pH 7.5) containing 0.3% Triton X-100 and then boiled with electrophoresis buffer for 4 minutes. All experiments were performed at room temperature under constant shaking. In another experiment, 10 μM rotenone was added to the reaction mixture before adding ADP and palmitoyl-carnitine when the influence of rotenone on the respiration supported palmitoyl-carnitine was measured. A sample of 1 mg was

withdrawn one minute after initiating respiration and the reaction was terminated by lowering the pH to 1. In a similar experiment, rotenone and palmitoyl-carnitine were replaced by 3 mM pyruvate.

3-Ketoacyl-CoA Thiolase Assay of Phosphorylated TOC

An old preparation of partially purified pig heart TOC was phosphorylated as described in Experimental Procedures. The 3-ketoacyl-CoA thiolase activity of phosphorylated TOC was measured by determining the formation of acetyl-CoA by HPLC. A standard assay mixture contained in 0.5 ml of 0.1 M Tris-HCl (pH 7.6), 15 μM 3-ketohexadecanoyl-CoA, 4 μg of phosphorylated or non-phosphorylated TOC, and 0.2 mM CoASH. The reaction was terminated after 3 minutes of incubation by adjusting the pH to 1-2. Subsequently, aliquots of reaction mixture were injected into HPLC.

Enzyme Assays of β-oxidation System A in Rat Heart Mitochondrial Membrane Vesicles

Fifty milligrams of frozen rat heart mitochondria were thawed at 4°C and suspended in 20 ml of 20 mM Tris-HCl (pH 7.5) containing 0.1 mM EGTA, 0.1 mM EDTA, 1 mM benzamidine, 2 mM merceptoethanol, leupeptin (1 μg/ml) and pepstatin A (1 μg/ml). After standing for 30 minutes on ice, the mixture was gently vortexed before being centrifuged at 100,000 x g for 40 minutes. The supernatant was discarded and the

pellet was gently homogenized in a glass-Teflon homogenizer in 5 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1 % sodium cholate. The resulting homogenate was centrifuged again at 100,000 x g for 30 minutes to obtain the pellet. Washing of the pellet with 10 mM Tris-HCl containing 0.1 % sodium cholate was repeated twice. Finally, the pellet (mitochondrial membrane) was gently homogenized with 10 mM Tris-HCl (pH 7.5) and then sonicated at 4°C for four 20-second bursts with an ultrasonic sonifier (Model 385) equipped with a microtip. This preparation of rat heart mitochondrial membrane vesicles was used for enzyme assays.

The complete β-oxidation cycle catalyzed by membrane-bound β-oxidation system A, consisting of very long-chain acyl-CoA dehydrogenase (VLCAD) and trifunctional β-oxidation complex (TOC), was assayed by measuring the formation of myristoyl-CoA from palmitoyl-CoA. The standard assay mixture contained in 0.5 ml of 0.1 M KPi (pH 7.4), 0.225 mg rat heart mitochondrial membrane vesicles, 0.2 mM ferricenium hexafluorophosphate, 1 mM NAD^+, 0.2 mM CoASH, and 15 μM palmitoyl-CoA. The assay mixture was incubated for different time intervals and then terminated by adjusting the pH to 1-2 with 6 N HCl. The reaction was allowed to proceed at room temperature with occasional shaking. The entire volume of the reaction mixture was subjected to HPLC analysis to quantify myristoyl-CoA and other metabolites. Long-chain enoyl-CoA hydratase of rat heart mitochondrial

membrane vesicles was assayed by an indirect method based on coupling the hydration of 2-*trans*-enoyl-CoA to the dehydrogenation and thiolytical cleavage of the products by the combined action of L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase in the presence of NAD^+ and CoASH. The reaction mixture of 0.5 ml contained 0.1 M KPi (pH 7.6), 0.225 mg rat heart mitochondrial membrane vesicles, 1 mM NAD^+, 0.2 mM CoASH, partially purified pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.3 units/0.5 ml), partially purified pig heart 3-ketoacyl-CoA thiolase (0.9 units/0.5 ml) and different concentrations of 2-*trans*-hexadecenoyl-CoA. The reaction mixture was incubated at room temperature with occasional shaking and then terminated by adjusting the pH to 1 with 6 N HCl. To determine the rate of the reaction, the formation of myristoyl-CoA was measured by HPLC.

Kinetic parameters (Km, Vmax) were determined by nonlinear curve fitting using the Sigma Plot program. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the conversion of 1 nanomole of substrate to product per minute.

HPLC Analysis

Prior to analysis by HPLC, the pH of the reaction was adjusted to 5.5 and the sample was filtered through a 0.22 μm (pore size) membrane. Aliquots were applied to a Waters Bondapak C18 reverse-phase column (3.9 cm x 3.9 mm) attached to the Water gradient HPLC system. The

absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile H_2O (9/1) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 5% to 80% in 50 minutes at a flow rate of 2 ml/min. The concentrations of the products were determined by the use of standard curves that were established with HPLC-purified acetyl-CoA and myristoyl-CoA.

SDS-PAGE and Autoradiography

SDS-PAGE was performed on 4-20% gradient polyacrylamide mini gels (Biorad) according to the general procedure of Laemmli (47). The gel was stained with Coomassie blue. Phosphorylated proteins were visualized by exposure to X-ray film (Kodak X-OMATAR).

Western Blot

Proteins of the rat heart mitochondrial membrane or subunits of partially purified TOC were separated by electrophoresis on gradient (4-20%) polyacrylamide gel in the presence of SDS and transferred to a nitrocellulose membrane by semi dry blotting (48). Thereafter, proteins were probed with diluted (1:1000) purified anti-TOC and visualized by incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase. (Nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate p-toludine were used as substrates for alkaline phosphatase.)

Protein Assay

Protein concentrations were determined as described by Bradford (49) and Wang and Smith (50) with bovine serum albumin as a standard.

RESULTS

Subcelluiar Location of CAT

The question addressed here is whether or not CAT is present in the cytosol of heart cell and if so, does it function in the energy-linked regulation of β-oxidation. The traditional approach of mechanically homogenizing tissues and separating subcellular fractions by centrifugation does not yield acceptable results in the case of the heart, an organ with fibrous connective tissue. The mechanical force required to break such tissue damages subcellular organelles, e.g. mitochondria, with the result that soluble enzymes are released from the organelle and appear in more than one fraction. To avoid such situation, adult rat cardiomyocytes were isolated and permeabilized with digitonin. The CAT activity detected in the cell suspension medium reflects the cytosolic activity of this enzyme. LDH and CS served as marker enzymes for the cytosolic and mitochondrial location, respectively. As is apparent from the data shown in Fig. 1, treatment with 40 μM digitonin caused the release of almost 80% of lactate dehydrogenase from these cells. It is also apparent that half of the cells became leaky during the time cells were incubated and separated from the incubation medium by centrifugation. However, both CS and CAT were not released except for a small amount representing 2% and 6% of their total activities,

respectively. This result agrees with the intramitochondrial location of these two enzymes. The small quantities of CAT and CS, which were detected extracellularly only when digitonin was present, most likely are due to their leakage from damaged mitochondria. Since no extracellular CAT activity was detected when half of the cellular lactate dehydrogenase had leaked out of the cells, a soluble CAT does not seem to be present in the cytosol of cardiomyocytes.

Determination of Membrane-bound or Membrane-associated Cytosolic CAT

It is however, possible that a CAT activity, different from the mitochondrial CAT, is associated with subcellular structures so that the enzyme is immobile but capable of catalyzing the conversion of acetylcarnitine to acetyl-CoA in the cytosol. For example, in liver, CAT activities are located in peroxisomes and microsomes in addition to being present in mitochondria (51). Such CAT activities, if present in cardiomyocytes, could generate acetyl-CoA in the cytosol even though they may be unable to exit from permeabilized cells. To assess this hypothesis, the CAT activity was measured in permeabilized cardiomyocytes by use of a radioactive assay procedure. Since LDH can exit from such cells, the reactant, [^{3}H]acetyl-CoA and CoASH, should be able to enter such cells. The results, shown in the Table 1, indicate that approximately 11% of the total CAT activity was detected by this approach. Half of this activity reflects soluble CAT, presumably of

mitochondrial origin as indicated by the presence of this part of the activity in the cell suspension medium. The residual activity of approximately 5% of the total activity (3.5 nmol min^{-1} mg of protein) may be due to membrane-bound or membrane-associated CAT with its active site facing the cytosol

Phosphorylation of TOC

Phosphorylation of pig heart TOC was first examined with a partially purified preparation which had been stored at -80°C for several years. This preparation is referred to as "old preparation" of TOC. In most of the phosphorylation assays, the incorporation of ^{32}P had a broad pH optimum ranging from 6.5 to 8.0. Therefore, a pH of 7.5 was used for further studies because pH of 7.0-7.5 is close to the physiological pH and optimal for many mitochondrial activities. The ATP concentration that yielded a maximum ^{32}P incorporation into TOC ranged from 0.1 to 0.3 mM. Thus, concentrations in this range were used in subsequent experiments. The time-dependent incorporation of ^{32}P was measured with the old preparation of partially purified TOC and results (data not shown) indicated that the incorporation of ^{32}P into TOC increased with time and reached a saturation level after about 30 minutes of incubation at 30°C. For that reason, all subsequent experiments were conducted at 30°C for 30 minutes to achieve maximum ^{32}P incorporation.

Phosphorylation of Partially Purified TOC in the Presence and Absence of cAMP-dependent Protein Kinase

Shown in Fig. 2 is the selective phosphorylation of the small subunit when partially purified pig heart TOC (old preparation) was incubated with [γ-^{32}P] ATP in the presence of Mg^{+2}. Incorporation of ^{32}P was not observed when Mg^{+2} was omitted from the reaction mixture as demonstrated in Fig. 2, (see lane 1 and lane 2). The Mg^{+2} dependency of TOC phosphorylation suggested the presence of an intrinsic protein kinase because most kinases are only active in the presence of Mg^{+2}. SDS-PAGE of partially purified pig heart TOC (old preparation) showed a protein band with a molecular mass close to 45 kDa besides the small subunit (47 kDa) and the large subunit (80 kDa) (see Fig. 2). The presence of a third protein band (45k kDa) in close proximity to the small subunit of TOC, could leave doubts about the precise location of the small subunit. This problem was solved by immunoblot analysis. Comparison of lane 3 and lane 4 of Fig. 2 clearly demonstrated that the protein band at 47 kDa was the small subunit of TOC.

The observed phosphorylation of the small subunit of TOC prompted the question of whether this modification affects the activity of this complex and specifically the activity of long-chain 3-ketoacyl-CoA thiolase and thereby controls fatty acid oxidation. This scenario justified a more detailed investigation of the phosphorylation of TOC. The accurate determination of the concentration of partially purified pig heart

TOC was problematic because the purification of membrane-bound TOC required Triton X-100 which interfered with the available protein assay procedures. Therefore, the precise determination of ^{32}P incorporation into TOC was difficult to establish. Thus, the relative phosphorylation in response to added casein kinase, protein kinase C and cAMP-dependent protein kinase, were determined with the old preparation of TOC. cAMP-dependent protein kinase was found to be more specific than the other two kinases (data not shown) because it caused the strong phosphorylation of the small subunit and resulted in the weak phosphorylation of the large subunit (Fig. 3) of TOC. Lane 2 in Fig. 3 shows an uncharacterized phosphoprotein of about 50 kDa that might be the autophosphorylated form of added cAMP-dependent protein kinase. Pyruvate dehydrogenase (PDH) of the pyuvate dehydrogenase complex (PDC), a mitochondrial enzyme was used in a control experiment to identify PDH and TOC when both were present in the same preparation. The ability to distinguish between TOC and PDH is clearly shown in lane 3 and lane 4 of Fig. 3.

Does the Phosphorylation of the Small Subunit of TOC Alter its Activity?

To monitor the effect of phosphorylation, 3-ketoacylCoA thiolase activity of the phosphorylated and non-phosphorylated forms of TOC (old preparation) were measured by HPLC. The results are shown in Table. 2. No difference was observed between the two forms of TOC. Similar

results were observed when TOC was phosphorylated by cAMP-dependent protein kinase and the activity of 3-ketoacyl-CoA thiolase was determined (data not shown). In another experiment, the phosphorylated form of TOC was dephosphorylated by acid phosphatase and the activities of both phosphorylated and dephosphorylated forms of TOC did not differ (data not shown).

The non-specific phosphorylation of the old preparation of TOC by exogenous kinases, except for cAMP-dependent protein kinase, and the unaltered activity of phosphorylated TOC left doubts about the metabolic significance of TOC phosphorylation. This situation prompted the isolation of a fresh batch of TOC. SDS-PAGE of fresh partially purified pig heart TOC is shown in lane 2 of Fig. 4. When the phosphorylation of the fresh preparation of partially purified pig heart TOC by endogenous kinase and exogenous cAMP-dependent protein kinase were investigated, no incorporation of ^{32}P into either subunit was detected except for a weak incorporation of ^{32}P in the presence of cAMP-dependent protein kinase, (see lane 4 and 5 of Fig. 4).

Effects of cAMP-dependent Protein Kinase and [Acetyl-CoA]/[CoASH] on the Phosphorylation of Rat Heart Mitochondrial Membrane Proteins

Prior to assaying the phosphorylation of TOC, its presence in rat heart mitochondrial membrane vesicles was determined by immunoblotting and activity measurements. Fig. 5 depicts the ^{32}P-

labeling pattern of proteins (see Fig. 5A) of rat heart mitochondrial membrane vesicles when incubated with [γ-^{32}P]-ATP under different conditions and resolution of proteins by SDS-PAGE (see Fig. 5B). The exogenous cAMP-dependent protein kinase was used to determine the substrate for phosphorylation in rat heart mitochondrial membrane vesicles as described in Experimental Procedures. Results shown in lane 6 of Fig. 5A, reveal the presence of several minor phosphoproteins on the autoradiogram beside two prominent phosphoproteins with molecular masses around 45 kDa and 30 kDa. The 45 kDa phosphoprotein was identified as the α subunit of PDH by running the phosphorylated form of partially purified PDH on the same gel. In another assay, partially purified pig heart TOC was added to rat heart mitochondrial membrane vesicles to increase its concentration and availability to a potential membrane-associated or membrane-bound kinase or exogenous cAMP-dependent protein kinase. Results are shown in lane 3 and lane 4 of Fig. 5A. Rat heart mitochondrial membrane vesicles were also used to determine endogenous kinase activity for endogenous substrate present in the membrane. Results are shown on lane 5 of Fig. 5A. A close look at Fig. 5A reveals phosphorylation of several proteins, most prominently α subunit of PDH and a uncharacterized phosphoprotein with an approximate mass of 30 kDa. Densitometric analysis of phosphorylated α subunit of PDH to determine the extent of the phosphorylation under all conditions showed the same extent of ^{32}P incorporation. None of the

conditions mentioned above resulted in a detectable TOC phosphorylation (see Fig. 5).

The influence of [acetyl-CoA]/[CoASH] on TOC phosphorylation was investigated with rat heart mitochondrial membrane vesicles. Varying [acetyl-CoA]/[CoASH] ratios were selected and the total concentration of acetyl-CoA plus CoASH was maintained at 1.2 mM. No TOC phosphorylation was detected at any of the [acetyl-CoA]/[CoASH] ratios (Fig. 6A). However, several other proteins were phosphorylated including the α subunit of PDH which was identified by authentic phosphorylated partially purified PDH.

Phosphorylation of Proteins under Different Metabolic Conditions Generated in Intact Rat Heart Mitochondria.

In this approach, intact mitochondria were used to generate different metabolic conditions that may result in the phosphorylation of TOC without disturbing the intramitochondrial organization of proteins necessary for phosphorylation. For that purpose, the internal pool of ATP of rat heart mitochondria was labeled by incubating them with inorganic phosphate (^{32}P). To achieve a maximum specific radioactivity, the phosphate content of the basal isoosmotic medium was reduced from 2 mM to 1 mM.

Phosphorylation of TOC was assayed in coupled rat heart mitochondria at different respiration states supported by hexadecanoyl-carnitine as a substrate. In coupled mitochondria in the presence of ADP

and phosphate, respiration changes from state 3 to state 4 when all ADP is converted to ATP. However, state 3 respiration can be maintained by reversing state 4 respiration by the addition of glucose and hexokinase to facilitate the conversion of ATP to ADP. When the respiratory state changes from 3 to 4, the matrix concentration of acetyl-CoA and NADH increases, whereas those of CoASH and NAD^+ decrease (52). The elevated ratios of [acetyl-CoA]/[CoASH] and [NADH]/[NAD^+] persist in the mitochondrial matrix during state 4 respiration. It is proposed that the flux of fatty acids through β-oxidation is controlled by the mitochondrial ratio of [acetyl-CoA]/[CoASH] and that the site of control is thiolase (9). Results obtained under these conditions are shown in Fig. 7. Presented in Fig. 7A are the recordings of respiration supported by palmitoyl-carnitine in coupled rat heart mitochondria. As indicated, samples were withdrawn at each respiration state and proteins were resolved on SDS-PAGE to locate phosphoproteins by autoradiography. The autoradiogram (see lane 1-5 in Fig. 7B) revealed a single phosphoprotein with a molecular mass close to 45 kDa, which was identified as the α subunit of PDH by running phosphorylated PDH on the same gel. TOC was not phosphorylated at any of the respiration states.

In another experiment, a state 4 condition was simulated by adding rotenone to prevent the palmitoyl-carnitine supported respiration in labeled coupled rat heart mitochondria. Fig. 8A shows the recording of respiration supported by palmitoyl-carnitine with and without

rotenone. The effect of state 4 respiration on the phosphorylation of rat heart mitochondrial proteins is shown in Fig. 8B. A single (lane 1 in Fig. 8B) phosphoprotein was found to be the α subunit of PDH with no indication of TOC phosphorylation. In a similar experiment, palmitoyl-carnitine was replaced by pyruvate and the phosphorylation of protein(s) were monitored. Results shown in lane 2 of Fig. 8B are the same as those observed under the other metabolic conditions.

Channeling of Long-chain Acyl-CoA Intermediates on the β-Oxidation System A in Rat Heart Mitochondrial Membrane Vesicles

Rat heart mitochondrial membrane vesicles were used as a system to study the channeling of intermediates of long-chain acyl-CoA between VLCAD and TOC. This system, referred to as β-oxidation system A, consists of VLCAD and TOC that catalyzed a complete cycle of β-oxidation. The activity of β-oxidation system A was determined with palmitoyl-CoA at a fixed coenzyme A concentrations of 0.2 mM and at 1 mM NAD^+. The ferricenium hexafluorophosphate replaced physiological electron acceptor, electron-transferring flavoprotein at 0.2 mM concentration. Before assaying the β-oxidation system A, the activities associated with VLCAD and TOC were estimated in rat heart mitochondrial membrane vesicles (data not shown).

Determination of Intermediates During the β-Oxidation Catalyzed by System A

Substrate, intermediates and products formed by the β-oxidation system A present in rat heart mitochondrial membrane vesicles were separated by HPLC and quantified by the use of standard curves established for each of the compounds with HPLC-purified acyl-CoA thioesters. The quantitative recovery of acyl-CoA thioesters was demonstrated by adding tridecanoyl-CoA (C_{13}) and heptadecanoyl-CoA (C_{17}) as internal standards to the reaction mixture and determining its concentration by HPLC (see Fig. 9). It is apparent from a time dependent study (Fig. 9) that all intermediates (I_1, I_2, I_3) formed during one cycle of β-oxidation of palmitoyl-CoA by system A were present in all incubations in addition to the substrate and products, myristoyl-CoA (C_{14}) and acetyl-CoA (not shown in chromatogram). Concentrations of I_1, I_2 and C_{14} were determined and are shown in Fig. 10. 3-Ketohexadecanoyl-CoA (I_3) was detectable in all incubations but was poorly separated from myristoyl-CoA to be quantified. The time course of C_{14} formation in rat heart mitochondrial membranes vesicles showed a significant lag (see Fig. 10) which is a characteristic feature of coupled reaction catalyzed by two enzymes that function independently. As can also be seen in Fig. 10, the concentration of I_1 increased during the first minute of incubation but then reached a steady state. L-3-Hydroxyhexadecanoyl-CoA showed a similar pattern but reached a steady state after 0.5 minute

of incubation.

To determine the effect of the substrate concentration on the appearance of intermediates in the incubation mixture, rat heart mitochondrial membrane vesicles were incubated with different concentrations of hexadecanoyl-CoA for one minute. The results shown in Fig. 11, demonstrate that at a low concentration of hexadecanoyl-CoA (10 μM or less), 3-ketohexadecanoyl-CoA (I_3) was not detectable in the incubation medium but its concentration together with those of the two intermediates, I_1 and I_2, increased as the concentration of hexadecanoyl-CoA was raised.

Comparison of Actual and Calculated Reaction Rates

The kinetic parameters of the reaction catalyzed by long-chain 2-enoyl-CoA hydratase of rat heart mitochondrial membrane vesicles was determined and the results are shown in Table. 3. The measured concentrations of I_1 during the β-oxidation by system A and the kinetic parameters of 2-enoyl-CoA hydratase (see Table. 3) were used to calculate rates. The calculated formation of myristoyl-CoA, based on the concentration of free 2-hexadecenoyl-CoA in the reaction mixture was slightly higher during the first minute of incubation than the observed formation of myristoyl-CoA in β-oxidation system A (see Fig 12). The observed lag in product formation and a higher calculated than observed rate do not support the concept of channeling between VLCAD and TOC

in rat heart mitochondrial membrane vesicles.

Activity of Acyl-CoA Dehydrogenases with 5-*cis*-tetradecenoyl-CoA

This study was conducted to determine if a *cis* 5,6 double bond present in a fatty acyl-CoA would interfere with the introduction of a 2,3-double bond during the first reaction of the β-oxidation cycle.

It had been previously demonstrated that acyl-CoA dehydrogenases present in the rat liver mitochondrial matrix more efficiently dehydrogenate long-chain unsaturated substrate than the dehydrogenases present in the membrane fraction (data not shown). The mitochondrial matrix is presumed to contain MCAD and LCAD while VLCAD resides in the mitochondrial membrane. This initial finding was further evaluated with partially purified MCAD, LCAD and VLCAD. 5-*cis*-Tetradecenoyl-CoA, a metabolite of oleic acid, was used as a substrate and for comparison tetradecanoyl-CoA was used. The results shown in Table. 4 demonstrates that LCAD acted equally well on these two substrates that differ only by the presence of the 5-*cis* double bond in the acyl chain of the unsaturated substrate. In contrast VLCAD acted poorly on the substrate that contained the 5-*cis* double bond. MCAD worked equally well on both 5-*cis*-tetradecenoyl-CoA and tetradecanoyl-CoA but its activity was 3-fold lower than the activity of LCAD.

DISCUSSION

Is CAT Involved in the Energy-Linked Regulation of β-Oxidation?

The subcellular location of CAT in the heart has not been determined unequivocally. Although the location of this enzyme in the mitochondrial matrix is well documented (51), its presence in the cytosol or to the outside of the inner mitochondrial membrane in heart and flight muscle has been considered (53-54) and disputed (55). Given this uncertainty and the proposed involvement of cytosolic CAT in the energy-linked regulation of cardiac fatty acid oxidation (10, 11), reassessment of the intracellular location of CAT is justified. The results of this study demonstrate that in rat heart approximately 5% of the total CAT activity might be residing in the cytosol in a membrane-bound or membrane-associated form with the active site facing the cytosol. Since the CAT-catalyzed formation of acetyl-CoA is significantly slower than the reverse reaction (data not shown) and since the cytosolic concentration of free CoA is estimated to be below 25 μM in the heart (10), the rate of acetyl-CoA formation in the heart cytosol may be as low as 0.3 nmol (min x mg of protein)$^{-1}$. The low CAT activity in the cytosol is unlikely to sustain a dynamic equilibrium between the mitochondrial and cytosolic pools of acetyl-CoA. Such intercompartmental communication would require

sufficiently high activities of intramitochondrial CAT, carnitine: acylcarnitine translocase, and cytosolic CAT (see Fig. 13)

Acetyl-CoA can also be exported from mitochondria by a sequence of reaction catalyzed by mitochondrial citrate synthase, tricarboxylate carrier, and ATP-citrate lyase. However, this reaction sequence is not well suited for facilitating the re-import of acetyl-CoA from the cytosol into mitochondria and therefore is unlikely to maintain a dynamic equilibrium between intramitochondrial and extramitochondrial acetyl-CoA. Moreover, the low activities of the tricarboxylate carrier (56-57) and ATP-citrate lyase (58-59) in the heart are unlikely to support a rapid adjustment of the cytosolic acetyl-CoA concentration. A report claiming high activity levels of ATP-citrate lyase in rat heart (60) is contradicted by reports that only trace level of activity (58) and mRNA (59) for this enzyme are present in rat heart. The export of mitochondrial acetyl-CoA in the form of citrate may be sufficient to provide cytosolic acetyl-CoA for the synthesis of malonyl-CoA. In fact, a recent report based on the use of the specific ATP-citrate lyase inhibitor, hydroxycitrate, is suggestive of a precursor-product relationship between citrate and malonyl-CoA (61). Taken together, the available evidence supports the notion that mitochondrial acetyl-CoA may be the precursor of cytosolic malonyl-CoA. But it does not fit a model for the energy-dependent regulation of fatty acid β-oxidation which requires the rapid export of mitochondrial acetyl-

CoA when the energy utilization is low and its rapid removal from the cytosol as the energy demand increases.

Two proposals aimed at explaining the energy-linked regulation of cardiac fatty acid oxidation are based on the assumption that the changes of the intramitochondrial acetyl-CoA concentration result in corresponding changes of acetyl-CoA in the cytosol. According to Neely and coworkers (10), an energy-dependent decrease in the β-oxidation of fatty acids, which causes the mitochondrial acetyl-CoA concentration to increase, would result in an elevated level of acetyl-CoA and a corresponding decrease of free CoA in the cytosol. As a consequence, rates of fatty acid activation and β-oxidation may decline. The second proposal by Lopaschuk and coworkers (11) assumes that a change of the acetyl-CoA concentration in the cytosol would result in a corresponding change of the malonyl-CoA concentration which might inhibit carnitine palmitoyltransferase I and thereby fatty acid oxidation (see Fig. 13). The proposed schemes constitute feedback loops that could explain the energy-linked regulation of cardiac fatty acid oxidation. However, in the absence or near absence of CAT from the cytosol it is unlikely that cardiac fatty acid oxidation is regulated by either of these mechanisms.

Does TOC Get Phosphorylated?

The phosphorylation of intracellular protein is a common mechanism of fundamental importance in biological regulation. Phosphorylation is a

rapid and reversible means of regulating enzyme activity and its efficiency is evident in many signal transduction pathways that use cascades of phosphorylation to effect cellular responses.

Several mitochondrial enzyme complexes are also regulated by phosphorylation. Most studied and the prime example of such regulation is pyruvate dehydrogenase complex (PDC), an important mitochondrial enzyme that converts pyruvate to acetyl-CoA for oxidation in the TCA cycle. PDC is regulated by both end product inhibition and phosphorylation. The trifunctional β-oxidation complex was also suspected of being phosphorylated. Thus, it seemed prudent to analyze TOC for this type of regulation.

Preliminary data from phosphorylation assays with an old preparation of TOC was promising, especially when only the small subunit of TOC was found to be phosphorylated. In addition, Mg^{+2} dependent phosphorylation of TOC suggested the presence of an intrinsic kinase in TOC as is the case with PDC. Moreover, end product inhibition of 3-ketoacyl-CoA thiolase of TOC had been observed (21) in agreement with a postulated energy-linked regulation of β-oxidation in heart. Therefore, the effect of phosphorylation on TOC activity was investigated to establish its role in the regulation of β-oxidation. As it has been shown in Table. 2, phosphorylation does not alter TOC activity. Since a stoichiometic analysis was not conducted because of difficulties in determining the protein concentration, the extent of TOC

phosphorylation could not be established. Much less than stiochiometric incorporation of ^{32}P in TOC could be one reason for the unchanged activity. For that purpose phosphorylation of TOC was optimized by adding cAMP-dependent protein kinase which was reported to be present in the inner mitochondrial membrane (?), and which was found to phosphorylate the smaller subunit of TOC selectively and heavily. However, phosphorylation of TOC by exogenous cAMP-dependent protein kinase did not result in a change of its activity. While these experiments were done an important change was noticed with the old preparation of TOC is that phosphorylation of TOC became less selective with time. This might be due to a conformational change or degradation of TOC that might expose amino acids susceptible to phosphorylation. To verify all observations, a fresh batch of TOC was prepared from pig heart and assayed for phosphorylation with and without cAMP-dependent protein kinase. Surprisingly, no phosphorylation was observed and only little incorporation of ^{32}P was detected in the presence of cAMP-dependent protein kinase. A possible explanation for these finding is the phosphorylation of a small percentage of conformationally altered TOC.

Immunogold labeling suggests the presence of cAMP-dependent protein kinase in the inner mitochondrial membrane/matrix, of liver, pancreas, skeletal muscles, heart and brain cell (62). If a kinase specific for TOC exists, it could have been lost during the purification of TOC which required detergent for its solubilization. To avoid such situation,

rat heart mitochondrial membrane vesicles were prepared under mild conditions. The results with rat heart mitochondrial membrane vesicles show the same pattern of phosphoproteins with and without cAMP-dependent protein kinase. There was no indication of TOC phosphorylation. The results obtained with such preparation are partially compatible with data presented by Techniko-Dobrova et al. (19). They observed three proteins with molecular masses of 44, 39, and 30 kDa in the bovine heart mitochondrial membrane, which they proposed were phosphorylated by cAMP-independent kinase. In rat heart mitochondrial membrane vesicles two phosphoproteins with molecular masses of approximately 45 kDa and 30 kDa were observed. The reason for the absence of the 3rd band at 39 kDa is not known. In the same study Techniko-Dobrova et al. presented evidence of three other phosphoproteins of 125, 19, and 6.5 kDa which they proposed were phosphorylated by cytoplasmic cAMP-dependent kinase A. In our experimental setup, cytosolic and mitochondrial matrix fractions were washed away when rat heart mitochondrial membrane vesicles were prepared. Therefore, the absence of the 125, 19, and 6.5 kDa phosphoproteins was not surprising.

The use of rat heart mitochondrial membrane vesicles has several disadvantages. One is the loss of molecule(s) that might serve as signal to activate or deactivate kinases or phosphatases. The loss of kinases or phosphatases themselves might occur if they are not tightly membrane-

bound. However, it was assumed that a TOC kinase either might be membrane- bound or an integral part of the enzyme. The best candidates for an effector molecule that might affect TOC kinase or phosphatase activity are acetyl-CoA and CoASH. These compounds or more precisely their concentration ratio also affects thiolase activity in the energy-linked regulation of β-oxidation in heart. Prompted by these ideas, the effect of the [acetyl-CoA]/[CoASH] ratio on the phosphorylation of TOC in rat heart mitochondrial membrane vesicles was assessed. Similar results to those observe with cAMP-dependent kinase were obtained.

It should be noted that experiments with purified TOC or mitochondrial membrane vesicles are simple and can be easily modified to stimulate certain conditions or situations. The disadvantage is that they are carried out under unphysiological conditions. As it has been mentioned before, phosphorylation is a tightly regulated process and a disturbance in the structural organization of proteins may produce a negative result. Therefore, phosphorylation of TOC was tested in intact mitochondria which contain protein kinases and substrates in their native state during the experiment and therefore the physiological relevance of any phosphorylation could not be questioned. A signal was required to affect the kinase activity for TOC and this was attempted by changing the energy state of heart mitochondria. Studies with coupled mitochondria permit adjustment of the energy state by limiting ADP

(state 4 respiration) or providing sufficient ADP (state 3 respiration) for oxidative phosphorylation. When the respiration state changes from 3 to 4, the ratios of [acetyl-CoA]/[CoASH] and [NADH]/[NAD^+] increase. Because these ratios change in response to a shift in the energy state, the effect of the phosphorylation of rat heart mitochondrial proteins was monitored as a function of the energy state. In search of other stimuli that could activate kinase activity for TOC, fatty acyl-CoA was replaced by pyruvate that deactivates PDC kinase. In another effort, an extreme state 4 respiration was created in rat heart mitochondria by using rotenone to block the electron transport chain. The resultant increase in the ratio of [NADH]/[NAD^+] leads to a suppression of β-oxidation (63). The depression of β-oxidation leads to an increased accumulation of intermediates of β-oxidation (63-68). Accumulation of long-chain intermediates of β-oxidation, a high ratio of [NADH]/[NAD^+] and other factors were considered as stimuli to activate kinase activity for TOC. All these conditions were not found to promote the phosphorylation of TOC. There was no sign of other phosphoproteins except for the α subunit of PDH. Most of the matrix proteins and proteins loosely associated with the mitochondrial membrane that could have been phosphorylated, were removed before performing SDS-PAGE. However, unlike the phosphorylation assay in rat heart mitochondrial membrane vesicles, a 30 kDa protein was not observed to be phosphorylated.

Based on all experiments with purified TOC, rat heart mitochondrial membrane vesicles and intact rat heart mitochondria, it is concluded that TOC is not phosphorylated. Therefore, product inhibition may be one of the possible ways that the activity of TOC is controlled in the energy-linked regulation of β-oxidation. Results with intact mitochondria in which different metabolic condition were generated without disturbing the integrity of organelle provide the most convincing evidence against TOC phosphorylation. Characterization of other phosphoproteins were beyond the scope of this work are therefore not being discussed.

Channeling of Long-chain Fatty Acyl-CoA on Rat Heart Mitochondrial Membrane Vesicles.

Channeling, refers to a mechanism where metabolites of a reaction sequence are transferred from one enzyme to the next enzyme without equilibrating with the bulk solution. The channeling of metabolites can be considered as direct transfer of intermediates or microcompartmentation of metabolite. The microcompartmentation is functionally an isolation of the intermediates from those in the rest of the compartment resulting in high local concentration of intermediates for efficient function of an enzyme (69). In Fig. 14, schematic diagrams are presented for the channeling and non-channeling situations of intermediates in β-oxidation system A. The diagram indicates that the channeling should allow the partial or complete transfer of intermediates between active sites. In contrast, a non-channeling situation indicates

that intermediates must equilibrate with the bulk medium before finding the second active sites by diffusion. Channeling of intermediates has been discovered with enzymes of β-oxidation. It has been tested with whole mitochondria and it was most convincingly shown in whole cells (27) that fatty acyl-CoAs intermediates are channeled between enzymes of β-oxidation. Evidence with whole cells strongly supports the notion of channeling in mitochondrial membrane bound long-chain β-oxidation system.

To study channeling in whole cell is complicated and requires accurate means of detection and quantification of intermediates and products. An attempt was made to detect channeling between VLCAD and TOC with inside-out vesicles of the rat heart mitochondrial membrane. By this approach, the integrity of membrane-bound β-oxidation system A and other membrane-bound proteins including proteins of the electron transport chain would remain intact and any interference by matrix enzymes would be minimal. Specially LCAD, which was found to dehydrogenate long-chain saturated and unsaturated fatty acyl-CoAs, could interfere in the channeling of long-chain intermediates between VLCAD and TOC in rat heart mitochondrial membrane vesicles.

The evidence presented here prompts the conclusion that vesicles prepared from rat heart mitochondrial membrane are not a suitable system to study the channeling of long-chain intermediates on

membrane-bound β-oxidation system A. Fig. 9 reveals the accumulation of all possible intermediates in incubation mixture. Most surprising is the observed accumulation of 2-hexadecenoyl-CoA and a small, but detectable amount of 3-ketohexadecanoyl-CoA. None of these intermediates have been observed to accumulate in previous studies with whole mitochondria and cells. However accumulation of L-3-hydroxyhexadecanoyl-CoA has been reported by several investigators (28-32). The observed accumulation of 3-ketohexadecanoyl-CoA in this study may be a consequence of the specific experimental setup with relatively high non-physiological concentration of hexadecanoyl-CoA. To test this possibility, the effect of different concentrations of hexadecanoyl-CoA on the accumulation of intermediates was monitored. It was found that high concentrations (>10 μM) of hexadecanoyl-CoA give rise to a small quantity of 3-ketohexadecanoyl-CoA and that its concentration increases with increasing concentration of hexadecanoyl-CoA. Since matrix enzymes of β-oxidation have a tendency to bind to the membrane, the presence of enzymes like LCAD and 3-ketoacyl-CoA thiolase in the vesicles could also be a reason for the accumulation of intermediates.

A lag (see Fig. 10) in the accumulation of the product of β-oxidation system A also is suggestive of non-channeling situation (see Fig. 14B), with 2-hexadecenoyl-CoA diffusing into the bulk medium before it reaches the second active site of long-chain 2-enoyl-CoA hydratase.

Accumulation of 2-hexadecenoyl-CoA might also slow down the rate of β-oxidation by system A by partially inhibiting VLCAD activity since acyl-CoA dehydrogenases are inhibited by 2-enoyl-CoAs (34, 70). Consequently, a lower observed rate of β-oxidation by system A compared to a calculated rate of the same reaction sequence is not surprising. Results with rat heart mitochondrial membrane vesicles do not agree with conclusions reached with whole mitochondria and cells (27). All these studies have provided strong evidence in support of intermediate channeling on the membrane bound β-oxidation system. The discrepancy between our results and evidence obtained with whole mitochondria or cells was not further explored. However, based on the findings of this study it is concluded that rat heart mitochondrial membrane vesicles are not a suitable model to study intermediates channeling on membrane bound β-oxidation system.

If intermediates of the membrane-bound β-oxidation system are not channeled, then each intermediate of β-oxidation would be present in the matrix in a finite amount and would reduce the available free CoASH needed for other mitochondrial processes. Moreover, if β-oxidation intermediates accumulate, they might inhibit enzymes of β-oxidation. In fact, 3-ketoacyl-CoA intermediates at nanomolar concentrations effectively inhibit acyl-CoA dehydrogenases (34, 70) and 3-hydroxyacyl-CoA dehydrogenase (71). In addition, acyl-CoA dehydrogenases are also inhibited by 2-enoyl-CoA (34, 70) and L-3-hydroxyhexadecanoyl-CoA

inhibits the 2-enoyl-CoA hydratase (71). Therefore, flux through the β-oxidation cycle might be strongly inhibited by intermediates. This situation does not exist *in vivo*. Channeling of intermediates on the membrane bound-β-oxidation system may play an important role in the energy-linked regulation of β-oxidation in non hepatic tissues. The [acetyl-CoA]/[CoASH] ratio was proposed to regulate thiolase (22) and that in turn could affect the activity of acyl-CoA dehydrogenases and other enzymes in the β-oxidation system without 3-ketoacyl-CoA accumulating in the matrix.

LCAD Might be an Essential Enzyme in β-Oxidation of Long-chain Unsaturated Fatty Acids

The dehydrogenation of long-chain unsaturated acyl-CoAs, especially those that have a 5-*cis* double bonds, by acyl-CoA dehydrogenases was studied. The results show that LCAD is almost equally active with saturated and unsaturated substrates that have acyl-chains with the same number of carbon atoms. This is also true for MCAD but its activity is approximately 3-4 fold lower with both saturated and unsaturated substrates when compared to LCAD. However, the lower activity of MCAD with tetradecanoyl-CoA and 5-*cis*-tetradecenoyl-CoA was expected based on the established carbon chain length specificities of acyl-CoA dehydrogenases. The effect of the 5-*cis* double bond on VLCAD activity is significant. VLCAD shows only 5% of the activity with 5-*cis*-tetradecenoyl-CoA when compared with the LCAD activity using the

same substrate. In a later study, Le et al. (personal communication) confirmed these results with a more sensitive fluorometric assay using electron transferring flavoprotein as electron acceptor. They also established that polyunsaturated acyl-CoAs with 5-*cis* double bonds are poor substrates of VLCAD. These results suggest that a double bond in close proximity to the thioester group interferes with the efficient dehydrogenation catalyzed by VLCAD. However this phenomenon is not apparent when the double bond is further removed from the site of the hydrogenation. The most important result of this study is the information it provides relevant to the metabolic function of LCAD. Here I propose that LCAD might be essential for the β-oxidation of long chain polyunsaturated fatty acids.

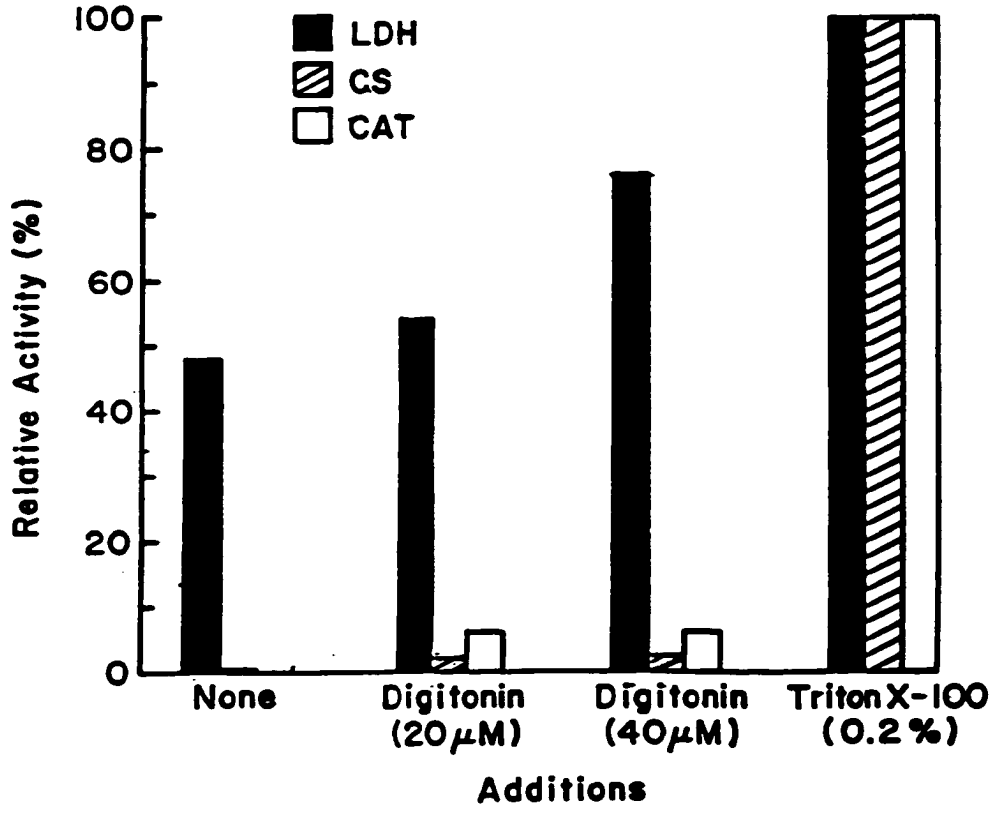

Figure 1. **Efflux of CAT, CS, and LDH from rat cardiomyocytes and cardiomyocytes treated with either digitonin or Triton X-100.** Enzyme activities were measured spectrophotometrically as described under **Methods and materials.**

Table 1. **CAT activity in permeabilized cardiomyocytes.**
CAT activity was determined by use of radioactive assay as described in Experimental procedures.

Experiment	Incubation time (min)	Deletion	Addition	Specific Activity (mU/mg of protein)	Rel. activity (%)
1	0	none	none	4.1*	6
2	4	cells	none	4.0*	6
3	4	carnitine	none	5.6 ± 0.1	9
4	4	none	none	12.8 ± 0.1	20
5	4	none	Triton X-100	64.0 ± 0.6	100

* average of two measurments

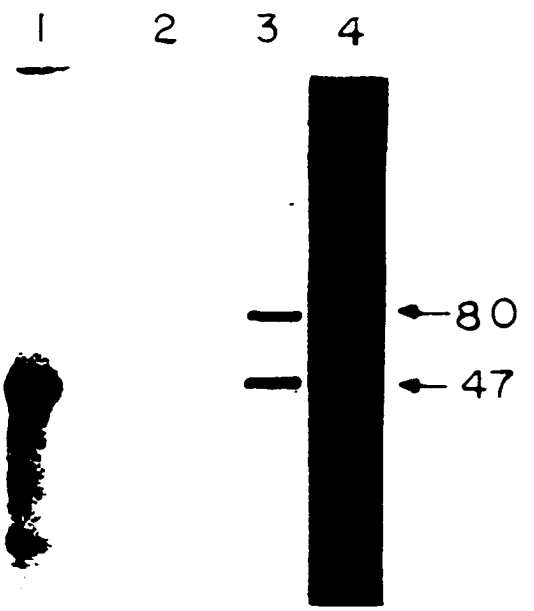

Figure 2. **Phosphorylation of partially purified pig heart trifunctional β-oxidation complex (TOC).** Lane 1, autoradiogram of partially purified pig heart TOC incubated with 10 mM Mg^{+2}; lane 2, same but without Mg^{+2}; lane 3, immunoblot of partially purified pig heart TOC; and lane 4, SDS-PAGE of partially purified pig heart TOC stained with Coomassie blue. Molecular masses are given in kDa.

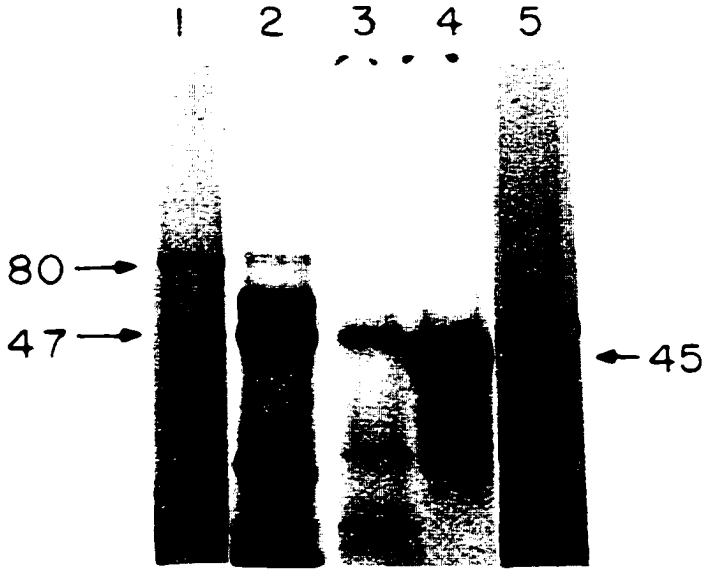

Figure 3. **Phosphorylation of partially purified pig heart TOC by partially purified bovine heart cAMP-dependent protein kinase.** Autoradiogram of partially purified pig heart TOC labeled with ATP^{32} in the presence of cAMP-dependent protein kinase. Lane 1 and 5, SDS-PAGE of partially purified pig heart TOC and partially purified porcine heart pyruvate dehydrogenase (PDH), respectively, stained with Coomassie blue. Lane 2 and 3, autoradiograms of partially purified pig heart TOC with and without partially purified bovine heart cAMP-dependent protein kinase, respectively. Lane 4, autoradiogram of partially purified PDH labeled with ATP^{32}. Molecular masses are given in kDa.

Table 2. **Effect of phosphorylation on 3-ketoacyl-CoA thiolase activity of partially purified pig heart TOC.**

Enzyme	Substrate	3-Ketoacyl-CoA thiolase Activity *U/mg*
Phosphorylated-TOC	3-ketohexadecanoyl-CoA	0.225
Non-Phosphorylated-TOC	3-ketohexadecanoyl-CoA	0.240

Partially purified pig heart TOC was phosphorylated by incubating with 0.1 mM ATP^{32} and 10 mM Mg^{+2} for 30 minutes. Mg^{+2} was omitted from the non-phosphorylated form of TOC. Assays were performed at a fixed concentration of 20 μM of 3-ketohexadecanoyl-CoA. For experimental details see Experimental procedures.

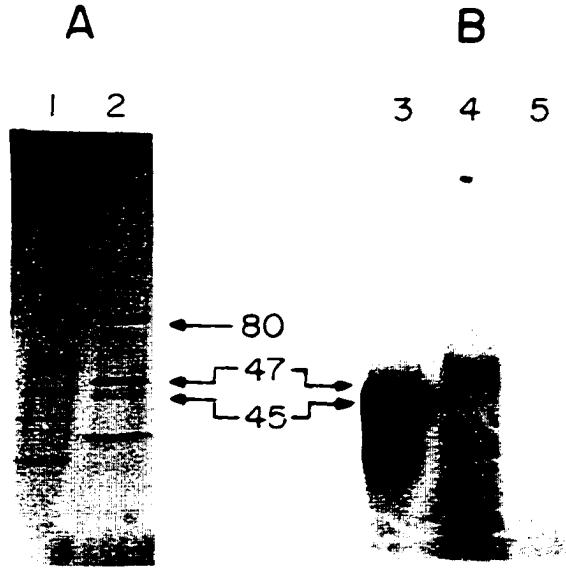

Figure 4. **Phosphorylation of partially purified pig heart TOC in the presence of exogenous partially purified bovine heart cAMP-dependent protein kinase (cAPK).** (A), SDS-PAGE of freshly prepared partially purified pig heart TOC and partially purified bovine heart PDH stained with Coomassie blue. Lane 1, 8 μg partially purified porcine heart PDH and lane 2, 3 μg partially purified pig heart TOC. (B), Autoradiogram of phosphorylated partially purified porcine heart PDH and partially purified pig heart TOC with and without cAPK. Lane 3, partially purified porcine heart PDH; lane 4, partially purified pig heart TOC with cAPK; and lane 5, partially purified pig heart TOC without cAPK. Molecular masses are given in kDa.

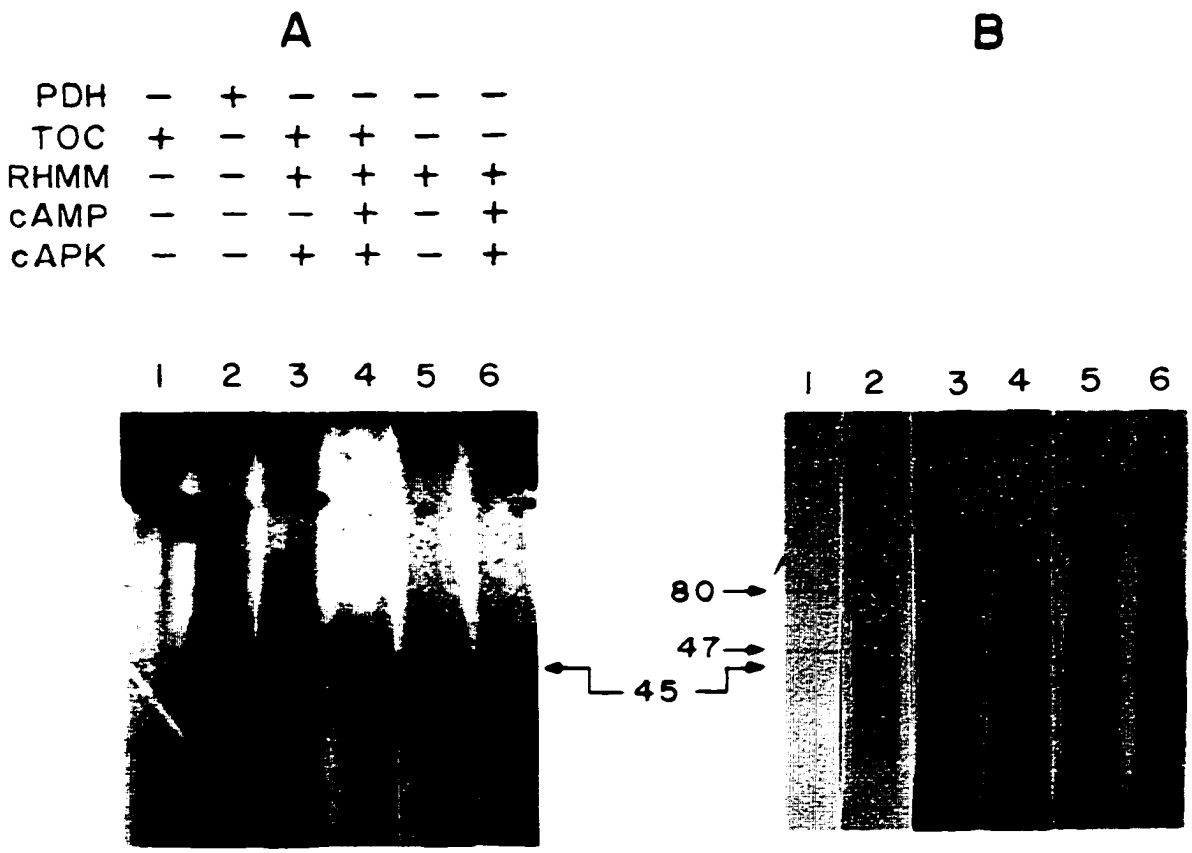

Figure 5. **Phosphorylation of rat heart mitochondrial membrane protein(s) in the presence of exogenous bovine heart cAMP-dependent protein kinase (cAPK).** For rat heart mitochondrial membrane preparations, kinase assay, and other experimental procedures see Experimental Procedures. (A), Autoradiogram of protein bands labeled by [γ-^{32}P] ATP in rat heart mitochondrial membrane with and without exogenous partially purified bovine heart cAMP-dependent protein kinase. (B), Coomassie blue stained SDS-PAGE of (A). Molecular masses are given in kDa.

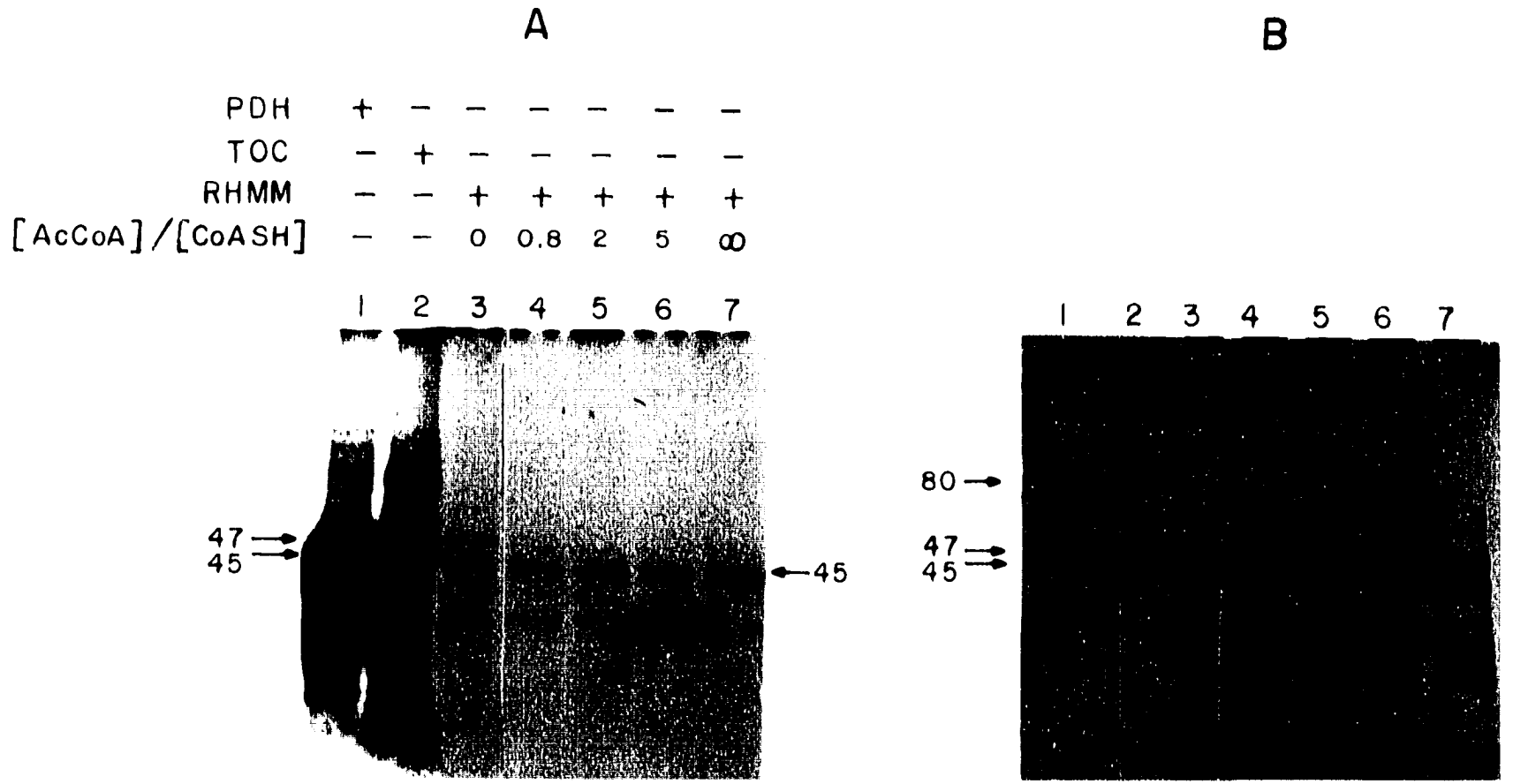

Figure 6. **Effect of [Acetyl-CoA]/[CoASH] on the phosphorylation of rat heart mitochondrial membrane protein(s).** The old preparation of partially purified pig heart TOC was used as reference for TOC detection in rat heart mitochondrial membrane. The concentration of acetyl-CoA plus CoASH was 1.2 mM and the ratios were as shown. (A), Autoradiogram and (B), SDS-PAGE stained with Coomassie blue of samples described in (A). Molecular masses are given in kDa.

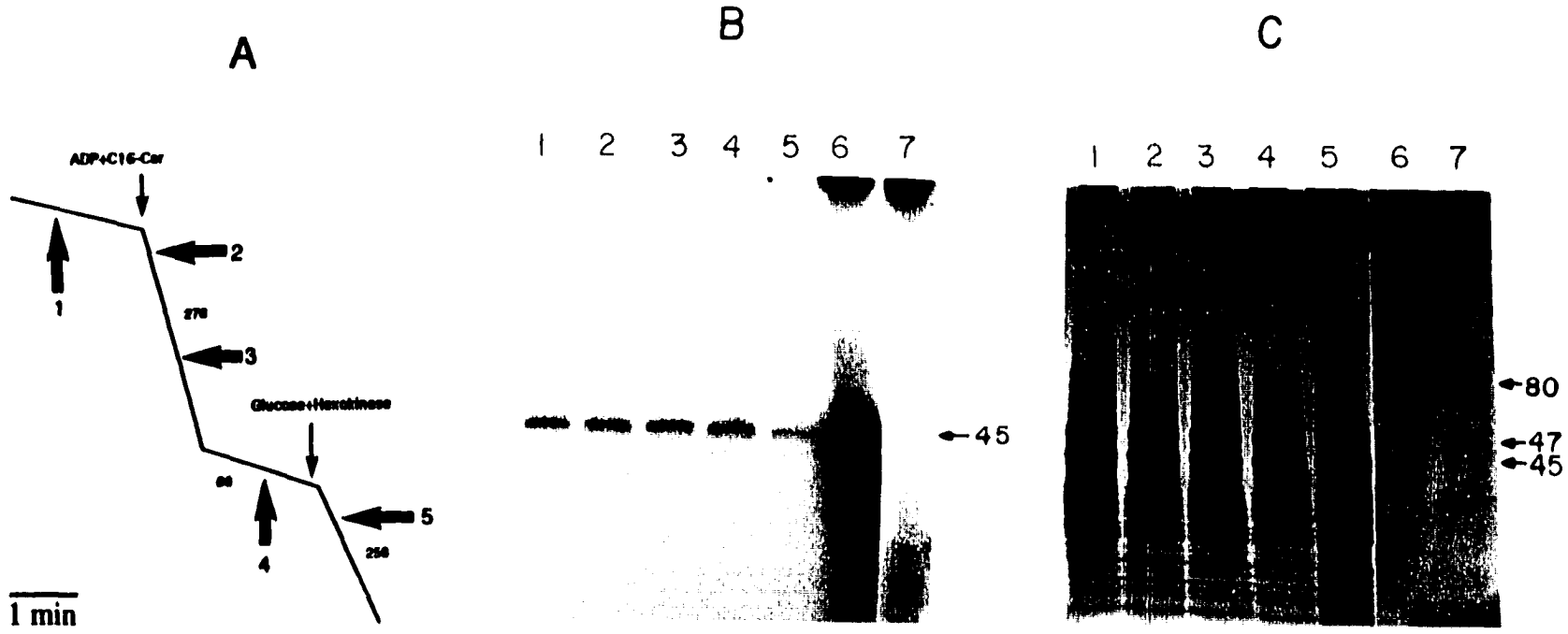

FIGURE 7. **Phosphorylation of proteins in rat heart mitochondria (RHM) incubated at respiration states 3 and 4 with palmitoylcarnitine as substrate.** At time zero, 13 mg of RHM prelabeled with inorganic phosphate (^{32}Pi), were added to 20 ml of a basal isoosmotic medium containing 0.5 mM malate and BSA (0.5 mg/ml). At the point indicated, 0.5 mM ADP and 20 μM palmitoyl-carnitine (C_{16}-car) were added. State-4 respiration was reversed to state-3 by adding 5 mM glucose and 6 units of hexokinase. Samples (2 mg) were withdrawn at various times as indicated by bold arrows and the reactions were terminated by lowering the pH to 1. (A), Trace of respiration supported by palmitoyl-carnitine. The numbers represent the rates of respiration in nmole of oxygen/min/mg of protein. (B), and (C), Autoradiogram and Coomassie blue staining of samples withdrawn at different respiration states. Lane 1, sample 5; lane 2, sample 4; lane 3, sample 3; lane 4, sample 2, lane 5, sample 1; lane 6, partially purified porcine heart PDH; and lane 7, partially purified pig heart TOC. Molecular masses of labeled proteins bands were determined from standard proteins run on the same gel. Molecular masses are given in kDa.

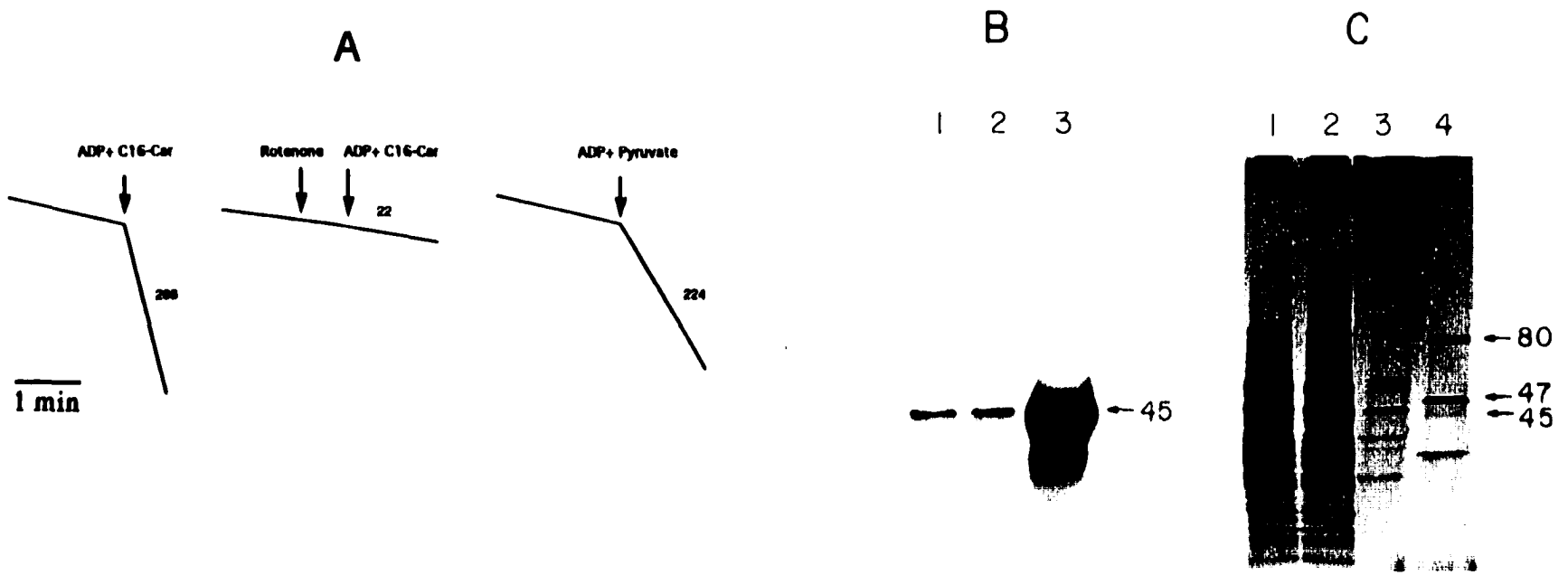

FIGURE 8. **Phosphorylation of rat heart mitochondrial proteins (RHM) during respiration supported by either palmitoylcarnitine in the presence of rotenone or pyruvate.** At time zero, 2 mg of RHM labeled with inorganic phosphate (^{32}Pi), were added to 4 ml of a basal isoosmotic medium containing 0.5 mM malate, 3 μM rotenone and BSA (0.5 mg/ml). Reaction mixtures were incubated for two minutes and at the point indicated, 0.5 mM ADP and 20 μM palmitoyl-carnitine were added. Samples (2 mg) were withdrawn and reactions were terminated after a minute by lowering the pH to 1. (A), Trace of respiration measurements. The numbers represent rates of respiration in nmole of oxygen/min/mg of protein. (B), and (C), Autoradiogram and Coomassie blue-stained SDS-PAGE of RHM incubated with palmitoyl-carnitine in the presence and absence of rotenone and RHM incubated with pyruvate, respectively. Lane 1, RHM incubated with pyruvate; lane 2, RHM incubated with palmitoyl-CoA and rotenone; lane 3, phosphorylated form of partially purified bovine heart PDH; lane 4, partially purified pig heart TOC. Molecular weights of labeled proteins bands were determined from standard protein run on the same gel. Molecular masses are given in kDa.

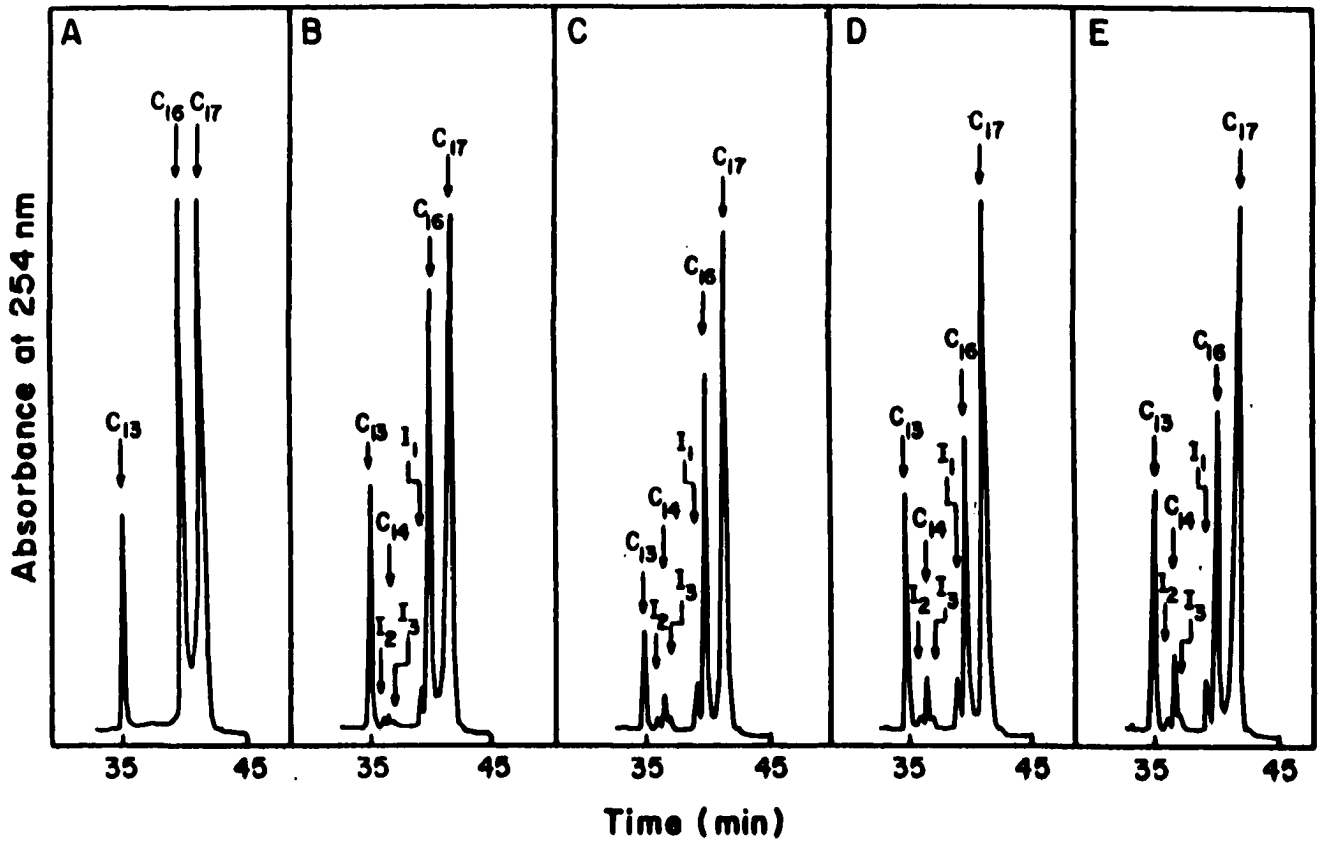

Figure 9. **HPLC profile of intermediates and products formed during the β-oxidation of hexadecanoyl-CoA by system A of rat heart mitochondrial membrane vesicles.** Half a milliliter of the assay mixture contained 0.1 M KPi (pH 7.0), 15 μM hexadecanoyl-CoA, 0.2 mM ferricenium hexafluorophosphate, 1 mM NAD^+, 0.2 mM CoASH, and 0.225 mg of rat heart mitochondrial membrane vesicles. The reaction was allowed to proceed for different periods of time and then were terminated by lowering the pH to1. Panel A, control at time zero. Panels B-E, after 0.5 min, 1 min, 1.5 min, 2 min of incubation, respectively. Peaks identified by use of authentic compounds were: I_1, 2-hexadecenoyl-CoA; I_2, L-3-hydroxyhexadecanoyl-CoA; I_3, 3-ketohexadecanoyl-CoA; C_{14}, myristoyl-CoA; C_{16}, hexadecanoyl-CoA; C_{13} and C_{17} were tridecanoyl-CoA and heptadecanoyl-CoA (internal standard), respectively.

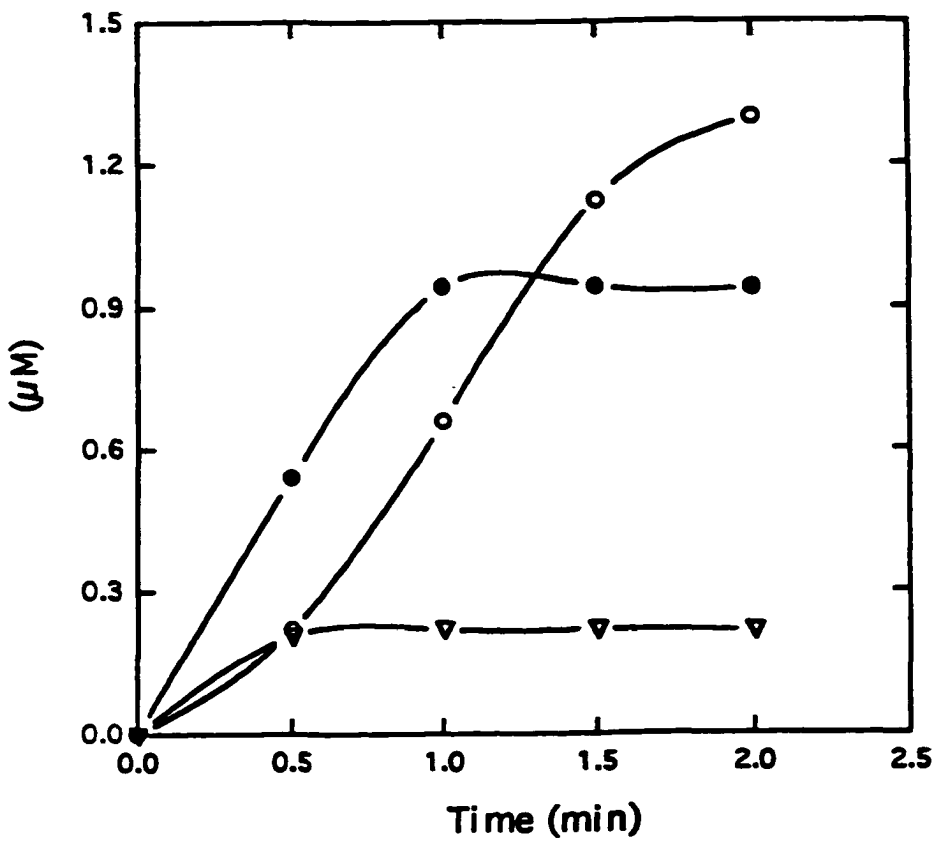

Figure 10. **Intermediate and product accumulation during the β-oxidation of hexadecanoyl-CoA by system A in rat heart mitochondrial membrane vesicles.** Accumulation of L-3-hydroxyhexadecanoyl-CoA (∇), 2-hexadecenoyl-CoA (•) and myristoyl-CoA (o) when 15 μM hexadecanoyl-CoA, 0.2 mM ferricenium hexafluorophosphate, 1 mM NAD^+ and 0.2 mM CoASH were incubated with 0.225 mg of rat heart mitochondrial membrane vesicles. The values are means based on two measurements.

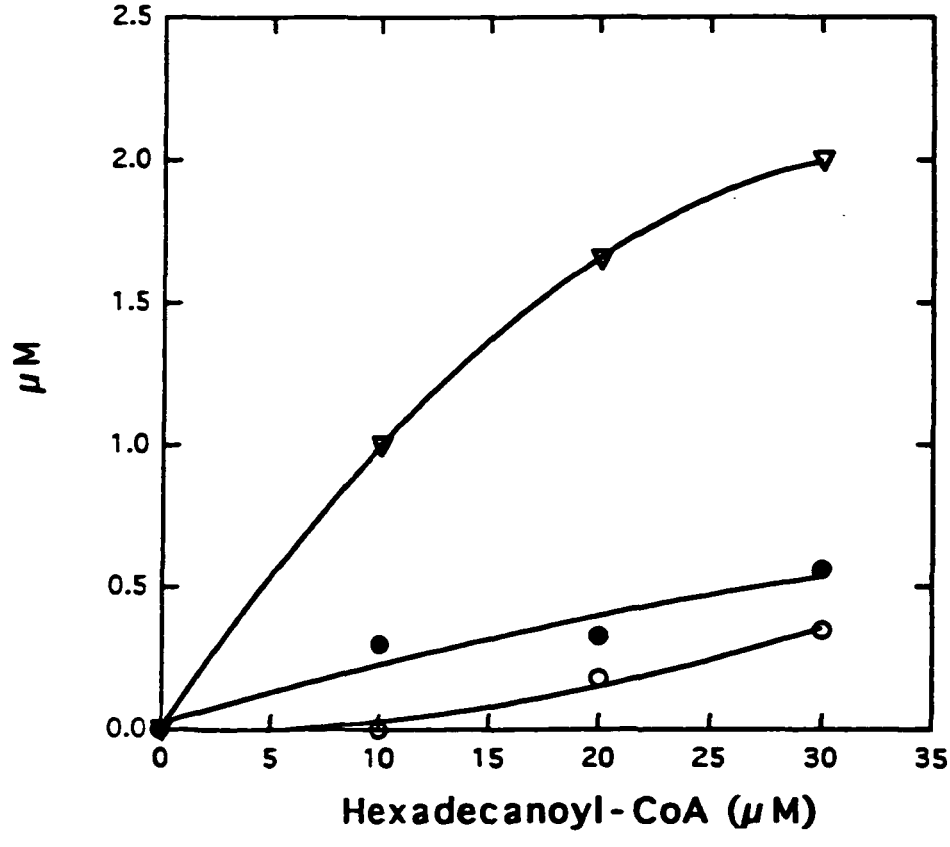

Figure 11. **Accumulation of intermediates in the β-oxidation of hexadecanoyl-CoA at different concentrations by system A in rat heart mitochondrial membrane vesicles.** Accumulation of 2-hexadecenoyl-CoA (∇), L-3-hydroxyhexadecanoyl-CoA (•) and 3-ketohexadecanoyl-CoA (o) when different concentrations of 15 μM hexadecanoyl-CoA were incubated with 0.2 mM ferricenium hexafluorophosphate, 1 mM NAD^+, 0.2 mM CoASH and 0.225 mg of rat heart mitochondrial membrane vesicles. The values are means based on two measurements.

Table 2. **Kinetic parameters of the enoyl-CoA hydratase present in rat heart mitochondrial membrane vesicles.**

Enzyme	**Substrate**	**Km[a]**	**Vmax[a]**
		μM	*Units/mg*
Hydratase[b]	2-Hexadecenoyl-CoA	6.5	24

[b]Enoyl-CoA hydratase activity was determined by the indirect method.
[a]Apparent Km and Vmax values are means of two determinations. Units are in nmole of product/min/mg of rat heart mitochondrial membrane vesicles.

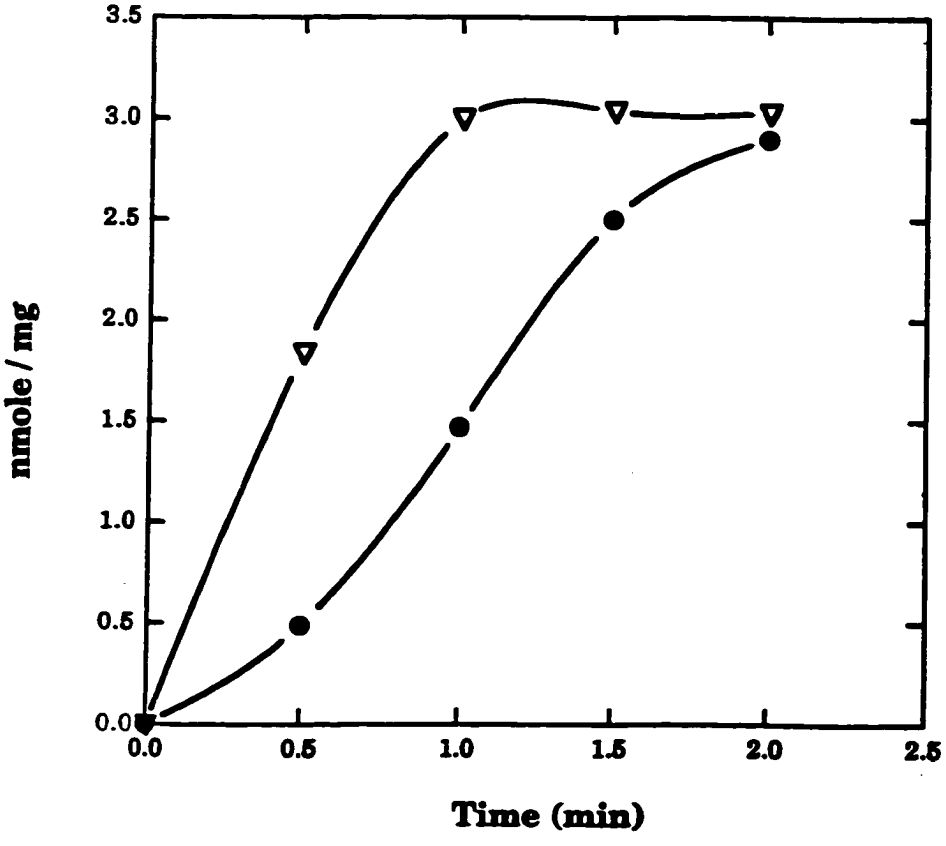

Figure 12. **Comparison of observed and calculated rates of β-oxidation by system A in rat heart mitochondrial membrane vesicles.** Observed rates (•) and calculated rates (∇) based on the accumulated concentration of 2-hexadecenoyl-CoA during β-oxidation by system A in rat heart mitochondrial membrane vesicles and kinetic parameters of 2-enoyl-CoA hydratase.

Table 3. **Influence of the 5-*cis*-double bond in long-chain acyl-CoA on the activities of acyl-CoA dehydrogenases.** For experimental details see Experimental Procedures.

Substrate	MCAD Sp. Act *U/mg*	LCAD Sp. Act *U/mg*	VLCAD Sp. Act *U/mg*	LCAD VLCAD *Ratio*	LCAD MCAD *Ratio*
Tetradecanoyl-CoA	0.211	0.710	0.300	2.4	3.3
5-*cis*-tetradecanoyl-CoA	0.200	0.683	0.035	19.5	3.4

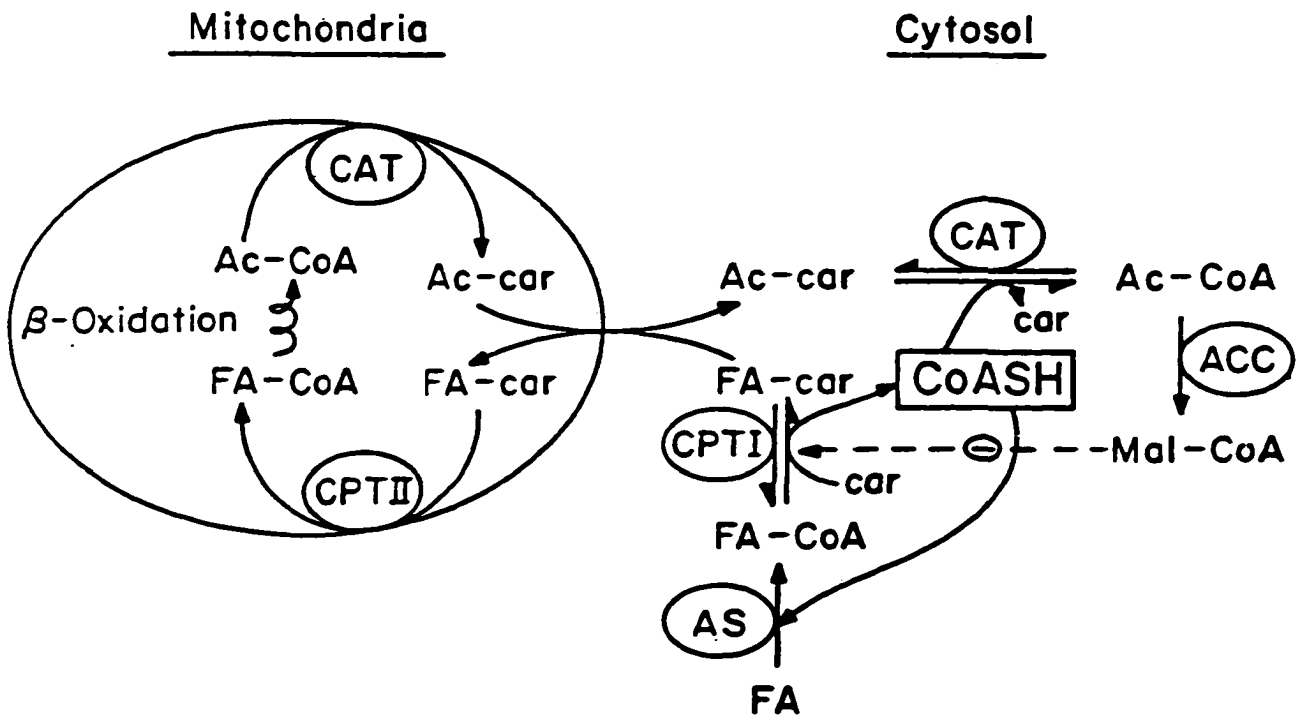

Figure 13. **Proposed regulation of fatty acid oxidation in rat heart in response to changes in the concentration of mitochondrial acetyl-CoA.** Ξ, inhibition. Abbreviation: CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase; ACC, acetyl-CoA carboxylase; AS, long-chain acyl-CoA synthetase; Ac-CoA, acetyl-CoA; FA-CoA, fatty acyl-CoA; Ac-car, acetylcarnitine; FA-car, fatty acylcarnitine; Mal-CoA, malonyl-CoA; FA, fatty acids.

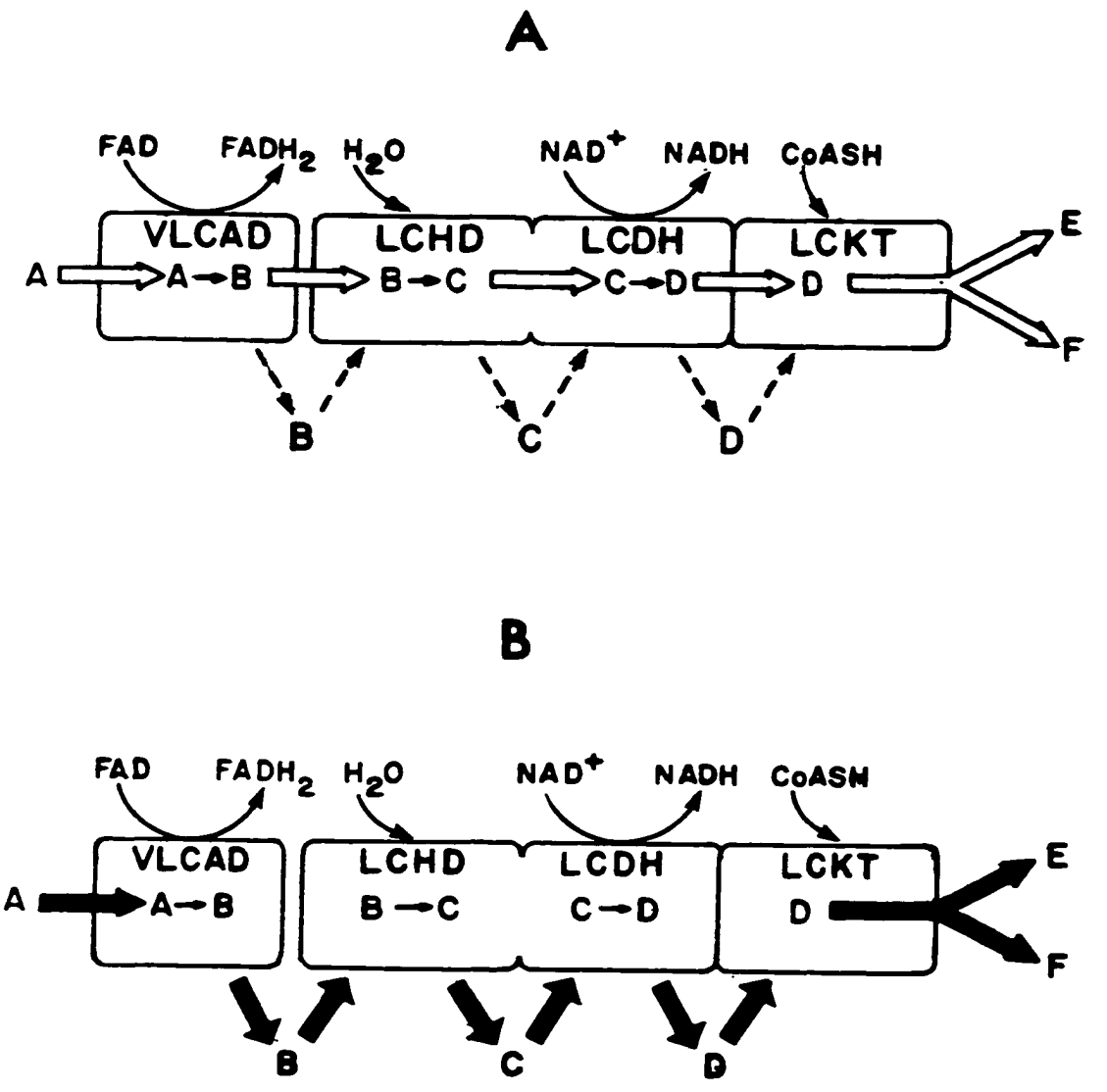

Figure 14. **Schematic presentation of two kinetic models for β-oxidation system A.** 14A, channeling of intermediates; 14B, non-channeling of intermediates. Open arrows (⇨) depict the movement of intermediates from one active site; broken arrows (- - ➤) depict the partial diffusion of intermediates into the bulk medium, whereas bold solid arrows (➡) indicate the diffusion of intermediates into the bulk medium. A, hexadecanoyl-CoA; B, 2-hexadecenoyl-CoA; C, L-3-hydoxyhexadecanoyl-CoA; D, 3-ketohexadecanoyl-CoA; E, acetyl-CoA; and F, tetradecanoyl-CoA.

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