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THE OCCURRENCE OF INTERMEDIATES IN MITOCHONDRIAL FATTY ACID OXIDATION

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Received 26 November 1973

1. Introduction

The pathway of fatty acid oxidation in mitochondria was first suggested in outline by Knoop [1]. Oxidation occurs by successive iterations of a sequence of four reactions. Each iteration results in the release of an acetyl-CoA molecule following the postulated formation of α-β unsaturated, β-hydroxy, and β-keto acyl-CoA intermediates. Dakin [2,3] was able to detect some of these compounds (excreted as free acids) during the oxidation of ω-phenyl fatty acids, but studies with ordinary fatty acids have failed to detect an accumulation of intermediates in mitochondria. Intravenous administration to rats of [6-^{14}C] or [11-^{14}C] palmitic acid (as triglyceride) revealed no difference in the time of appearance of $^{14}CO_2$. Weinman et al. [4] therefore proposed that once the process of breakdown is initiated a palmitic acid molecule is oxidised to completion without the release of intermediates. Garland et al. [5] could account for almost all of the mitochondrial CoA during palmitoylcarnitine oxidation as either free CoA, acetyl-CoA or $HClO_4$-insoluble (long-chain) fatty acyl-CoA, and concluded that short-chain acyl-CoA intermediates could only occur in very small quantities (less than 0.02 nmol/mg of mitochondrial protein). This led to the tentative suggestion [6,7] that fatty acid oxidation may be catalysed by a multienzyme complex. More recently, Bremer and Wojtczak [8] have detected β-hydroxy palmitoylcarnitine formation by mitochondria oxidising palmitoylcarnitine, but significant quantities were only found in the presence of free carnitine and a very high NADH/NAD$^+$ ratio.

Aqueous extracts of acetone-dried mitochondria have already been shown to oxidise short-chain fatty acyl-CoA with the formation of saturated and, to a smaller extent, β-hydroxy acyl-CoA intermediates [9]. This communication demonstrates the existence of intermediates during the oxidation of palmitoylcarnitine by intact mitochondria.

2. Materials and methods

2.1. *[16-^{14}C] palmitoyl-L-carnitine chloride*

This was prepared by a modification of the method of Chase and Tubbs [10]. In order to attain an adequate yield on a small scale it was necessary to dry all glassware and chemicals very thoroughly before use. [16-^{14}C] palmitic acid was supplied by C.E.A. (91, Gif-sur Yvette, France).

The palmitoylcarnitine was separated from unreacted palmitic acid and carnitine on a 3 × 1 cm column of silicic acid (Mallinckrodt Chemical Works, St. Louis, USA) containing 50% by weight of Hyflo Supercel (Hopkins & Williams Ltd., Chadwell Heath, Essex, England). The upper phase of n-butanol:acetic acid:water (4:1:5, by vol) was used as solvent and the radioactive peak eluted from the column was shown to be free from palmitic acid and carnitine by t.l.c. on silica gel G(E. Merck, Darmstadt, Germany) developed in the same solvent; hydrolysis and radio—gas chromatography gave rise to a single peak with the retention time of methyl palmitate.

2.2. *O_2 electrode incubations*

Rat liver mitochondria were isolated, washed twice and resuspended in 0.25 M sucrose containing 10 mM

Tris–HCl buffer, pH 7.2. Incubations were carried out at 18°C in an O_2 electrode vessel (Rank Bros., Bottisham, Cambs., U.K.) containing 20 mM Tris–HCl (pH 7.2), 3.3 mM $MgCl_2$, 3.3 mM potassium phosphate (pH 7.0), 80 mM KCl, 40 mM sucrose, 1 mM ADP, 3.3 mM potassium malonate and 4 mg/ml defatted bovine serum albumin. The volume of the incubation was 3 ml and the concentration of mitochondrial protein was 11 mg/ml. Oxidation was initiated by adding 144 nmoles of palmitoylcarnitine containing 0.1 μCi of ^{14}C and allowed to continue until 12–20% of the oxygen calculated to convert the palmitoylcarnitine into acetyl-CoA had been consumed.

2.3. *Extraction of radioactive products*

Oxidation was terminated by transferring the contents of the electrode vessel, together with a wash of 1 ml of water, into 1 ml of 4 M KOH at 20°C. After 30 min 0.5 ml of 10 M HCl was added and the mixture extracted 3 times with 5 ml of diethyl ether. The ether was dried over anhydrous Na_2SO_4, transferred to a new vessel and evaporated in a stream of N_2. The extracted fatty acids were then methylated in 2 ml of 14% BF_3-methanol [11] for 15 min at 50°C. After hydrolysis of excess reagent the methyl esters were extracted in 2 × 4 ml of n-hexane. Just before analysis the hexane was evaporated and the sample dissolved in 0.1 ml CS_2.

2.4. *Gas chromatography*

Separation of methyl esters was achieved in a Pye Unicam Series 104 Chromatograph (W.G. Pye and Co. Ltd., York Street, Cambridge, U.K.) fitted with 1.5 m × 6 mm (o.d.) glass columns containing 15% Apiezon L coated on 80–100 mesh Phasesep N AW-BW solid support (Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, U.K.). Half the sample from one incubation was injected onto the column. 90% of the effluent gas was led through a combustion tube to a gas flow proportional counter which continuously monitored $^{14}CO_2$. The argon carrier gas was supplemented with 2% propane in order to facilitate counting, which was performed by a Nuclear Chicago ratemeter (333, East Howard Avenue, Des Plaines, Illinois, USA.). The remaining 10% of the effluent gas was passed through a flame ionisation detector.

3. Results and discussion

Fig. 1 (a) shows the gas chromatography of radioactive products derived from the mitochondrial oxidation of [16-^{14}C] palmitoyl-L-carnitine. The conditions of hydrolysis used to extract fatty acids from the mitochondria were designed to be quantitative for acyl-CoA esters. These conditions were sufficiently severe to hydrolyse about 40% of the unoxidised palmitoylcarnitine (and also large quantities of unlabelled fatty acid esters). Consequently the radioactive peak with the retention time of methyl palmitate is derived from both the intramitochondrial palmitoyl-CoA, and also from residual palmitoylcarnitine substrate. For the sake of clarity the mass traces have been omitted from fig. 1. In fig. 1(b) 0.13 μmoles of rotenone were added prior to the substrate.

Since no free CoA or carnitine was added in either incubation the observed intermediates presumably existed as thioesters within the mitochondria. Oxidation of palmitoylcarnitine by any broken mitochondria present is unlikely to be significant in the absence of CoA and NAD [8].

It can be seen in fig. 1(a) that intact mitochondria oxidising palmitoylcarnitine do contain considerable quantities of saturated acyl-CoA intermediates. Sufficient oxygen was consumed in this case to account for 18% of the palmitoylcarnitine being completely oxidised to acetyl units. The observed intermediates, however, appear to be mainly of long chain length. No β-hydroxy or unsaturated acyl-CoA intermediates were observed unless rotenone was added. In fig. 1(b), after 13% of the calculated oxidation of the substrate in the presence of rotenone, β-hydroxy and unsaturated intermediates have been produced, although inhibition of the β-hydroxy acyl-CoA dehydrogenase was by no means complete.

Addition of carnitine to an incubation containing rotenone caused the amount of β-hydroxy intermediates to increase at the expense of the saturated intermediates (data not shown). Hydrolysis in this case was performed at 50°C in order to ensure complete breakdown of carnitine esters. In the absence of rotenone carnitine caused a large increase in the amount of myristate, but still no β-hydroxy intermediates were observed.

These results demonstrate the occurrence of some intermediates during β-oxidation in mitochondria.

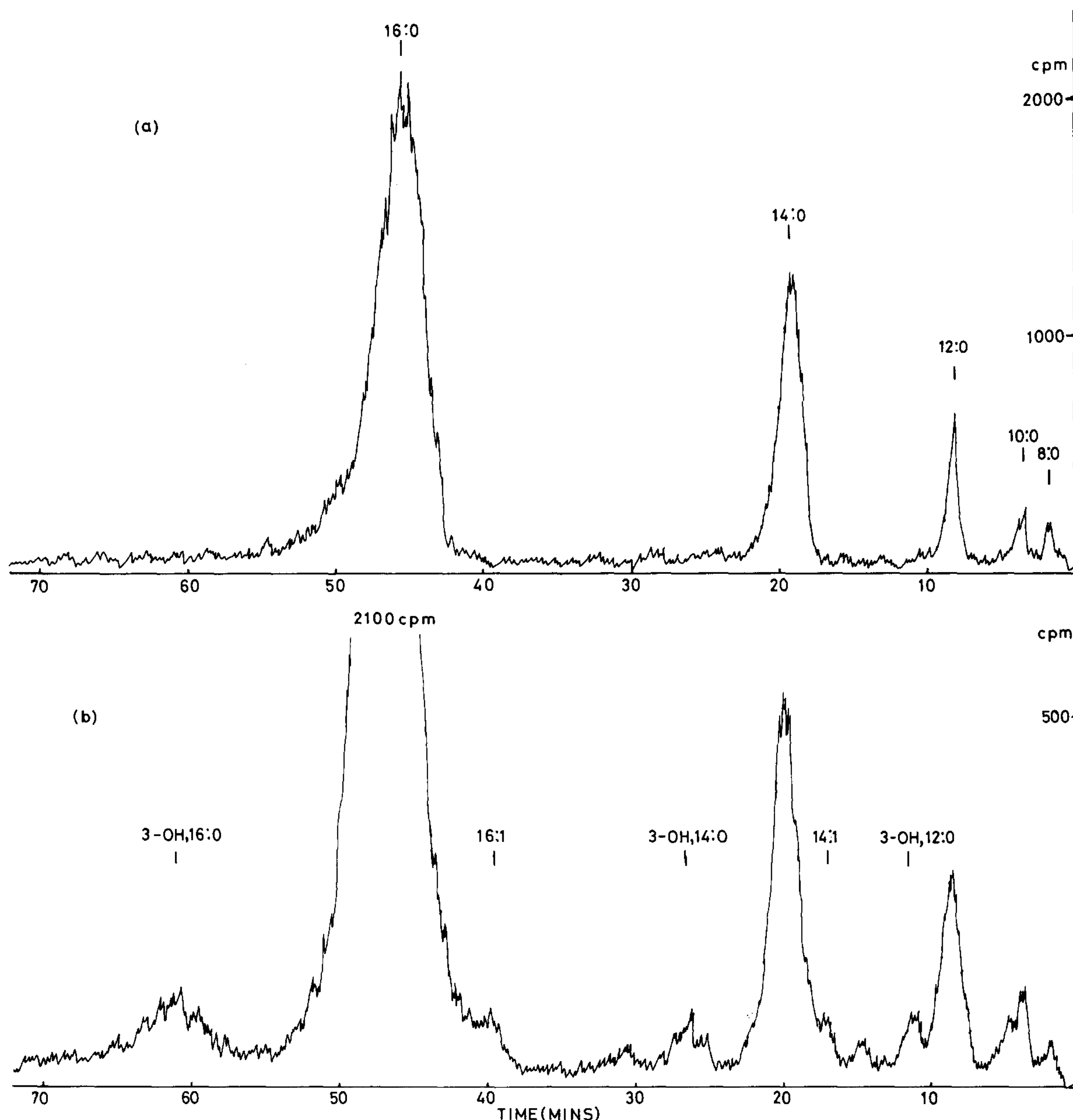

Fig. 1. Intermediates of faty acid oxidation by rat liver mitochondria. Gas chromatograms of radioactive products of [16-^{14}C] palmitoyl-L-carnitine oxidation by rat liver mitochondria. Flow rate of argon was 60 ml/min and the temperature 200°C. (a) Standard incubation (b) rotenone added before substrate. Standard methyl esters: 16:0; 3-OH, 16:0; 16:1 show retention times of methyl palmitate and its β-hydroxy and 2-unsaturated derivatives. Shorter homologues are indicated similarly.

Waterson and Hill [12] suggested that enoyl-CoA hydratase is rate limiting in the presence of acetoacetyl-CoA, but mitochondria oxidising palmitoylcarnitine in the presence of malonate evidently do not contain detectable quantities of unsaturated intermediates. The preponderance of saturated acyl-CoA intermediates may therefore indicate that acyl-CoA dehydrogenase is rate limiting in vivo, or it may reflect some degree of organisation of the enzymes of β-oxidation.

Acknowledgements

K.K.S. thanks the Medical Research Council for a research training grant. The expense of this work was partly met by grants from the Science Research Council (to P.K.T.) and the Medical Research Council of Canada (to Professor H.B. Stewart; ref. [9]).

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