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Intramitochondrial control of the oxidation of hexadecanoate in skeletal muscle

A study of the acyl-CoA esters which accumulate during rat skeletal-muscle mitochondrial β-oxidation of [U-^{14}C]hexadecanoate and [U-^{14}C]hexadecanoyl-carnitine

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1. We describe the acyl-CoA and acyl-carnitine esters which arise from the incubation of well-coupled State 3 rat skeletal-muscle mitochondrial fractions with [U-^{14}C]hexadecanoate and [U-^{14}C]hexadecanoyl-carnitine. 2. Acyl-CoA ester intermediates of chain length 16, 14, 12, 10 and 8 carbons were detected. 3. Although incubations were in steady state in respect of oxygen consumption, $^{14}CO_2$ production and generation of acid-soluble radioactivity, quantitative analysis of acyl-CoA esters showed that steady state was not achieved in respect of all intermediates. 4. 3-Hydroxyacyl- and 2-enoyl-CoA and -carnitine esters were found under normoxic conditions. 5. Direct measurement of NAD^+ and NADH shows that under identical incubation conditions our observations cannot be explained by gross perturbation of the [NAD^+]/[NADH] ratio. 6. We hypothesize that there is a small pool of rapidly recycling NAD^+ channelled between complex I of the respiratory chain and the newly described mitochondrial-inner-membrane-associated β-oxidation trifunctional enzyme [Uchida, Izai, Orii and Hashimoto (1992) J. Biol. Chem. **267**, 1034–1041].

INTRODUCTION

Mitochondrial β-oxidation of long-chain fatty acids is a complex multi-enzyme process subject to regulation at several sites (Kunz, 1991). There is increasing evidence for organization of some of the enzymes of β-oxidation; although there is no direct evidence for the formation of a complex involving all of the enzymes, some degree of supramolecular organization has been suggested from analysis of chain-shortened acyl groups (i.e. the 'leaky hose-pipe' model of Stanley and Tubbs, 1974, 1975). Thus Kerner and Bieber (1990) have shown that a preparation of carnitine palmitoyltransferase (CPT) was associated with a complex containing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase activities. Very recently a trifunctional enzyme which has long-chain 3-hydroxyacyl-CoA dehydrogenase, long-chain 2-enoyl-CoA hydratase and long-chain 3-oxoacyl-CoA thiolase activities has been isolated and characterized from rat and human liver and is present in all tissues studied (Uchida et al., 1992; Carpenter et al., 1992) and we have described children deficient in this trifunctional enzyme (Jackson et al., 1991, 1992). β-Oxidation of long-chain fatty acids is regulated by a number of different factors including substrate supply, the recycling of cofactors and the disposal of acetyl-CoA (Oram et al., 1973). The entry of acyl groups into the matrix space is catalysed by the carnitine acyltransferases (CPT) and the carnitine/acylcarnitine translocase. In muscle CPT I is very sensitive to inhibition by malonyl-CoA (Saggerson and Carpenter, 1981), but the physiological importance of this inhibition is uncertain, as fatty acid synthesis is not active in muscle and there is no obvious source for malonyl-CoA, because acetyl-CoA carboxylase activity is low and muscle mitochondria lack a citrate transporter to generate cytosolic acetyl-CoA (England and Robinson, 1969).

The acyl-CoA dehydrogenases are regulated by product inhibition and by the redox state of the mitochondria. The acyl-CoA dehydrogenases are inhibited by 2-enoyl-CoA, 3-hydroxyacyl-CoA and particularly by 3-oxoacyl-CoA esters ($K_i = 0.07\ \mu M$ for 3-oxodecanoyl-CoA; Davidson and Schulz, 1982), and it has been suggested that 3-oxoacyl-CoA esters may function *in vivo* as feedback inhibitors of fatty acid oxidation. Reducing equivalents generated by the acyl-CoA dehydrogenases are transferred to the ubiquinone pool and complex III of the respiratory chain via electron-transfer flavoprotein (ETF) and ETF:coenzyme Q oxidoreductase. As ETF semiquinone is a potent inhibitor of the acyl-CoA dehydrogenases (Beckman et al., 1981) and electrons can flow between the NAD^+/NADH, succinate/fumarate and ETF (oxidized form)/ETF semiquinone pools (Frerman, 1987), the acyl-CoA dehydrogenases can be controlled by the redox state of mitochondria (Kunz, 1988).

The most active of the enzymes of β-oxidation is short-chain 2-enoyl-CoA hydratase, and it has been suggested that this enzyme might be important in the regulation of β-oxidation because of the wide range of activities in relation to substrate chain length and sensitivity to inhibition by acetoacetyl-CoA ($K_i = 1.6\ \mu M$; Waterson and Hill, 1972). However, the presence of an acetoacetyl-CoA-insensitive 2-enoyl-CoA hydratase with optimal activity for oct-2-enoyl-CoA (Fong and Schulz, 1981) and the demonstration of long-chain hydratase activity of the trifunctional protein (Uchida et al., 1992) suggest that the short-chain enzyme has little impact on the control of the pathway.

The 3-hydroxyacyl-CoA dehydrogenases are predominantly regulated by the redox state. β-Oxidation flux is impaired by rotenone inhibition of complex I of the respiratory chain (Bremer and Wojtczak, 1972), in mitochondria from patients with inherited disorders of complex I (Watmough et al., 1990) and in the hypoxic isolated perfused myocardium (Moore et al., 1982). It

Abbreviations used: CPT, carnitine palmitoyltransferase; ETF, electron-transfer flavoprotein.

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has been argued that in all these situations the [NAD^+]/[NADH] ratio is unphysiologically low and that therefore little control of the pathway is exerted by the 3-hydroxyacyl-CoA dehydrogenases under normal conditions (Bremer and Wojtczak, 1972; Kunz, 1988). However, in a more recent publication Kunz (1991) has demonstrated that in isolated liver mitochondria control of the pathway shifts with flux and that at high rates of flux there is significant control exerted at the 3-hydroxyacyl-CoA dehydrogenase step.

In view of the importance of muscle β-oxidation in respect of whole-body fat metabolism (Felig and Wahren, 1975) and the aetiology of inborn errors of fatty acid oxidation (Bartlett et al., 1991), it is important to study the intramitochondrial regulation of β-oxidation in muscle tissues. Furthermore, the recent discovery of a trifunctional protein and its localization in the mitochondrial inner membrane (Uchida et al., 1992; Carpenter et al., 1992) has rekindled interest in the relation of β-oxidation to the respiratory chain. We have developed techniques for the analysis of radiolabelled intact CoA esters (Watmough et al., 1989) and carnitine esters (Bhuiyan and Bartlett, 1988; Bhuiyan et al., 1992) derived from the oxidation of [U-^{14}C]hexadecanoate. These methods have been applied to the study of inherited disorders of mitochondrial β-oxidation in fibroblast and muscle mitochondria (Singh Kler et al., 1991; Jackson et al., 1992) and of the respiratory chain in muscle mitochondria (Watmough et al., 1990). In the present study we have used these techniques to further our understanding of the intramitochondrial control of β-oxidation in rat skeletal muscle by measuring flux, intermediates and the [NAD^+]/[NADH] ratio during substrate-limited pulses of [U-^{14}C]hexadecanoate oxidation.

EXPERIMENTAL

Materials

CoA, crotonase, cytochrome *c*, ADP, ATP, NADPH and L-carnitine were from Sigma Chemical Co., Poole, Dorset, U.K. Fatty acids were from Aldrich Chemical Co., Gillingham, Dorset, U.K. Acyl-CoA synthetase, acyl-CoA oxidase, NADH, NAD^+, $NADP^+$ and BSA (fraction V, fatty acid free) were obtained from Boehringer Corp., Mannheim, Germany. DEAE-Sephacel was from Pharmacia, Uppsala, Sweden. Acetonitrile (S grade) was from Rathburn Chemicals, Walkerburn, Scotland, U.K. H.p.l.c.-grade water, KH_2PO_4, triethylamine, H_3PO_4, KCl and all other chemicals (AnalaR grade) were from BDH, Poole, Dorset, U.K. Soluble bovine liver CPT (Ramsay et al., 1987) was a gift from Dr. J. P. Derrick, Department of Biochemistry, University of Cambridge, Cambridge, U.K. [U-^{14}C]Hexadecanoate (sp. radioactivity 928 μCi/μmol) was from Amersham International, Amersham, Bucks., U.K., and was used for radiochemical experiments as a BSA complex (hexadecanoate:albumin molar ratio 5:1; Sherratt et al., 1988).

Synthesis of CoA and carnitine esters

Acyl-CoA standards were synthesized as described by Watmough et al. (1989) or by the mixed-anhydride method of Benert and Sprecher (1977). Carnitine esters were synthesized as described by Bhuiyan et al. (1987) or were gifts from Dr. M. Pourfarzam. [U-^{14}C]Hexadecanoyl-carnitine was synthesized enzymically in two stages, by using acyl-CoA synthetase and CPT. [U-^{14}C]Hexadecanoate (2.06 μmol, sp. radioactivity 120 μCi/μmol) was dissolved in 300 μl of 1 M KOH at 40–50 °C, and incubated with 0.7 unit of acyl-CoA synthetase for 3 h at 30 °C in medium containing 50 mM KH_2PO_4, 0.1 % (v/v) Triton X-100, 10 mM $MgCl_2$, 10 mM ATP and 3 mM CoA, pH 7.2 and final volume 5.0 ml (Pourfarzam and Bartlett, 1991). The reaction was terminated by addition of HCl to pH 2. Excess substrate was extracted with diethyl ester, the pH adjusted to 5 by the careful addition of 1 M KOH and the mixture purified by using a C_{18} Bond-Elut cartridge (Pourfarzam and Bartlett, 1991). The purity was checked by radio-h.p.l.c. as described by Watmough et al. (1989). The yield was 95 %. [U-^{14}C]Hexadecanoyl-carnitine was synthesized by using purified CPT in a reaction mixture containing 1.57 μmol of [U-^{14}C]hexadecanoyl-CoA, 10 mM L-carnitine and 50 mM Hepes, pH 7.0. The incubation was maintained at 25 °C for 14 h. [U-^{14}C]Hexadecanoyl-carnitine was extracted with 2 × 2 ml of butanol saturated with water. The extracts were combined and free carnitine was removed with 2 ml of water saturated with butanol. Butanol was removed under N_2 at 40–45 °C. The product was purified from [U-^{14}C]hexadecanoyl-CoA that had not reacted by using a DEAE-Sephacel column as described by Watmough et al. (1989). The purity was checked by radio-h.p.l.c. of the 4-bromophenacyl derivative as described by Pourfarzam and Bartlett (1991); a single radiochemical peak was obtained. The yield was 90 % with respect to [U-^{14}C]-hexadecanoyl-CoA. The product was diluted to 60 μCi/μmol with hexadecanoyl-carnitine.

Mitochondrial preparation

Mitochondria were prepared from hind-leg muscle or from the hearts of 18 h-fasted male Wistar rats as described by Watmough et al. (1988). Mitochondrial protein was determined by the modified Lowry method of Peterson (1977), by using Boehringer Precimat standards.

Polarography

Oxygen uptake was measured polarographically at 30 °C and pH 7.4 in a final volume of 0.74 ml of medium containing 1–3 mg of mitochondrial protein, 120 mM KCl, 10 mM Hepes, 10 mM potassium phosphate and 1 mM EDTA supplemented with BSA (2 mg/ml) and cytochrome *c* (0.2 mg/ml). Respiratory control ratios were measured with 10 mM glutamate plus 1 mM malate (Sherratt et al., 1988), and with glutamate plus malate plus hexadecanoyl-carnitine (50 μM) in the absence of BSA.

Radiochemical incubation conditions

All incubations were carried out at 30 °C in a shaking water bath (160 strokes/min). Incubations with [U-^{14}C]hexadecanoyl-carnitine were in 1.0 ml of medium containing 110 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 1 mM EGTA, 0.2 mg/ml cytochrome *c*, 100 μM CoA and 5 mM ADP, pH 7.4, with mitochondrial suspension (1–3 mg of protein), and after 5 min preincubation reactions were started with 30 nmol of substrate (sp. radioactivity 60 μCi/μmol). Incubations with [U-^{14}C]hexadecanoate were carried out in the above medium plus 100 μM CoA/5 mM ATP/0.5 mM carnitine with (1–3 mg of) skeletal muscle or heart mitochondrial protein. After 5 min preincubation, reactions were started by addition of 60 nmol of [U-^{14}C]hexadecanoate (sp. radioactivity 60 μCi/μmol, as a 5:1 BSA complex).

All incubations were quenched with 200 μl of acetic acid. $^{14}CO_2$ and total acid-soluble radioactivity were measured as described by Watmough et al. (1988), and acyl-CoA and acyl-carnitine ester fractions were separated and measured by radio-h.p.l.c. as described by Singh Kler et al. (1991), except that a

Hypersil 5ODS column (Technicol) was used for analysis of CoA esters in the experiments using [U-^{14}C]hexadecanoyl-carnitine and a cartridge was used for the experiments using [U-^{14}C]-hexadecanoate. Hypersil 5MOS or Techsphere hexyl columns (HPLC Technology) were used for the analysis of acyl-carnitines.

Measurement of mitochondrial NAD^+ and NADH concentrations

Incubations were carried out with unlabelled hexadecanoate as described for the radiochemical experiments. Samples for NAD^+ analysis were quenched with 100 μl of 5 M $HClO_4$, centrifuged, and the supernatant was neutralized with 5 M KOH. After re-centrifugation, 200 μl of the supernatant was analysed by h.p.l.c. as below. Samples for NADH analysis were quenched with 100 μl of 0.5 M KOH/50 % (v/v) ethanol/35 % (w/v) CsCl, centrifuged, and the supernatant was adjusted to pH 8 with 0.5 M triethanolamine/0.4 M KH_2PO_4/0.1 M K_2HPO_4. After re-centrifugation, the supernatant was analysed by h.p.l.c.

H.p.l.c. was carried out with a Spectra-Physics SP8700 solvent-delivery system fitted with a Rheodyne 7125 loop injector, a Pye Unicam LC3 u.v. detector (detection at 260 nm) and Shimadzu RF-535 fluorescence detector (excitation and emission wavelengths at 339 and 460 nm respectively) connected in series, with a Techsphere 5 μm SAX column (from HPLC Technology). The gradient of 5 mM KH_2PO_4 (pH 4.0), 0.5 M KH_2PO_4 (pH 5.0) and 1 M KCl was as used by Reiss et al. (1984), with a flow rate of 1.5 ml/min. The column was maintained at 27 °C.

RESULTS

Polarography

Respiratory control ratios greater than 3 were obtained with 10 mM glutamate plus 1 mM malate and did not decrease with the inclusion of hexadecanoyl-carnitine in a medium without BSA, thus showing that mitochondria were not uncoupled by the substrate.

Products and intermediates of β-oxidation of [U-^{14}C]hexadecanoyl-carnitine

The measurement of total acid-soluble radioactivity (including tricarboxylic-acid-cycle intermediates and short-chain acyl-carnitines) from [^{14}C]hexadecanoate has been previously proposed as a better measure of β-oxidation flux than $^{14}CO_2$ release in muscle (Veerkamp et al., 1986; Watmough et al., 1988) and liver (Sherratt and Osmundsen, 1976). This finding was confirmed for muscle mitochondria oxidizing [U-^{14}C]hexadecanoyl-carnitine (results not shown). $^{14}CO_2$ was a minor product in most cases and formed a good measure of flux only in the absence of both 1 mM malate and 2 mM carnitine, similar to rat skeletal-muscle mitochondria oxidizing free [U-^{14}C]hexadecanoate (Watmough et al., 1988).

The most striking finding from our analysis of the CoA ester fraction from all the incubations with [U-^{14}C]hexadecanoyl-carnitine was the presence of 3-hydroxyacyl- and 2-enoyl CoA esters (Figure 1a). The identity of hexadec-2-enoyl-CoA was confirmed by spectral analysis using photodiode-array detection (Watmough et al., 1989). However, the chromatographic resolution was not sufficient to quantify these peaks separately. Similarly, hexadec-2-enoyl-carnitine and 3-hydroxyhexadecanoyl-carnitine were found in the carnitine ester fraction in incubations made in the presence of 0.5 mM carnitine (Figure 1b). The amounts of long-chain CoA esters (hexadec-2-enoyl-CoA, tetradecanoyl-CoA, 3-hydroxyhexadecanoyl-tetradec-2-enoyl-CoA) increased with time even though flux measured as total acid-soluble radioactivity, $^{14}CO_2$ production or oxygen consumption suggested that steady-state conditions were prevailing.

CoA and carnitine esters derived from β-oxidation of [U-^{14}C]hexadecanoate

In view of the unexpected accumulation of 3-hydroxyacyl- and 2-enoyl-CoA and carnitine esters, the experiments were repeated with [U-^{14}C]hexadecanoate to see if the results were due to the use of [U-^{14}C]hexadecanoyl-carnitine as a substrate. In addition, a chromatographic method was developed to measure NAD^+ and NADH concentrations.

The CoA esters occurring during β-oxidation of [U-^{14}C]-hexadecanoate are shown in Figure 2. Levels of hexadecanoyl-CoA reached approx. 9 nmol/mg of protein within 2 min, in both the presence and the absence of 1 mM malate, showing that the activation of hexadecanoate by acyl-CoA synthetase was not rate limiting. The concentration of hexadecanoyl-CoA remained high throughout the incubations, except when malate was present, when it declined to 1 nmol/mg of protein, owing to substrate depletion. Acetyl-CoA was only detected in the absence of malate, rising to 1.2 nmol/mg of protein after 8 min. Both 3-hydroxyacyl-CoA and 2-enoyl-CoA esters were detected, as was

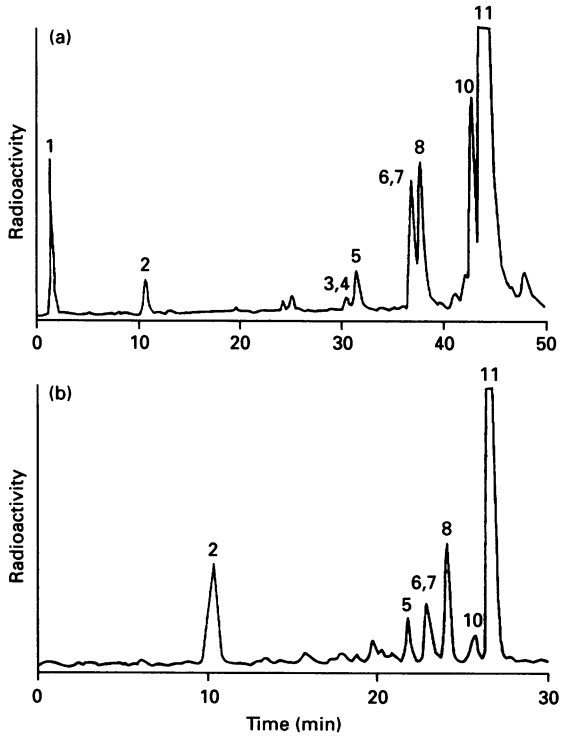

Figure 1 Radio-h.p.l.c. analysis of (a) acyl-CoA esters and (b) acyl-carnitine esters arising from the incubation of skeletal-muscle mitochondria with [U-^{14}C]hexadecanoyl-carnitine (in the presence of 0.5 mM carnitine in b)

The identities of the peaks are as follows: 1, tricarboxylic-acid-cycle intermediates; 2, acetyl; 3, dodec-2-enoyl; 4, 3-hydroxytetradecanoyl; 5, dodecanoyl; 6, tetradec-2-enoyl; 7, 3-hydroxyhexadecanoyl; 8, tetradecanoyl; 10, hexadec-2-enoyl; 11, hexadecanoyl. The conditions of incubation and analysis were as described in the Experimental section.

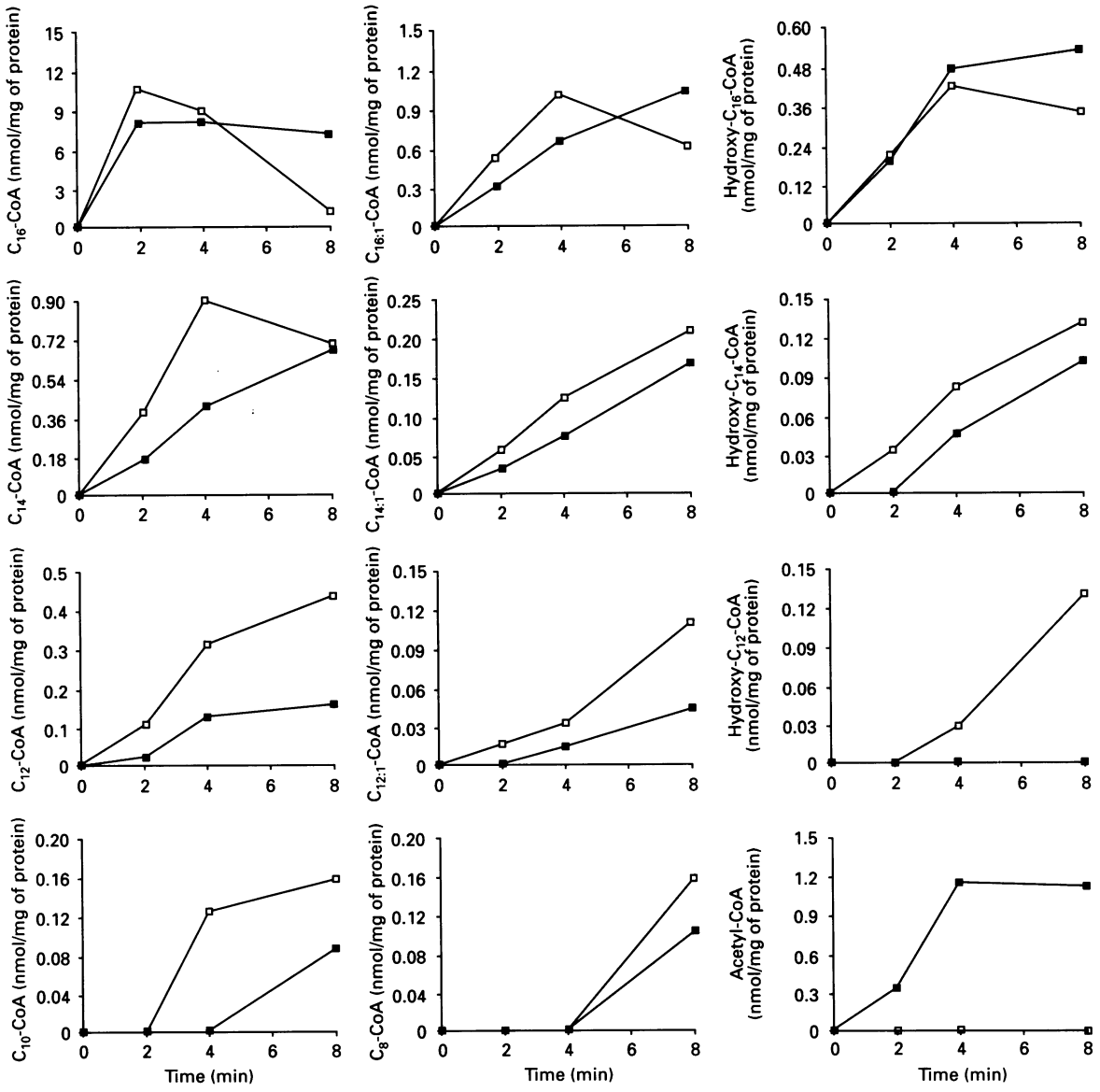

Figure 2 Generation of acyl-CoA esters by skeletal-muscle mictochondria incubated with [U-^{14}C]hexadecanoate in the presence (□) or absence (■) of malate (1 mM)

Conditions of assay and analysis were as described in the Experimental section.

observed in the incubations with [U-^{14}C]hexadecanoyl-carnitine. The resolution of (C_n)3-hydroxyacyl-CoA and (C_{n-2})2-enoyl-CoA esters was improved, so that quantification of these CoA esters was possible. Typical chromatograms are shown in Figure 3. The 3-hydroxyacyl-CoA and 2-enoyl-CoA esters for each chain length were comparable in amount, owing to the equilibrium of 2-enoyl-CoA hydratase, and were each approximately one-third of the amount of the corresponding saturated acyl-CoA esters. As in the incubations with [U-^{14}C]hexadecanoyl-carnitine, the concentrations of some CoA esters increased throughout the time course, rather than a steady state being reached (see, for example, Figure 2; tetradec-2-enoyl-CoA and 3-hydroxytetradecanoyl-CoA). There was a lag phase in the production of the shorter-chain CoA esters which was more pronounced in the absence of malate, and increased with decreasing chain length.

The carnitine esters detected during β-oxidation of [U-^{14}C]-hexadecanoate are shown in Figure 4. Apart from saturated acyl-carnitines, hexadec-2-enoyl-carnitine and 3-hydroxyhexadecanoyl-carnitine were also observed. Hexadecanoyl-carnitine was formed rapidly, rising to 3.2 nmol/mg of protein within 2 min in the presence of 1 mM malate and 6 nmol/mg of protein in the absence of malate, and therefore it is unlikely that CPT I has high control strength under these experimental conditions. Hexadec-2-enoyl-carnitine and 3-hydroxyhexadecanoyl-carnitine were both formed faster in the absence of malate than in its presence, whereas the other carnitine esters seen were present in greater concentrations in the presence of 1 mM-malate, although the amounts of tetradecanoyl-carnitine and dodecanoyl-carnitine had begun to decrease in the later time points in the presence of malate. As well as the carnitine esters shown, some shorter-chain-length esters were also seen at the 8 min time

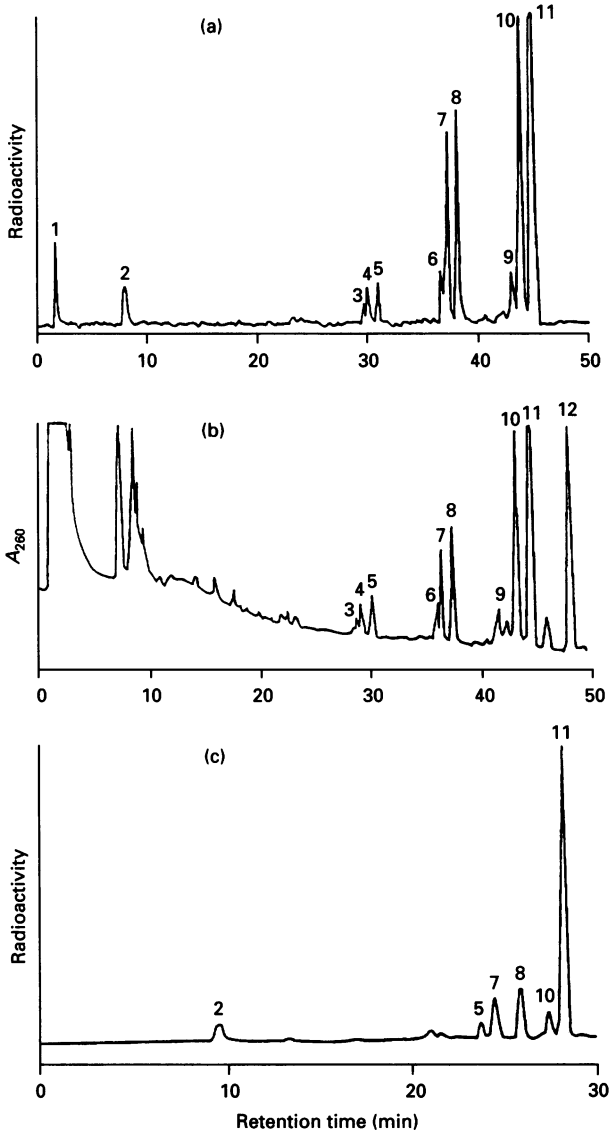

Figure 3 H.p.l.c. analysis of acyl-CoA esters (a, radio-chromatogram; b, 260 nm u.v. chromatogram) and acyl-carnitine esters (c, radiochemical chromatogram) resulting from the incubation of skeletal-muscle mitochondria with [U-^{14}C]hexadecanoate in the absence of malate

The identities of the peaks are as follows: 1, tricarboxylic-acid-cycle intermediates; 2, acetyl; 3, dodec-2-enoyl; 4, 3-hydroxytetradecanoyl; 5, dodecanoyl; 6, tetradec-2-enoyl; 7, 3-hydroxyhexadecanoyl; 8, tetradecanoyl; 9, hexadec-3-enoyl; 10, hexadec-2-enoyl; 11, hexadecanoyl; 12, heptadecanoyl (internal standard). The conditions of incubation and analysis were as described in the Experimental section.

point in the presence of malate (for example octanoyl-carnitine, 1.6 nmol/mg of protein).

Measurement of NAD$^+$ and NADH during hexadecanoate oxidation

Incubations were carried out as described for [U-^{14}C]-hexadecanoate, but with 60 nmol of unlabelled hexadecanoate as the substrate in the presence and absence of 2.5 μM rotenone. Incubations were quenched at intervals after the start of pre-incubation, and the NAD$^+$, NADH and NADPH concentrations are shown in Figure 5. Muscle mitochondria as isolated were partially reduced (NADH was 0.44 nmol/mg of protein, representing 18% reduction), presumably owing to low oxygenation of the concentrated mitochondrial suspension. This reduction disappeared to immeasurable concentrations of NADH within 2 min of preincubation. After addition of substrate, NADH concentrations rose slightly to about 0.11 nmol/mg of protein (representing 4% reduction) and without NADPH accumulation. In the presence of rotenone, NADH increased to 1.1 nmol/mg of protein during the preincubation, probably owing to the utilization of endogenous substrates. NADPH concentrations also increased, to 0.39 nmol/mg of protein, owing to the action of the energy-dependent NADH–NADPH transhydrogenase. After addition of substrate to the rotenone-inhibited mitochondria, NADH concentrations rose to 2.1 nmol/mg of protein (84% reduction) over 8 min. Measurement of the corresponding NAD$^+$ concentrations was difficult, as they were near the limit of detection, but the results correlated well with the NADH, with amounts of nearly 2.4 nmol/mg of protein during hexadecanoate oxidation, but falling to 0.4 nmol/mg of protein in rotenone-inhibited mitochondria, representing an [NAD$^+$]/[NADH] ratio of about 0.2 at 8 min. NADP$^+$ could not be quantified, as it was not resolved from other u.v.-absorbing compounds.

DISCUSSION

The radio-chromatographic methods used in the present study allow the measurement of intramitochondrial acyl-CoA ester intermediates; other workers, by the measurement of free acids after alkaline hydrolysis, measure in addition acyl-carnitine esters accumulated extramitochondrially owing to the action of CPT and the translocase (e.g. Veerkamp et al., 1983; Jin et al., 1992). Similarly, the higher amounts of acyl-carnitine esters compared with the corresponding acyl-CoA esters reflect differences in distribution. Mahadevan et al. (1970) and Al-Arif and Blecher (1971) have shown, at least for the forward direction, that although 3-hydroxyacyl-CoA, 2-enoyl-CoA and 3-oxoacyl-CoA esters are substrates for CPT, their rates of transfer are lower than those of the corresponding saturated acyl-CoA esters. Hence the pattern seen in the carnitine ester fraction may not directly reflect the CoA ester fraction within the mitochondrial matrix. Indeed, our results are consistent with this, since the ratio between 2-enoyl or 3-hydroxyacyl and the corresponding saturated CoA esters is higher than that of the carnitine esters. Furthermore, in many incubations 3-hydroxyhexadecanoyl-carnitine and hexadec-2-enoyl-carnitine were the only carnitine esters detected in addition to saturated acyl-carnitines, even though shorter-chain 3-hydroxyacyl-CoA and 2-enoyl-CoA esters were present.

The production of 3-hydroxyacyl- and 2-enoyl-CoA esters by rat skeletal-muscle mitochondria oxidizing [U-^{14}C]hexadecanoyl-carnitine or [U-^{14}C]hexadecanoate, in the absence of respiratory poisons, was unexpected. Previous studies of acyl groups formed from hexadecanoate (as free acids after alkaline hydrolysis) have shown generation of 3-hydroxyacyl and 2-enoyl groups only under conditions when disposal of reducing equivalents via the respiratory chain is impaired, e.g. State 4 conditions, ischaemia or impaired activity of complex I owing to rotenone inhibition or enzyme deficiency (Bremer and Wojtczak, 1972; Stanley and Tubbs, 1974, 1975; Lopez-Cardozo et al., 1978; Moore et al., 1980; Watmough et al., 1989, 1990). Most of these studies, however, were carried out on liver mitochondria. Previous studies of rat muscle mitochondria have shown production of tetradecanoate, dodecanoate and decanoate from hexadecanoate

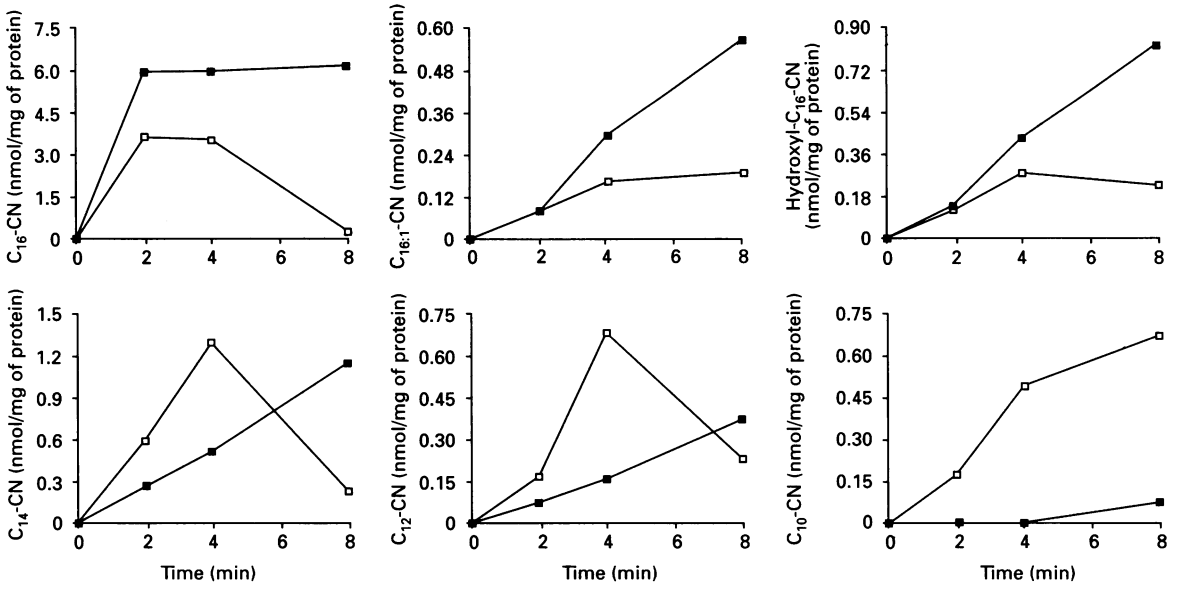

Figure 4 Generation of acyl-carnitine esters by skeletal-muscle mitochondria incubated with [U-^{14}C]hexadecanoate in the presence (□) or absence (■) of malate

Conditions of assay and analysis were as described in the Experimental section. Abbreviation: CN, carnitine.

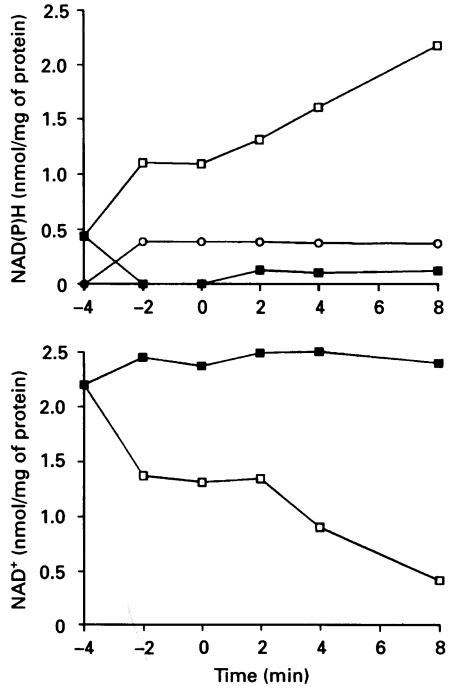

Figure 5 Time course of NAD^+ (lower panel) and NADH (□, ■) and NADPH (○) (upper panel) generation by skeletal-muscle mitochondria incubated with hexadecanoate (60 μM) in the absence (■) or presence (□, ○) of rotenone

Conditions of incubation and analysis were as described in the Experimental section.

(Veerkamp et al., 1983). Moore et al. (1982), using rabbit heart mitochondria, showed 3-hydroxyacid production from hexadecanoyl-carnitine in the absence of rotenone and in the presence of 10 mM malate, and this accumulation increased approx. 10-fold with 10 μM rotenone. However, studies using human muscle (Watmough et al., 1990; Singh Kler, 1991; Jackson et al., 1992), using the same techniques as those used in the present study, showed production of only saturated acyl-CoA and carnitine esters in control muscle, a similar pattern to that demonstrated by Stanley and Tubbs (1974, 1975) in rat liver.

There are a number of possible mechanisms for the accumulation of 3-hydroxyacyl- and 2-enoyl-CoA esters. Inadequate oxygenation of the incubations is unlikely; polarographic studies have shown that pulses can be obtained with hexadecanoyl-carnitine at very low oxygen concentrations (owing to the low K_m of complex IV for oxygen, approx. 2 μM; Chance, 1957). State-4 accumulation of 3-hydroxyacyl- and 2-enoyl-carnitines has been reported for liver (Lopez-Cardozo et al., 1978); however, in the present study incubations with [U-^{14}C]hexadecanoyl-carnitine were carried out under State-3 conditions (5 mM ADP), and even though the [U-^{14}C]hexadecanoate incubations were carried out with no additional ADP, the inclusions of 5 mM $MgCl_2$, which activates contaminating myofibrillar ATPase, maintains mitochondria in State 3 (Hedman, 1965). In addition, measurement of the NAD^+ and NADH concentrations in mitochondria showed that gross changes in nicotinamide redox state, which could account for the accumulation of 3-hydroxacyl- and 2-enoyl-CoA esters and would also result from State 4 or inadequate oxygenation, did not take place, although such changes were observed in incubations made in the presence of rotenone. The NADH/NAD^+ ratios obtained here were in good agreement with those obtained by Moore et al. (1982) in rabbit heart mitochondria, and by Latipaa (1989) in perfused rabbit heart, showing that the h.p.l.c. method used is a fast and sensitive direct method for analysis of the nicotinamide redox state which compares well with enzymic methods or estimation from enzyme equilibria.

An explanation for the occurrence of 3-hydroxyacyl- and 2-enoyl-CoA esters may be the physiological activity ratios of the β-oxidation enzymes in muscle. If long-chain 3-hydroxyacyl-CoA dehydrogenase activity was such that 3-hydroxyacyl-CoA

esters accumulated, 2-enoyl-CoA esters would accumulate because of the equilibrium of the hydratase reaction, and the acyl-CoA dehydrogenases would be product-inhibited (Davidson and Schulz, 1982; Powell et al., 1987). The full range of β-oxidation enzyme activities has not been reported from rat muscle; Reichmann and De Vivo (1991) compared the enzyme activities from a variety of tissues, and found the acyl-CoA dehydrogenases to have by far the lowest activities, as found by Jackson et al. (1991) in human muscle. However, Reichmann and De Vivo (1991) measured 3-hydroxyacyl-CoA dehydrogenase activity in the non-physiological direction with a short-chain substrate. Yang et al. (1987) measured the full range of β-oxidation enzymes in rat heart mitochondria and observed that the activity of 3-hydroxyacyl-CoA dehydrogenase (measured in the physiological direction with 3-hydroxydecanoyl-CoA as substrate) was about 10 times that of the acyl-CoA dehydrogenases. We have demonstrated that rat heart mitochondrial incubations generated acyl-CoA intermediates very similarly to incubations of skeletal-muscle mitochondria (results not shown), and it is probable that the relative enzyme activities in two tissues are also similar. We conclude that it is most unlikely that 3-hydroxyacyl-CoA esters are observed because of low amounts of the 3-hydroxyacyl-CoA dehydrogenases. However, it is difficult to interpret enzyme-activity data in terms of intramitochondrial control, because of differences in electron acceptors, assay conditions and the preparations measured, and also because the measurement of enzyme activity in homogenates or disrupted mitochondria will not reflect any supramolecular organization which may be present within the mitochondrion.

There may be species differences in the control of β-oxidation in muscle, since Watmough et al. (1990) and Singh Kler et al. (1991) showed that in control human muscle, when very similar incubation and analysis conditions to those in the present study were used, only saturated CoA ester and carnitine esters could be detected. Moore et al. (1982) have shown the accumulation of 3-hydroxy-alkanoic and -2-enoic acids in rabbit heart mitochondria. More importantly, the physiological relevance of our results is supported by the findings of Moore et al. (1980) and Latipaa (1989), who detected 3-hydroxyacyl and 2-enoyl derivatives in normoxic perfused hearts (rabbit and rat respectively). The disposal of acetyl-CoA by the tricarboxylic acid cycle or export as acetyl-carnitine has been invoked as a mechanism for the control of muscle β-oxidation (Pande, 1971; Veerkamp et al., 1983, 1986; Watmough et al., 1988; Wang et al., 1991). However, the mechanism of the presumed feedback inhibition is poorly understood. Wang et al. (1991) observed that purified 3-oxoacyl-CoA thiolase is inhibited by acetyl-CoA and suggested that this is the mechanism for acetyl-CoA inhibition of β-oxidation. However, we did not detect 3-oxoacyl-CoA esters under conditions when acetyl-CoA was observed, even though flux was stimulated 3-fold by malate under which conditions acetyl-CoA was not observed.

There have been recent reports of the isolation from mitochondrial-inner-membrane fractions of a complex involving the latter three enzyme activities of the β-oxidation cycle either alone (Uchida et al., 1992) or in association with CPT (Kerner and Bieber, 1990) and of children with inherited disorders of this complex (Jackson et al., 1991, 1992). It is possible that some control of mitochondrial β-oxidation flux resides in the properties of this complex and its relation to other components of the mitochondrial inner membrane, such as complex I of the respiratory chain, rather than in the properties of isolated enzymes. Such a model could explain our observation of 3-hydroxyacyl-CoA and 2-enoyl-CoA esters in the presence of NAD^+, if it is assumed that there exists a small rapidly cycling pool of NAD^+/NADH which is tightly channelled between complex I and the trifunctional enzyme. If this small pool became highly reduced, it would not be apparent by measurement of the total mitochondrial nicotinamide pool. It has recently been demonstrated that the control of β-oxidation in isolated uncoupled rat liver mitochondria is located at CPT I at low flux rates, but at high flux rates control is divided between the respiratory chain and enzymes of β-oxidation (Kunz, 1991). Furthermore, there is evidence for the interaction of (short-chain) 3-hydroxyacyl-CoA dehydrogenase with complex I of the respiratory chain (Sumegi and Srere, 1984; Fukushima et al., 1989).

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