Biochimica et Biophysica Acta, 501 (1978) 286—295

BBA 47428

ON THE RATE-LIMITING STEP IN THE TRANSFER OF LONG-CHAIN ACYL GROUPS ACROSS THE INNER MEMBRANE OF BROWN ADIPOSE TISSUE MITOCHONDRIA

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(Received July 11th, 1977)

Summary

Brown adipose tissue mitochondria predominantly oxidize fatty acids in order to generate heat for non-shivering thermogenesis, and have an unusually high capacity for net transfer of long-chain fatty acyl groups from the outer to the inner (matrix) compartment.

The activities of the "outer" and "inner" carnitine long-chain acyltransferases have been estimated in isolated mitochondria of cold-acclimated guinea pigs by the continuous spectrophotometric recording of the redox level of flavoproteins in the acyl-CoA dehydrogenase pathway. This redox level is determined by the intramitochondrial content of acyl-CoA under the selected experimental conditions. The apparent initial rate of the "inner" acyltransferase (palmitoyl-L-carnitine added) is three order of magnitudes higher than the "outer" acyltransferase (palmitoyl-CoA added), and this difference is not influenced by the substrate concentration, pH and reaction temperature. Thus, the "outer" acyltransferase reaction is rate limiting in the transfer of long-chain acyl groups across the inner membrane of these mitochondria and catalyzes a non-equilibrium reaction in the intact organelle.

Estimates of the absolute rate of the "outer" long-chain acyltransferase indicate that it exceeds that of rat liver mitochondria by a factor of 20.

Introduction

It is now well established that the rate of fatty acid oxidation is the major determinant of metabolic energy generated in the brown adipose tissue to maintain non-shivering thermogenesis during cold acclimation (for review, see

Abbreviations: BES, *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; TTFA, 1-[Thenoyl-(2′)]-3,3,3-trifluoroacetone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

ref. 1). For this reason, a detailed knowledge of the regulation of fatty acid metabolism in this tissue, notably the oxidative pathway, is important in order to understand thermogenesis.

Experimental evidence has been presented in favour of a carnitine-dependent and a carnitine-independent oxidation of long-chain fatty acids in isolated mitochondria from brown adipose tissue (for review, see ref. 2) as well as from liver and heart. The carnitine-dependent pathway has been proposed to be very active in brown adipose tissue mitochondria [3] and this transfer reaction requires an activation of long-chain fatty acids to their acyl-CoA derivatives which largely takes place in the mitochondrial outer membrane [4]. The net transfer of long-chain acyl groups into the matrix compartment involves the carnitine/carnitine long-chain acyltransferase system [5,6]. Two pools of carnitine long-chain acyltransferases have been described [5,7—11] as well as a long-chain acylcarnitine-carnitine exchange carrier or translocator [12—14]. The enzyme catalyzing the formation of acyl-L-carnitine from acyl-CoA and L-carnitine (transferase A or "outer" transferase) appears to be loosely associated with the external surface (cytosolic side) of the inner membrane of rat liver mitochondria [11]. The other enzyme which catalyzes the formation of acyl-CoA from the translocated acyl-L-carnitine and intramitochondrial CoA (transferase B or "inner" transferase) appears to be firmly bound to the matrix side of the inner membrane [11]. It has been proposed [15] that the "outer" acyltransferase is rate limiting in the oxidation of long-chain fatty acids by rat liver mitochondria, but it is difficult to draw any definite conclusions from the experiments published so far. No information is yet available regarding the acyltransferases in brown adipose tissue mitochondria and the possible regulatory function of the "outer" acyltransferase [1].

The purpose of the present paper is to report on the rates of the long-chain acyltransferases of intact brown adipose tissue mitochondria as determined from the rate of reduction of flavoproteins in the acyl-CoA dehydrogenase pathway induced by palmitoyl-CoA (dependent on both acyltransferases as well as the exchange carrier) and by palmitoyl-L-carnitine (dependent on the exchange carrier and the "inner" acyltransferase). Our studies support the conclusion that the "outer" acyltransferase is a primary regulator of the rate of transfer of long-chain acyl groups across the inner membrane of brown adipose tissue mitochondria, and that this enzyme catalyzes a non-equilibrium reaction favouring a unidirectional transport of acyl groups in this organelle.

Materials and Methods

Animals. Weaned guinea pigs of the Pir/Srr/c strain were used. Approx. 4 weeks old, the animals were transferred from an environment of 22 to 5°C where they remained for at least 14 days to obtain an optimal increase in the mitochondrial mass [16].

Chemicals. Palmitoyl-CoA, antimycin A, oligomycin, rotenone and BES were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Palmitoyl-L-carnitine chloride was purchased from Supelco Inc. (Bellefonte, Pa., U.S.A.) and L-carnitine chloride was a product of Koch Light Laboratories Ltd. (Colnbrook, England). 1-[Thenoyl-(2')]-3,3,3-trifluoroacetone (TTFA) was ob-

tained from Merck (Darmstadt, W. Germany). All other chemicals were of the highest purity commercially available.

Isolation of mitochondria. Brown adipose tissue mitochondria were isolated by differential centrifugation essentially as described [17,18].

Assay of carnitine long-chain acyltransferase activities. Assay of carnitine long-chain acyltransferase activities was based on a continuous spectrophotometric measurement of reduction of the flavoproteins in the acyl-CoA dehydrogenase pathway [19,20]. Palmitoyl-L-carnitine and palmitoyl-CoA were used as the substrates for the "inner" and "outer" long-chain acyltransferase, respectively. The reaction mixture contained in a final volume of 1 ml: 135 μmol sucrose, 2.5 μmol $MgCl_2$, 40 μmol BES, pH 7.5, 5 nmol rotenone, 5 nmol 1-[Thenoyl-(2′)]-3,3,3-trifluoroacetone (TTFA), 0.74 μg antimycin A, 3.3 μmol cyanide, 5 μmol ascorbate, 90 nmol *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and 2 μg oligomycin. All acids were pH adjusted by KOH. The reaction was started by the addition of 20—30 μM (40—75 nmol/mg protein) palmitoyl-L-carnitine or palmitoyl-CoA and the net amount of acyl groups transferred from the outer to the inner mitochondrial compartment was estimated from the degree of flavoprotein reduction as measured by the decrease in absorbance at 455 nm relative to 510 nm (see Fig. 1) using an Aminco-Chance dual-wavelength spectrophotometer with both monochromators calibrated at an accuracy of ±0.2 nm (for wavelength setting, see Results). The temperature was thermostatically controlled (35°C unless otherwise stated).

The rapid reduction of flavoproteins by palmitoyl-L-carnitine, which was used as a measure of the "inner" acyltransferase activity, was also followed by the decrease in absorbance at 455 nm relative to 510 nm when a solution of palmitoyl-L-carnitine was mixed with a mitochondrial suspension using a dual-wavelength/stopped flow technique [21]. The dual-wavelength spectrophotometer was operated at a chopper frequency at 60 Hz, but was modified electronically to give a shorter response time (i.e. $t_{1/2}$ = 5 ms) than in the original design. The mixing device was an unequal volume mixer (the relative volume of the two syringes was approx. 10 to 1) and the optical pathlength of the 80 μl flow cell (from Helma GmbH and Co., Germany) was 1.0 cm. The output of the photomultiplier tube was connected to a Model 514 A Transient Recorder (Bryans Southern Instruments Ltd., London, England). A model 1100 W + W recorder from Kontron, Switzerland was used to draw the replots of the stored data of the transient recorder.

Other analytical methods. Protein was determined using the Folin-Ciocalteux reagent [22].

Results

Reduced minus oxidized difference spectrum of flavoproteins in the acyl-CoA dehydrogenase pathway

In order to eliminate the spectral contributions from other components of the respiratory chain, the terminal part of the chain was reduced by ascorbate and TMPD in the presence of cyanide. Reversed electron flow was inhibited by antimycin A, and rotenone and TTFA was added to inhibit forward flow through the NADH and succinate dehydrogenase, respectively. The reduced

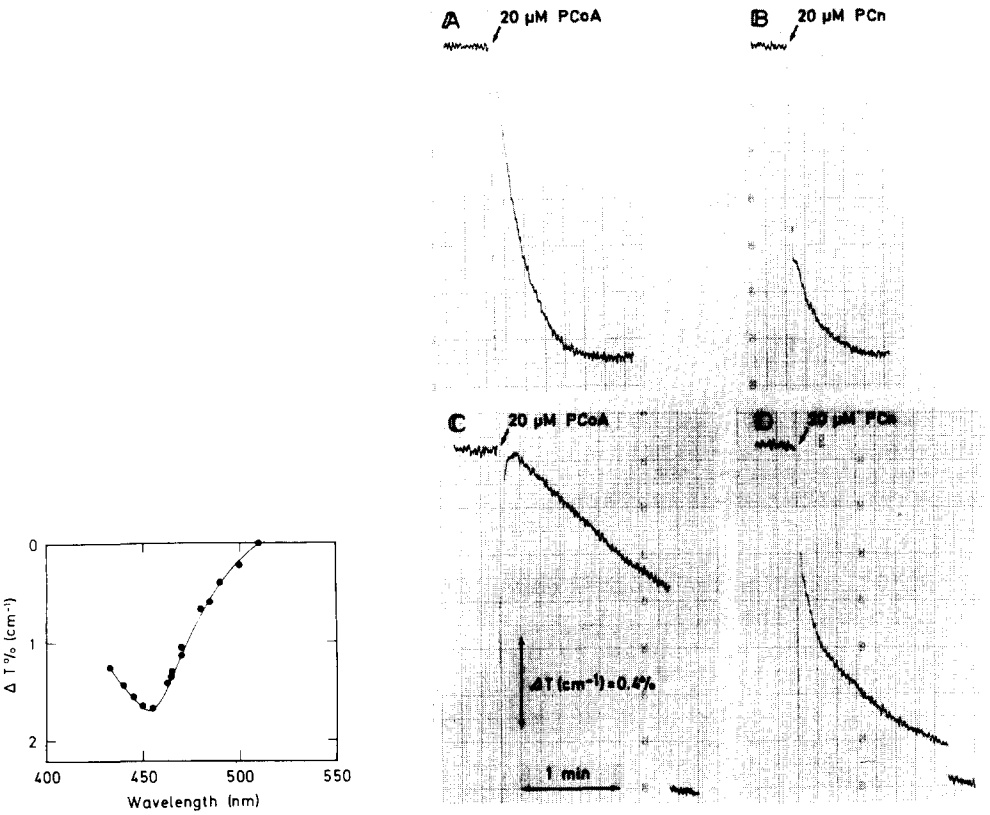

Fig. 1. Kinetic absorption spectrum of flavoproteins in the acyl-CoA dehydrogenase pathway of brown adipose tissue mitochondria reduced by the addition of palmitoyl-L-carnitine at 35°C. The mitochondria were suspended in the standard incubation medium (see Materials and Methods) at 0.41 mg of mitochondrial protein/ml. The reduction was initiated by the addition of 30 nmol palmitoyl-L-carnitine, and the steady-state change in transmission ($\Delta T(\%)$) was recorded with the reference wavelength set at 510 nm and the measuring wavelength as indicated on the abscissa.

Fig. 2. The reduction of the flavoproteins in the acyl-CoA dehydrogenase pathway of brown adipose tissue mitochondria induced by the addition of 71 nmol palmitoyl-CoA (PCoA)/mg protein (plus 0.6 mM L-carnitine) at 35°C (A) and 5°C (C), and by 71 nmol palmitoyl-L-carnitine (PCn)/mg protein at 35°C (B) and 5°C (D). Standard incubation medium was used (see Materials and Methods). The change in transmission, $\Delta T(\%) = \Delta(T_{455\,nm} - T_{510\,nm})$. 0.44 mg mitochondrial protein were used.

minus oxidized difference spectrum thus induced by 30 μM (73 nmol/mg protein) palmitoyl-L-carnitine (Fig. 1) therefore represents a summation spectrum with rather selective contributions from the long-chain acyl-CoA dehydrogenase [23], electron transfer flavoprotein [24], and electron transfer flavoprotein dehydrogenase [16,25]. As seen from Fig. 1 the spectrum is similar to that previously reported for rat liver mitochondria [19] except that the spectral change is approx. 6-fold greater on a protein basis.

Time course of flavoprotein reduction induced by palmitoyl-CoA plus carnitine and palmitoyl-L-carnitine

Typical progress curves of flavoprotein reduction induced by palmitoyl-L-carnitine and palmitoyl-CoA plus L-carnitine are shown in Fig. 2. In the case of

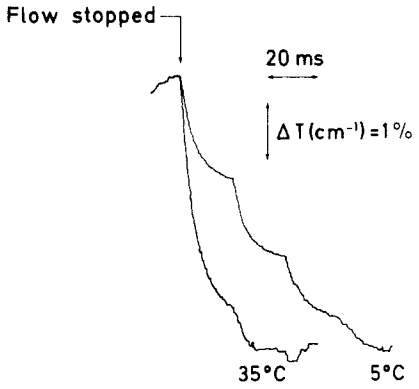

Fig. 3. The reduction of the flavoproteins in the acyl-CoA dehydrogenase pathway of brown adipose tissue mitochondria induced by the addition of 71 nmol palmitoyl-L-carnitine/mg protein at 35 and 5°C measured by the stopped-flow technique as described in Materials and Methods. The standard incubation mixture given in Materials and Methods was used; 0.44 mg mitochondrial protein were used. The change in transmission, $\Delta T(\%) = \Delta(T_{455nm} - T_{510nm})$.

palmitoyl-L-carnitine the progress curves (Figs. 2B and 2D) are clearly biphasic at 35 and 5°C. The initial rapid phase was not measurable by a conventional spectrophotometer in contrast to the progress curve of palmitoyl-CoA plus carnitine (Figs. 2A and 2C) which was almost linear for about 30 s.

From Fig. 2 it is seen that there is a marked effect of temperature on the rate of flavoprotein reduction induced by palmitoyl-CoA plus carnitine, but apparently little effect on that induced by palmitoyl-L-carnitine. It should also be noticed that, in contrast to e.g. rat liver mitochondria [26], the apparent activity of the "inner" acyltransferase was so high even at 5°C that it was just at the limit of detection by our stopped-flow spectrophotometric technique. Thus, from Fig. 3 it is seen that the $t_{1/2}$ value for the flavoprotein reduction induced by palmitoyl-L-carnitine was approx. 25 ms at 5°C and approx. 5 ms at 35°C; the latter is exactly the response time of the instrument (see Materials and Methods). It is also seen that the progress curve reflects the beam chopper frequency of 60 Hz.

This relationship between the rate of flavoprotein reduction induced by acylcarnitine and acyl-CoA plus carnitine was also found for other species of long-chain fatty acids, but C_{16} was choosen since maximal activities were obtained with these derivatives.

It has been reported [27] that about 100 nmol palmitoyl-D-carnitine per mg of mitochondrial protein induce complete fragmentation of heart mitochondria. At our experimental conditions, however, we could not attribute any of the spectral changes to a detergent effect of the substrates. A detergent effect was not detectable by light scattering measurements until the concentration of palmitoyl-L-carnitine reached a value of >140 nmol/mg of protein.

The "outer" acyltransferase activity revealed almost maximal initial rates (V) at the palmitoyl-CoA concentration, pH and temperature selected in the standard incubation conditions. A temperature of 35°C was selected since it is close to the physiological temperature of the animal. The initial rates of the "outer" and "inner" acyltransferase were tested at different pH values (range 6—8),

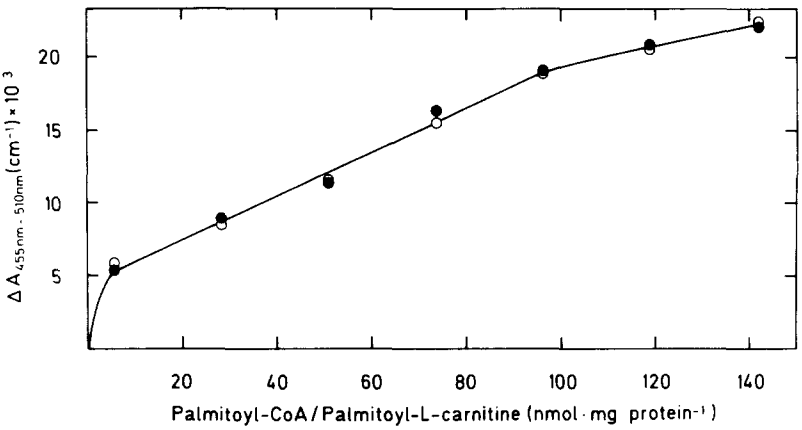

Fig. 4. The reduction of the flavoproteins in the acyl-CoA dehydrogenase pathway of brown adipose tissue mitochondria induced by the addition of increasing amounts of palmitoyl-L-carnitine (○), and palmitoyl-CoA (●) (plus 0.6 mM L-carnitine). The experimental conditions are given in Materials and Methods; 0.44 mg mitochondrial protein were used. The change in transmission, $\Delta T(\%) = \Delta(T_{455nm} - T_{510nm})$.

temperatures (range 5—35°C) and substrate concentrations (4—140 nmol/mg protein), and in all cases the "inner" acyltransferase activity was too fast to be measured by a conventional spectrophotometric technique. The difference in the initial rates of the two acyltransferases, however, was of the same order of magnitude as in the standard assay procedure (data not shown).

Estimation of specific activity of the "outer" acyltransferase

In order to be able to express the spectral changes (reduction of flavoproteins) induced by the acyl-CoA and acyl-L-carnitine esters in conventional enzymic activities (nmol substrate · min^{-1} · mg protein^{-1}) calibration curves were obtained by measuring the steady-state spectral change (Fig. 2) as a function of the amount of added acyl esters (Fig. 4). It is seen that increasing equimolar amounts of palmitoyl-CoA (in the presence of L-carnitine) and palmitoyl-L-carnitine induce identical spectral changes at 455—510 nm.

TABLE I

THE ACTIVITIES OF "OUTER" LONG-CHAIN ACYLTRANSFERASE IN MITOCHRONDRIA ISOLATED FROM DIFFERENT TISSUES OF THE RAT AND FROM BROWN ADIPOSE TISSUE OF COLD-ACCLIMATED GUINEA PIGS

Mitochondria	"Outer" acyltransferase activity (nmol · min^{-1} · mg protein^{-1})	Method	T (°C)	Ref.
Rat liver	12.1 *	Dual-wavelength spectrophotometry	25	26
Rat liver	13.0	Isotope exchange	30	28
Rat kidney	8.5	Isotope exchange	30	28
Rat heart	23.0	Isotope exchange	30	28
Rat adipose tissue	4.4	Isotope exchange	30	28
Guinea pig brown adipose tissue	256 (n = 4) (221—289)	Dual-wavelength spectrophotometry	35	Present study

* Calculated as initial rate from the progress curve of flavoprotein reduction; data given in Figs. 3 and 4 of ref. 26.

Based on these calibration curves the initial rate of the "outer" acyltransferase reaction was calculated to be 256.3 nmol · min^{-1} · mg $protein^{-1}$ (n = 4) at 35°C and palmitoyl-CoA concentration of 40 nmol/mg protein (0.6 mM L-carnitine). As can be seen from Table I this value is approx. 20 times higher than previously measured by the same method in rat liver mitochondria and 10—50 times higher than measured by the isotope exchange method in mitochondria in a number of rat tissues.

Discussion

In recent years new data have accumulated on the fatty acid metabolism of the brown adipose tissue (for review, see ref. 1). Since fatty acids are supposed to be the main fuel utilized by this tissue for thermogenesis [2,29], a detailed knowledge of the fatty acid metabolism is important to understand thermogenesis. In the present study we have focused on the carnitine-dependent oxidation of long-chain fatty acids which seems to be very active in brown adipose tissue mitochondria [3]. This pathway consists of a series of enzyme-catalyzed reactions, but we have here limited our study to the sequential reactions responsible for the transfer of the acyl moieties across the inner membrane [5,6], i.e. those catalyzed by the "outer" acyltransferase [5,7—11], the acylcarnitine-carnitine exchange carrier or translocator [12—14] and the "inner" acyltransferase [5,7—11]. A number of studies have been carried out in order to determine the site(s) of regulation of mitochondrial fatty acid oxidation [30—32], and experimental evidence has been presented to support the conclusion that the activity of the "outer" long-chain acyltransferase is the rate-limiting step [15]. However, it is difficult to draw any definite conclusions from the experiments published so far, but the question is of particular interest in the brown adipose tissue where the mitochondrial oxidation of long-chain fatty acids represents the major energy supply in order to maintain non-shivering thermogenesis during cold acclimation (for review, see ref. 1).

Most studies on carnitine long-chain acyltransferase have so far been based on (1) measurement of mitochondrial oxidation of acyl-CoA and acylcarnitine derivatives [33], (2) an isotope exchange method [6] or (3) a direct assay either of the forward or the backward reaction (for review, see ref. 34). Furthermore, different methods have been used to selectively measure the "outer" and "inner" acyltransferase in isolated, intact mitochondria, i.e. by selective inhibition of the "outer" acyltransferase by either 2-bromoacyl-CoA derivatives [35], digitonin treatment [10,36], sonication [37] or phospholipase treatment and specific antibodies [26]. The approach selected in the present study represents a modification of the spectrophotometric method introduced by Garland et al. [19]. The main advantage of this method is that it measures the initial rates of the forward reaction in the intact mitochondria using the redox state of flavoproteins in the acyl-CoA dehydrogenase pathway as an estimate of the intramitochondrial acyl-CoA level. When acyl-CoA is used as the acyl donor, the measurement involves a reaction sequence consisting of three steps, i.e.

$$\text{acyl-CoA}_{out} + \text{L-carnitine}_{out} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{acyl-L-carnitine}_{out} + \text{CoASH}_{out} \quad (1)$$

$$\text{acyl-L-carnitine}_{out} + \text{L-carnitine}_{in} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{acyl-L-carnitine}_{in} + \text{L-carnitine}_{out} \quad (2)$$

$$\text{acyl-L-carnitine}_{in} + \text{CoASH}_{in} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{acyl-CoA}_{in} + \text{L-carnitine}_{in} \quad (3)$$

where Reactions 1 and 3 are catalyzed by the "outer" and "inner" acyltransferases respectively and Reaction 2 by the acylcarnitine-carnitine exchange carrier. When acylcarnitine is used as the substrate, only the last two reactions are involved. The absence of any lag-phase in the assay progress curve with acyl-CoA as the substrate (Fig. 2), already indicates that $k_1 << k_2, k_3$. It was therefore not unexpected to find that the flavoproteins were reduced approx. 1000 times faster with palmitoyl-L-carnitine than with palmitoyl-CoA as the substrate. Thus, in contrast to previous proposals for liver mitochondria [6,38], it is evident that the "outer" acyltransferase in brown adipose tissue mitochondria catalyzes a non-equilibrium reaction which is of particular interest from a regulatory point of view. This finding as well as the vectorial organization of the "outer" and "inner" acyltransferases to the C-side and M-side, respectively, of the inner membrane [26], are only compatible with a unidirectional transfer of long-chain acyl groups across the inner membrane. This conclusion is also supported by the finding that even high concentrations of $CoASH_{out}$ do not inhibit the "outer" acyltransferase activity (Normann, P.T. and Flatmark, T., unpublished results). In previous measurements, based on an isotope exchange method and using partly purified enzyme, the finding of an equilibrium constant $K_{eq} = 0.45$ for the "outer" acyltransferase [38] supported an equilibrium reaction, but in this case the localization of the enzyme to the mitochondrial inner membrane and its participation in a sequential reaction was not considered from a kinetic point of view. Thus, in the brown adipose tissue mitochondria, it is even more clearly shown than in rat liver mitochondria [20], that the "outer" acyltransferase indeed is the rate-limiting step in the overall transfer of long-chain acyl groups across the inner membrane. This conclusion, however, does not mean that this reaction is rate limiting in the oxidation of fatty acids in this tissue. Thus, preliminary studies in this laboratory indicate that the long-chain acyl-CoA synthetase of the outer membrane may represent a site for regulation of fatty acid oxidation in this tissue (Normann, P.T. and Flatmark, T., unpublished results).

Our determination of the specific activity of the "outer" long-chain acyltransferase is based on the assumption that binding of added long-chain fatty acyl-CoA to soluble proteins or membrane surfaces in the outer compartment is negligible in freshly prepared mitochondria. That this assumption is satisfied is supported by the almost linear relationship between the spectral change induced and the amount of palmitoyl-CoA added except at very low concentrations (Fig. 4). Furthermore, for the measurement of the specific activity of the "outer" acyltransferase it is assumed that the spectral change measured is proportional to the amount of acyl-CoA_{in} since the long-chain acyl-CoA dehydrogenase has been found to be localized to the matrix side of the inner membrane [39]. From an analytical point of view it should be noted that phosphate buffers should be avoided in the assay of acyltransferase activities in intact mitochondria. Thus, even short-time incubation in a medium containing, e.g.

5 mM phosphate lowered the initial rate as well as the extent of flavoprotein reduction, whereas no such inhibitory effect was observed on preincubation in the absence of phosphate (Normann, P.T. and Flatmark, T., unpublished results). This effect of phosphate is explained by induction of a leakage of CoASH as first shown by Bremer et al. [40] in rat liver mitochondria. With these assumptions in mind it is seen from the data presented in Table I that the specific activity of the "outer" acyltransferase is 10—20-fold higher than reported for rat liver and heart mitochondria. This result is in good agreement with the notion that these mitochondria are specialized for fatty acid oxidation (for review, see ref. 1).

Acknowledgements

This work was supported by the Norwegian Research Council for Science and the Humanities (C.11.14-3) and the Norwegian Cancer Society. We are indebted to Siv. ing. B. Hovdenes for assistance with instrumentation.

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