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# ORGANIZATION AND REGULATION OF FATTY ACID OXIDATION IN MITOCHONDRIA OF BROWN ADIPOSE TISSUE

Z. DRAHOTA, A. ALEXANDRE, C. R. ROSSI AND N. SILIPRANDI

*Institute of Biological Chemistry, Centro per lo Studio della Fisiologia dei Mitocondri, University of Padova, Padova (Italy) and Institute of Physiology, Czechoslovak Academy of Sciences, Prague (Czechoslovakia)*

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SUMMARY

In mitochondria from brown adipose tissue no GTP-dependent oleate synthetase could be detected. The ATP-dependent acyl-CoA synthetase is probably the only enzyme involved in oleate oxidation.

In the absence of added carnitine, oleate oxidation proceeds only in the presence of malate and of phosphate, and only for a few minutes. This is apparently due to the very low ATP/AMP ratio which is unfavourable for the synthetase activity. The substrate level phosphorylation supports continuous oleate oxidation by dramatically increasing the ATP/AMP ratio. This effect probably depends on the transphosphorylation of GTP with AMP.

In the presence of added carnitine, oleate oxidation is stimulated by phosphate and is inhibited by atractyloside. These effects are probably due to a direct action of these substances on the ATP-dependent synthetase.

## INTRODUCTION

The main function of brown adipose tissue, which can be found in newborn animals, cold-adapted animals and hybernators, is heat production[1,2]. Considerable data indicate that lipids are the main fuel for this thermogenesis[1-5]. However, the mechanism of fatty acid oxidation in brown adipose mitochondria is very poorly understood. For instance, during maximal physiological activity, *i.e.* during thermogenesis, most of the energy is dissipated as heat[1], while ATP synthesis is very limited[6,7]. An unanswered question is how energy is supplied to maintain this high rate of fatty acid oxidation, a process known to be ATP requiring. Also, since brown fat mitochondria are very active in fatty acid oxidation they can serve as a good model for studying the general mechanism of the organization and regulation of fatty acid utilization.

In the present paper the main characteristics of fatty acid oxidation and the mechanism of its regulation are reported.

## EXPERIMENTAL PROCEDURE

Mitochondria were prepared from brown adipose tissue of 20–30-day-old Wistar strain albino rats by the procedure of SCHNEIDER AND HOGEBOOM in 0.25 M sucrose[8]. The fatty layer, which appeared after the first low-speed centrifugation, was removed by filtration through gauze.

$O_2$ uptake was measured with a Clark oxygen electrode as described by KIELLEY AND BRONK[9].

Acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) was assayed as previously described[10]. Protein concentration was determined by the biuret reaction[11]. Potassium oleate was prepared as a fine emulsion at 60° and adjusted to pH 8.0. A 20-mM suspension was found to be stable for several months. The concentrations of ATP, ADP, and AMP in mitochondria were measured enzymically. ATP was determined fluorimetrically by using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) according to the method of STEINER AND WILLIAMS[12]. ADP was determined by the pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) system previously described[13]. AMP was determined under the same conditions as ADP except that adenylate kinase (EC 2.7.4.3) was added. Reagents were analytical grade. The enzymes used for analysis were purchased from Boehringer. Uniformly labelled [$^{14}$C]oleate was obtained from Radiochemical Centre, Amersham.

Atractyloside was kindly given by Prof. Santi (University of Padova, Italy).

## RESULTS

Preliminary experiments showed that fatty acid oxidation in brown adipose mitochondria, as in mitochondria of other tissues, occurs in the absence of added carnitine but that it is stimulated by the addition of this substance. For convenience a "carnitine-independent" oxidation and a "carnitine-dependent" oxidation will be described separately. The rate of the "carnitine-dependent" pathway was much higher (average, 150 natoms/min per mg protein) than the "carnitine-independent" path (average, 50 natoms/min per mg protein). Brown adipose mitochondria prepared by the procedure of SCHNEIDER AND HOGEBOOM[8] were in the uncoupled state, and it was not possible to obtain coupled preparations even if the mitochondria were isolated and incubated in the presence of defatted serum albumin.

### *"Carnitine-independent" oleate oxidation*

*Effect of phosphate.* Mitochondria from brown adipose tissue exhibited a high rate of oleate oxidation in the presence of malate. Added ATP was not required, indicating that endogenous ATP or GTP were being utilized for the oleate oxidation (Fig. 1, Trace A). Omission of phosphate from the incubation mixture resulted in a large decrease of the respiratory rate (Fig. 1, Trace B). The activating effect of phosphate can be explained by its effect on the ATP-dependent mitochondrial acyl-CoA synthetase[10]. Table I shows that the rate of formation of acyl-CoA in a solubilized preparation of brown adipose mitochondria was increased 2-fold by the addition of 8 mM phosphate. Phosphate at 50 mM did not inhibit the rate of oleate oxidation in brown adipose mitochondria. This is in contrast with the inhibitory action of phosphate at this concentration on oleate oxidation in uncoupled liver[14] and kidney[15]

mitochondria. This difference could be explained by the absence of a GTP-dependent thiokinase in brown adipose mitochondria which has been shown to be inhibited by high phosphate concentrations in the kidney and liver mitochondria[15,16]. Our attempts to show the presence of a GTP-dependent thiokinase in brown adipose tissue have proved unsuccessful. We conclude that the ATP-dependent thiokinase is probably the only enzyme involved in oleate activation in brown adipose mitochondria.

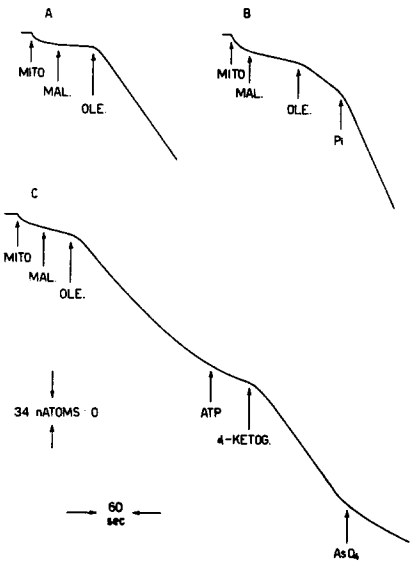


Fig. 1. Requirements for oleate oxidation. The incubation system contained 80 mM KCl, 15 mM Tris–HCl buffer (pH 7.3), 6 mM $MgCl_2$, and 12 mM sucrose. At the points indicated by arrows, 2 mg of mitochondrial protein (MITO), 3 mM malate (MAL.), 0.1 mM oleate (OLE.), 4 mM ATP, 0.5 mM α-ketoglutarate (α-KETOG.), 20 mM arsenate ($AsO_4$), and 30 mM phosphate ($P_i$) were added. Total volume of the reaction mixture was 2 ml. The temperature was 25°. In Traces A and C, 5 mM phosphate was present in the medium.

TABLE I

ATP-DEPENDENT ACYL-CoA FORMATION BY SOLUBILIZED MITOCHONDRIAL PROTEIN

The preparation of solubilized mitochondrial protein, the incubation system and the method of analysis were performed as previously described[10]. The substrate was oleate. Incubations were carried out for 4 min at 38°.

| *Addition* | *Acyl-CoA formed (nmoles/mg protein per min)* |
|---|---|
| None | 3.7 |
| 8 mM phosphate | 7.8 |

*Effect of α-ketoglutarate.* Since mitochondria from brown adipose tissue are completely uncoupled the only process for the production of ATP, which is necessary for fatty acid oxidation, is substrate-level phosphorylation. Evidence in support of this assumption is the observation that arsenate inhibited oleate oxidation almost

completely: the residual $O_2$ uptake was presumably due to α-ketoglutarate oxidation (Fig. 1, Trace C).

The rate of the carnitine-independent oleate oxidation decreased gradually, and after 3–4 min completely stopped (Fig. 1, Trace C; Fig. 2). Added ATP did not

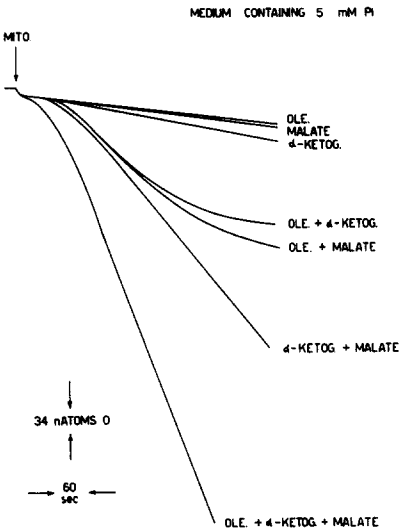


Fig. 2. Effect of α-ketoglutarate on oleate oxidation. Experimental conditions were the same as for Fig. 1. Phosphate, 5 mM, was present in the medium. Additions of substrates to the incubation mixture are indicated on the right of the figure. The concentrations of the added substrates were the same as in Fig. 1.

TABLE II

OXIDATION OF UNIFORMLY LABELLED [$^{14}$C]OLEATE

Experimental conditions as for Fig. 1. Phosphate (5 mM) was present in the medium. Incubations were carried out in Warburg vessels at 30° in the presence of 0.2 μmole of uniformly labelled [$^{14}$C]oleate ($4 \cdot 10^5$ counts/min). $^{14}CO_2$ was trapped by 0.2 ml of 1 M hyamine hydroxide. After 15 min of incubation the reaction was stopped by the addition of 0.2 ml of 20% $H_2SO_4$ (v/v). The shaking was continued for an additional 15 min. After the addition of 0.1 ml of conc. $HClO_4$, both hyamine $^{14}CO_2$ and the $HClO_4$-soluble activity were determined by liquid-scintillation counting. The acid-soluble activity represents the metabolites derived from the acid-insoluble oleate.

| *Additions* | *Counts/min per mg per 15 min* | | |
|---|---|---|---|
| | $^{14}CO_2$ (*A*) | *Acid-soluble activity* (*B*) | *A* + *B* |
| (*A*) *Effect of malate and α-ketoglutarate* | | | |
| Oleate | 70 | 800 | 870 |
| Oleate + 3 mM malate | 280 | 18 370 | 18 650 |
| Oleate + 0.5 mM α-ketoglutarate | 100 | 2 200 | 2 300 |
| Oleate + 3 mM malate + 0.5 mM α-ketoglutarate | 510 | 29 770 | 30 280 |
| (*B*) *Effect of carnitine* | | | |
| Oleate + 3 mM malate + 1 mM ATP | 290 | 19 120 | 19 410 |
| Oleate + 3 mM malate + 3 mM carnitine | 260 | 17 800 | 18 060 |
| Oleate + 3 mM malate + 1 mM ATP + 3 mM carnitine | 704 | 42 000 | 42 704 |

restore the original respiratory rate (Fig. 1, Trace C). A constant respiratory rate could be maintained by the addition of small amounts (0.5 mM) of α-ketoglutarate (Fig. 1, Trace C; Fig. 2). Evidence that α-ketoglutarate activates oleate oxidation, and not merely serves as an oxidizable substrate, is shown by the results in Table II. When oleate oxidation was determined by the amount of $^{14}CO_2$ and $^{14}C$-labelled acid-soluble metabolites formed from uniformly labelled [$^{14}C$]oleate it was found that α-ketoglutarate had a significant activating effect (Table II). The action of α-ketoglutarate could be detected only in the presence of malate (Fig. 2) and could not be elicited by other oxidizable substrates such as succinate or α-glycerophosphate. Since the stimulatory effect of α-ketoglutarate was completely abolished by arsenate (Fig. 1, Trace C), the function of the substrate appears to be the promotion of substrate-level phosphorylation.

The shift induced by α-ketoglutarate on the concentrations of intramitochondrial ATP and AMP can explain the activating effect evoked by this substrate on oleate oxidation.

TABLE III

CHANGES IN THE DISTRIBUTION OF "ENDOGENOUS" MITOCHONDRIAL NUCLEOTIDES

The incubation conditions are the same as those reported in Fig. 1. Phosphate, 5 mM, was present in the incubation solution. Results are expressed as nmoles/10 mg protein.

| *Experimental conditions* | *ATP* | *ADP* | *AMP* |
|---|---|---|---|
| No incubation | 5.5 | 22 | 44.0 |
| 5 min incubation with 3 mM malate and 0.1 mM oleate | 5.0 | 18 | 51.0 |
| 5 min incubation with 3 mM malate and 0.5 mM α-ketoglutarate | 50.0 | 23 | 0.6 |
| 5 min incubation with 3 mM malate, 0.1 mM oleate and 0.5 mM α-ketoglutarate | 40.0 | 23 | 3.0 |
| 5 min incubation with 1 mM ATP* | 12.0 | 20 | 43.0 |

* In this experiment the incubation mixture was diluted 15-fold with ice-cold 0.25 M sucrose. The endogenous adenine nucleotide was determined in the mitochondrial pellets recovered after centrifugation for 5 min at 20000 × *g*.

As shown in Table III, in freshly isolated mitochondria over 60 % of the adenine nucleotide pool is in the form of AMP while less than 8 % is ATP. This ratio is unfavourable for the activity of the ATP-dependent thiokinase which is inhibited by AMP[10,17]. Incubation of the mitochondria with oleate and malate did not modify the unfavourable adenine nucleotide ratio (Table III). However, the addition of α-ketoglutarate dramatically changed the ATP/AMP ratio (Table III) resulting in a very favourable condition for oleate activation. Incubation of mitochondria with added ATP did not appreciably modify the original ATP/AMP ratio (Table III). This could explain why externally added ATP is unable to stimulate oleate oxidation (Fig. 1, Trace C).

*"Carnitine-dependent" oleate oxidation*

In mitochondria from brown adipose tissue, as well as in liver and kidney mitochondria, fatty acid oxidation is stimulated by addition of ATP *plus* carnitine[15,18-21]

(Fig. 3, Trace A). The "carnitine-dependent" process was also stimulated by 5 mM phosphate[15,18] but inhibited by 1 mM atractylate[15,18]. The inhibitory effect of atractylate was almost complete in the absence of inorganic phosphate but reduced to about 30 % in the presence of 5 mM phosphate (not reported in the figures). In the presence of phosphate, added CoA restored the maximal rate of atractyloside-inhibited respiration (Fig. 4, Trace B). Similar results were obtained in the absence of phosphate.

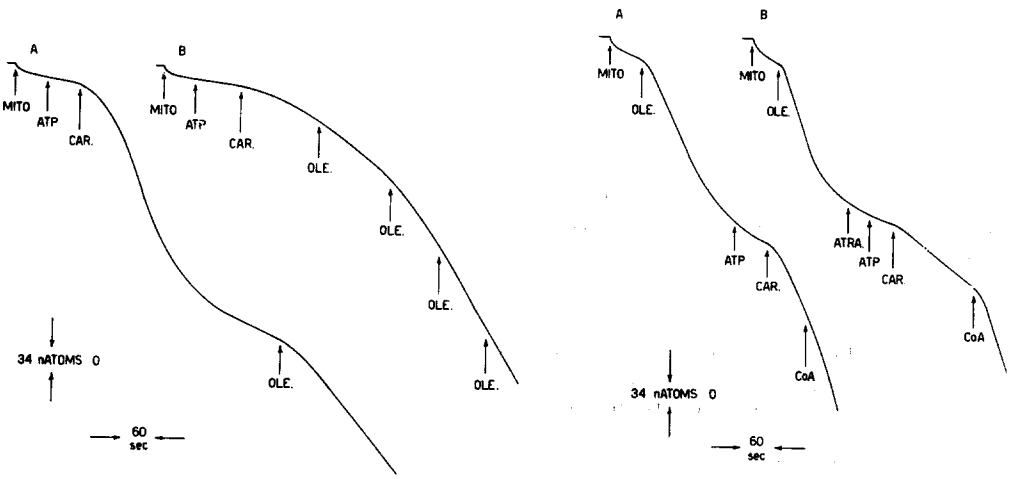


Fig. 3. Fatty acid oxidation in the presence of added ATP and carnitine. Experimental conditions were the same as for Fig. 1. Phosphate, 5 mM, and malate, 3 mM, were present in the medium. At the points indicated by arrows, 2 mg of mitochondrial protein (MITO), 1 mM ATP, 1 mM carnitine (CAR.), and 0.1 mM oleate (OLE.) were added. The system in Trace B contained 10 mg of bovine serum albumin.

Fig. 4. Effect of atractyloside on oleate oxidation. Experimental conditions were the same as for Fig. 1. Phosphate, 5 mM, and malate, 3 mM, were present in the medium. At the points indicated by arrows, 2 mg of mitochondrial protein (MITO), 0.1 mM oleate (OLE.), 1 mM ATP, 1 mM carnitine (CAR.), 0.05 mM CoA, and 1 mM atractyloside (ATRA.) were added.

In the "carnitine-dependent" system the maximal rate of respiration was obtained even in the absence of added fatty acid[20,21] (Fig. 3, Trace A). Serum albumin completely abolished the endogenous respiration[20], and subsequent additions of oleate induced a progressive increase in the respiratory rate (Fig. 3, Trace B). These results suggest that, in the carnitine-linked mechanism, brown adipose mitochondria oxidize the available endogenously-free fatty acids.

## DISCUSSION

From the results reported in this paper it appears that brown adipose mitochondria actively oxidize free fatty acids by a "carnitine-dependent" and a "carnitine-independent" mechanism. The existence of the two mechanisms implies, as in liver and kidney, the existence of two different sites of fatty acid activation[10,15,18,19], one being external and the other internal to the so-called "carnitine barrier" (which could be localized in the inner membrane[22]). In liver and kidney mitochondria the "external" activation of fatty acids is ATP-dependent while the "internal" activation is both ATP- and GTP-dependent[14,15], indicating the existence in the internal site of

two distinct acyl-CoA synthetases, one ATP- and the other GTP-dependent. In brown adipose mitochondria, activation of fatty acid is probably only ATP-dependent. This means that, in the internal site as well as the external site, only the ATP acyl-CoA synthetase is operative. Since in isolated mitochondria ATP is present only in the "internal" site, the "external" thiokinase is not operative unless ATP, necessary for fatty acid activation, and carnitine, necessary for the translocation of acyl-CoA, are added together. In the presence of ATP and carnitine the external system becomes active and provides additional acyl groups for the oxidative enzymes located in the "internal" site. In other words while in the "carnitine-independent" pathway only internally activated acyl groups are oxidized, in the "carnitine-dependent" pathway the "externally" activated acyl groups are also oxidized. This situation is operative in brown adipose mitochondria where the "carnitine-dependent" process has a higher fatty acid oxidation rate than the "carnitine-independent" path.

A remarkable observation is that fatty acid oxidation *via* the "carnitine-independent" system proceeds for only a few minutes and then stops. This is apparently due to the very low ATP/AMP ratio which is unfavourable for acyl-CoA synthetase activity[10]. This interpretation is supported by the observation that small amounts of α-ketoglutarate dramatically increased the ATP/AMP ratio and supported continuous fatty acid oxidation. Since brown adipose mitochondria are uncoupled, it must be assumed that ATP produced upon α-ketoglutarate addition can only be derived from substrate-level phosphorylation[23]. Added ATP, unlike α-ketoglutarate, is unable to stimulate fatty acid oxidation and to modify the ATP/AMP ratio. This observation can be explained by the previous observations that the mitochondrial adenylate kinase (EC 2.7.4.3) is located in the outer mitochondrial membrane[24–26], while a specific transphosphorylation between GTP and AMP takes place in the mitochondrial matrix[26]. The only way mitochondrial AMP, which is sequestered within the inner membrane, can be phosphorylated is by transphosphorylation with GTP[26]. The following sequence of nucleotide reactions is postulated to occur upon α-ketoglutarate addition:

$$GTP + AMP \leftrightharpoons GDP + ADP \quad (1)$$

$$GTP + ADP \leftrightharpoons GDP + ATP \quad (2)$$

$$2GTP + AMP \leftrightharpoons 2GDP + ATP$$

This sequence of reactions is also in agreement with our observation that the level of mitochondrial ADP does not change upon the addition of α-ketoglutarate (Table III).

The requirement of malate to obtain the activation of fatty acid oxidation by α-ketoglutarate can be explained by the previous observation that malate facilitates the permeation of α-ketoglutarate through the inner mitochondrial membrane[27,28].

The other pathway of fatty acid oxidation is the "carnitine-dependent pathway" which is studied in the presence of added ATP and carnitine. The stimulatory effect of phosphate and the inhibitory action of atractylate on this process can be explained by the action of these substances on the ATP-specific thiokinase[10]. As discussed above, in mitochondria from brown adipose tissue the "carnitine-dependent" pathway is much higher than the "carnitine-independent" pathway.

Free fatty acids present in freshly isolated mitochondria, and which are easily absorbed by bovine serum albumin, are oxidized only through the "carnitine-dependent" pathway. This would suggest that these endogenous fatty acids are localized outside the "carnitine barrier".

Finally, considering that the ATP necessary for the "carnitine-dependent" process must be available outside the carnitine barrier, it is very likely that physiologically this ATP is provided by glycolysis, a process which has been shown to be very efficient in brown adipose cells[1].

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