THE ACTION OF ATRACTYLOSIDE AND AMP ON LONG-CHAIN FATTY ACID OXIDATION AND ON THE ATP-DEPENDENT FATTY ACID THIOKINASE

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1. Introduction

It is known that rat liver mitochondria oxidize long-chain fatty acids provided that sufficient ATP is available for activation. Pretreatment of mitochondria with DNP, which depletes endogenous ATP, results in an inhibition of the ATP-dependent fatty acid oxidation [1, 5]. Moreover, addition of ATP to DNP-treated mitochondria has no effect in inducing respiration unless oligomycin * [4, 5, 8, 15] or carnitine * [1, 3, 5, 6, 8] is also added.

It is known that atractyloside inhibits both the "oligomycin-activated" and the "carnitine-activated" systems [5, 6, 8] and that addition of CoA removes the atractyloside inhibition only in the latter system [5, 6, 8, 15]. In our experience [5] the atractyloside induced inhibition on the "carnitine-activated" system can be removed not only by addition of CoA but also by addition of inorganic phosphate.

In the present communication further details on the "carnitine-activated" system are reported. These results unequivocally show that inorganic phosphate can replace CoA in the reactivation of the atractyloside-inhibited oxidation. Furthermore, present results indicate that the inhibitory action of atractyloside on fatty acid oxidation depending on carnitine and ATP is strongly enhanced by added AMP and that such an inhibition is due to a direct action on the ATP-dependent fatty acid thiokinase.

* For convenience the DNP-inhibited system reactivated by addition of ATP plus oligomycin or carnitine will be designated "oligomycin-activated" system and, respectively, "carnitine-activated" system.

2. Experimental procedure

Liver mitochondria were prepared from Wistar strain albino rats essentially by the procedure of Schneider [9]. Oxygen uptake was measured with a Clark oxygen electrode as described by Kielley and Bronk [10]. Long-chain fatty acid thiokinase was essayed by measuring fatty acyl-CoA formation with acyl-CoA dehydrogenase according to Tubbs and Garland [11]. The essay incubation mixture contained, in a final volume of 0.20 ml, the following reagents: Tris-HCl, pH 7.4, 50 μmoles; oleate, 200 μmoles; CoA, 0.1 μmole; ATP, 2 μmoles; $MgCl_2$, 3 μmoles. In the blank ATP was omitted. Incubations were carried out for 3–4 min at 38°C.

All reagents were analytical grade. CoA was completely reduced before use with sodium amalgam [12]. Atractyloside was a kind gift from Prof. R.Santi, University of Padova, Padova, Italy. Acyl-CoA dehydrogenase was prepared from beef liver mitochondria according to Tubbs and Garland [11].

3. Results

The oxygen traces reported in fig. 1 show that in the presence of DNP and atractyloside, oleate was oxidized at the maximum rate provided that ATP, carnitine and inorganic phosphate, or CoA, were added (fig. 1, trace A). However, when 1 mM AMP was added to the incubation mixture, the addition of inorganic phosphate *and* CoA was required in order to promote oxygen uptake (fig. 1, trace B). It has to be emphasized that in the absence of atractyloside

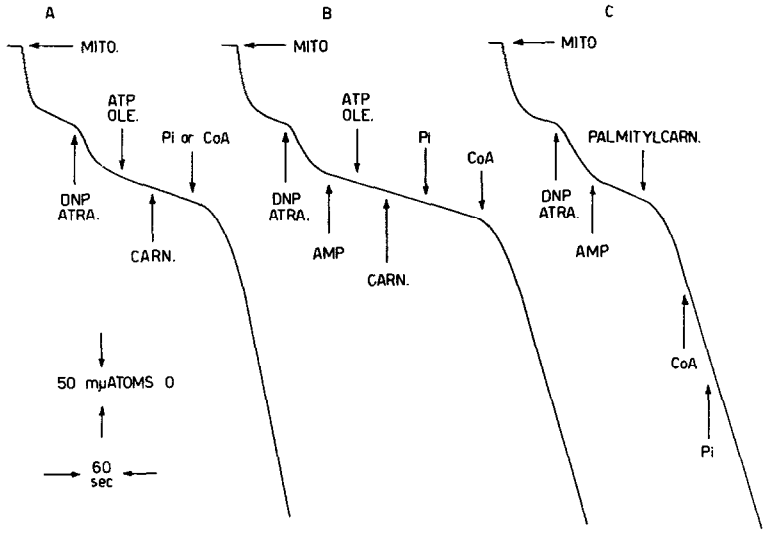

Fig. 1. Polarographic traces of oleate oxidation. The incubation mixture contained 10 mM Tris-HCl buffer (pH 7.4), 26 mM NaCl, 58 mM KCl and 6 $MgCl_2$. At the points indicated by arrows 8 mg of mitochondrial protein (MITO), 0.05 μmole of DNP, 1.5 μmole of atractyloside, 2 μmoles of ATP, 0.2 μmole of oleate, 1 μmole of carnitine, 0.1 μmole of CoA, 16 μmoles of orthophosphate, 2 μmoles of AMP, 1 μmole of DL-palmitylcarnitine were added. Total volume 2.0 ml; temperature 20°C.

no inhibition ensued to the addition of 1 mM AMP. Since in all conditions tested palmitylcarnitine was oxidized at the maximum rate (fig. 1, trace C) it can be deduced that all the above reported inhibitions occur at the stage of fatty acid activation.

In table 1 the action of inorganic phosphate, AMP and atractyloside on the ATP-dependent fatty acid thiokinase solubilized from rat liver mitochondria is reported. It is shown that the enzyme was activated by inorganic phosphate and partially inhibited by atractyloside and AMP; the highest inhibition was obtained in the presence of atractyloside and AMP and in the absence of inorganic phosphate.

The microsomal fatty acid thiokinase exhibited similar behaviour, as depicted in fig. 2, where oleyl-CoA production is plotted versus CoA concentration, in the presence and absence of inorganic phosphate, atractyloside and AMP.

4. Discussion

The reported results clearly show that the atractyloside inhibition on the "carnitine-activated" fatty

Table 1

Effect of orthophosphate, atractyloside and AMP on long-chain fatty acid thiokinase solubilized from rat liver mitochondria.

Assay conditions	Oleyl-CoA formation (mμmoles/mg/min)
No phosphate	2.32
No phosphate + atractyloside	1.50
No phosphate + AMP	1.60
No phosphate + AMP + atractyloside	0.40
Plus phosphate	3.20
Plus phosphate + atractyloside	2.06
Plus phosphate + AMP	2.10
Plus phosphate + AMP + atractyloside	1.0

The packed rat liver mitochondria pellets were suspended in a small volume of water and lyophilized. The lyophilized material was resuspended in 0.1 M Tris-HCl (pH 7.4), 0.5% aqueous Triton X-100, 5×10^{-3} M mercaptoethanol and sonically disrupted (20 kilocycles, Sonifer apparatus) for 1 min at a temperature between 0° and 5°. The suspension was centrifuged at 80000 × *g* for 30 min and the clear supernatant (240 μg of protein) was used for enzymic assay. Thiokinase activity was measured with oleate as substrate, as described under Experimental procedure. Where indicated inorganic phosphate (8 mM), atractylate (1.5 mM) and AMP (1 mM) were added. Incubation was carried out for 4 min at 38°C.

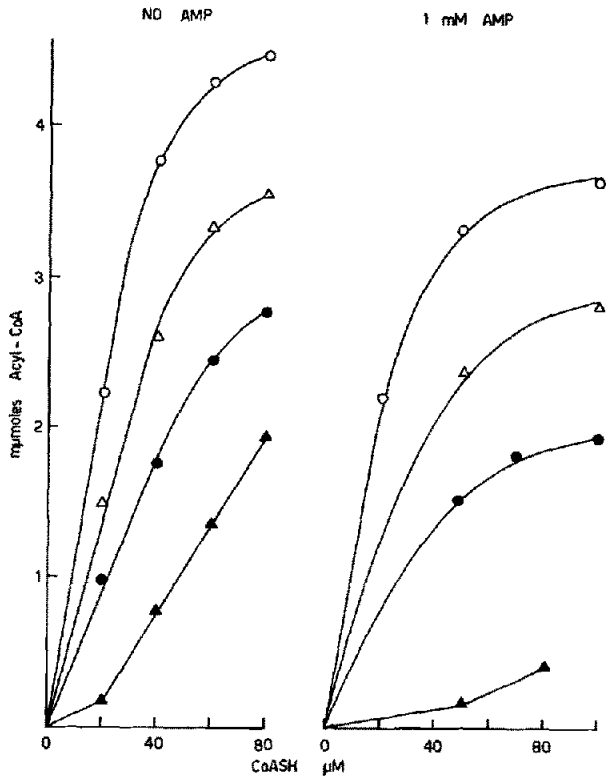

Fig. 2. Acyl-CoA synthesized by rat liver microsomes versus CoA concentration. Incubation and assay system as in table 1. Rat liver microsomes (93 μg of protein) were incubated for 3 min at 38°C. The curves were obtained with 8 mM orthophosphate and no atractylate (○), 8 mM orthophosphate and 1.9 mM atractylate (△), no orthophosphate and no atractylate (●), no orthophosphate and 1.5 mM atractylate (▲). Experiments were performed in the absence (left traces) and presence of 1 mM AMP (right traces).

acid oxidation in rat liver mitochondria can be relieved by addition of CoA or, alternatively, by addition of inorganic phosphate (fig. 1, trace A). These findings are apparently in contrast with those reported by Van den Bergh [8, 15], showing that the addition of CoA is absolutely necessary also in the presence of inorganic phosphate in order to obtain fatty acid oxidation. However, Van den Bergh's experiments were carried out in the presence of added AMP which is known to inhibit the ATP-dependent fatty acid thiokinase [13, 14]. In fact, the inhibition induced by atractyloside is reversed by phosphate or by CoA (fig. 1, trace A), while the inhibition induced by the combined addition of atractyloside and AMP can be reversed only by addition of CoA plus inorganic phosphate (fig. 1, trace B).

These results are explained by the data obtained with mitochondrial or microsomal ATP-dependent fatty acid thiokinase (table 1 and fig. 2): the enzyme appears to be inhibited by atractyloside and by AMP, and stimulated by inorganic phosphate. The highest inhibitory effect is observed in the absence of phosphate, when both atractyloside and AMP are added to the incubation mixture. Under these conditions a progressively increasing synthesis of acyl-CoA is achieved by addition of increasing amount of CoA.

These results cannot support the hypothesis of Van den Bergh on a dual carnitine-dependent site of fatty acid activation [8, 15].

In fact, the effect of inorganic phosphate, atractyloside and AMP on the "carnitine-activated" oxidation of fatty acid at the mitochondrial level can be rationalized by the parallel behaviour of fatty acid thiokinase.

It is interesting to remark that in intact mitochondria inhibited by atractyloside and AMP, palmitylcarnitine is constantly oxidized at the maximum rate independently of the addition of CoA or of phosphate (fig. 1, trace C). Since this substrate requires, like fatty acid, free CoA for its oxidation, it can be concluded that in mitochondrial preparations CoA is available in sufficient amount for palmitylcarnitine oxidation.

As is illustrated in fig. 3, such CoA appears to be confined "within" the "inner" mitochondrial compartment where the β-oxidation takes place. According to the same scheme, in the "carnitine-activated" system fatty acid, unlike acylcarnitine, in order to reach the "inner" pool of CoA must be activated externally. This "external" activation requires availability of ATP and of an external pool of CoA. The successive transport of externally activated acyls into the "inner" compartment requires carnitine. It can be postulated that the external pool of CoA would not be present as free coenzyme but it would derive from acyl-CoA already present into the mitochondria and continuously generated in its free form through the carnitine transacylase system (see scheme in fig. 3).

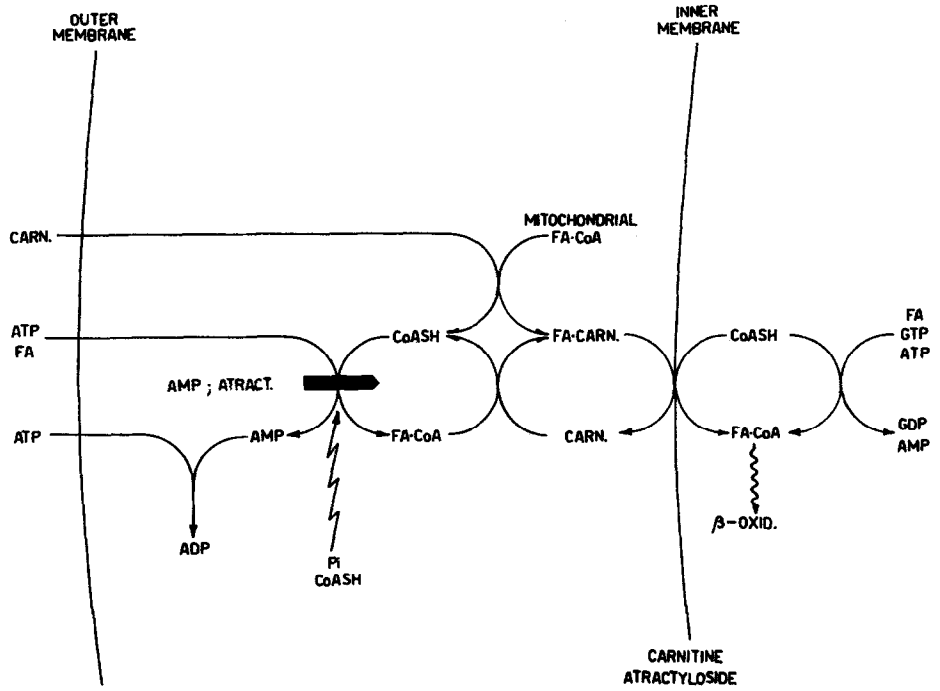

Fig. 3. Proposed scheme for mitochondrial organization of fatty acid activation.

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