INHIBITION BY FATTY ACYL ESTERS OF ADENINE NUCLEOTIDE TRANSLOCATION IN RAT-LIVER MITOCHONDRIA

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1. Introduction

At low substrate concentrations linoleate oxidation in isolated rat-liver mitochondria can be stimulated by addition of phosphate and phosphate acceptor [1]. With ADP as phosphate acceptor this State-3 linoleate oxidation is strongly inhibited after addition of CoASH. Inhibition by CoASH itself seems rather unlikely. However, in media containing ADP and Mg^{2+} the long-chain acyl-CoA synthetase present in the outer mitochondrial membrane can form linoleyl-CoA [cf. 2], ATP being derived from ADP through the action of adenylate kinase. Since extramitochondrial acyl-CoA cannot be oxidized in the absence of added carnitine [3], small amounts of linoleyl-CoA could accumulate, giving rise to the observed inhibition of State-3 respiration. Indeed, linoleate oxidation is not inhibited by CoASH under conditions preventing linoleate activation outside of the mitochondrial matrix (e.g. replacement of ADP by AMP, omission of Mg^{2+}, or addition of excess hexokinase + glucose). Moreover, CoASH does not inhibit State-3 respiration with substrates like succinate, oxoglutarate or glutamate + malate [4]. Obviously linoleyl-CoA is the inhibitor of State-3 linoleate oxidation. From other observations [5] it was concluded that this inhibition is effected through an inhibition of mitochondrial ADP uptake.

At about the same time Pande and Blanchaer [6] published data on the inhibition of adenine nucleotide (AdN) uptake in rat-heart mitochondria by palmityl-CoA and stearyl-CoA. More recently Shug et al. [7] described experiments indicating an inhibition of AdN transport in rat-liver mitochondria by oleyl-CoA. In the present work the inhibitory effect of some acyl-CoA esters on AdN translocation is reported. This effect is measured as an inhibition either of ADP uptake or of dinitrophenol (DNP)-induced ATPase activity in rat-liver mitochondria. Evidence is presented to show a close relationship between the chain-length of acyl-CoA esters and their inhibitory effect on AdN translocation.

Since earlier reports in the literature [8,9] pointed to an inhibition by palmitylcarnitine of AdN uptake, the effect of some acylcarnitines was also investigated. Metabolic implications of the present results are discussed in a subsequent paper [10].

2. Methods and materials

The activity of the AdN translocator was determined in forward-exchange of ^{14}C-ADP against the endogenous AdN pool by measuring the amount of radioactivity accumulated in the mitochondria during incubations at 0°. Plotting ln (100 −% exchange) against incubation time, the rate of exchange (nmoles/min/mg protein) was derived from the formula [11]:

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$$\text{rate of exchange} = \frac{1}{a} \cdot \frac{1}{t_{1/2}} \cdot \frac{\text{E I}}{\text{E + I}} \cdot \ln 2$$

where a, $t_{1/2}$, E and I represent mg protein present, time needed for 50% exchange, external AdN pool and amount of exchangeable intramitochondrial AdN, respectively.

Rat-liver mitochondria were isolated according to Myers and Slater [12] and washed once more to remove all endogenous carnitine. Protein was determined with the biuret method as described by Cleland and Slater [13]. P_i liberated in ATPase experiments was estimated according to Fiske and Subbarow as modified by Sumner [14].

L-Palmitylcarnitine was synthesized as described by Bremer [15]. L-carnitine chloride was a gift of Otsuka Pharm. Fact. (Osaka). L-octanoylcarnitine and L-linoleylcarnitine were kindly provided by Dr. J. Bremer and Dr. B.O. Christophersen (Oslo). L-linoleylcarnitine contained the antioxidant Santoquin [16]. In preliminary experiments it could be demonstrated that Santoquin *per se* has no influence on ATPase activity. Fatty acids, atractyloside and bovine serum albumin (BSA), fraction V, were purchased from Sigma; acyl-CoA esters from P-L Biochemicals; (8-^{14}C)-ADP from the Radiochemical Centre; D,L-acetylcarnitine, CoASH, phosphoenolpyruvate, pyruvate kinase and hexokinase from Boehringer. The enzyme suspensions were dialyzed before use and BSA was defatted according to Chen [17]. Micellar solutions of fatty acids were prepared by neutralizing aqueous solutions of the potassium salts. Solutions of palmitate and palmitylcarnitine were heated to 70° before use.

3. Results and discussion

The sensitivity of the DNP-induced ATPase of rat-liver mitochondria to acyl-CoA esters of different chain-length is illustrated in fig. 1. For comparison the inhibitory effect of free palmitate is also shown. Acetyl-CoA and octonoyl-CoA are almost inactive, whereas palmityl-CoA is strongly inhibitory. Previous experiments had shown that the concentration of palmityl-CoA should be expressed in a relative (nmoles per mg protein) rather than in an absolute (μM) manner, indicating accumulation of pamityl-CoA in the membrane phase. The severe inhibition of the ATPase activity by palmityl-CoA can be explained by a direct inhibition of ATP translocation. The data in table 1, showing a direct inhibition of ADP exchange by palmityl-CoA support this explanation. A good agreement exists between the degree of inhibition by acyl-CoA compounds in both types of experiments. It should be noted that relatively low concentrations of palmityl-CoA suffice for half-maximal inhibition of ADP exchange.

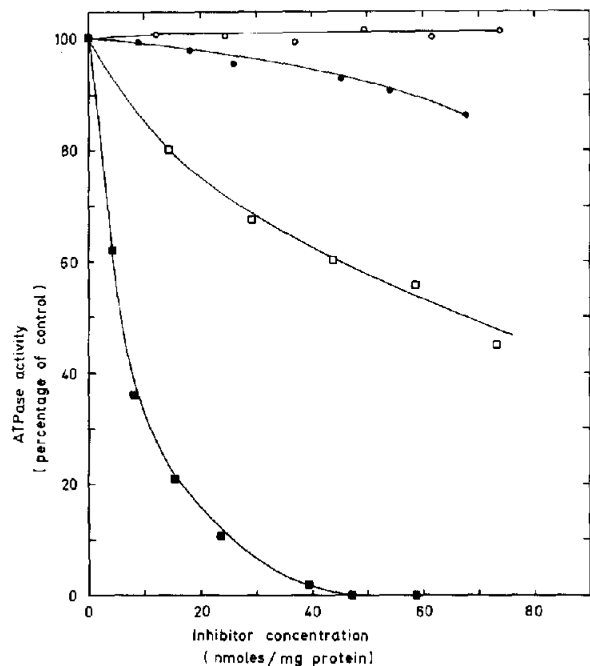

Fig. 1. Inhibition of DNP-induced ATPase by acyl-CoA esters. The standard reaction medium contained 15 mM KCl, 2 mM EDTA, 50 mM Tris-Cl pH 7.4, 50 mM sucrose, 0.1 mM DNP and 4 mM ATP. Reactions were started by addition of mitochondria (0.58 mg protein). Final vol, 1.5 ml. Temp., 25°. Reaction time, 10 min. (o——o——o) acetyl-CoA; (•——•——•) octanoyl-CoA; (□——□——□) palmitate; (■——■——■) palmityl-CoA.

Fig. 2 shows that the effect of palmityl-CoA can be mimicked by palmitate *plus* a fixed amount of CoASH. As might be expected from the Mg^{2+}-dependency of the "external" fatty acid-activating enzyme [18], CoASH enhances the inhibition of the DNP-stimulated ATPase by palmitate only if Mg^{2+} is present. Similar results are obtained replacing

Table 1
Inhibition of ADP exchange by acyl-CoA esters.

Expt. No.	Additions	Concentration (nmoles/ mg protein)	Rate of exchange (nmoles/min/mg)	(%)
1	None	–	0.72	100
	Acetyl-CoA	24.0	0.74	103
	Octanoyl-Coa	24.0	0.68	94
	Palmityl-CoA	24.0	0.00	0
2	None	–	0.60	100
	Palmityl-CoA	1.7	0.43	72
	Palmityl-CoA	3.4	0.34	57
	Linoleate	39.0	0.23	38

Mitochondria were incubated at 0° in 1.5 ml of a medium containing 15 mM KCl, 4 mM EDTA, 50 mM triethanolamine pH 7.4 and 75 mM sucrose. After 1 min ^{14}C-ADP (203,000 cpm) was added. Final ADP concentration, 0.4 mM. The reaction was stopped by millipore filtration in samples (0.10 ml) withdrawn at 0.5, 1, 2, 3, 5, 7, 10, 15 and 20 min after ^{14}C-ADP addition, respectively. After being washed with 0.15 M NaCl, the filters were dried and radioactivity was measured in a liquid scintillation counter. Mitochondrial protein, 2.81 mg (Expt. 1) and 3.28 mg (Expt. 2).

palmitate by linoleate (not shown). Obviously, small amounts of long-chain acyl-CoA formed during the incubations can block the AdN translocator effectively.

Some time ago Font and Gautheron [19] reported an inhibition of ADP uptake by CoASH in pig-heart mitochondria. This effect can be explained by activation of endogenous long-chain fatty acids. In line with this explanation we found that addition of CoASH in the presence of Mg^{2+} strongly inhibits the DNP-stimulated ATPase activity of aged mitochondrial preparations (not shown), considerable amounts of endogenous fatty acids being released during aging [20].

The inhibition of the DNP-induced ATPase by acylcarnitines is shown in fig. 3. At room temp. the presence of rotenone is strictly required because otherwise acylcarnitines are oxidized too fast to give any inhibition [cf. 6,8]. Only addition of long-chain acylcarnitines leads to an appreciable inhibition of ATPase activity. Similar results were obtained in exchange experiments (not shown). Inhibition of the DNP-induced ATPase by palmitylcarnitine and by palmityl-CoA can be reversed by addition of BSA during the incubation, whereas complexation with BSA prior to incubation

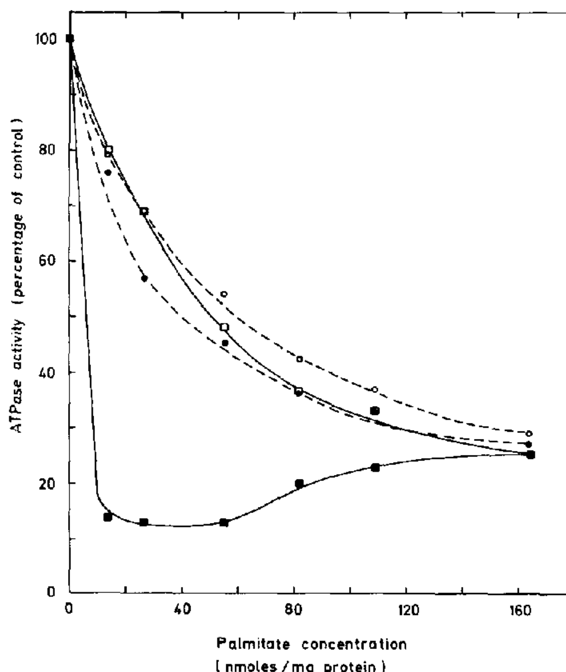

Fig. 2. Inhibition of DNP-induced ATPase by palmitate in the presence of added CoASH. Standard medium as in fig. 1. In experiments with $MgCl_2$ an ATP-regenerating system (7.5 I.U. pyruvate kinase and 5 mM phosphoenolpyruvate) was added. Mitochondrial protein, 0.63 mg. (○---○---○) no further additions; (●---●---●)+ CoASH (60 μM); (□---□---□)+ $MgCl_2$ (5 mM); (■——■——■)+ CoASH + $MgCl_2$.

completely prevents the inhibition (not shown).

Fig. 4 shows the effect of palmitylcarnitine on the mitochondrial ATPase under various conditions. The unmasking of the latent ATPase [cf. 21,22] at concentrations higher than about 50 nmoles palmitylcarnitine/mg protein is found similarly with palmityl-CoA. It can be explained by increasing disruption of the inner membrane, due to the surface-active properties of both palmityl-esters. Under these conditions the AdN translocater is by-passed and the latent ATPase is inhibited neither by the acyl esters themselves nor by, e.g., atractyloside. It therefore appears that comparable concentrations of palmityl-CoA and palmitylcarnitine exert almost identical non-specific detergent effects, whereas palmityl-CoA is a much stronger inhibitor of the translocator. Moreover, complete inhibition of AdN translocation occurs at palmityl-CoA concentrations that are lower than those required to stimulate the latent ATPase. Hence it may

be concluded that the inhibition of AdN translocation by palmityl-CoA is not related to its surface-active properties.

A kinetic examination of the inhibition of State-3 succinate oxidation by palmityl-esters is given in fig. 5. Palmityl-CoA and palmitylcarnitine behave like competitive inhibitors with respect to ADP (fig. 5a). From a $1/v$ against palmityl-CoA concentration plot (fig. 5b) an apparent K_i of 0.5 nmole palmityl-CoA/mg protein can be derived. It is difficult to assess, however, whether monomeric, dimeric or micellar forms of palmityl-CoA are responsible for its inhibitory action. In these kinetic experiments almost all palmityl-CoA is probably bound to mitochondrial protein, whereas the critical micellar concentration (3 to 4 μM) attributed to palmityl-CoA [23] is given in absolute concentration terms. In addition the critical micellar concentration of ionic detergents is strongly dependent on the salt concentration of the medium [24].

In table 2 the effectiveness of palmitate, palmityl-

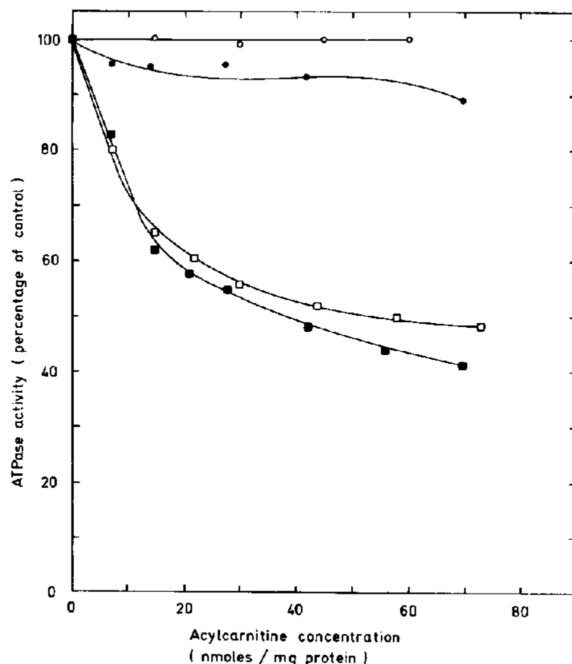

Fig. 3. Inhibition of NDP-induced ATPase by acylcarnitine esters. Standard medium as in fig. 1 with 0.3 μg rotenone. Mitochondrial protein, 0.61 mg. (○——○——○) D,L-acetylcarnitine; (●——●——●) L-octanoylcarnitine; (□——□——□) L-palmitylcarnitine; (■——■——■) L-linoleylcarnitine.

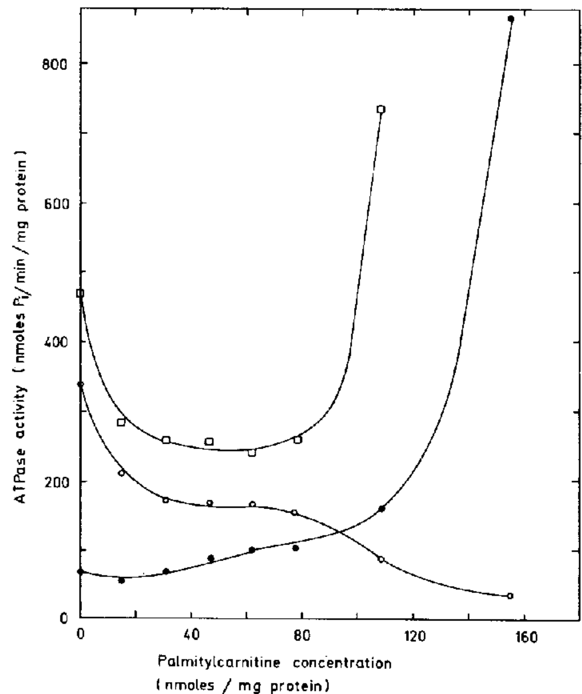

Fig. 4. Dependence of ATPase activity on L-palmitylcarnitine concentration. Standard medium as in fig. 3. Mitochondrial protein, 0.65 mg. (○——○——○) no further addition or omissions; (□——□——□) + 5 mM $MgCl_2$ (+ ATP-regenerating system); (●——●——●) + 5 mM $MgCl_2$ (+ ATP-regenerating system), but DNP omitted.

carnitine and palmityl-CoA is compared within the same mitochondrial preparation, both in exchange experiments and in ATPase experiments. It follows that activation of palmitate to palmityl-CoA increases the inhibitory action on the translocator manifold. Subsequent conversion of palmityl-CoA into palmitylcarnitine will fully reverse this effect. Since there are no indications that palmityl-CoA and linoleyl-CoA differ much in inhibitory power, the presence of double bonds in acyl-CoA esters probably is not a critical factor. The same conclusion does hold for long-chain acylcarnitines (fig. 3). On the other hand, well-known inhibitors of AdN translocation like atractyloside and bongkrekic acid, having one and six double bonds, respectively, lose much, if not all, of their activity after hydrogenation [25,26].

Obviously, the presence of a long hydrocarbon chain is a prerequisite for inhibition of the translocator by acyl esters thereby indicating [27,28] some degree of Van

Table 2
Inhibition of AdN translocation by palmitate, L-palmityl-carnitine and palmityl-CoA.

Additions	Concentration (nmoles/mg protein)	Rate of exchange (A) (nmoles/min/mg)	(%)	ATPase (B) (%)
None	–	0.80	100	100
Palmitate	53.0	0.50	63	35
Palmityl-carnitine	53.0	0.53	66	55
Palmityl-CoA	53.0	0.00	0	0
Palmityl-CoA	5.3	0.49	61	54
CoASH	53.0	1.21	151	

Expt. A, Inhibition of ^{14}C-ADP exchange. Experimental details as in table 1. Expt. B, Inhibition of DNP-induced ATPase activity. Same standard conditions as in fig. 3. Mitochondrial protein, 3.06 mg (A) and 0.69 mg (B).

der Waals interaction between the hydrocarbon tail and non-polar entities of the inner membrane. The influence of the polar groups should, however, not be overlooked as can be concluded from comparison of palmityl-CoA (with three strongly anionic groups), palmitate (with one weakly anionic group) and palmitylcarnitine (with a zwitterionic structure) at physiological pH. In this connection it may be worth mentioning that substitution of the carboxylic group of atractyloside by a methyl group leads to a complete loss of activity [25], whereas introduction of a second carboxylic group in atractyloside enhances the activity [29,30].

For the moment one can only speculate about the mechanism of action of pamityl-CoA as compared to those of atractyloside and bongkrekic acid [31]. Recently the suggestion was put forward that bongkrekic acid should first be converted into a CoA derivative before being able to inhibit AdN translocation [32]. Whether or not such an intimate link exists between the action of bongkrekic acid and palmityl-CoA, it

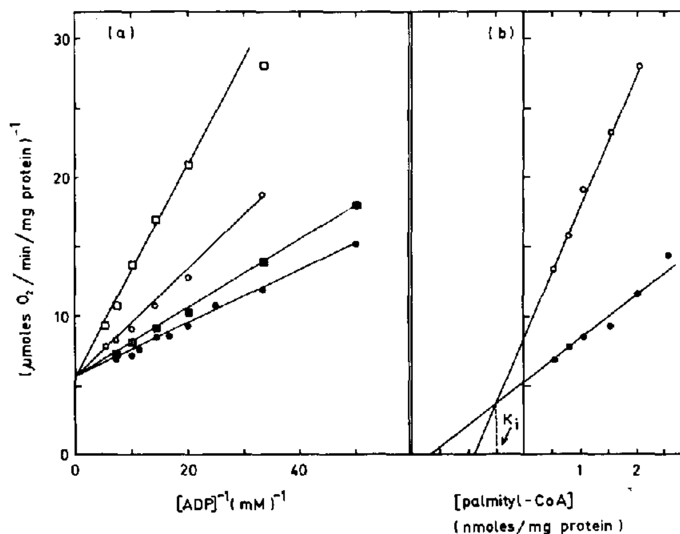

Fig. 5. Inhibition of succinate oxidation by palmityl-esters. Mitochondria (3.43 mg) were added to a medium containing 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-Cl pH 7.4, 10 mM potassium phosphate pH 7.4, 10 mM succinate, 2 μg rotenone, 10 mM glucose, 10 I.U. of hexokinase and fatty acyl esters as indicated below. After 1 min ADP was added and oxygen uptake was measured using a vibrating platinum electrode. Final reaction vol, 2.0 ml Temp., 25°. (a) (●——●——●) none; (■——■——■) L-palmitylcarnitine (2.7 nmoles/mg protein); (○——○——○) palmityl-CoA (1.0 nmole/mg protein); (□——□——□) palmityl-CoA (2.1 nmoles/mg protein). (b) (○——○——○) 30 μM ADP; (●——●——●) 140 μM ADP.

seems worthwile to investigate whether palmityl-CoA like bongkrekic acid [31,33] can influence AdN binding sites on the inside of the inner membrane, since intramitochondrial palmityl-CoA concentrations up to 1 nmole/mg protein have been observed [34].

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