ON THE MECHANISM OF UNCOUPLER-DEPENDENT INHIBITION OF ACYL-CARNITINE OXIDATION BY RAT LIVER MITOCHONDRIA

H. OSMUNDSEN and J. BREMER
Institute for Medical Biochemistry, The University of Oslo, Karl Johansgate 47, Oslo, Norway

Received 16 August 1976

1. Introduction

The mechanism by which acyl-carnitines penetrate the inner mitochondrial membrane has for some time been the subject of experimental investigation. The apparent impermeability of the inner membrane of rat liver mitochondria to carnitine [1,2] together with the observed low concentration of carnitine in these mitochondria (less than 0.1 nmol/mg protein) [1], have led to the hypothesis that the inner mitochondrial carnitine palmitoyl-transferase (EC 2.3.1.21) is vectorial, catalysing a direct exchange between external carnitine and matrix CoA [1]. In heart mitochondria, however, an exchange of carnitine across the inner membrane apparently takes place [3]. It has been suggested from experiments on liver mitochondria [4] and blowfly flight-muscle mitochondria [5] that the penetration of acyl-carnitines is dependent on the presence of the proton gradient generated by oxidative phosphorylation.

We have examined the uncoupler induced inhibition of palmitoyl-carnitine oxidation, as described by Levitsky and Skulachev [4], and found this to be critically dependent on the composition of the incubation medium. This has led us to suggest that the inhibitory effect of the uncoupler, which is expressed only in sucrose containing media, is primarily due to a collapse of the osmotic potential of the matrix. Analogous conclusions have been reached both with respect to the dinitrophenol (DNP) + valinomycin induced inhibition of glutamate/malate oxidation by rat liver mitochondria in sucrose media [6], and the slow rate of palmitoyl-carnitine oxidation by brown adipose tissue mitochondria suspended in iso-osmolar sucrose media [7]. The presence of a proton gradient across the inner membrane is therefore at best of secondary significance.

2. Materials and methods

Liver mitochondria were prepared from 160–200 g male albino Wistar rats using a medium containing 300 mM sucrose 5 mM Hepes*, 1 mM EDTA, adjusted to pH 7.2 with Tris base. If the mitochondria were to be incubated in a KCl containing medium, they were given a final wash in that medium. 1-[^{14}C]Palmitoyl-carnitine was prepared as described by Bremer et al. [8], and had a specific activity of 1.2×10^3 c.p.m./nmol.

Mitochondrial β-oxidation of 1-[^{14}C]palmitoyl-carnitine was followed by measurement of $HClO_4$ (2.5%) soluble radioactivity. The incubation medium contained 1.5 mg/ml defatted bovine serum albumin, 1 mM Tris/malonate, 0.1 mM EDTA 5 mM Hepes, pH 7.2. If not otherwise indicated, the medium contained either 300 mM sucrose or 130 mM KCl. Sucrose containing media were pH adjusted using Tris base. Proteins were determined by the method of Lowry [9].

3. Results and discussion

The stimulatory effect of K^+ on mitochondrial respiration has long been recognised [10]. In a medium containing a low K^+ concentration the addition of DNP, or better DNP + valinomycin, led to inhibition of glutamate/malate oxidation by rat liver

* 2(*N*-hydroxyethylpiperazin-*N*-yl)ethanesulphonate

Table 1
The effect of 300 mM sucrose and 130 mM KCl on the oxidation of 1-[^{14}C]palmitoyl-carnitine (63 μM) by rat liver mitochondria

Medium	Additions to the incubation medium/c.p.m. × 10^{-2} in acid soluble supernatant					
	None	ADP	ADP+P_i	ADP+P_i+DNP	ADP+DNP	P_i+DNP
300 mM Sucrose	4.5 (100)	4.3 (96)	6.4 (142)	5.9 (131)	2.9 (65)	4.8 (106)
130 mM KCl	34.0 (100)	36.4 (107)	96.9 (285)	95.9 (282)	95.2 (280)	87.0 (256)

The composition of the media is described in the methods section. ADP and P_i were included each at a concentration of 1 mM, and DNP to a concentration of 100 μM. After 5 min incubation at 30°C, in a total volume of 2 ml containing 3.4 mg protein, the reaction was stopped by the addition of 2 ml of ice-cold 5% $HClO_4$. After centrifugation 0.5 ml of the supernatant was counted in a Packard Model 3375 Scintillation Counter. The numbers in brackets represent the acid soluble radioactivity expressed as % of the activity obtained in the absence of any additions.

mitochondria. This inhibition became progressive with increasing concentrations of sucrose [6].

Table 1 shows that the oxidation of 1-[^{14}C]palmitoylcarnitine is markedly inhibited by the use of 300 mM sucrose, instead of 130 mM KCl, in the incubation medium. This applies to all incubation conditions used. From these data it is also apparent that the action of 100 μM DNP, in the presence of 300 mM sucrose, is to further inhibit the oxidation of palmitoyl-carnitine, as observed by Levitsky and Skulachev [4]. This effect persists in the presence of ADP. When KH_2PO_4 (P_i) is included in the incubation medium, together with ADP or alone, the DNP induced inhibition is abolished. In the presence of 200 mM sucrose there is a general stimulation of palmitoyl-carnitine oxidation, compared to that taking place in 300 mM sucrose medium, and DNP now causes a slight stimulation (see fig.2). In the KCl medium DNP was strongly stimulatory, and ADP and P_i had no additional effect.

The effect of DNP, and of various concentrations of P_i and KCl, on palmitoyl-carnitine oxidation in the 300 mM sucrose medium is illustrated in fig.1. These results show that DNP is inhibitory only in the absence of P_i, and that P_i has a much more marked stimulatory effect than similar low concentrations of KCl. In view of the known swelling properties of P_i [11], it is likely that its protective effect is due to the prevention of the mitochondrial contraction, which otherwise may take place on the addition of DNP.

The inhibitory effect of DNP on palmitoyl-carnitine oxidation is in fig.2 shown to be dependent on the molarity of the sucrose medium, as is also the absolute rate of palmitoyl-carnitine oxidation. The increase in respiratory rate correlates well with increased mitochondrial swelling (i.e. decreased optical density). Similarly with increased swelling, the effect of DNP becomes slightly stimulatory.

These results are in good accord with results obtained from studies on brown adipose tissue mitochondria, where good correlations were found

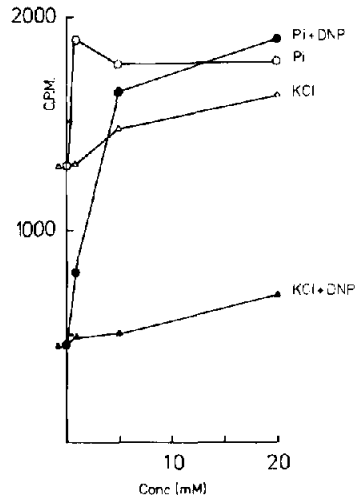

Fig.1. The effect of various concentrations of KCl and KH_2PO_4 on the DNP induced inhibition of 1-[^{14}C]palmitoyl-carnitine (63 μM) oxidation in 300 mM sucrose medium. Where appropriate DNP was included to a concentration of 30 μM, and KH_2PO_4 and KCl as indicated in the figure. To maintain constant osmolarity water had been added to the various media. Each sample of 2 ml contained 3.5 mg protein. Other experimental details are described in the legend to table 1.

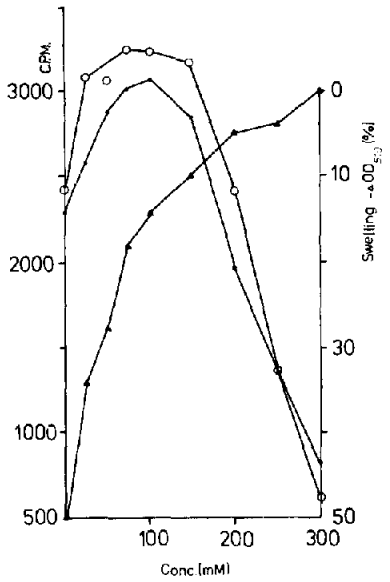

Fig.2. The effect of sucrose concentration on mitochondrial swelling, and the rate of 1-[^{14}C]palmitoyl-carnitine oxidation. In samples containing DNP this was included to a concentration of 30 μM. The basic medium contained 5 mM Hepes, 0.1 mM EDTA, adjusted to pH 7.2 with Tris base, and 5 mM sucrose contributed by the addition of the mitochondrial suspension. Each 2 ml sample contained 5.8 mg mitochondrial protein. (▲) Swelling, (●) palmitoyl-carnitine oxidation in the absence of DNP, (○) palmitoyl-carnitine oxidation in the presence of DNP. Other experimental details are described in the legend to table 1.

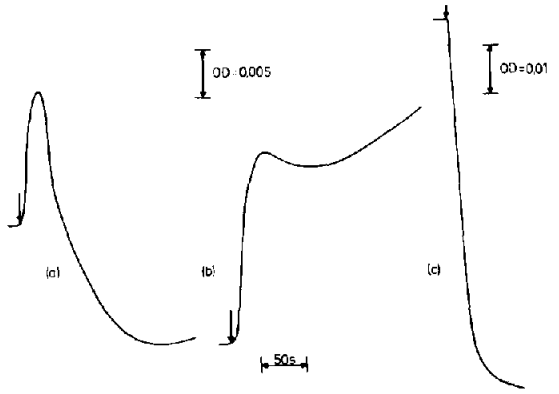

Fig.3. The effect of FCCP on mitochondrial size. Mitochondrial suspensions (6.8 mg protein in 6 ml medium) were divided equally into two cuvettes. FCCP (1 μM) was added to the suspension placed in the sample beam of an Aminco DW-2 spectrophotometer, operated in the split beam mode. Changes in optical density at 510 nm were recorded. The position of the arrow indicates the time of addition of FCCP. Typical traces of changes observed in a medium containing 25 mM sucrose, 300 mM sucrose and 130 mM KCl are represented by (a), (b) and (c), respectively.

between decreased respiratory rates and sucrose impermeable spaces [7]. Increasing concentrations of sucrose have also been associated with decreased glutamate/malate induced NADH fluorescence [12], which has been taken as evidence that substrate transport into the matrix has been inhibited. Alternatively, it is possible that the activity of the oxidative enzymes of the matrix has decreased, due to dehydration of the matrix space.

We have measured the activities of the internal and external palmitoyl-carnitine transferases by the procedure of Solberg [13], and found their activities in a medium containing 300 mM sucrose to be about 50% of those obtained in the KCl medium (results not shown), while the rate of palmitoyl-carnitine oxidation was about 10% of that obtained in the KCl medium (table 1). The acyl-carnitine transfer through the inner membrane was therefore not rate limiting for β-oxidation when substrate concentrations of palmitoyl-carnitine were used. It is therefore likely that the activity of the β-oxidation system has been markedly diminished following dehydration of the matrix.

The effect of uncoupler (FCCP) on mitochondrial size, when added to suspensions of mitochondria in 300 mM and 25 mM sucrose media, and 130 mM KCl medium, is illustrated in fig.3. In the sucrose media an instantaneous contraction occurs on addition of the uncoupler. This is presumably due to loss of osmotically active ions, e.g. K^+ and anions, following the collapse of the membrane potential. Uncoupler induced loss of K^+ from rat liver mitochondria has previously been demonstrated by Kimmich et al. [14]. In a medium containing 25 mM sucrose this contraction is followed by extensive swelling as the ions from the initial leak equilibrate according to the osmotic potentials prevailing on either side of the inner membrane. In the presence of 300 mM sucrose, however, the initial contractile leak is not reversed. On the contrary, it is superceded by a slower, but progressive, contraction. This is probably due to the high osmotic potential of the medium, and the absence

of significant concentrations of penetrant ions in the medium.

Levitsky and Skulachev [4] observed that pre-incubation of the mitochondria with palmitoyl-carnitine prior to addition of uncoupler led to lesser inhibition of respiration, than when the order of addition was reversed. These authors also showed that palmitoyl-carnitine caused swelling when added to coupled mitochondria, but not when added to uncoupled mitochondria. Thus pre-swelling of the mitochondria may have led to lesser matrix contraction by DNP, and consequently to lesser inhibition of palmitoyl-carnitine oxidation.

The inhibition by FCCP of acetyl-carnitine oxidation by blowfly flight muscle mitochondria incubated in a KCl medium has recently been demonstrated [5]. Severe inhibition was, however, only temporary, and during the subsequent period of respiratory acceleration the mitochondria were reported to be swelling. This indicates that inhibition may be caused by contraction, and that FCCP brought about a loss of osmotically active ions, which only slowly can penetrate back into the matrix.

Acknowledgements

We thank Mr T. Gjelvold for excellent technical assistance. This work was supported by the Royal Norwegian Council for Scientific and Industrial Research.

References

[1] Yates, D. W. and Garland, P. B. (1966) Biochem. Biophys. Res. Commun. 23, 460–465.
[2] Brosnan, J. T. and Fritz, I. B. (1971) Biochem. J. 125, 94P–95P.
[3] Ramsay, R. R. and Tubbs, P. K. (1975) FEBS Lett. 54, 21–25.
[4] Levitsky, D. O. and Skulachev, V. P. (1972) Biochim. Biophys. Acta 275, 33–50.
[5] Danks, S. M. and Chappel, J. B. (1975) FEBS Lett. 59, 230–233.
[6] Nicholls, D. G. and Lindberg, O. (1972) FEBS Lett. 25, 61–64.
[7] Nicholls, D. G. and Grav, H. J. and Lindberg, O. (1972) Eur. J. Biochem. 31, 526–533.
[8] Bremer, J. and Wotjczak, A. B. (1972) Biochim. Biophys. Acta 280, 515–530.
[9] Lowry, O. H., Rosebrough, H. J., Farr, A. L. and Randall, R. J. (1951) J. biol. Chem. 193, 265–275.
[10] Pressman, B. C. and Lardy, H. A. (1955) Biochim. Biophys. Acta 18, 482–486.
[11] Chappel, J. B. and Crofts, A. (1966) in: Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), Vol. 7, pp. 239–316. Elsevier, Amsterdam.
[12] Gomez-Puyou, A., Sandoval, M., Tuena de Gomez-Puyou, M., Pena, A. and Chavez, E. (1972) Biochemistry 11, 97–102.
[13] Solberg, H. E. (1974) Biochim. Biophys. Acta 360, 101–112.
[14] Kimmich, G. A. and Rasmussen, H. (1967) Biochim. Biophys. Acta 131, 413–420.