BINDING OF GUANINE NUCLEOTIDES TO THE OUTER SURFACE OF THE INNER MEMBRANE OF GUINEA PIG BROWN FAT MITOCHONDRIA IN CORRELATION WITH THE THERMOGENIC ACTIVITY OF THE TISSUE

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1. Introduction

Energy coupling in mitochondria isolated from brown adipose tissue (BAT) is closely correlated with the calorigenic activity of the tissue, i.e. the mitochondria are extremely uncoupled when prepared from brown fat during active thermogenesis [1,2]. Recoupling of respiration and ATP-production in these mitochondria is essentially influenced by externally added nucleosidediphosphates or triphosphates [3,1]; GDP especially reveals an extensive recoupling effect on oxidative phosphorylation which could be only demonstrated with BAT mitochondria and appears specific for the reversal of uncoupling induced by nonesterified fatty acids [4]. This communication reports on studies of the incorporation of purine and pyrimidine nucleotides into mitochondria from guinea pig BAT and from rat liver. Results are interpreted as indicating nucleotide binding to the outer face of the inner mitochondrial membrane. GDP binding by BAT mitochondria is 5 times higher than that by rat liver mitochondria and is significant for the thermogenic activity of the tissue.

2. Methods and materials

Guinea pigs classified as newborn were less than 24 h old. The age of fetal guinea pigs was determined as described by Draper [5]. Mitochondria from guinea pig BAT were isolated as earlier described [3] in 20 mM triethanolamine, pH 7.2, 1 mM EDTA and 250 mM sucrose. Rat liver mitochondria were prepared in the same medium employing the same isolation procedure.

The incorporation of nucleotides into the mitochondria was determined by radioactivity measurements. For this purpose the mitochondria were incubated in the isolation medium supplemented with 5 mM potassium phosphate, 2 mM $MgCl_2$ and 100 μM ^{3}H-labeled nucleotides (specific activity 15 Ci/mol). The temperature was 0 1°C. After varying times of incubation (5–600 s) the mitochondria were separated from the reaction mixture by silicone oil layer centrifugation, as described by Heldt et al. [6] in a Beckman Microfuge, using silicone oil from Wacker Chemie, München, AR 100/AR 150 = 2/5. Radioactivity was measured in a Philips liquid scintillation analyzer. The values were corrected for the nucleotide contained in the portion of external medium carried through the silicone oil by the mitochondria which was determined by marking the medium with [^{14}C]sucrose.

The mitochondrial protein was determined by a modified biuret method [1].

Biochemicals were from Boehringer Mannheim GmbH, Mannheim, Germany. ^{3}H-labeled nucleotides and [^{14}C]sucrose were obtained from Amersham Buchler GmbH, Braunschweig, Germany. All other chemicals were of analytical grade.

3. Results and discussion

As reported in a preliminary note [7], isolated

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Table 1
Incorporation of nucleotides into mitochondria from guinea pig BAT and rat liver

Externally added nucleotide	Nucleotide incorporation (nmol/mg mitoch. protein)	
	BAT mitochondria	Rat liver mitochondria
GMP	0.34 ± 0.13 (6)	0.11 ± 0.03 (6)
GDP	0.61 ± 0.16 (12)	0.15 ± 0.04 (8)
GTP	0.55 ± 0.10 (8)	0.18 ± 0.04 (6)
CDP	0.27 ± 0.04 (6)	0.14 ± 0.03 (6)
UDP	0.14 ± 0.04 (6)	0.08 ± 0.03 (8)
ADP	1.97 ± 0.16 (10)	8.56 ± 0.65 (10)

The concentration of externally added nucleotides was 100 μM. 0.8–1.2 mg mitochondrial protein/ml were incubated for 180 s at 0–1°C. The medium and experimental details are described in the Methods section. Data present mean values ± S.D.; the number of experiments is given in parenthesis.

BAT mitochondria from newborn guinea pigs are able to incorporate significantly higher amounts of externally added GDP than mitochondria from rat liver under the same experimental conditions. This incorporation is defined by the amount of radioactive labeled nucleotide carried through the silicone oil layer by the mitochondria. This finding appears noteworthy in view of the drastic recoupling influence of guanine nucleotides on oxidative phosphorylation in mitochondria from thermogenic active BAT. As demonstrated in table 1, the incorporation of guanine nucleotides into BAT mitochondria is 3–5 times higher than that measured with liver mitochondria, whereas the incorporation of CDP and UDP is about twice as high as the incorporation of these nucleotides into mitochondria from liver. Since the recoupling influence of CDP, UDP and GMP on BAT mitochondria is considerably smaller than that of GTP and especially of GDP [8], the greater incorporation of GDP and GTP may indicate a correlation between the nucleotide incorporation into BAT mitochondria and the recoupling effect of the incorporated nucleotides.

The significance of guanine nucleotide incorporation into BAT mitochondria becomes particularly obvious from the results presented in fig.1. The amount of GDP incorporated into isolated BAT mitochondria decreases considerably during the postnatal development of the guinea pigs, when the animals are kept in a warm environment (22°C). Data measured with mitochondria from newborn guinea pigs are 4–5 times higher than values obtained with mitochondria from 20-day-old animals. BAT mitochondria isolated from fetal guinea pigs yield similar results as mitochondria from 20-day-old animals.

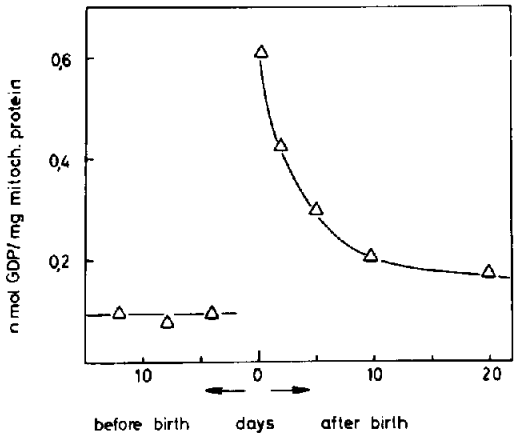

Fig.1. Incorporation of GDP into BAT mitochondria from guinea pigs during the pre- and postnatal development of the animals. The reaction mixture contained 0.7–1.2 mg mitochondrial protein/ml and 100 μM extramitochondrial GDP. The temperature was 0–1°C; the incubation was finished after 180 s. Experimental details are described in the methods section. Values present means from 3–7 experiments. No measurements were possible immediately before the birth of the animals, since the age of fetal guinea pigs cannot be determined exactly in the perinatal period.

From the tightly coupled oxidative phosphorylation in BAT mitochondria prepared from fetal guinea pigs it may be concluded that thermogenesis does not occur in BAT during the prenatal period. The thermogenic activity of the tissue attains its maximum in newborn guinea pigs and decreases during the first 10 days after the birth of the animals, kept at 22°C [9]. It appears from the demonstrated results that the amount of GDP incorporated into isolated BAT mitochondria corresponds to the thermogenic activity of the tissue.

The question arises whether the observed incorporation of guanine nucleotides into BAT mitochondria represents a transfer of the nucleotides into the matrix space of the mitochondria or wether it reflects binding of the nucleotides to the mitochondrial inner membrane. External guanine nucleotides might enter the matrix of BAT mitochondria by an unspecific transfer action of the adenine nucleotide carrier. The properties of the adenine nucleotide translocator are similar in mitochondria from BAT and liver with respect to the adenine nucleotide-specificity of the carrier and its sensitivity to atractyloside [10]. Similar to rat liver mitochondria [11] the incorporation of [^{3}H]ADP into BAT mitochondria (table 1) reflects an almost complete equilibration of the external radioactive label with the intramitochondrial ADP and ATP after 3 min of incubation at 0°C. The incorporation of radioactivity into BAT mitochondria is smaller than that into liver mitochondria which is due to the smaller pool of ADP plus ATP occurring in BAT mitochondria [12]. A transfer of guanine nucleotides across the mitochondrial inner membrane via adenine nucleotide translocator should be largely depressed by ATP and should be inhibited by atractyloside. However, as shown in table 2, the inhibition of GDP incorporation by a nine-fold excess in ATP is only about 60%, and atractyloside virtually causes no inhibition of the incorporation of GDP or the other investigated guanine and pyrimidine nucleotides (not shown here). Moreover, guanine nucleotides do not interfere with the adenine nucleotide translocation in mitochondria from liver [13] and from BAT [10]. These findings clearly show that the incorporation of guanine nucleotides into BAT mitochondria is not due to an unspecific transport by the adenine nucleotide carrier. This does not rule out the possibility that GDP transverses the inner membrane of BAT mitochondria by some other mechanism which is not inhibited by atractyloside.

On the other hand, it has been shown earlier with rat liver mitochondria that externally added GDP is not phosphorylated by substrate level phosphorylation [14]. Apparently the extramitochondrial GDP does not mix with the intramitochondrial guanine nucleotide pool. Therefore at least the GDP incorporation observed with rat liver mitochondria (tables 1 and 2) cannot be due to a transport into the mitochondrial matrix. This agrees also with results by Pfaff and Klingenberg [15], who found no appre-

Table 2
The influence of atractyloside and purine nucleotides on the incorporation of GDP into BAT mitochondria from newborn guinea pigs and into rat liver mitochondria

Additions	GDP incorporation (nmol/mg mitoch. protein)	
	BAT mitochondria	Rat liver mitochondria
–	0.61	0.15
33 μM Atractyloside	0.58	0.14
100 μM GTP	0.48	0.10
300 μM GTP	0.27	0.08
300 μM ATP	0.31	0.11
900 μM ATP	0.22	0.07

The reaction mixture contained 0.8 mg mitochondrial protein/ml and 100 μM externally added GDP. The temperature was 1–0°C, the incubation was finished after 180 s. Experimental conditions are described in Methods.

ciable transport of guanine nucleotides into rat liver mitochondria. Furthermore, there exists strong indication that the site of the nucleotide action on oxidative phosphorylation in BAT mitochondria is located on the outer face of the mitochondrial inner membrane. As reported earlier [3,16], energy coupling is also restored by externally added ATP or by ATP formed by substrate level phosphorylation within the mitochondrial matrix. Recoupling achieved by extramitochondrial ATP is not influenced by atractyloside [8], whereas recoupling caused by internally generated ATP is completely blocked by the inhibitor [16]. This clearly shows that the site of ATP-induced recoupling is only accessible from the outside but not from the matrix space of the mitochondria. Likewise GDP-induced recoupling is only observed with externally added GDP, whereas the intramitochondrial GDP has no restoring effect on energy coupling in BAT mitochondria [4]. It is concluded from these findings that the recoupling of oxidative phosphorylation induced by guanine nucleotides and by ATP is caused by the binding of these nucleotides to the outer face of the mitochondrial inner membrane.

Further evidence on the existence of nucleotide binding sites different from the adenine transport binding sites becomes obvious from the competition of nucleotides, including adenine nucleotides, for being incorporated into the mitochondria. Whereas GDP does not depress adenine nucleotide translocation [10], a significant decrease in GDP incorporation occurs in the presence of externally added GTP or ATP (table 2). In other experiments (not shown here) a similar repression of the incorporation of GMP and GTP by other guanine nucleotides and by adenine nucleotides was found. Apparently all these nucleotides compete with each other for binding sites on the mitochondrial membrane. In principle this is also observed with liver mitochondria (table 2). Preliminary studies to characterize these binding sites indicated the existence of high-affinity and low-affinity GDP binding sites in mitochondria from BAT as well as from liver*. GDP-specific binding by BAT mitochondria from hamsters was recently reported by Nicholls [17]. Incorporation of guanine nucleotides, UDP and CDP into rat liver mitochondria is generally smaller than incorporation of these nucleotides into BAT mitochondria (table 1). On the basis of nucleotide binding to the mitochondrial membrane this may be explained by differences in the cristae membrane surface which is considerably smaller in liver mitochondria than in mitochondria from BAT [1].

The amount of CDP and GDP incorporated into rat liver mitochondria (table 1) corresponds with data measured by Duee and Vignais [13]. These authors, however, inferred a transmembrane exchange of CDP and GDP with intramitochondrial nucleotides. Since no difference was found in the properties of nucleotide incorporation into mitochondria from rat liver and BAT, except in nucleotide quantities, our experiments are likely to indicate a mode of nucleotide binding by liver mitochondria similar to that by BAT mitochondria, thus confirming that no other nucleotides, except ADP and ATP are able to pass through the mitochondrial membrane [14,15].

The nucleotide-induced restitution of energy coupling in BAT mitochondria has been characterized by various criteria. Nucleotides, first of all GDP, were shown to 'normalize' the respiratory control and oxidative phosphorylation as well as the ion permeability of isolated BAT mitochondria (for review see [18]); no information, however, was achieved on the mechanism of this recoupling effect. The available results ([16] this paper) indicate that the recoupling is most likely caused by nucleotides approaching the outer face of the mitochondrial inner membrane. The number of nucleotide binding sites on the outer face of the membrane depends on the thermogenic activity of BAT. Further investigations on the nature of this binding may provide a way for characterizing the recoupling mechanism.

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