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ISOLATION OF THE GDP BINDING PROTEIN FROM BROWN ADIPOSE TISSUE MITOCHONDRIA OF SEVERAL ANIMALS AND AMINO ACID COMPOSITION STUDY IN RAT

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SUMMARY: The nucleotide binding protein (uncoupling protein, GDP binding protein) of brown adipose tissue mitochondria has been isolated from cold adapted rat, newborn guinea pig and newborn rabbit. The purification, using hydroxyapatite in sucrose gradient centrifugation, follows the procedures established previously for the isolation of this protein from cold adapted hamster. A similar degree of purification was obtained, reaching 60 µmol GDP bound/g protein. In SDS gel electrophoresis the purified protein gave a single band of M_r 32 000 from all species.

INTRODUCTION:

In the brown adipose tissue (BAT) from newborn mammals, adult cold adapted mammals or hibernating species, energy coming from oxidation of substrates is dissipated as heat by uncoupled mitochondria (see review in 1). Coupling can be restored by addition of di- or triphosphate purine nucleotides (2,3) which specifically bind to the mitochondria and inhibit the abnormal ion conductance of the inner membrane (1). In BAT from cold adapted rats first a striking increase of M_r 32 000 protein in SDS gels of mitochondria was observed (4). Looking at further hibernating and other species it was concluded that a high thermogenic status of BAT was characterized

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Abbreviations: BAT, brown adipose tissue; SDS, sodium dodecylsulfate; MOPS, morpholinopropane sulfonic acid.

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by a large portion of the M_r 32 000 protein (5,6,7). At this time, using photo-labeling with azido-nucleotides it was demonstrated that the binding site of nucleotides was also an M_r 32 000 component which was differentiated from the ADP/ATP translocator with M_r 30 000 (5).

From the suggestion that there might exist some similarity between nucleotide binding protein of BAT mitochondria (also called uncoupling protein (8) or GDP-binding protein) and the ADP/ATP carrier, the first purification of the uncoupling protein from cold adapted hamster has been developed by Lin and Klingenberg (8). Previously a partial purification of the protein from cold adapted rat had been described, using affinity chromatography on GDP-Agarose (9). But with this procedure the yield is very low and the isolation of the pure protein seems to be difficult. This paper reports on the isolation and characterization of the nucleotide binding protein from BAT mitochondria of three sources, cold-adapted rat, newborn guinea pig and newborn rabbit, using the hydroxyapatite procedure (8).

MATERIALS AND METHODS:

Male adult Sprague-Dawley rats were adapted to cold (4-5°C) for 3 or 4 weeks. Newborn guinea pigs and rabbits were 0 to 1 day old.

Mitochondria were isolated from interscapular brown adipose tissue in a medium containing 250 mM sucrose, 10 mM Tris pH 7.2, 2 mM EDTA.

Protein content was determined by Lowry's method in the presence of 1% SDS (10) using bovine serum albumin as standard. Triton X-100 was measured by absorption at 274 nm in 0.1% SDS solution.

SDS polyacrylamide gel electrophoresis was run according to (4) or to (11) in 10% acrylamide and 0.1% bis-acrylamide. Staining was carried out by Coomassie brilliant blue G or R-250.

The isolation of the uncoupling protein was performed as described by Lin and Klingenberg (8) using hydroxyapatite columns at room temperature and sucrose gradient centrifugation (385 000 g for 15 h at 5°C).

The binding of GDP was assayed by equilibrium dialysis with a "Dianorm" apparatus for 5 h at 5°C with (8-H^3) GDP (N.E.N. or Amersham).

The amino acid composition of the purified protein was determined as in (12).

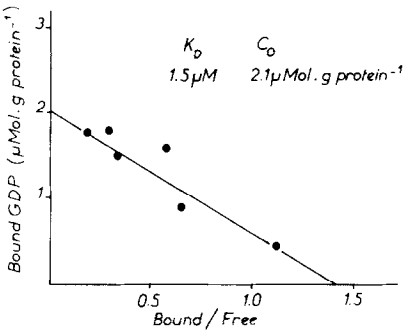

Figure 1: Binding of (8-H^3)GDP to Triton X-100 extract from rat brown adipose tissue mitochondria. Scatchard plot. Lubrol-WX treated mitochondria were extracted with Triton X-100 (Triton/mitochondrial protein ratio 2.5). The binding activity of Triton extract was determined in the following medium: 20 mM MOPS pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA, 20 mg Triton X-100/ml, 0.64 mg extracted protein/ml. K_D, dissociation constant; C_o, binding capacity.

RESULTS:

The purification of the nucleotide binding protein was followed by measuring the GDP binding, as shown in Figure 1 for the crude Triton extract from BAT mitochondria of cold adapted rat. The mass action plot of the binding reveals the existence of a single type of binding sites with a dissociation constant $K_D = 1.5 \times 10^{-6}$ M. Based on these binding measurements the purification is monitored during the different steps as shown in Table I. Here the isolation of the nucleotide binding protein from BAT mitochondria of cold adapted rat and newborn rabbits is recorded. After extraction of soluble proteins from mitochondria by Lubrol WX, the membrane proteins are solubilized by Triton X-100 under conditions of pH, ionic strength and Triton concentration as described previously (8)(Fig. 2). A striking increase of the purification is obtained after passing the Triton extract through hydroxyapatite with a recovery of 50 to 60% of the binding activity. After sucrose gradient centrifugation the specific binding is still further increased from 9 to 15 or 17 μmol/g protein. The recovery is 20 to 30% of the total binding

Table I. Purification steps of the nucleotide binding protein from brown adipose tissue mitochondria

	Total Protein (mg)		Specific GDP binding (µmol/ g protein)		Purification (fold)		Recovery (%)	
	a	b	a	b	a	b	a	b
Mitochondria	39	85	0.95	0.78	1	1	100	100
Triton X-100 extract	10	22	2.4	2.2	2.5	2.8	62	73
Hydroxyapatite	2.1	4.4	9.6	9.3	10	12	54	62
Sucrose gradient	0.5	1.2	15.3	16.9	16	22	21	30

a: cold-adapted rat; b: newborn rabbit. Similar data were obtained with newborn guinea pig.

After Lubrol treatment of mitochondria for 30 min at 2°C (w/vol 3.2%) the 140 000 g pellet was extracted with Triton X-100 for 30 min at 2°C (mitochondrial protein/Triton X-100 ratio 1.5 to 2.5 w/w).

The binding activities were measured in 20 mM MOPS, pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA with 0.25 M sucrose for mitochondria 20 mg Triton. ml^{-1} for Triton extract, 10 mg Triton. ml^{-1} for hydroxyapatite pass-through and 1 mg Triton. ml^{-1} for peak fraction from sucrose gradient.

activity existing in the original BAT mitochondria. Similar results are obtained for the purification of the nucleotide binding protein from BAT mitochondria of newborn guinea pigs.

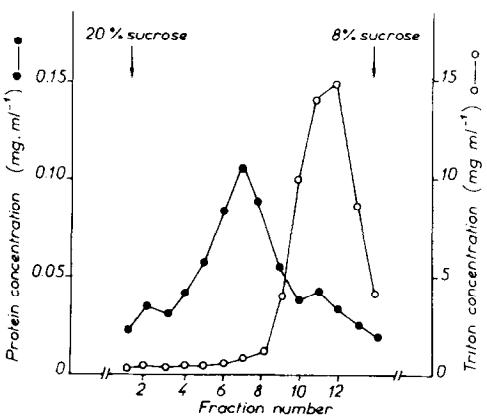

Figure 2: Sucrose density gradient centrifugation of the nucleotide binding protein. 10.6 ml 8-20% w/w linear sucrose gradient was prepared. 0.6 ml of concentrated breakthrough of the hydroxyapatite column corresponding to 0.75 mg protein was layered upon the gradient. After centrifugation, 0.9 ml fractions were collected. The binding activity of the peak fractions was determined according to Lin and Klingenberg (8). The results are given in Table 1.

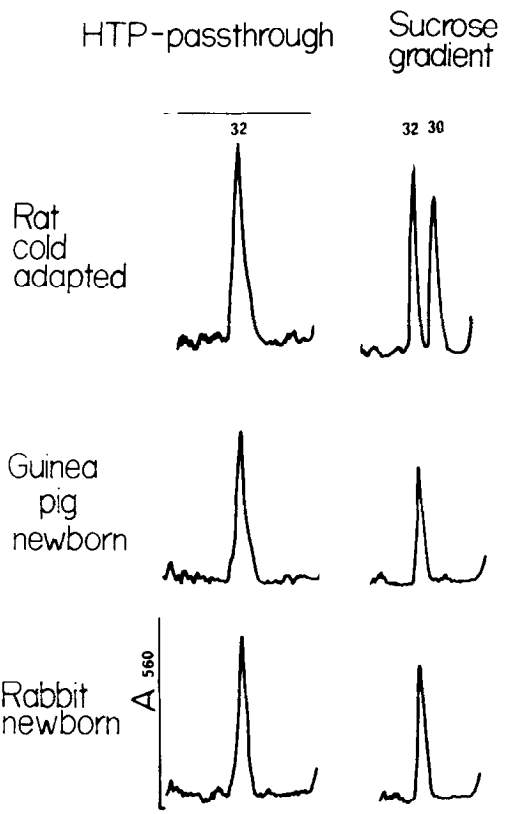

Figure 3: SDS polyacrylamide gel electrophoresis of purified extracts from BAT mitochondria. Densitometric traces of the gels from the stage of hydroxyapatite pass-through and sucrose gradient centrifugation. In one gel 3 µg of the peak fraction from gradient were coelectrophoresed with 2 µg of purified ADP/ATP carrier from beef heart mitochondria.

SDS-polyacrylamide gel electrophoresis of the breakthrough of the hydroxyapatite column shows mainly one peak corresponding to M_r 32 000, at an identical position in the preparation from the three different sources. A small shoulder may correspond to some residual amounts of ADP/ATP carrier. After sucrose gradient centrifugation the peak fractions containing the large pure uncoupling protein yield the M_r 32 000. The degree of purification appears to be similar in the preparation from all three sources(Fig. 3).

The amino acid composition of the purified nucleotide binding protein from cold adapted rat is given in Table II.

Table II. Amino acid composition of the nucleotide binding protein from rat brown adipose tissue mitochondria (mole %)

Asx	5.9	Gly	10.6	Ileu	5.0	Lys	4.8
Thr	6.9	Ala	6.6	Leu	10.3	Arg	3.9
Ser	7.2	Val	6.6	Tyr	3.8		
Glx	9.3	Cys	1.7	Phe	5.0		
Pro	5.5	Met	2.8	His	4.0	Polarity	42

The polarity was calculated according to (14).

The composition is remarkably similar to that obtained with the purified protein from cold adapted hamster (12).

DISCUSSION:

Purification of the nucleotide binding protein from BAT mitochondria of three different animal species can successfully be performed by the same procedure. Most important is the solubilization by Triton and the hydroxyapatite step. This agrees with the idea that the uncoupling protein and the ADP/ATP carrier have some similarity (8). Further support comes from the present studies which extend the same isolation procedure to the nucleotide binding protein to BAT from newborns. In agreement with the results from cold adapted hamster the binding capacity in the purified protein for GDP is 15 to 17 µmol/g protein. This corresponds to a functional M_r 60 000 to 66 000 per binding site. Assuming a purity of about 90%, the M_r of the protein from the various species is around 63 000 corresponding to a dimer of 32 000 subunits, in agreement with the M_r determined by SDS gel electrophoresis. For the protein from cold adapted hamster, evidence had been obtained by sedimentation equilibrium runs that the protein in fact occurs as a dimer with approximately M_r 65 000 (13). Again this is analog to the ADP/ATP carrier consisting of two subunits of M_r 30 000 with only one binding site for various inhibitor ligands.

Similar as in cold adapted hamster the protein share of the nucleotide binding protein in BAT mitochondria from the three different sources amounts to about 60% of the total mitochondrial protein and 15% of the inner membrane protein. The amino acid composition of nucleotide binding protein from the cold adapted rat is similar to that from cold adapted hamster. It differs markedly from that of the ADP/ATP carrier from beef heart mitochondria. In particular there is a reduced proportion of alanine and lysine and an increased content of threonine and histidine.

In summary, the nucleotide binding protein from BAT mitochondria of various rodents can be isolated by the same procedure under similar properties. Still it remains to be seen whether the isolated protein produces the uncoupling effect by forming an ion channel through the membrane. Differences between the protein from various sources may be demonstrated by immunological studies and finally by the complete amino acid sequence.

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