ISOLATION OF THE UNCOUPLING PROTEIN FROM BROWN ADIPOSE TISSUE MITOCHONDRIA

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1. Introduction

The thermogenesis of brown adipose tissue has been related to the uncoupling of respiration in their mitochondria [1–4]. Coupling can be induced by adding purine nucleotides such as GDP, ADP, GTP and ATP [5,–7]. The peculiar uncoupling in these mitochondria was related to a high ion conductance of the inner mitochondrial membrane and addition of purine nucleotides largely suppressed this conductance [4–8]. A specific binding site for the purine nucleotides was determined on the outer surface of the inner membrane of brown adipose tissue mitochondria [9,10].

Using azido-ADP a 32 000 MW protein was identified in high abundancy only in brown adipose tissue (BAT) mitochondria [11–13] and differentiated from ADP, ATP carrier with carboxyatractylate (CAT) [13]. It was proposed that this purine nucleotide binding protein is responsible for the uncoupling and also for the control by purine nucleotides.

An isolation of this protein was first reported by Ricquier using Triton X-100 and GDP affinity chromatography [14]. The purification was partial and data on yields and properties of the isolated product were not yet given. A functional analogy of the 'uncoupling protein' as an anion carrier to the ADP, ATP carrier of mitochondria, as well as the fact that both proteins bind specifically nucleotides and have about the same MW suggested certain similarities between this protein and the ADP, ATP carrier. Therefore, it seemed reasonable to apply the unusually simple and efficient purification methods, developed by us for the isolation of the ADP, ATP carrier [15,16], to the purification of the uncoupling protein from BAT mitochondria. The present paper reports on the isolation procedure yielding a more than 90% pure protein which has fully retained the original binding activity for GDP.

Abbreviations: BAT, brown adipose tissue; CAT, carboxyatractylate; MOPS, morpholinopropane sulfonic acid

2. Material and methods

Hydroxylapatite was prepared according to Tiselius as described by Bernardi [17]. For cold adaption 3-week-old BR 46-Wistar II rats and golden hamsters were exposed to 4°C, for the duration of 2–3 weeks (rats) and 3–4 weeks (hamsters).

Mitochondria were isolated from interscapular BAT of rats and from interscapular, subscapular, axillary and dorsal cervical BAT of hamsters. Muscle, connective tissue and white fat were cut off carefully at 4°C. The medium for isolation of mitochondria contained 300 mM sucrose, 10 mM Tris, pH 7.2, 2 mM EDTA.

Triton X-100 was determined by absorption at 280 nm in 0.1% SDS solution. SDS polyacrylamide gel electrophoresis was carried out as described in [18]; 5 × 100 nm gels with 11% acrylamide and 0.1% bisacrylamide were used.

For the isolation of the uncoupling protein, mitochondria were extracted first with 3.2% Lubrol WX and then with Triton X-100. The extract was applied directly to hydroxylapatite columns (2 mg protein/ml column) at room temperature and eluted with 20 mM MOPS, pH 6.7. The breakthrough fractions were concentrated by pressure dialysis and subjected to sucrose gradient centrifugation (see fig.4). GDP binding was determined by equilibrium dialysis, using a

'Dianorm' apparatus. Dialysis was carried out at 5°C for 5 h.

3. Results

Specific binding of GDP to the uncoupling protein seems to be the most suitable way of following the protein after solubilization and during the isolation steps. Furthermore, the binding capacity and the affinity should be a measure of the intactness of the solubilized protein. For extraction of the protein, Triton X-100 was chosen which had proven to be also the best suitable detergent for the isolation of the ADP, ATP carrier. Binding with [^{3}H]GDP in a crude extract obtained with Triton X-100 is shown in fig.1. This binding plot is compared with that measured in the mitochondria. The mass action plots demonstrate the remarkable result that GDP binds to the solubilized extract in a quantitatively similar fashion as to the mitochondria. In both cases linear relations are observed reflecting a single family of binding sites. The dissociation constant of the extract is even lower ($K_D = 2.7 \times 10^{-6}$ M). This indicates that the native conformation of the protein is largely retained. Also there seemed to be no loss of binding capacity since the number of sites measured in the mitochondria is largely recovered in the Triton extract.

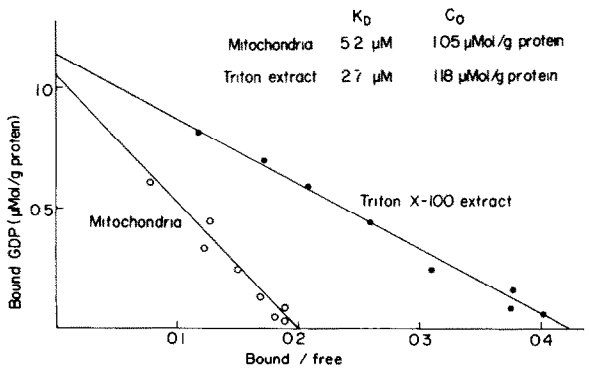

Fig.1. Binding of [^{3}H]GDP to mitochondria and to Triton X-100 extract. Mass action plot. Triton extract was prepared from intact mitochondria. 90% of total mitochondrial protein were extracted. For the binding mitochondria were incubated in 20 mM MOPS, pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA, 100 mM sucrose, 17 mg protein/ml. The binding activity of Triton extract was determined in a medium containing 20 mM MOPS, pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA, 19 mg Triton X-100/ml, 7 mg extracted protein/ml. K_D, dissociation constant; C_O, binding capacity.

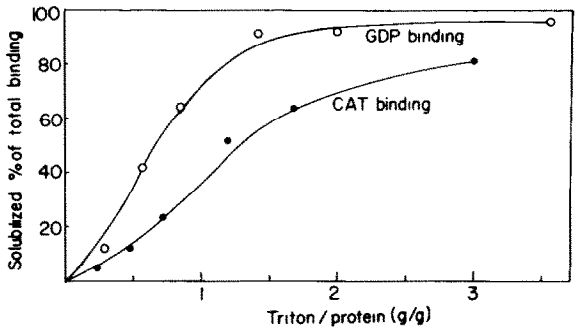

Fig.2. The solubilization of uncoupling protein and CAT binding protein by Triton X-100. Mitochondria were preloaded with [^{3}H]CAT, washed three times and then extracted with increasing concentrations of Triton X-100 for 30 min at 0°C. The 100 000 × *g* supernatant was taken for measuring GDP binding, as assayed by equilibrium dialysis, and [^{3}H]CAT. The medium contained 20 mM MOPS, pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA.

The conditions of extraction, concentration of Triton, salt and pH have been optimized for the extraction of the uncoupling protein. A partial separation from the ADP, ATP carrier already at this step was achieved (fig.2). On increasing the amount of Triton, the uncoupling protein (as assayed by GDP binding) is released more easily than the CAT binding protein. At a Triton/protein ratio of 1.4 ~90% of uncoupling protein and 55% of CAT binding protein are extracted. The easier extraction of the uncoupling protein is more striking with respect to the required salt concentration. The uncoupling protein is almost fully extracted at 20 mM Na_2SO_4, whereas the extraction of the CAT binding protein was shown to be strongly dependent on ionic strength [16].

The binding of GDP to the solubilized protein is strongly inhibited by high salt concentration similar as in the mitochondria. Furthermore, the binding of GDP is sensitive to high pH and the affinity largely decreases above pH 7.2. The sensitivity to pH in the extract is similar to that of whole mitochondria [9]. On the basis of these results the conditions for the extraction were chosen as follows: Triton to mitochondrial protein = 1.2–2 w/w, 20 mM MOPS, pH 6.7., 20 mM Na_2SO_4, 1 mM EDTA.

The uncoupling protein is not solubilized by alkyl-type polyoxyethylene detergents such as Lubrol WX, Brij, similar to the CAT binding protein [16]. Lubrol, however, releases most of the soluble matrix proteins. The initial pretreatment of the BAT mitochondria with

Lubrol results in a 3-fold enrichment of the uncoupling protein in the residual membranes.

The most efficient purification step for the isolation of the ADP, ATP carrier is passing the Triton-solubilized extract through a hydroxylapatite column [15,16]. Similarly for purifying the uncoupling protein, the crude Triton extracts of mitochondria were applied to a hydroxylapatite column which adsorbed most of the mitochondrial protein but permitted the uncoupling protein to pass through freely. The recovery of the uncoupling protein in the breakthrough amounted to 75 to 95%. This fraction contained only about 2.5 μmol/g protein CAT as shown in parallel experiments with extracts from CAT loaded mitochondria whereas for GDP 12 μmol/g protein binding sites were determined. Previous experiments with beef heart mitochondria [19] indicate that relatively more CAT binding protein can be expected to be adsorbed at the hydroxylapatite column when mitochondria are not loaded with CAT. Therefore, for the isolation of the GDP-binding protein no CAT was added to the mitochondria.

Upon gel electrophoresis of the breakthrough, the 32 000 MW subunit (fig.3) of the uncoupling protein is the only dominant peak with a relatively small shoulder around 30 000 which may be attributed to the ADP, ATP carrier. The drastic enrichment from the crude extract is well illustrated.

The next step is a surcose density gradient centrifugation which gives an additional purification and also permits at the same time to remove the bulk of the free Triton (fig.4). An intervening gel filtration on

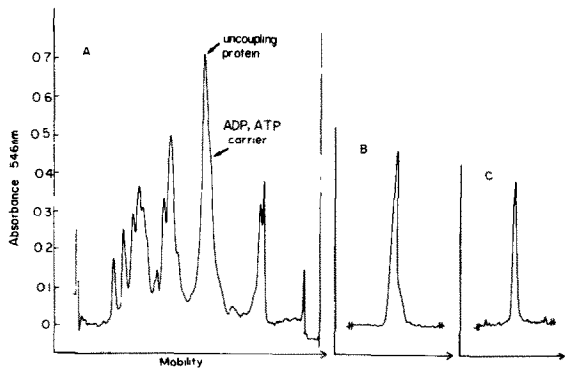

Fig.3. Densitometric traces of SDS-polyacrylamide gel electrophoresis. (A) Triton X-100 extract of Lubrol-pretreated mitochondria; (B) breakthrough of the hydroxylapatite columns; (C) peak fraction upon sucrose gradient centrifugation.

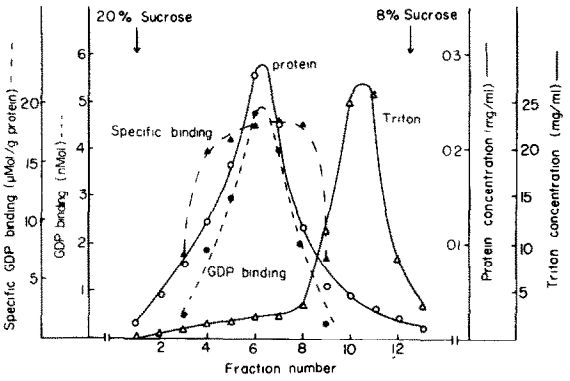

Fig.4. Sucrose density gradient centrifugation of the uncoupling protein. 11 ml 8–20% w/w linear sucrose gradient was prepared in a 13 ml centrifuge tube. 0.5 ml of concentrated column breakthrough fraction, containing 1.2 mg protein, was layered on top of the gradient. The sucrose gradient contained 0.1% Triton X-100. After centrifugation at 384 000 × *g* for 15.5 h at 5°C, 0.90 ml fractions were collected. The binding activity was determined after 4 h dialysis at 4°C against 20 mM MOPS, pH 6.7, 20 mM Na_2SO_4, 0.16 mM EDTA.

Sepharose 6B or AcA 34, as used for the isolation of the CAT binding protein, turned out to be unnecessary. Gel electrophoresis gives evidence for the purity of the resulting product (fig.3).

The purification steps of the uncoupling protein from BAT mitochondria from cold-adapted hamsters and rats are evaluated in table 1. The binding capacity for GDP serves as a measure of purification. Already in the Triton extract the uncoupling protein is enriched more than 3-fold. A major purification is obtained by hydroxylapatite. The sucrose gradient step comes close to a pure protein. The recovery, based on the original mitochondria protein, approached 30%.

4. Discussion

The uncoupling or GDP-binding protein from BAT mitochondria can be isolated by a relatively simple procedure, giving high yields of what we consider to be an undenatured product. The basis for the purification is the retention of the full GDP binding capacity after solubilization with Triton X-100. Therefore, it was unnecessary to covalently bind nucleotides to the protein, for example by photoaffinity label, as was first used by Heaton et al. for identification of the protein in the mitochondria [13].

Table 1
Purification steps of uncoupling protein

	Volume ml	Protein concentration (mg/ml)	Specific binding (μmol/g prot.)	Total protein (mg)	Total binding (nmol)	Purification (fold)	Recovery (%)
Mitochondria: hamster (rat)	1.6 (1.6)	58.1 (48.9)	0.91 (0.93)	93 (78)	84 (72)	1	100
Lubrol extract	4.1 (4.35)	15.1 (11.8)	– –	62 (51)	– –	–	–
Triton X-100 extract	4.6 (4.7)	4.73 (4.5)	3.43 (2.5)	22 (21)	74 (54)	3.8 (2.7)	88 (75)
Hydroxylapatite	19.7 (14.1)	0.27 (0.23)	11.8 (12.7)	5.4 (3.41)	64 (42)	13 (14)	76 (58)
Sucrose gradient	66.6	0.18	17.5	1.25	21	19	26

Mitochondria from hamsters were used. Data in parentheses are for rat mitochondria

Our working hypothesis that the uncoupling protein has functional and molecular similarities to the ADP, ATP carrier is fully substantiated by the similarity of the purification procedure. There are some differences, for example, the stability of the GDP binding capacity, whereas it has not been possible to detect any binding of ADP to the solubilized ADP, ATP carrier. The uncoupling protein can be isolated without protection by the ligand GDP, whereas the ADP, ATP carrier deteriorates in relatively short time when not being protected by CAT [16,19].

The molecular weight of the polypeptide is only tentatively determined to be 32 000 by SDS gel electrophoresis. If there is one binding site for GDP per 32 000 MW protein, a binding capacity of 31 μmol/g protein should be measured in the purified protein. The value of only 16 μmol GDP/g protein in our final preparation argues that either the effective MW per binding site is 64 000, or that the protein is only 50% pure. The fact that a single 32 000 band in SDS gel is registered and the analogy to the ADP, ATP carrier lets us propose that the protein is pure and has a MW of 64 000 with two subunits of 32 000 for each GDP binding site. Direct proof for this MW will be given in a subsequent paper.

From the number of GDP binding sites in whole mitochondria (which ranges from 0.9 to 1.2) and the MW of 64 000, the concentration of the uncoupling protein can be determined. In the presently used mitochondria, isolated from cold-adapted hamsters and rats, the uncoupling protein was calculated to represent from 5.8 to 7.7% of the total protein. Based on the total integral protein of the inner mitochondrial membrane the share of the uncoupling protein reaches 15 to 20%, considering that more than 60% of the total mitochondrial protein can be extracted by Lubrol. The eminent importance of the uncoupling protein to the function of BAT mitochondria is drastically demonstrated by these values.

Acknowledgements

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