Uncoupling protein in human brown adipose tissue mitochondria

Isolation and detection by specific antiserum

M.E.J. Lean and W.P.T. James*

Dunn Clinical Nutrition Centre, Medical Research Council and University of Cambridge, Addenbrooke's Hospital, Trumpington Street, Cambridge CB2 1QE, England and **Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, Scotland*

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A protein of M_r 32000 has been isolated from human infant brown adipose tissue mitochondria following the procedure used to purify the uncoupling protein from rat brown adipose tissue mitochondria. A specific antiserum has been raised against the human 32 kDa protein, and used to detect it by probing mitochondrial proteins separated by SDS–PAGE. The protein is present in large amounts in brown adipose tissue but is undetectable in human liver, heart or white adipose tissue. It has strong immunological cross-reactivity with rat brown adipose tissue uncoupling protein.

Infant *Brown adipose tissue* *Uncoupling protein* *Mitochondria* *Antiserum* *Radioimmunoassay*

1. INTRODUCTION

Brown adipose tissue (BAT) has attracted a great deal of interest recently because of its important role in the energy regulation of rodents. It is also suggested that there may be a similar mechanism in man which could have a bearing on the development of obesity [1,2].

It has been shown in rodents that BAT is unique in that its mitochondria have the capacity to dissociate, or uncouple, substrate oxidation from the production of adenosine triphosphate. This results in the production of heat which allows the mitochondria to play a major role in cold acclimation and arousal from hibernation [3]. It has also been suggested that heat production by BAT mitochondria is defective in genetically obese rodents [4,5] and an important factor in their high metabolic efficiency. Increased heat production may also explain the low metabolic efficiency of rodents overfed on a cafeteria type of diet [6]. The uncoupling of BAT mitochondria is achieved by a high proton conductance across the mitochondrial membrane. The pathway is blocked in vitro by exogenous purine nucleotides [7], whose binding site has been identified as a 32-kDa protein, variously called GDP binding protein, uncoupling protein, or thermogenin [3], in the inner mitochondrial membrane [8]. It has been suggested that this protein is directly responsible for the physiological uncoupling of BAT mitochondria although purine nucleotides do not seem to be the functional regulators in vivo [9].

The use of a radioimmunoassay for this protein has now shown that where heat production is increased, the mass of uncoupling protein is elevated, in accordance with the documented enhanced in vitro binding capacity for GDP [10,11].

The recognition of BAT and its histological similarity with that of rodents, suggests that it might be involved in heat production in the human infant [12]. With time, however, lipid rapidly accumulates in BAT (or 'embryonal adipose tissue')

Abbreviation: BAT, brown adipose tissue

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and most of it takes on an appearance indistinguishable from white adipose tissue. Thus infant BAT has also been considered merely an immature, lipid-depleted, storage tissue [13]. Whether or not BAT has any physiological relevance to human energy metabolism is therefore not clear. Some evidence for the persistence of active BAT in adult man may be inferred from the high level of GDP binding to mitochondria from periadrenal adipose tissue obtained from patients with phaeochromocytoma, when, with a high circulating catecholamine level, this tissue resembles BAT histologically [14,15]. Additional evidence for the presence of BAT mitochondrial uncoupling protein has been reported very recently in a patient with a phaeochromocytoma by immunohistochemistry using anti-rat-BAT-uncoupling protein serum, and by photoaffinity labelling of mitochondria [16].

We here report the isolation of the 32-kDa protein from BAT mitochondria from human infants, and the detection and measurement of this protein using antiserum raised against it.

2. MATERIALS AND METHODS

2.1. *Samples and preparation*

Tissue was obtained from 3 infants (2 male) dying between 0 and 6 months from either cot death (2) or intraventricular haemorrhage (1). None had congenital abnormalities, growth abnormalities or a history of illnes or drug treatment before death. Tissue was removed at routine postmortem examination 1 or 2 days following death, the bodies having been stored at 4°C. In these, and other preliminary dissections, tissue with gross appearance of BAT was plentiful in the axillary and cervical regions, and in the peri-adrenal and perirenal sites; the interscapular region contained very little. White adipose tissue was taken from the abdominal subcutaneous site. Samples were stored at −20°C prior to mitochondrial preparation.

BAT was dissected free of any macroscopic white adipose, vascular and connective tissue. Mitochondria from BAT, liver and heart were prepared by a standard method [17] with a fast centrifugation step of 11000 × *g*. The entire mitochondrial preparation procedure was conducted at 0–4°C. Mitochondrial protein concentration was measured by a modification [18] of the procedure in [19].

2.2. *Isolation of 32-kDa protein from BAT mitochondria*

The method for isolating uncoupling protein from mitochondria of hamsters and rats described in [20] was followed using the pooled BAT obtained from the 3 infants. The purity of the isolated protein following hydroxyapatite column chromatography was verified, and its M_r-value estimated by one-dimensional SDS–PAGE, largely following the method in [21]. For increased sensitivity a silver-staining technique was used (Bio-Rad Laboratories). Bio-beads SM-2 (Bio-Rad) were used to remove excess Triton X-100 from uncoupling protein where necessary [22].

2.3. *Antiserum, activity and specificity*

Six-month old Dutch rabbits were first bled to obtain pre-immune serum and then inoculated with a total of 100 μg of 32-kDa protein emulsified using Freund's complete adjuvant, divided into 4 sites, 2 intramuscular and 2 subcutaneous. Two weeks later, a booster dose of 60 μg of 32-kDa protein was given, emulsified in Freund's incomplete adjuvant, divided into one intramuscular and one subcutaneous site. Samples of serum were heated to 56°C for 30 min, filtered using a 0.22 nm filter unit (Millex-GS Millipore) and stored at −20°C. The radioimmunoassay method for antiserum activity was the same as that described for measurement of rabbit anti-rat-uncoupling protein activity [10], employing a 96-well microtitre plate to immobilise the 32-kDa protein (treated with Bio-beads); antiserum binding was detected with ^{125}I-labelled Protein A.

Specificity of the immune serum was examined by probing mitochondrial proteins from BAT, liver, heart and white adipose tissue, which had been separated by SDS–PAGE and transferred to mitrocellulose paper by electrophoretic blotting [10]. Antibody binding was detected by incubation with ^{125}I-labelled Protein A. After overnight autoradiography to identify the positions of the protein tracks, each track was cut up into 5-mm slices which were counted for radioactivity. An adjacent track on the SDS–PAGE containing M_r-markers was cut off and stained.

2.4. *Quantification of 32-kDa protein in mitochondria from different tissues*

The electro-blotting technique was further used to quantify the 32-kDa protein content of mitochondria from BAT, liver, heart and white adipose tissue. The counts from ^{125}I-labelled Protein A at the 32-kDa protein peak – two adjacent slices of 0.5 mm – were compared with counts from known amounts of purified 32-kDa protein.

3. RESULTS

3.1. *Isolation of the 32-kDa protein from human BAT mitochondria*

From a total of 26.4 g of BAT, which was noted to have a high lipid content compared with that used previously from rats, the mitochondrial yield was 120 mg mitochondrial protein and the suspension on electron microscopy was found to contain about 75% intact mitochondria (not shown). The final yield of purified protein was 2.34 mg.

SDS–PAGE revealed a single band with apparent M_r very slightly lower than purified rat BAT mitochondrial uncoupling protein when run in adjacent tracks. When equal amounts of the proteins were run together, they were found to co-migrate, with a single stained band. The purity of the preparation was further checked using a silver-staining technique considered to be 10–50-times as sensitive in detecting proteins as Coomassie blue R-250. Again, only a single band was found (fig.1).

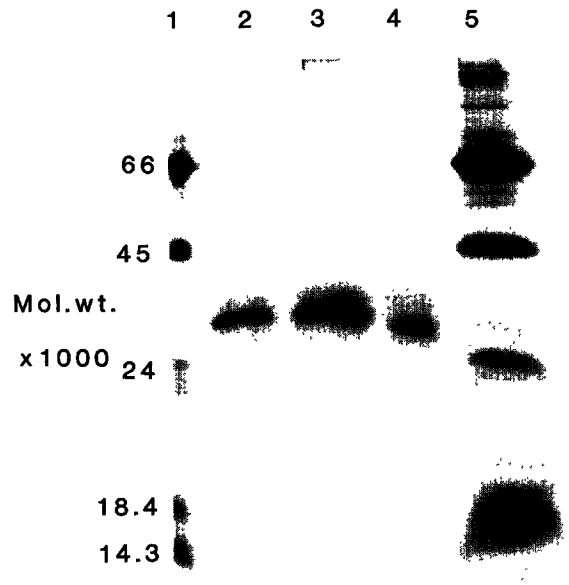

Fig.1. SDS–PAGE of purified human 32-kDa protein and rat uncoupling protein. Track 1 and 5 contain protein standards. Track 2 contains rat uncoupling protein 3 μg. Track 4 contains human 32-kDa protein 3 μg. Track 3 contains both proteins, 3 μg each, run together. Samples were dissociated in a solution with final concentration SDS 5% β-mercaptoethanol 5%, by heating to 70°C for 30 min. Stacking gel contained 3%, and resolving gel 15% acrylamide. Electrophoresis was carried out at 50 V for 12 h. Proteins were stained with Coomassie brilliant blue® and subsequently with Bio-Rad silver stain. M_r-values were estimated from a plot of relative mobility against log M_r of standards.

3.2. *Anti-human 32-kDa protein serum: quantification of 32-kDa protein in mitochondrial samples from various tissues*

Fig.2a shows the titration of rabbit anti-human 32-kDa protein against human 32-kDa protein. Pre-immune serum showed negligible binding.

The use of immune serum to probe a nitrocellulose blot of complete BAT mitochondrial proteins, separated by SDS–PAGE, revealed only one major labelled peak at a position corresponding to a M_r-value of 32000 (fig.3,4). The background counts in regions corresponding to M_r-values <32000 were close to zero. However, in regions with proteins of M_r-values >32000, levels up to 10% of the peak were found. There was also a minor peak at M_r 64000. This finding may reflect protein aggregation or even a true dimer as suggested in [23]. The effects of increased concentration of SDS and of different sample preparation methods were examined in an attempt to increase the dissociation of the 32-kDa protein monomer, but no satisfactory improvement was found.

From a standard plot of binding to known amounts of the purified protein, the concentration of 32-kDa protein in mitochondria of BAT, pooled from the 3 infants, was found to be between 78 and 100 μg/mg mitochondrial protein. Liver and heart and white adipose tissue mitochondria did not contain quantifiable 32-kDa protein. With a double load of 50 μg mitochondrial protein from white adipose tissue, a very small increase in counts above baseline was seen at the 32-kDa position.

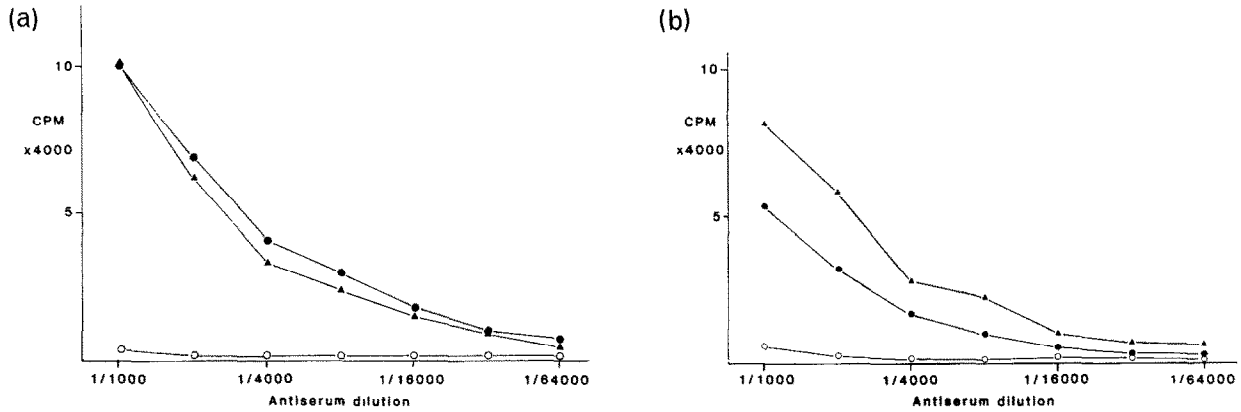

Fig.2. Antiserum dilution curves for anti-human 32-kDa protein activity (a) and anti-rat uncoupling protein activity (b). Wells of microtitre plates were coated with 32-kDa protein by incubation with 50 μl (20 μg/ml) overnight at 4°C. After washing and blocking with radioimmunoassay (RIA) buffer (PBS, pH 7.6, containing bovine serum albumin 1% sodium azide 0.1%), serial dilutions of pre-immune serum (○), anti-human 32-kDa protein serum (●) or anti-rat uncoupling protein serum (▲) were added to the wells and incubated for 1 h at 23°C. Antibody was detected after washing with RIA buffer by incubating with ^{125}I-labelled protein A in RIA buffer 30000 cpm/well for 1 h. Against rat uncoupling protein, rabbit-anti-human 32-kDa protein serum is lower in activity than anti-rat-uncoupling protein by a factor of 2. Pre-immune serum showed negligible binding.

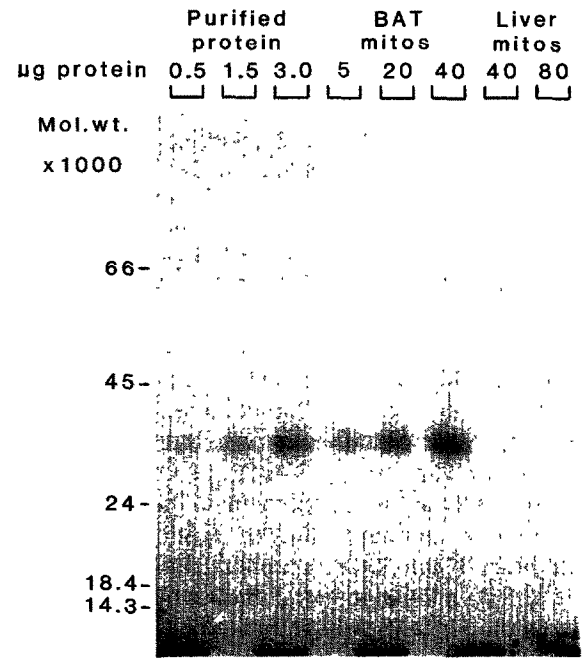

Fig.3. Autoradiograph showing antibody binding to purified human 32-kDa protein and human brown adipose tissue mitochondria. No binding to human liver mitochondria could be detected.

This might have been through contamination, but the assessed specific protein content amounted maximally to only 2% of that in BAT (fig.4a).

3.3. *Cross-immunoreactivity of human BAT 32-kDa protein and rat BAT uncoupling protein*

Rabbit anti-human 32-kDa protein serum and rabbit anti-rat-uncoupling protein serum were shown by solid phase radioimmunoassay to have very similar activities against BAT mitochondrial 32-kDa proteins, from both human and rat (fig.2). Probing nitrocellulose blots of SDS–PAGE-separated proteins showed the two immune sera to have almost identical activity against human BAT mitochondria, with a major peak at M_r 32000 (fig.5), both having negligible activity against mitochondria from other tissues. The anti-human 32-kDa protein serum, tested against mitochondria from rat BAT, again showed a clear peak at M_r 32000, but the background binding was a little higher than seen with human BAT mitochondria tested simultaneously and its binding to rat heart and liver mitochondria was substantially higher than that to human heart, liver or white adipose tissue mitochondria, which were considered to show no binding (fig.4).

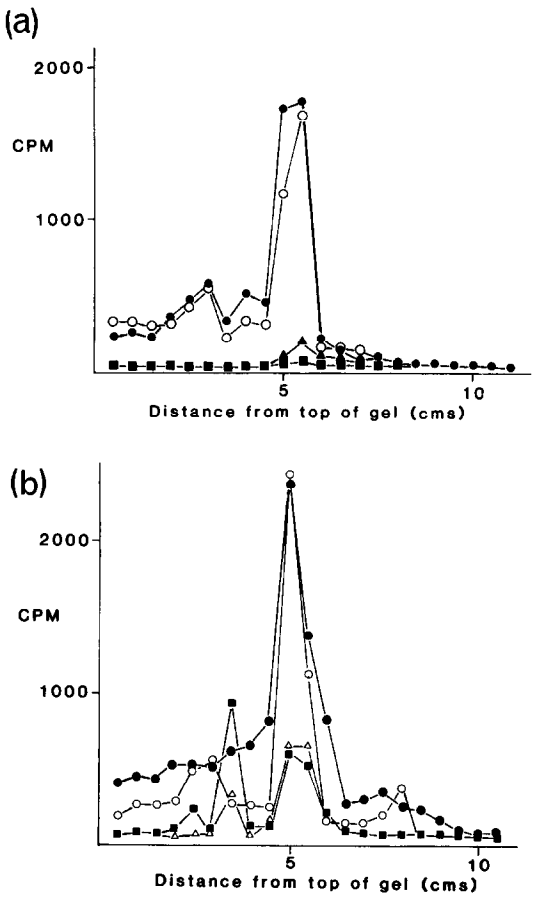

Fig.4. Rabbit anti-human 32-kDa protein serum activity against mitochondrial proteins from human (a) and rat (b), separated by SDS–PAGE and electroblotted onto nitrocellulose paper. Binding to total mitochondrial proteins from BAT 25 μg (●), heart 50 μg (■), liver (△), and white adipose tissue (▲), are compared with binding to the purified 32-kDa uncoupling protein 3 μg (○). Binding to human heart and liver mitochondrial proteins gave identical flat traces.

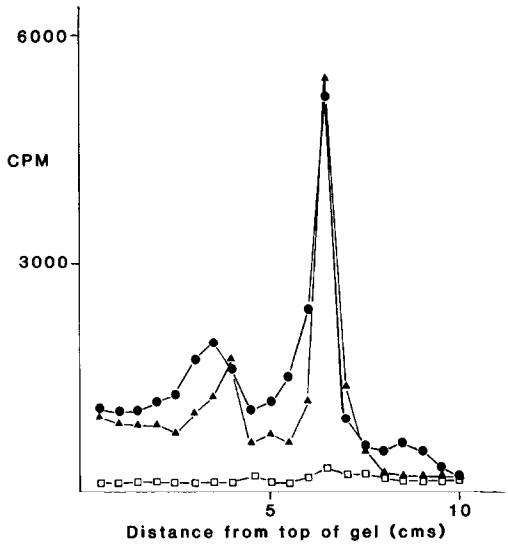

Fig.5. Comparison of anti-human 32-kDa protein serum (●) and anti-rat uncoupling protein serum (▲) activity against human BAT mitochondria (40 μg mitochondrial protein) following SDS–PAGE and electroblotting. Both showed negligible activity against 40 μg mitochondrial protein from human liver (□).

4. DISCUSSION

This work shows that BAT mitochondria from the human infant contain a protein which, isolated by the same methods, co-migrates with rat BAT mitochondrial uncoupling protein on one-dimensional SDS–PAGE, and that the two proteins cross-react strongly with immune serum raised against the other. It seems reasonable therefore to conclude that the human 32-kDa protein is indeed a human uncoupling protein, and that infant BAT is probably capable of the same kind of thermogenesis as that of rodents.

It is encouraging for future work that this protein, which has been reported as being remarkably stable in hamsters [24], can be isolated in relatively large amounts from human post mortem tissue.

The antiserum raised against the human uncoupling protein proved to be very specific, with no immunoreactive protein evident in mitochondria from liver, heart or white adipose tissue. The electroblot and antiserum probe method suggest that the uncoupling protein content of human infant BAT is 78–100 μg/mg mitochondrial protein. This is of the same order as that of cold acclimated rats and mice [10,11]. Preliminary studies using a solid phase radioimmunoassay for human uncoupling protein, however, show a lower figure of 40 μg/mg mitochondrial protein. This is nearer the value for warm acclimated mice [11]. Using the solid phase radioimmunoassay uncoupling protein was undetectable at <4 μg/mg mitochondrial protein in samples from human liver, heart and white adipose tissue. It has often been suggested that BAT becomes atrophic and functionally inactive after the first few months of life [25,26], although

routine histological studies do suggest that it remains in some sites, e.g., the perirenal area and intercostal spaces, until late in life [12,27], and it is recognized that BAT hypertrophies in individuals exposed to cold conditions before death [28,29] and under other stimulatory conditions such as that found with phaeochromocytoma [14,15]. This assay now provides a method for detecting in man an important component of changes in the mitochondrial mass with time and in different physiological and pathological conditions, and a means for assessing the capacity of tissues to generate heat.

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