THE MITOCHONDRIAL ATPase OF BROWN ADIPOSE TISSUE

Purification and Comparison with the Mitochondrial ATPase from Beef Heart

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1. Introduction

In brown adipose tissue (BAT), as in other tissues, heat production is the result of an increase in respiration [1,2]. An obligatory coupling of mitochondrial respiration to ATP synthesis [3] implies that if respiration in BAT is to markedly increase a similar increase must concomitantly occur in ATP synthesis. Thus, either the capacity of the ATP synthesizing system parallels the respiratory system, or respiration must become uncoupled from the constraints imposed by limited ATP synthesis. Available evidence suggests that a form of physiological uncoupling, specific for the tissue, occurs [4–6]. An alternative hypothesis, recently reiterated, has, however, proposed that coupling remains intact and the high amount of ATP which would be formed is utilized by a cytoplasmic membrane ATPase [7–10].

The hydrolytic activity of the ATPase of BAT-mitochondria is low relative to that of rat liver mitochondria [11,12]. Also, addition of uncoupler to mitochondria respiring in State 3 produces only a 30% increase in respiration in rat liver but a 500% stimulation in BAT [13]. This indicates a low activity of the ATP synthesizing system with respect to exogenous ADP in intact BAT-mitochondria. Additionally, Lindberg et al. [5] were not able to demonstrate the characteristic protrusions on the inner side of the inner mitochondrial membrane believed to correspond to the ATPase complexes, under conditions which revealed them in other tissues [14].

In view of these observations we have found it important to isolate and quantitate the ATPase from BAT-mitochondria and to compare it with other mitochondrial ATPases. The method described is suitable for the isolation of the soluble ATPase from small amounts of mitochondria. The work also demonstrates that an ATP synthesizing system which could parallel the capacity of the respiratory system during thermogenesis is not present in the mitochondria from hamster brown adipose tissue.

2. Materials and methods

2.1. *Isolation of mitochondria*

BAT-Mitochondria were isolated from golden hamsters, cold acclimated for at least 3 weeks, by the method described for rat BAT-mitochondria [15]. Rat liver and beef heart mitochondria were isolated by conventional methods [16,17].

2.2. *Purification of the mitochondrial ATPases from BAT and beef heart*

All operations were performed at 2–5°C. Mitochondria, prepared in 0.25 M sucrose, were stored at a concentration of 50 mg protein/ml under nitrogen at −20°C until required. In a typical isolation procedure

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250 mg mitochondrial protein was thawed, and diluted with 1 vol buffer 1 mM Tris–HCl, pH 8, containing 2.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 1 mM ATP and 20% (v/v) methanol. The diluted mitochondria were centrifuged at 45 000 × *g* for 10 min, the yellow supernatant discarded, and the pellet resuspended in about 18 ml of the above buffer.

This suspension was sonicated in a manner similar to that described by Horstman and Racker [18]. A Branson sonifier fitted with a small tip was used at maximum power. The sonication was performed such that the temperature of the solution rose to 35°C during the first 7.5 min, and to 50°C during the subsequent 7.5 min, after which time it was returned to 0°C. The sonicate was then centrifuged at 150 000 × *g* for 45 min. The pellet was discarded and to each 10 ml of clear yellow supernatant 3 ml of 50% (w/w) aqueous poly-(ethylene glycol) (av. mol. wt 6000) was added slowly while the mixture was stirred. The solution was then brought to 2.5 mM $MgCl_2$ and the resultant precipitate was collected by centrifugation at 40 000 × *g* for 15 min. The precipitate was dissolved in about 1 ml 50 mM Tris–HCl, pH 8.0, containing 20% methanol and 2.5 mM 2-mercaptoethanol. The BAT-enzyme was then applied to a column of Sepharose 6B (1.5 × 100 cm) and eluted with the same buffer. Fractions containing ATPase activity were pooled and loaded onto a column of Sephadex A-25 (1 × 4 cm) (Whatman DE-52 will also work) equilibrated with the above buffer. The enzyme was eluted with a linear gradient formed from 0.15 M and 0.5 M Tris–HCl, pH 8, both containing 20% methanol and 2.5 mM 2-mercaptoethanol, 20 ml of each solution.

The beef heart material yielded a homogeneous ATPase after DEAE-chromatography without the Sepharose 6B filtration step. Fractions containing enzyme activity were pooled and concentrated either by poly(ethylene glycol) precipitation as described above or in an Amicon Diaflo all using a PM10 membrane.

2.3. *Other procedures*

ATP synthesis [19], ATPase activity [20] and respiration rates [21] were measured by published procedures.

Antimycin A concentration was calculated from its absorbance [22]. Energy coupling in BAT mitochondria was established by addition of 1 mM GDP and 20 mg/ml bovine serum albumin (Pentex, fatty acid poor) [23]. Procedures for gel electrophoresis and staining were essentially as described for the *Escherichia coli* ATPase [20].

Antibodies against beef heart ATPase were prepared by intramuscular injection of 600 μg purified enzyme in 2 ml 0.1 M potassium phosphate (pH 7) mixed with an equal volume of Freund's complete adjuvant into each of two rabbits. Intramuscular booster injections were performed at 4 and 6 weeks, using 2 mg antigen each time. Seven days after the last injection, about 30 ml blood was collected from the ear vein of each rabbit. After clotting the blood serum was collected by centrifugation.

Aurovertin was a gift from Dr R. B. Beechy.

3. Results and discussion

3.1. *Evaluation of respiratory chain and ATPase components*

Evidence for an increased amount of respiratory chain components in BAT-mitochondria compared to rat liver mitochondria was obtained by inhibition studies with Antimycin A. Antimycin A combines specifically and stoichiometrically with cytochrome *b* (one per two cytochrome *b* molecules) [24]. Figure 1

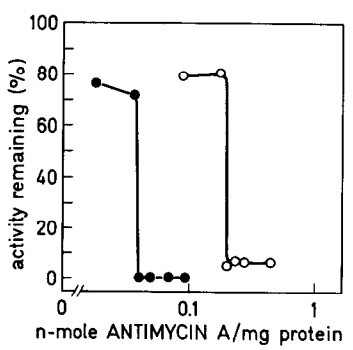

Fig.1. Antimycin A inhibition of uncoupled respiration in mitochondria from hamster brown adipose tissue and rat liver. Incubation medium: 100 mM KCl, 20 mM K-TES, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.2. Mitochondria – brown adipose tissue 0.9 mg/ml, rat liver 1.8 mg/ml. The mitochondria were stored in 100 mM KCl, 20 mM K-TES, pH 7.2. Additions – succinate 10 mM (rat liver) or *s,n*-glycerol-3-phosphate, 20 mM (brown adipose tissue), rotenone 4 μM, FCCP 0.4 μM and antimycin A at the concentrations indicated. (•) Rat liver, (○) Brown adipose tissue.

shows, that a five-fold higher titre of antimycin A was required to inhibit uncoupled respiration in BAT-mitochondria than in rat liver mitochondria. This suggests, that BAT-mitochondria have about five-times more respiratory-chain components compared to rat liver mitochondria, in good agreement with the earlier reports on cytochrome contents [25–27].

Inhibitors of the mitochondrial ATPase, oligomycin and aurovertin, were then employed to study the amount of this enzyme present in BAT-mitochondria. Both interact with the mitochondrial ATPase in a 1:1 molar ratio when the enzyme is in its synthetic mode, and have therefore previously been used to quantitate ATPase [28]. The results of titration studies of the degree of inhibition of incorporation of $^{32}P_i$ into ATP in the presence of varying concentrations of inhibitor are shown in fig.2A and B. It is evident that less inhibitor is required per mg protein for BAT-mitochondria than for rat liver mitochondria, and 50% inhibition in BAT occurred with both inhibitors at approximately one-third the concentration needed for rat liver. The results indicate a lower amount of ATPase/mg mitochondrial protein in BAT than in rat liver.

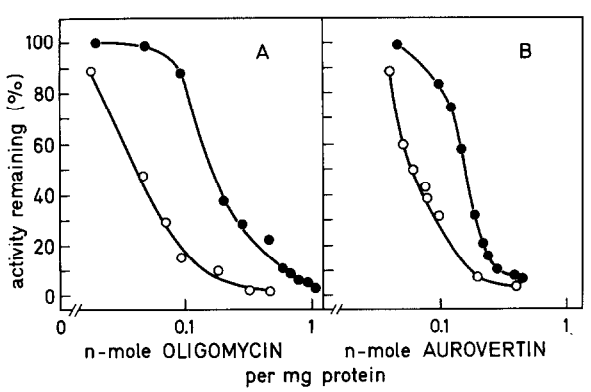

Fig.2. Inhibition of ATP synthesis in mitochondria from brown adipose tissue and rat liver by (A) Oligomycin and (B) aurovertin. Incubation medium as in the legend to fig.1 and in addition $^{32}P_i$, 10 mM glucose, 5 units hexokinase, 35 μM palmitoyl-l-carnitin and 3 mM malate. For brown adipose tissue the medium contained additionally 2 mM GDP and 20 mg/ml bovine serum albumin [23]. Aliquots, 0.2 ml, from 1 ml samples were taken at 0.5 min and 10.5 min and analyzed for organic phosphate according to [19]. (•) Rat liver, (○) Brown adipose tissue.

3.2. *Comparison of ATPases from BAT and beef heart mitochondria*

3.2.1. Isolation

Previously reported methods for the isolation of mitochondrial ATPase were unsuccesful with BAT [18,36,37]. The isolation procedure described in Materials and methods was therefore developed and the results of a typical procedure are shown in table 1. The specific activity of the BAT-enzyme was comparable with that from beef heart and always between 35–50 units/mg protein at 27°C. The low ATPase activity of the intact mitochondrial membranes is thus clearly a consequence of there being only a very small amount of enzyme protein/mg mitochondrial protein. The isolated enzyme is entirely stable for at least three months when stored in liquid nitrogen. The procedure can be readily scaled up for the beef heart ATPase yielding about 50 mg homogeneous enzyme starting with 2 g mitochondrial protein.

3.2.2. Properties

The ATPase from beef heart mitochondria was pure and homogeneous after the above isolation, as judged from non-dissociating polyacrylamide gel electrophoresis. The BAT-preparation contained a protein having the same mobility as the beef heart material, but in addition a minor component of higher mobility. ATPase activity in the gels was localized by staining and found to correspond in both cases to the single protein band visible with the beef heart enzyme after Coomassie Blue treatment. The molecular weights of the enzymes were estimated after electrophoresis in polyacrylamide-gradient gels by comparison with standard proteins, and found to be about 360 000 for both enzymes.

The subunit composition of the enzymes was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis. The resultant polypeptide patterns are shown in fig.3. It is apparent that the three major polypeptides in the beef heart enzyme, having approximate mol. wt 56 000, 52 000 and 32 000, correspond to the visible bands in the BAT-enzyme. Polypeptides corresponding to the two smaller subunits from the beef heart enzyme were occasionally visible in the BAT-preparation, when sufficiently concentrated material could be applied. An additional polypeptide of mol. wt 40 000 was evident in the BAT-preparation and further purifica-

Table 1

Initial	Brown adipose tissue (230 mg frozen mitochondria)				Beef heart (250 mg frozen mitochondria)			
	Protein (mg)	Specific activity (units/mg)	Total activity (units)	Volume (ml)	Protein (mg)	Specific activity (units/mg)	Total activity (units)	Volume (ml)
Crude sonicate	122	0.25	30	18	110	1.8	165	16
Supernatant	9.9	2.7	26	16.5	10.2	12.4	126	16
Redissolved PEG precipitate	8.5	2	16	1.2	8.8	16.2	143	2
Sepharose eluate	0.33	42	14	16.5	–	–	–	–
A-25 eluate	0.21	48	10	5	2.2	59	130	9.5

1 unit ATPase is equivalent to the amount of enzyme which catalyses the hydrolysis of 1 μmol ATP/min at 27°C at pH 8

tion attempts have failed to completely remove this protein. It corresponds to the second band seen in non-dissociating polyacrylamide gels which lacks ATPase activity. This was confirmed by two-dimensional gel electrophoresis. The first-dimension was run under non-dissociating conditions, and the second in the presence of sodium dodecylsulphate.

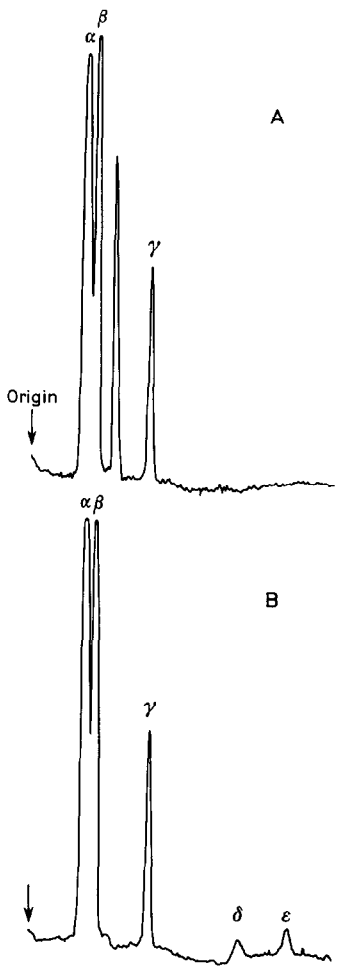

Fig.3. Densitometric traces after gel electrophoresis in the presence of sodium dodecyl sulphate. (A) Mitochondrial ATPase of brown adipose tissue. (B) Mitochondrial ATPase of beef heart. Samples were incubated for 1 min in 1% dodecyl sulphate and 1% 2-mercapto ethanol in boiling water. Electrophoresis in polyacrylamide gel (10%), in the presence of 0.1% dodecyl sulphate, was carried out for about 6 h at 6 mA/tube. About 15 μg protein were applied /tube and gels were stained for protein with Coomassie Brilliant Blue R-250. Densitrometric traces were made in a Joyce-Loeble-Microdensitometer Mk III B.

Antiserum was prepared against the purified beef heart enzyme. Both beef heart- and BAT-ATPase activities were inhibited 60% by addition of the antiserum and slight precipitation was visible in both cases. No inhibition was found with control serum. In Ouchterlony double-diffusion plates a single precipitin line was formed with the beef heart enzyme, although this was hardly visible with the BAT-enzyme, presumably because of the extreme dilution of the latter. Aurovertin inhibited both enzyme activities 50–55% and a fluorescence enhancement was observed in both cases. This is similar to previous observations [31,32].

The results presented are consistent with the role of brown adipose tissue as a thermogenic, rather than an energy conserving organ, and show that significant heat production can only occur by uncoupling between ATP synthesis and respiration.

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References

[1] Dawkins, M. J. R. and Hull, D. (1964) J. Physiol. (London) 172, 216–238.
[2] Joel, C. D. (1965) in: Handbook of Physiology, Section 5. Adipose Tissue (Renold, A. E. and Cahill, G. F. eds) pp. 59–84, Amer. Physiol. Soc., Washington.
[3] Lardy, H. A. and Wellman, H. (1952) J. Biol. Chem. 195, 215–224.
[4] Smith, R. E., Roberts, J. C. and Hittelman, K. J. (1966) Science 154, 653–654.
[5] Lindberg, O., de Pierre, J., Rylander, E. and Afzelius, B. A. (1967) J. Cell Biol. 34, 293–310.
[6] Christiansen, E. N., Pedersen, J. I. and Grav, H. J. (1969) Nature (London) 222, 857–860.
[7] Girardier, L., Seydoux, J. and Clausen, T. (1968) J. Gen. Physiol. 52, 925–940.
[8] Horwitz, B. A., Horowitz, J. M. and Smith, R. E. (1969) Proc. Natl. Acad. Sci. USA 64, 113–120.
[9] Horwitz, B. A. (1975) in: Temperature Regulation and Drug Action (Lomax, P., Schönbaum, E. and Jacob, J. eds) pp. 150–158, S. Karger, Basel.

[10] Horowitz, J. M., Fuller, C. A. and Horwitz, B. A. (1976) in: Regulation of Depressed Metabolism and Thermogenesis (Jansky, L. and Musacchia, X. J. eds) pp. 3–25, Charles Thomas Publ., Springfield, Ohio.
[11] Bulychev, A., Kramar, R., Drahota, Z. and Lindberg, O. (1972) Exp. Cell Res. 72, 169–187.
[12] Drahota, Z. and Houštěk, J. (1975) in: Symposium on Depressed Metabolism and Cold Thermogenesis, Prague (Jansky, L. ed) Charles University Press, in press.
[13] Lindberg, O., Bieber, L. L. and Houštěk, J. (1976) in Regulation of Depressed Metabolism and Thermogenesis (Jansky, L. and Musacchia, X. J. eds) pp. 117–136, Charles Thomas Publ., Springfield, Ohio.
[14] Fernández-Morán, H., Oda, T., Blair, P. V. and Green, D. E. (1964) J. Cell Biol. 22, 63–100.
[15] Hittelman, K. J., Lindberg, O. and Cannon, B. (1969) Eur. J. Biochem. 11, 183–192.
[16] Hogeboom, G. H., Schneider, W. C. and Palade, G. H. (1948) J. Biol. Chem. 172, 619–635.
[17] Löw, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–374.
[18] Horstman, L. L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344.
[19] Lindberg, O. and Ernster, L. (1956) in: Meth. Biochem. Anal., Vol. 1 (Glick, D. ed) pp. 1–22, Interscience, New York.
[20] Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208–216.
[21] Prusiner, S. B., Cannon, B. and Lindberg, O. (1968) Eur. J. Biochem. 6, 15–22.
[22] Slater, E. C. (1973) Biochim. Biophys. Acta 301, 129–154.
[23] Rafael, J., Klaas, D. and Hohorst, H.-J. (1968) Hoppe Seyler's Z. Physiol. Chem. 349, 1711–1724.
[24] Wilson, D. F. and Dutton, P. L. (1971) in: Electron and Coupled Energy Transfer in Biological Systems (King, E. T. and Klingenberg, M. eds) pp. 221–242, Marcel Dekker, Inc., New York.
[25] Cannon, B., Houštěk, J. and Drahota, Z. (1975) Abstracts, 10th FEBS Congress, Paris.
[26] Joel, C. D. and Ball, E. G. (1962) Biochemistry 1, 281–287.
[27] Lišková, Z., Strunecká, A. and Drahota, Z. (1974) Physiol. Bohemoslov. 23, 229–234.
[28] Bertina, R. M., Schrier, P. I. and Slater, E. C. (1973) Biochim. Biophys. Acta 305, 503–518.
[29] Catterall, W. A. and Pedersen, P. L. (1971) J. Biol. Chem. 246, 4987–4994.
[30] Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D. and Munn, E. A. (1975) Biochem. J. 148, 533–537.
[31] Chang, T. and Penefsky, H. S. (1973) J. Biol. Chem. 248, 2746–2754.
[32] Van de Stadt, R. J. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 253–263.