ATPase-inhibitor proteins of brown-adipose-tissue mitochondria from warm- and cold-acclimated rats

Esther W. YAMADA,*§ Norman J. HUZEL,* Ratna BOSE,† Anna-Lisa KATES‡ and Jean HIMMS-HAGEN‡

*Department of Biochemistry and Molecular Biology, and †Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3, and ‡Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

1. A group of male Sprague–Dawley rats (5–6 weeks old) was cold-acclimated at 4 °C for 4 weeks. Warm-acclimated controls remained at 24 °C. Total protein content of brown adipose tissue (BAT) increased more than 3-fold and total uncoupling protein (UCP) content increased more than 6-fold upon cold-acclimation. The concentration of UCP in isolated BAT mitochondria almost doubled. 2. Specific ATPase activity of the non-thermogenic BAT mitochondria (from warm-acclimated controls) was low and increased about 6-fold on addition of 1 μM -Ca²⁺, which raised free Ca²⁺ levels (measured by Fura-2) in the incubation media from $1.32 \pm 0.28 \mu\text{M}$ (mean $\pm$ S.E.M.) to $2.29 \pm 0.39 \mu\text{M}$ [at which the Ca²⁺-binding ATPase-inhibitor protein (CaBI) is inactivated]. Correspondingly, the specific ATP synthetase activity of the non-thermogenic BAT mitochondria was high and was decreased by 74% by addition of 1 μM -Ca²⁺. 3. In contrast, specific ATPase activity of thermogenic BAT mitochondria (from cold-acclimated rats) was 5 times that of the control group, and addition of Ca²⁺ had only a small stimulatory response. Correspondingly, the specific ATP synthetase activity of the thermogenic BAT mitochondria was low, and the decrease by Ca²⁺ was small, albeit significant. 4. Extracts of BAT mitochondria from both groups of animals contained significant amounts of the ATPase-inhibitor protein of Pullman and Monroy (PMI) as well as of CaBI, as shown by gel electrophoresis. Kinetic studies of inhibition of mitochondrial ATPase activity showed that PMI activity was unaltered in extracts from the thermogenic BAT mitochondria, whereas CaBI activity was slightly but significantly increased. 5. The presence of active ATPase-inhibitor proteins in BAT mitochondria was shown for the first time. We conclude that uncoupling of oxidative phosphorylation occurs in thermogenic BAT mitochondria, even in the presence of the ATPase-inhibitor proteins.

INTRODUCTION

Brown adipose tissue (BAT) is characterized by numerous mitochondria and multilocular lipid droplets. The function of BAT is to produce heat (thermogenesis) in response to stimulation by cold or the diet (Nicholls & Locke, 1984; Cannon & Nedergaard, 1985; Himms-Hagen, 1986, 1989, 1990). This function is controlled by the release of noradrenaline from sympathetic nerve terminals within the BAT. Hydrolysis of triacylglycerols, stored in the lipid droplets, to non-esterified fatty acids results, to provide the major fuel for increased thermogenesis.

Increased thermogenesis in BAT mitochondria occurs in preference to ATP synthesis through the action of a unique 32 kDa uncoupling protein (UCP), which is located in the mitochondrial inner membrane (Klingenberg, 1990; for review, see Himms-Hagen, 1990). UCP is induced as a result of the action of noradrenaline (Ricquier & Bouillaud, 1986). The thermogenically active state of UCP is achieved by the binding of endogenous fatty acids which over-ride the inhibitory action of endogenous purine nucleotides (ATP, ADP, GDP) (see Nicholls, 1983). Active UCP dissipates the electrochemical gradient through leakage of protons, thus by-passing the ATP synthetase complex and resulting in acceleration of operation of electron transport.

Early work on isolated BAT mitochondria indicated that the capacity for oxidative phosphorylation is relatively low (Lindberg *et al.*, 1967; Skaane *et al.*, 1972; Bulychev *et al.*, 1972). The low F₁-ATPase activity of hamster BAT mitochondria is unaltered

by acclimation to cold (Yacoe, 1981), in contrast with the large increases in cytochrome *c* concentration (Yacoe, 1981) and in UCP concentration (Trayhurn *et al.*, 1983; Sundin *et al.*, 1987). BAT mitochondria of cold-acclimated hamsters contain a much lower concentration of ATP synthetase protein than do mitochondria of heart (bovine) or liver (rat) (Cannon & Vogel, 1977; Houstek & Drahota, 1977).

Mitochondria of other tissues are known to contain at least two ATPase-inhibitor proteins (see Ernster *et al.*, 1979; Schwerzmann & Pedersen, 1986; Hashimoto *et al.*, 1990; Yamada & Huzel, 1992) which may be involved in control of the energy-transducing ATP synthetase complex. The first of these (PMI) was isolated from bovine heart mitochondria (Pullman & Monroy, 1963). Addition of PMI to uncoupled submitochondrial particles of bovine heart resulted in inhibition of ATPase activity concomitantly with an increase in P:O ratios. Whether PMI has a direct effect on ATP synthesis as well as on ATP hydrolysis was debated in later research (see Panchenko & Vinogradov, 1985; Schwerzmann & Pedersen, 1986; Lippe *et al.*, 1988a,b; Hashimoto *et al.*, 1990). It is important to recognize that, since ATP synthesis and hydrolysis are two aspects of the same reaction and are both catalysed by the same ATP synthetase complex, inhibition of ATP hydrolysis by an inhibitor protein without affecting ATP synthesis would pose a thermodynamic paradox. Such a paradox is not apparent for the second ATPase-inhibitor protein, a Ca²⁺-binding protein (CaBI) (Yamada & Huzel, 1988), which was found to influence ATPase and ATP synthetase activities of the energy-transducing complex of submitochondrial particles of heart in a reciprocal manner,

Abbreviations used: PMI, Pullman and Monroy mitochondrial ATPase-inhibitor protein; CaBI, Ca²⁺-binding mitochondrial ATPase-inhibitor protein; BAT, brown adipose tissue; UCP, uncoupling protein of BAT.

§ To whom correspondence should be addressed.

its activity being regulated by Ca^{2+} (Yamada & Huzel, 1989).

The objective of the present work was to find out whether an altered amount or activity of either PMI or CaBI in BAT mitochondria might be involved in the regulation of the state of coupling of oxidative phosphorylation. We therefore studied BAT mitochondria from warm-acclimated and cold-acclimated rats. The mitochondria from BAT of warm-acclimated rats have a low concentration of UCP (see Himms-Hagen, 1986) and are known to be isolated in a coupled state (see Himms-Hagen, 1970); they are referred to here as 'non-thermogenic BAT mitochondria'. The mitochondria from BAT of cold-acclimated rats are known to have a high concentration of UCP (see Himms-Hagen, 1986) and to be isolated in an uncoupled state; they are referred to here as 'thermogenic BAT mitochondria'. It was expected that comparison of ATPase-inhibitor proteins in BAT mitochondria in these two distinct states might reveal an indication of their possible regulatory roles in mitochondrial function.

EXPERIMENTAL

Materials

CaCl_2 (Suprapur) was obtained from British Drug Houses. Glass-redistilled deionized water was used throughout. ^{125}I -Protein A (2–10 $\mu\text{Ci}/\mu\text{g}$) was obtained from New England Nuclear.

Preparation of BAT mitochondria

Male Sprague-Dawley rats (5–6 weeks old) were obtained from Charles River. Ten rats were maintained for 1 week at 24 °C with free access to food (Purina rat chow 5012) and water under lights for 12 h per day (07:30–19:30 h). Then four of the rats (nos 7–10) were transferred to 4 °C for 4 weeks; the others were maintained at 24 °C. The warm- (control) and cold-acclimated rats were killed by decapitation. Interscapular BAT was removed, placed in ice-cold isolation medium (0.25 M-sucrose, 1 mM-Hepes and 0.2 mM-EDTA buffer, pH 7.2), cleaned of white adipose tissue and muscle, weighed, and homogenized in fresh isolation medium. Samples of homogenate were frozen in liquid N_2 and stored at –70 °C for determination of protein and UCP later.

Mitochondria were isolated from the homogenate in 0.25 M-sucrose/0.2 mM-potassium EDTA/1 mM-Hepes buffer (pH 7.2), followed by two washes in this buffer (Desautels *et al.*, 1978). The final wash and suspension buffer was 10 mM-Tris/HCl (pH 7.4) containing 0.25 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-dithiothreitol (5–10 mg of mitochondrial protein/ml). Samples to be used for UCP assays were frozen at –70 °C. The remainder was freeze-dried and stored at –70 °C until used for assays of enzyme and inhibitor-protein activities.

Assays of UCP

Protein was estimated by a modified Lowry method (Schacterle & Pollack, 1973) with BSA as standard. UCP was measured by solid-phase radioimmunoassay as described previously, with purified rat UCP as a standard (Kates & Himms-Hagen, 1990). Extracts of BAT homogenates and mitochondria were prepared in 5% Triton X-100. Rabbit antiserum was prepared against purified hamster UCP, and immunocomplexes were detected with ^{125}I -Protein A.

Calculation of mitochondrial recovery

The total UCP in interscapular BAT was calculated from the measured concentration of UCP in homogenates ($\mu\text{g}/\text{mg}$ of homogenate protein) and the volume of homogenate containing the total tissue. Total mitochondrial protein in BAT was calculated from the concentration of UCP in isolated mitochondria ($\mu\text{g}/\text{mg}$ of mitochondrial protein) and the total UCP in the interscapular BAT. The proportion of total mitochondrial protein recovered was the total amount in the mitochondrial suspension as a percentage of the total mitochondrial protein in the homogenate.

Mitochondrial ATPase and ATP synthetase activities

BAT mitochondria were stored at –70 °C and thawed once just before use. ATP synthetase activities of BAT mitochondria were measured by a luminescence assay, patterned after that of Lemasters & Hackenbrock (1976), under the optimal conditions described previously (Yamada & Huzel, 1989). Briefly, the reaction mixture consisted of 0.25 M-sucrose, 8 mM- MgSO_4 , 5 μM -cytochrome *c*, 6.25 μM -rotenone, 120 μM - P^i P^5 -di-(adenosine 5')pentaphosphate, 50 mM-Tris/acetate buffer, 20 mM-potassium phosphate buffer (pH 7.5), 1.2 mM-ADP and enzyme, with or without (controls) 5 mM-succinate in a final volume of 0.2 ml. After preincubation for 10 min at 30 °C (Klein & Vignais, 1983; Lippe *et al.*, 1988a) in the absence of ADP, the reaction was initiated by ADP and incubation was continued for an additional 4 min with shaking. The reaction was terminated by a 20-fold dilution into 0.1 M-Tris/acetate buffer (pH 7.75) containing 0.1% Triton X-100 and 2 mM-EDTA. ATP content of the samples was then determined by the ATP-monitoring reagent (luciferin-luciferase) as described previously (Yamada & Huzel, 1989). Other controls lacked enzyme or ADP; all assays were in duplicate. Buffer media were aerated for 10 min before preincubation. ATP synthesis in the absence of succinate was negligible.

ATPase activity of the mitochondria was measured by the standard procedure patterned after the method of Monk & Kellerman (1976). The reaction mixture (final volume 1 ml) containing 0.36 M-mannitol, 50 mM-Tris/sulphate buffer (pH 7.7), 1.67 μg of antimycin, 2 mM-phosphoenolpyruvate, 8.5 units of pyruvate kinase (Type II; Sigma), 15 units of lactate dehydrogenase (Type II; Sigma), 2 mM-MgATP and 0.205 μmol of NADH (NADH had to be omitted in assays of free Ca^{2+} by Fura-2) was equilibrated for 10 min with shaking, followed by termination with 0.33% SDS and measurement of the A_{340} (Yamada *et al.*, 1980). Controls lacked enzyme, which was added just before termination of the reaction.

In some assays of ATP synthetase and ATPase activities, 1 μM - Ca^{2+} (Suprapur) was added to the incubation media before preincubation.

Heat-stable protein fractions and ATPase-inhibitor protein activities

The fractions were prepared at 0–4 °C essentially as described previously (Yamada & Huzel, 1988). Briefly, mitochondria (5 mg of protein/ml) were heated in deionized water containing 1 mM-phenylmethanesulphonyl fluoride (5 mg/ml) at 75 °C for 10 min and ice-cooled. Heat-denatured protein was sedimented by centrifugation at 185000 *g* for 10 min (Spinco rotor 70.1 Ti). The pellet was washed with water; the wash and supernatant fractions were combined. Protein was precipitated from the combined fraction with 10% trichloroacetic acid in ice; the precipitate (20200 *g*, 10 min, Sorvall SS-34 rotor) was washed once with 10% trichloroacetic acid and dissolved in 0.25 M-sucrose (pH adjusted to 7.0). Ice-cold ethanol was added to a final con-

centration of 70 % (v/v); after centrifugation for 10 min, the pellet was dissolved in 0.25 M-sucrose. Ethanol precipitation was repeated only once, owing to the smallness of the samples. The final fractions were dialysed for 22 h in 0.25 M-sucrose/0.25 mM-EDTA / 5 mM-2-mercaptoethanol / 10 mM-Tris/sulphate buffer (pH 8.0) and stored at -70°C . Protein of the fractions was determined by the Pierce protein assay kit. Gel electrophoresis (Laemmli, 1970) of the heat-stable protein fractions, staining, and destaining were as described previously (Yamada & Huzel, 1989).

ATPase-inhibitor protein activity of the heat-stable protein fractions from BAT mitochondria was determined by the standard assay (Yamada & Huzel, 1989). To estimate PMI activity, $1\text{ }\mu\text{M-Ca}^{2+}$ was included in the preincubation medium to inactivate CaBI (Yamada & Huzel, 1988, 1989). Total inhibitor protein activity was determined in the absence of exogenous Ca^{2+} . A unit of ATPase-inhibitor protein activity is defined as the amount that resulted in 50 % inhibition of 0.2 unit of ATPase activity under the conditions specified (Horstman & Racker, 1970; Yamada & Huzel, 1988).

Measurement of free Ca^{2+} concentrations

Ca^{2+} contamination of glassware, solutions and tissue extracts has proved to be troublesome in studies of the role of Ca^{2+} in metabolism. Some of the problems were solved by the introduction of the chelator EGTA to buffer Ca^{2+} . However, other problems arose from the use of Ca^{2+} -EGTA buffers such as loss of mitochondrial Ca^{2+} to the external EGTA sink during incubation (see Williamson *et al.*, 1981). Because of this and other anomalies introduced by Ca^{2+} -EGTA buffers, they were not used in the present work, particularly in view of reports that EGTA could replace the Ca^{2+} -binding protein calmodulin, or interact directly with Ca^{2+} -transport systems (see Berman, 1982; Kotagal *et al.*, 1983; Trosper & Philipson, 1984). Instead, redistilled deionized water and reagents of highest purity were used throughout this study. Ca^{2+} concentration of the water was estimated by atomic absorption to be less than $0.1\text{ }\mu\text{M}$.

Free Ca^{2+} levels of the incubation media were measured by the ratio method of Gryniewicz *et al.* (1985) with the fluorescent indicator Fura-2 (for review see Cobbold & Rink, 1987) and a Jasco Ca^{2+} analyser (model CAF-102). A calibration curve with Ca^{2+} -EGTA solutions (1 nM – 0.1 mM-Ca^{2+}) was in agreement with the ratio method of assay. An internal standard was included also in which $1\text{ }\mu\text{M-CaCl}_2$ (Suprapur) was added to each incubation mixture to be analysed by Fura-2 at the temperature used for incubation (30°C). Measurements were in duplicate, and each of the various incubation media was assayed at least twice.

Statistical analysis

A mainframe computer (VM/370) and statistics package SAS (SAS Institute Inc., Statistical Analysis System, Cary, NC, U.S.A.) were used. The GLM (general linear model) for unbalanced ANOVA (analysis of variance) in which the number of samples (n) per group is unequal was used. The *post-hoc* test was Scheffe's; the level of significance was $P < 0.05$.

RESULTS

Cold-acclimation of rats and UCP

Cold-acclimation of the rats significantly increased BAT weight, total protein content and total mitochondrial protein content (Table 1). However, body weight of the cold-acclimated

rats was less than that of the controls, for growth is less rapid despite an increased food intake. The concentration of mitochondrial UCP almost doubled, in terms of both homogenate protein and mitochondrial protein (Table 1). The proportion of mitochondrial protein relative to total homogenate protein was not altered by cold-acclimation, and the recovery of mitochondria was the same (about 25 %) in both groups of rats (Table 1).

ATP synthetase, ATPase activities and Ca^{2+}

Estimates by the Fura-2 fluorescence method gave the basal concentration of Ca^{2+} in the incubation media to be $1.32 \pm 0.28\text{ }\mu\text{M}$ (mean $\pm$ S.E.M.). After addition of $1\text{ }\mu\text{M-Ca}^{2+}$, the concentration rose to $2.29 \pm 0.39\text{ }\mu\text{M}$. Addition of mitochondria (up to $10\text{ }\mu\text{g}$ of protein) would not add significantly to the Ca^{2+} levels.

Table 2 shows that the specific ATPase activity of BAT mitochondria of warm-acclimated rats was low. When Ca^{2+} ($1\text{ }\mu\text{M}$) was included in the preincubation media of these control BAT mitochondria, the result was a 5.5-fold increase in specific ATPase activity. A similar increase in ATPase activity was found for submitochondrial particles of heart or skeletal muscle after the removal or inactivation of CaBI (Yamada & Huzel, 1989; Yamada, 1990). In contrast, the mean specific ATPase activity of the thermogenic BAT mitochondria from the cold-acclimated rats was already 5 times that of the non-thermogenic controls and almost as high as that found after addition of $1\text{ }\mu\text{M-Ca}^{2+}$ (Table 2). Only a small but significant increase in ATPase activity of the thermogenic BAT mitochondria occurred with $1\text{ }\mu\text{M}$ exogenous Ca^{2+} .

The ATP synthetase activities of the thermogenic and non-thermogenic BAT mitochondria were inversely proportional to the ATPase activities. Table 2 shows that the specific ATP synthetase activity of the non-thermogenic BAT mitochondria is high, comparable with that found for mitochondria of other tissues (Yamada & Huzel, 1992). Addition of $1\text{ }\mu\text{M-Ca}^{2+}$ resulted in a decrease of 74 % in the specific ATP synthetase activity of the non-thermogenic BAT mitochondria. For the thermogenic BAT mitochondria, the specific ATP synthetase activity was only 30 % of that of the non-thermogenic BAT mitochondria. There was only a small but significant decrease in specific ATP synthetase activity of the thermogenic BAT mitochondria when $1\text{ }\mu\text{M-Ca}^{2+}$ was added.

ATPase inhibitor protein activity

Studies were undertaken next to determine whether the high ATPase and low ATP synthetase activities as well as the decreased responses to Ca^{2+} by the thermogenic BAT mitochondria might be due to loss or inactivation of the ATPase-inhibitor proteins. The high protein content of crude mitochondrial lysates interferes with assays of the ATPase-inhibitor proteins. Advantage can be taken, however, of the heat-stability of both inhibitors (see Yamada & Huzel, 1988, 1992). Accordingly, heat-stable protein fractions were prepared from the mitochondria. From these, the ATPase-inhibitor proteins can be determined by densitometer tracings of stained polyacrylamide gels and by assays of the inhibition of mitochondrial ATPase activity. There is consistent agreement between the two methods (Yamada & Huzel, 1989). Total inhibitor protein activity is given with Ca^{2+} omitted from the preincubation medium. With added Ca^{2+} ($1\text{ }\mu\text{M}$), the activity of PMI alone is measured, the difference giving the activity of CaBI.

No significant difference was found in the amount of heat-stable protein recovered from the non-thermogenic and thermogenic BAT mitochondria, the concentrations (mg/mg of mitochondrial protein; means $\pm$ S.E.M.) being 0.136 ± 0.009 and

Table 1. Effect of cold-acclimation on rats

$n = 4$ for cold-acclimated rats and 6 for warm-acclimated rats. * Statistically significant effect of cold-acclimation.

Parameter	Warm-acclimated rats	Cold-acclimated rats
Body wt. (g)	376.7 ± 11.3	342.0 ± 5.2*
Interscapular BAT wt. (mg)	514 ± 48.1	1021 ± 87.0*
Interscapular BAT protein (mg)	36.2 ± 2.19	127.4 ± 9.4*
Total UCP in BAT (mg)	0.536 ± 0.036	3.478 ± 0.121*
UCP in BAT (μg/mg)	15.2 ± 1.65	27.7 ± 2.04*
Total mitochondrial protein (mg)	16.5 ± 1.48	57.4 ± 4.88*
(% of total protein)	45.8 ± 3.95	46.0 ± 5.12
Mitochondrial protein recovered (mg)	4.12 ± 0.56	13.87 ± 2.24*
(% of total)	25.4 ± 2.72	24.3 ± 3.54
UCP in BAT mitochondria (μg/mg)	33.5 ± 2.76	61.6 ± 4.48*

Table 2. Effect of cold-acclimation on ATP synthetase and ATPase activities of BAT mitochondria

Enzyme activities were determined by the methods described in the Experimental section. These are the same animals described in Table 1. Values are means ± S.E.M.; numbers of animals are given in parentheses. * Statistically significant by Scheffe's test. ** Statistically significant (plus Ca^{2+} compared with no Ca^{2+}).

Animals	ATPase activity (μmol/min per mg)		ATP synthetase activity (μmol/min per mg)	
	No Ca^{2+}	Plus 1 μM- Ca^{2+}	No Ca^{2+}	Plus 1 μM- Ca^{2+}
(A) Warm-acclimated (6)	0.091 ± 0.008	0.497 ± 0.013**	1.436 ± 0.110	0.369 ± 0.016**
(B) Cold-acclimated (4)	0.441 ± 0.075	0.598 ± 0.005**	0.426 ± 0.036	0.303 ± 0.012**
<i>P</i> (B compared with A)	< 0.0010*	< 0.0003*	< 0.0001*	< 0.0222*

Table 3. ATPase-inhibitor protein activity of BAT mitochondria from warm- and cold-acclimated rats

Heat-stable protein fractions were prepared and inhibitor protein activity was determined by the standard assay as described in the Experimental section. These are the same animals described in Tables 1 and 2. Values are means ± S.E.M.; numbers of animals are given in parentheses. * Statistically significant (Scheffe's test).

Animals	Total specific ATPase-inhibitory activity (units/mg)	CaBI specific activity (units/mg)	PMI specific activity (units/mg)	Ratio CaBI/PMI
(A) Warm-acclimated (6)	741.9 ± 2.95	504.7 ± 3.77	237.2 ± 1.48	2.13 ± 0.027
(B) Cold-acclimated (4)	791.8 ± 2.20	551.6 ± 1.51	239.0 ± 0.35	2.31 ± 0.004
<i>P</i> (B compared with A)	< 0.0001*	< 0.0001*	< 0.3690	< 0.0009*

0.131 ± 0.019 respectively. Table 3 shows that the specific inhibitory activity of CaBI was about 2 times that of PMI in extracts from the non-thermogenic BAT mitochondria. Upon cold-acclimation, the total specific ATPase-inhibitory activity of the extracts from the thermogenic BAT mitochondria was increased, owing to a small (9%) but significant increase in the specific inhibitory activity of CaBI while that of PMI remained unchanged. Thus the data indicate a small gain of CaBI activity upon cold-acclimation, rather than a loss as suggested by the studies of the whole mitochondria and the effect of 1 μM- Ca^{2+} .

Gel electrophoresis of heat-stable protein fractions from BAT mitochondria

The heat-stable protein fractions from BAT mitochondria were dialysed overnight in sample buffer (Laemmli, 1970) at 4 °C

in Spectrapor tubing (3.5 kDa cut-off) before application to 15%-acrylamide gels. Staining was overnight rather than for 1 h as before (Yamada & Huzel, 1989). Fig. 1 shows that the extracts from both thermogenic and non-thermogenic BAT mitochondria contain both CaBI and PMI. Extracts from the other warm-acclimated controls gave results similar to that found for rat no. 6. There was no noticeable difference in the amounts of CaBI and PMI in the fractions from the thermogenic as compared with those from the non-thermogenic mitochondria. Of significance was the lack of a visible band at 32 kDa (Fig. 1), corresponding to UCP, in any of the extracts from the control BAT mitochondria or even from those from the thermogenic BAT mitochondria in which the concentration of UCP had doubled. Thus it is apparent that UCP did not survive the heat and ethanol treatments by which the protein fractions were prepared.

Fig. 1. Gel electrophoresis of heat-stable protein fractions from BAT mitochondria

Lane 1, marker proteins (kDa), from top to bottom: ovalbumin (45.0), α -chymotrypsinogen (24.0), β -lactoglobulin (18.0), lysozyme (16.0), bovine trypsin inhibitor (6.0) and insulin, A and B chains (3.0); lane 2, 2 μ g of CaBI; lane 3, 2 μ g of PMI; lane 4, 10 μ g from non-thermogenic BAT mitochondria (rat no. 6); lanes 5–8, 10 μ g each from thermogenic BAT mitochondria (rats 7–10). The heat-stable protein fractions were prepared as described in the Experimental section.

DISCUSSION

As expected, cold-acclimation of rats resulted in large increases in total BAT protein and mitochondrial protein, accompanied by a specific increase in the concentration of UCP in the mitochondria. The concentration of UCP in mitochondria from BAT of the cold-acclimated rats in the present experiments is similar to that reported by others (Ashwell *et al.*, 1985) and by us in another study (Park & Himms-Hagen, 1988). The concentration of UCP in mitochondria from BAT of the warm-acclimated rats is somewhat higher than that described by others (Ashwell *et al.*, 1985) and by us (Park & Himms-Hagen, 1988), perhaps because of the use of a lower environmental temperature (24 °C, versus 26 °C used by Park & Himms-Hagen, 1988) or a different strain of rat (Sprague-Dawley rats, versus Zucker rats in Ashwell *et al.*, 1985).

Although the specific ATP synthetase activity of the non-thermogenic BAT mitochondria from warm-acclimated rats was comparable with that of other tissues (Yamada & Huzel, 1992), the activity of the ATP synthetase in thermogenic BAT mitochondria from cold-acclimated rats was much decreased. Others also have found low ATP-synthesis rates in thermogenic BAT mitochondria from cold-adapted and newborn animals (Lindberg *et al.*, 1967; Skaane *et al.*, 1972; Bulychev *et al.*, 1972). Accompanying the great decrease in specific ATP synthetase activity of thermogenic BAT mitochondria of cold-acclimated rats was a great increase in specific ATPase activity. This result is in contrast with the report that ATPase activity of hamster BAT mitochondria was unaltered by acclimation to cold (Yacoe, 1981). However, since the ATP synthetase complex catalyses a reversible reaction, the lowering of synthetic activity should lead to an increase in ATPase activity (see Nicholls & Akerman, 1982).

Ca^{2+} -induced uncoupling of oxidative phosphorylation could not be the reason for the observed effects of Ca^{2+} on ATP synthetase and ATPase activities of BAT mitochondria in present studies. It is true that Ca^{2+} uptake at high, unphysiological, concentrations (10–100 μM) lowers $\Delta\psi$ of liver mitochondria to such an extent that ATP synthesis is prevented (Mitchell & Moyle, 1969; Heaton & Nicholls, 1976; Akerman, 1978; Nicholls & Akerman, 1982). As a consequence, ATPase activity increases

to add to the protons extruded by the respiratory chain giving 'super-stoichiometric' uptake of Ca^{2+} in relation to the oxygen consumed (Brand & Lehninger, 1975; Reynafarje & Lehninger, 1974). It is true also that Ca^{2+} concentrations estimated by Fura-2, as in present work, may be half as high as those estimated by the alternative indicator of choice, Quin-2 (Mazorow & Millar, 1990). There are directly opposing views as to which of the two indicators gives the true absolute values of Ca^{2+} (see Mazorow & Millar, 1990; Cobbold & Rink, 1987). In either case, our Ca^{2+} concentrations are far below the 0.1 mM- Ca^{2+} that caused uncoupling of oxidative phosphorylation of BAT mitochondria from guinea pigs (Christiansen, 1971) and of mitochondria of other tissues (see above and Lehninger, 1962; Lehninger *et al.*, 1967; Lotscher *et al.*, 1980; Gunter & Pfeiffer, 1990).

Since the ATPase-inhibitor proteins regulate ATPase activity of the mitochondrial ATP synthetase complex, it follows that they must be displaced from the complex in order for ATPase activity to increase. One way to determine how ATPase and ATP synthetase activities of isolated mitochondria are influenced by the ATPase-inhibitor proteins is to add micromolar concentrations of Ca^{2+} to the assay media in order to inactivate CaBI but to leave PMI intact, for PMI is not sensitive to Ca^{2+} (Klein *et al.*, 1982; Wong *et al.*, 1982). This method was used successfully in studies of mitochondria of bovine heart and skeletal muscle and other tissues (Yamada *et al.*, 1981; Yamada & Huzel, 1988, 1989, 1992). As expected, the specific ATPase activity of the non-thermogenic BAT mitochondria of warm-acclimated rats was greatly increased by Ca^{2+} , and the specific ATP synthetase activity was correspondingly greatly decreased. In contrast, with thermogenic BAT mitochondria of cold-acclimated rats, the already high specific ATPase activity and low ATP synthetase activity hardly responded to the presence of added Ca^{2+} . Christiansen (1971) also observed that respiration of BAT mitochondria from cold-stressed guinea pigs showed little response to Ca^{2+} , unlike the warm-acclimated controls, in which the increase in respiration in response to Ca^{2+} was considerable. The high specific ATPase activity of the thermogenic mitochondria of cold-acclimated rats indicated that the amount or activity of the ATPase-inhibitor proteins might be decreased in comparison with the non-thermogenic BAT mitochondria of warm-acclimated rats. The much decreased stimulation of ATPase activity of the thermogenic BAT mitochondria by Ca^{2+} suggested that it was CaBI that was missing or ineffective.

The use of a second method of studying the ATPase-inhibitor proteins, by direct assay of specific inhibitory activities of extracts from mitochondria (i.e. heat-stable protein fractions) (Yamada & Huzel, 1989), showed unexpectedly that CaBI activity was slightly increased in extracts from thermogenic BAT mitochondria, PMI activity remaining unchanged. In addition, the amounts of PMI and CaBI protein in the heat-stable fractions, as measured by polyacrylamide-gel electrophoresis, appeared to be relatively unchanged in the thermogenic BAT mitochondria from cold-acclimated rats. This is the first time that the presence and activity of the ATPase-inhibitor proteins have been demonstrated to be present in BAT mitochondria.

The major difference between the assays of intact mitochondria and those of the heat-stable extracts is that UCP and possibly other factors are absent from the extracts. Thus we conclude that the decrease in ATP synthetase activity and the increase in ATPase activity in thermogenic BAT mitochondria of cold-acclimated rats are not brought about by changes in the amount of either CaBI or PMI. Rather, it seems likely that the elevated concentration of UCP, together with its stimulatory effector, fatty acids, can bring about an uncoupling of oxidative phosphorylation that is not sensitive to the actions of CaBI and PMI, which normally inhibit ATP hydrolysis and promote ATP

synthesis by the ATP synthetase complex. On the other hand, there does appear to be a role for CaBI in addition to PMI in the regulation of the state of coupling of non-thermogenic BAT mitochondria of warm-acclimated rats, as in mitochondria of other tissues.

The possibility that there is excess Ca^{2+} in the intact thermogenic BAT mitochondria which is interfering with the action of CaBI, thus leading to elevated specific ATPase activity and decreased ATP synthetase activity, is unlikely, for these isolated mitochondria have in fact a lower Ca^{2+} concentration compared with the non-thermogenic BAT mitochondria (Greenway & Himms-Hagen, 1978). Christiansen (1971) likewise found a low content of Ca^{2+} in thermogenic BAT mitochondria of newborn and cold-stressed guinea pigs. In the work of Das & Harris (1990a,b), in which mitochondrial ATPase activity was taken to be a measure of ATP synthetase activity, reversible Ca^{2+} -dependent hormonal activation of mitochondrial ATPase of cultured Ca^{2+} -tolerant heart myocytes was reported. Since the cytosolic and intramitochondrial Ca^{2+} levels were not determined and the ATPase activity was already elevated in the absence of exogenous Ca^{2+} , comparison with our and other work is not possible.

Normally, Ca^{2+} uptake into mitochondria via the Ca^{2+} uniport is driven by the Ca^{2+} concentration gradient plus the membrane potential generated by substrate oxidation or by ATPase activity (Lehninger *et al.*, 1967; Carafoli *et al.*, 1980; Nicholls & Akerman, 1982). Rates of influx and efflux of Ca^{2+} were calculated to be about equivalent at an extramitochondrial concentration of $1\ \mu\text{M}\text{-Ca}^{2+}$ (Nicholls, 1978; Carafoli, 1987). Despite their high ATPase activity, thermogenic BAT mitochondria are capable of high rates of Ca^{2+} uptake only when they are recoupled after isolation with exogenous succinate, GDP (or ADP to a lesser extent) and albumin (Hittelman *et al.*, 1967; Christiansen, 1971; Trayhurn & Fraser, 1983). If left uncoupled, the rates of Ca^{2+} uptake are about one-third of those of the non-thermogenic BAT mitochondria (Trayhurn & Fraser, 1983). In addition, depletion of Ca^{2+} in thermogenic BAT mitochondria probably results as a consequence of noradrenaline-induced Na^{+} -dependent release of Ca^{2+} (Connolly *et al.*, 1984). In our studies, ADP, but not GDP, is present in the reaction mixture for ATP synthesis. Still, it is possible that the thermogenic mitochondria, because of their uncoupled state and lowered $\Delta\psi$, may not be transporting as much Ca^{2+} as the non-thermogenic mitochondria. However, lower intramitochondrial Ca^{2+} alone, in the presence of physiological concentrations of Mg^{2+} , would not affect the activity of CaBI unless the K_a for Ca^{2+} of CaBI (estimated for purified CaBI to be $3\ \mu\text{M}$; Yamada, 1990) has been altered downward by the uncoupled state. A case in point, although not entirely analogous, is the finding that the $K_{0.5}$ for Ca^{2+} activation of pyruvate dehydrogenase activity of BAT mitochondria was about doubled in the uncoupled compared with the coupled state (McCormack & Denton, 1980).

This work was supported by grants from the Medical Research Council of Canada (E.W.Y. and J.H.-H.).

REFERENCES

- Akerman, K. E. O. (1978) *Biochim. Biophys. Acta* **502**, 359–366
- Ashwell, M., Holt, S., Jennings, G., Stirling, D. M., Trayhurn, P. & York, D. A. (1985) *FEBS Lett.* **179**, 233–237
- Berman, M. C. (1982) *J. Biol. Chem.* **257**, 1953–1957
- Brand, M. D. & Lehninger, A. L. (1975) *J. Biol. Chem.* **250**, 7958–7960
- Bulychev, A., Kramer, R., Drahotka, Z. & Lindberg, O. (1972) *Exp. Cell Res.* **72**, 169–187
- Cannon, B. & Nedergaard, J. (1985) in *New Perspectives in Adipose Tissue Structure, Function, and Development* (Cryer, A. & Van, R. L. R., eds.), pp. 223–270, Butterworths, London
- Cannon, B. & Vogel, G. (1977) *FEBS Lett.* **76**, 284–289
- Carafoli, E. (1987) *Annu. Rev. Biochem.* **56**, 395–433
- Carafoli, E., Gavilanes, M., Affolter, H., Tuena de Gomez-Puyou, M. & Gomez-Puyou, A. (1980) *Cell Calcium* **1**, 255–265
- Christiansen, E. N. (1971) *Eur. J. Biochem.* **19**, 276–282
- Cobbold, P. H. & Rink, T. J. (1987) *Biochem. J.* **248**, 313–328
- Connolly, E., Nanberg, E. & Nedergaard, J. (1984) *Eur. J. Biochem.* **141**, 187–193
- Das, A. M. & Harris, D. A. (1990a) *Am. J. Physiol.* **259**, H1264–H1269
- Das, A. M. & Harris, D. A. (1990b) *Cardiovasc. Res.* **24**, 411–417
- Desautels, M., Zaron-Behrens, G. & Himms-Hagen, J. (1978) *Can. J. Biochem.* **56**, 378–383
- Ernster, L., Carlsson, C., Hundal, T. & Nordenbrand, K. (1979) *Methods Enzymol.* **55**, 399–407
- Greenway, D. C. & Himms-Hagen, J. (1978) *Am. J. Physiol.* **234**, C7–C13
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Gunter, T. E. & Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786
- Hashimoto, T., Yoshida, Y. & Tagawa, K. (1990) *J. Bioenerg. Biomembr.* **22**, 27–38
- Heaton, G. M. & Nicholls, D. G. (1976) *Biochem. J.* **156**, 635–646
- Himms-Hagen, J. (1970) *Adv. Enzyme Regul.* **8**, 131–151
- Himms-Hagen, J. (1986) in *Brown Adipose Tissue* (Trayhurn, P. & Nicholls, D. G., eds.), pp. 214–268, Arnold, London
- Himms-Hagen, J. (1989) *Prog. Lipid Res.* **28**, 67–115
- Himms-Hagen, J. (1990) *FASEB J.* **4**, 2890–2898
- Hittelman, K. J., Fairhurst, A. S. & Smith, R. E. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 697–702
- Horstman, L. L. & Racker, E. (1970) *J. Biol. Chem.* **245**, 1336–1344
- Housteck, J. & Drahotka, Z. (1977) *Biochim. Biophys. Acta* **484**, 127–139
- Kates, A.-L. & Himms-Hagen, J. (1990) *Am. J. Physiol.* **258**, E7–E15
- Klein, G., Satre, M., Zaccari, G. & Vignais, P. V. (1982) *Biochim. Biophys. Acta* **681**, 226–232
- Klein, G. & Vignais, P. V. (1983) *J. Bioenerg. Biomembr.* **15**, 347–362
- Klingenberg, M. (1990) *Trends Biochem. Sci.* **15**, 108–112
- Kotagal, N., Colca, J. R. & McDaniel, M. L. (1983) *J. Biol. Chem.* **258**, 4808–4813
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehninger, A. L. (1962) *Physiol. Rev.* **42**, 467–517
- Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Adv. Enzymol.* **29**, 259–320
- Lemasters, J. J. & Hackenbrock, C. R. (1976) *Eur. J. Biochem.* **67**, 1–10
- Lindberg, O., DePierre, J., Rylander, E. & Afzelius, B. A. (1967) *J. Cell Biol.* **34**, 293–310
- Lippe, G., Sorgato, M. C. & Harris, D. A. (1988a) *Biochim. Biophys. Acta* **933**, 1–11
- Lippe, G., Sorgato, M. C. & Harris, D. A. (1988b) *Biochim. Biophys. Acta* **933**, 12–31
- Lotscher, H. R., Winterhalter, K. H., Carafoli, E. & Richter, C. (1980) *Eur. J. Biochem.* **110**, 211–216
- Mazorow, D. L. & Millar, D. B. (1990) *Anal. Biochem.* **186**, 28–30
- McCormack, J. G. & Denton, R. M. (1980) *Biochem. J.* **190**, 95–105
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
- Monk, B. C. & Kellerman, G. M. (1976) *Anal. Biochem.* **79**, 544–552
- Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474
- Nicholls, D. G. (1983) *Biosci. Rep.* **3**, 431–441
- Nicholls, D. G. & Akerman, K. (1982) *Biochim. Biophys. Acta* **683**, 57–88
- Nicholls, D. G. & Locke, R. M. (1984) *Physiol. Rev.* **64**, 1–64
- Panchenko, M. V. & Vinogradov, A. D. (1985) *FEBS Lett.* **184**, 226–230
- Park, I. R. A. & Himms-Hagen, J. (1988) *Am. J. Physiol.* **255**, R874–R881
- Pullman, M. E. & Monroy, G. C. (1963) *J. Biol. Chem.* **238**, 3762–3769
- Reynafarje, B. & Lehninger, A. L. (1974) *J. Biol. Chem.* **249**, 6067–6073
- Ricquier, D. & Bouillaud, F. (1986) in *Brown Adipose Tissue* (Trayhurn, P. & Nicholls, D. G., eds.), pp. 86–104, Arnold, London
- Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654–655
- Schwerzmann, K. & Pedersen, P. L. (1986) *Arch. Biochem. Biophys.* **250**, 1–18
- Skaane, O., Christiansen, E. N., Pedersen, J. I. & Gray, H. J. (1972) *Comp. Biochem. Physiol.* **42B**, 91–107
- Sundin, U., Moore, G., Nedergaard, J. & Cannon, B. (1987) *Am. J. Physiol.* **252**, R822–R832

- Trayhurn, P. & Fraser, D. R. (1983) *Biochem. J.* **214**, 171–175
- Trayhurn, P., Richard, D., Jennings, G. & Ashwell, M. (1983) *Biosci. Rep.* **3**, 1077–1084
- Trosper, T. L. & Philipson, K. D. (1984) *Cell Calcium* **5**, 211–222
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295
- Wong, S. Y., Galante, Y. M. & Hatefi, Y. (1982) *Biochemistry* **21**, 5781–5787
- Yacoe, M. E. (1981) *Biochem. J.* **194**, 653–656
- Yamada, E. W. (1990) *Int. J. Purine Pyrimidine Res.* **1**, 7–11
- Yamada, E. W. & Huzel, N. J. (1988) *J. Biol. Chem.* **263**, 11498–11503
- Yamada, E. W. & Huzel, N. J. (1989) *Biochemistry* **28**, 9714–9718
- Yamada, E. W. & Huzel, N. J. (1992) *Biochim. Biophys. Acta* **1139**, 143–147
- Yamada, E. W., Shiffman, F. H. & Huzel, N. J. (1980) *J. Biol. Chem.* **255**, 267–273
- Yamada, E. W., Huzel, N. J. & Dickison, J. C. (1981) *J. Biol. Chem.* **256**, 10203–10207

Received 13 April 1992; accepted 22 April 1992