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Regulation of GDP binding and uncoupling-protein concentration in brown-adipose-tissue mitochondria

The effects of cold-acclimation, warm-reacclimation and noradrenaline

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We have used a specific immunoassay for uncoupling protein and [^{3}H]GDP binding to study the acute and chronic responses of brown-adipose-tissue (BAT) mitochondria of warm-acclimated rats to housing at 4 °C and cold-acclimated rats to housing at 27 °C. These studies have shown the following. (1) In the cold-exposed rat the increase in mitochondrial uncoupling-protein concentration parallels the increase in GDP binding from 1 day to 5 days, but that acutely (initial 4 h) the increase in GDP binding is not associated with any change in uncoupling-protein concentration. 2. In the cold-acclimated rat rehoused at 27 °C, GDP binding fell by over 50% in the first 2 days, without any change in uncoupling-protein concentrations. 3. Noradrenaline acutely (30 min) increased BAT mitochondrial GDP binding of lean and obese Zucker rats, without any change in uncoupling-protein concentrations. 4. The increases in GDP binding in cold-exposed rats were associated with increases in the rate of swelling of mitochondria in the presence of valinomycin and potassium acetate. The evidence supports the hypothesis that the acute response of the rat to changes in environmental temperature are associated with unmasking or remasking of uncoupling protein, whereas chronically changes in uncoupling-protein concentration predominate.

INTRODUCTION

Functional brown adipose tissue (BAT) mitochondria are able to adapt their thermogenic capacity in response to both environmental temperature (Foster & Frydman, 1978) and dietary signals (Rothwell & Stock, 1983). Increased heat production is achieved by allowing proton re-entry into the mitochondrial matrix, via an alternative pathway to that associated with ATP synthesis. Thus, respiration rate, no longer determined by the cell's requirement for ATP, proceeds at an unrestrained rate (for review, see Nicholls & Locke, 1984). This loose coupling of BAT mitochondria has been attributed to a unique M_r-32000 protein, in the mitochondrial inner membrane, known as 'thermogenin' or 'uncoupling protein' (Heaton *et al.*, 1978). The protein is thought to be a proton conductance channel (Klingenberg & Winkler, 1985), which can be inhibited by a purine nucleotide binding to a site exposed on the outer surface of the mitochondrial inner membrane (Nicholls & Locke, 1984). The specific binding of [^{3}H]GDP has been used extensively as an indication of the thermogenic state of the tissue. Sympathetic stimulation of BAT, as a result of, for example, cold exposure, increases GDP binding. Chronic cold exposure has been shown to increase the concentration of uncoupling protein in parallel with the increase in GDP binding (Desautels *et al.*, 1978; Ashwell *et al.*, 1983; Nedergaard & Cannon, 1985). The mechanism of the acute response to cold is, however, unclear. It has been suggested that acute stimulation results in unmasking of more GDP-binding sites on previously inactive uncoupling protein (Desautels *et al.*, 1978; Swick & Swick, 1986), although others have disputed the existence of unmasking and remasking of functional uncoupling protein.

The obese Zucker (*fa/fa*) rat has a lower [^{3}H]GDP-binding capacity at room temperature, which, in the adult rat, is associated with a decrease in the concentration of uncoupling protein. In the younger animal, however, the decrease in GDP binding is thought to be attributed to a masking of the uncoupling protein (Ashwell *et al.*, 1985).

We have investigated the existence of this unmasking/remasking process by studying the effect of cold exposure on extents of GDP binding and uncoupling-protein concentration (determined by immunoassay) in BAT mitochondria of Zucker rats. The time course of the unmasking response and of the reverse process (remasking) has been studied. In addition, the acute response to noradrenaline treatment of lean and obese Zucker rats has been investigated, to illustrate that the sympathetic nervous system mediates the unmasking of the uncoupling protein and to compare the degree of unmasking in lean and obese animals.

EXPERIMENTAL

Animals

Lean male Zucker (*Fa/?*) rats were housed in wire-mesh cages at 23 °C with a 12 h-light/12 h-dark lighting schedule. At 5–6 weeks of age, rats were moved in single cages to a 4 °C environment for 2, 3, 4 and 24 h and 5 days. In a second experiment, rats were caged individually and, after exposure to 4 °C for 1 week, were placed at 27 °C for 3 or 24 h and 2, 5 and 8 days. All animals were

Abbreviation used: BAT, brown adipose tissue.

allowed free access to rat laboratory chow (Christopher Hill, Poole, Dorset, U.K.) and water. In both experiments the precise age at which the animals were initially housed at 4 °C was varied such that all rats were 7 weeks old when killed. In a third experiment, obese (*fa/fa*) and lean (*Fa/?*) Zucker rats 7 weeks old were injected, subcutaneously, with either 80 μg of noradrenaline bitartrate/100 g body wt. or saline (0.9% NaCl) vehicle and killed 30 min after injection for study of BAT function.

BAT mitochondria

All rats were killed by cervical dislocation while at the temperature at which they were last housed. BAT mitochondria from the interscapular site were prepared and washed with 2% (w/v) defatted bovine serum albumin to remove endogenous non-esterified fatty acids (Cannon & Lindberg, 1979). The final mitochondrial pellet was resuspended in 0.25 M-sucrose at a final concentration of 1–3 mg/ml.

Determination of specific GDP binding to BAT mitochondria

[^{3}H]GDP binding was measured by incubation of freshly prepared BAT mitochondria with 10 μM-[8-^{3}H]GDP (sp. radioactivity 18 Ci/mmol) at room temperature (Nicholls, 1976). Non-specific binding was assessed by the addition of unlabelled 200 μM-GDP to the incubation medium. [^{3}H]GDP and [U-^{14}C]sucrose (sp. radioactivity 350 mCi/mmol) used to assay inter-mitochondrial space were obtained from Amersham International (Amersham, Bucks., U.K.).

Scatchard analysis was performed on pooled samples of mitochondria from a number of experimental groups, with [^{3}H]GDP concentrations of 0.025–10 μM. All data were analysed on an Apple II computer by using a modified ligand-binding program. All Scatchard plots best-fitted a one-site model (Munson & Rodbard, 1980).

Purification of uncoupling protein

Uncoupling protein was solubilized and purified from BAT mitochondria prepared from cold-adapted (3 weeks at 4 °C) 8–10-week-old lean Zucker rats. The method of Lin & Klingenberg (1982) was used, with the omission of the final sucrose-gradient-density centrifugation. The uncoupling protein was concentrated to approx. 1 mg/ml (Lowry *et al.*, 1951) and stored at −70 °C. The purity of the protein was confirmed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). The purified uncoupling protein was used for the immunoassay after removal of excess Triton X-100 with Bio-Beads SM-2-(Bio-Rad) as described by Holloway (1983).

Antiserum preparation

An antibody to the purified uncoupling protein was raised in New Zealand White rabbits. The specificity of the antisera to the uncoupling protein was confirmed by an immunoblot of BAT mitochondrial proteins by using the biotin–streptavidin system (Amersham International). There was no interaction with the proteins of rat liver mitochondria.

Immunoassay of uncoupling protein

The concentration of the uncoupling protein in mitochondrial preparations was determined by competitive indirect solid-phase radioimmunoassay (Lean *et al.*, 1983). For this, 96-well micro-titre plates (Flow Laboratories, Cambridge, U.K.) were coated with Bio-Bead-treated purified uncoupling protein (1 μg/well) diluted in phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.102% Na_2HPO_4 and 0.02% KH_2PO_4, pH 7.2) and incubated overnight at 4 °C. BAT mitochondrial samples (1 mg/ml) were solubilized in Triton X-100 (0.5%, w/v) and diluted 5-fold with phosphate-buffered saline containing 1% (w/v) bovine serum albumin. Bio-Bead-treated purified uncoupling protein, used as standard, and solubilized mitochondrial samples were incubated with antibody (1:1500 final dilution) for 2 h at room temperature. The standard curve was constructed over the range 0–20 ng. Triplicate portions of standard and sample were incubated in the wells overnight (4 °C). After washing the plates with phosphate-buffered saline containing 1% albumin and 0.05% (w/v) Tween 20, ^{125}I-labelled Protein A (sp. radioactivity 30 mCi/mg; Amersham International), was added to the plates (30000 c.p.m./well) and incubated for approx. 5 h (4 °C). Plates were washed (phosphate-buffered saline/1% albumin/0.05% Tween 20), and the wells were cut out and individually counted for radioactivity on an automatic γ-radiation counter (LKB 1272 Clinigamma).

In the noradrenaline study in lean and obese Zucker rats, a competitive indirect enzyme-linked immunosorbent assay ('ELISA') was performed. The procedure for the radioimmunoassay was used, except that the standard curve was constructed over the range 0–200 ng/well and the ^{125}I-Protein A was replaced by the biotin–streptavidin system (Amersham International). The plate was incubated with biotinylated donkey anti-rabbit immunoglobulin (1:1800 dilution) for 90 min at room temperature. Streptavidin–biotin–peroxidase was used as conjugate (1:1500 dilution, 20 min at room temperature) and *o*-phenylenediamine as substrate for the peroxidase. The plates were analysed by a micro-elisa auto reader (MR580; Dynatech Instruments, Torrance, CA, U.S.A.) at 495 nm.

Mitochondrial swelling

Mitochondrial permeability to protons was determined as the initial rate of swelling of non-respiring mitochondria in iso-osmotic media containing valinomycin (Nicholls & Lindberg, 1973). Mitochondria were prepared by the procedure outlined above but in 250 mM-sucrose/5 mM-Tes/1 mM-EDTA, pH 7.2 at 4 °C. Mitochondria (0.25–0.35 mg/ml) were incubated in 150 mM-potassium acetate/10 mM-Tes/5 μM-rotenone, pH 7.0, at 23 °C. Swelling was initiated by the addition of 0.5 μM-valinomycin, and the decrease in A_{540} was monitored in a Pye–Unicam SP8-400 spectrophotometer. To determine the rate of GDP-inhibitable swelling, mitochondrial swelling was assayed in the presence and absence of 200 μM-GDP.

Protein was assayed by the method of Lowry *et al.* (1951). Data was analysed by either two-way or one-way ANOVA, followed by a Tukey test for individual statistical significances.

RESULTS

Rats exposed to 4 °C for 2 h exhibited an increase in [^{3}H]GDP binding to BAT mitochondrial protein (Table 1). As the period of cold exposure increased, GDP

Table 1. Effect of different times of cold exposure (4 °C) on GDP binding and uncoupling-protein concentration in BAT mitochondria from lean Zucker rats

Values are means ± S.E.M., with the numbers of animals shown in parentheses. ANOVA showed F values of 31.5 (DF5) ($P < 0.01$), 27.6 (DF5) ($P < 0.01$) and 6.1 (DF5) ($P < 0.01$) for GDP binding, uncoupling protein and molar binding ratio respectively. Individual differences from mean values at zero time were then assessed by the Tukey test. $*P < 0.05$, $**P < 0.01$, compared with control rats at 23 °C.

Time at 4 °C (h)	GDP binding (pmol/mg of protein)	Uncoupling protein (μg/mg of protein)	GDP bound (mol/mol of uncoupling protein)
0	343.3 ± 35 (11)	22.2 ± 2.9 (11)	0.55 ± 0.06 (11)
2	507.3 ± 24 (4)*	19.5 ± 0.3 (4)	0.83 ± 0.03 (4)**
3	540.5 ± 17 (4)*	20.7 ± 2.0 (3)	0.88 ± 0.03 (3)**
4	696.5 ± 12 (4)**	27.8 ± 0.6 (4)	0.81 ± 0.02 (4)**
24	666.25 ± 28 (4)**	43.3 ± 3.8 (4)*	0.52 ± 0.04 (4)
120	968.5 ± 19 (4)**	75.0 ± 5.7 (4)**	0.42 ± 0.03 (4)

Table 2. Effect of returning rats to a warm environment (27 °C), after cold-acclimation (4 °C), on GDP binding and concentration of uncoupling protein in BAT mitochondria of lean Zucker rats

Values represent means ± S.E.M. for the numbers of rats shown in parentheses. ANOVA showed F values of 87.4 (DF5) ($P < 0.01$), 19.8 (DF5) ($P < 0.01$) and 35.1 (DF5) ($P < 0.01$) for GDP binding, uncoupling protein and molar binding ratio respectively. Individual differences of means from zero time were statistically tested by the Tukey test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with rats at 4 °C (1 week).

Time at 27 °C (h)	GDP binding (pmol/mg of protein)	Uncoupling protein (μg/mg of protein)	GDP bound (mol/mol of uncoupling protein)
0	726.8 ± 52.7 (4)	78.0 ± 3.5 (4)	0.30 ± 0.02 (4)
3	446.3 ± 23.1 (4)**	89.8 ± 5.3 (4)	0.16 ± 0.01 (4)**
24	396.8 ± 12.8 (8)***	90.5 ± 6.0 (8)	0.14 ± 0.01 (8)***
48	337.0 ± 8.0 (4)***	94.8 ± 7.4 (4)	0.11 ± 0.01 (4)***
120	227.1 ± 6.0 (8)***	49.8 ± 3.5 (8)***	0.15 ± 0.01 (8)***
192	235.3 ± 4.3 (4)***	62.3 ± 3.4 (4)*	0.12 ± 0.01 (4)***

binding progressively increased, to 2-fold after 4 h and 3-fold after 5 days (Table 1). As found by Desautels *et al.* (1978), GDP binding reached a plateau before markedly increasing after 24 h of cold exposure. A parallel increase in the amount of uncoupling protein present in BAT mitochondria was not initially observed, as uncoupling-protein concentrations remained constant during the initial 4 h of cold exposure (Table 1). A significant increase in the amount of uncoupling protein had occurred by 24 h at 4 °C, a concomitant increase in uncoupling protein with extent of GDP binding being observed between 24 h and 5 days (Table 1).

The ratio of GDP bound to the uncoupling protein may be calculated, since the immunoassay and the GDP-binding measurements were performed on the same mitochondrial samples. On initial cold exposure the ratio mol of GDP bound/mol of uncoupling protein increased from 0.55 to over 0.8 (Table 1). This high molar binding ratio was observed for at least 4 h of cold exposure, after which it fell progressively back to control (pre-cold exposure) values as the amount of uncoupling protein increased.

In the second experiment, the effects of rewarming cold-acclimated rats was studied. After 7 days at 4 °C, mitochondrial GDP binding was slightly lower than that observed in rats housed at 4 °C for 5 days in the previous experiment (726 ± 19 and 968 ± 19 pmol/mg respectively). This difference probably reflected the extra mitochondrial proliferation during longer cold-acclimation, since total tissue GDP binding was highest in the 7-day cold-acclimated rats (results not shown). When animals were returned to a warm environment (27 °C) after 1 week at 4 °C, there was a rapid decrease in BAT mitochondrial GDP binding (Table 2). A marked decrease in GDP binding occurred in the first 3 h, and this continued to decrease until a plateau was reached after 5 days at 27 °C, somewhat below the GDP binding observed in the rats housed at 23 °C in the previous experiment. This would be expected, since exposure to warmer environments has been shown to decrease BAT mitochondrial GDP binding (Ashwell *et al.*, 1983). The initial decrease in GDP binding was not paralleled by a decrease in amount of uncoupling protein, which remained constant at approx. 90 μg/mg of mitochondrial protein for at least 2 days after animals were returned to 27 °C. Between 2 and 5 days (27 °C) the uncoupling-protein concentration decreased to approx. 50 μg/mg, but did not fall below this in the time course studied here (Table 2).

The degree of unmasking of the uncoupling protein, as reflected in the molar binding ratio of GDP to uncoupling protein, was rapidly decreased when animals were

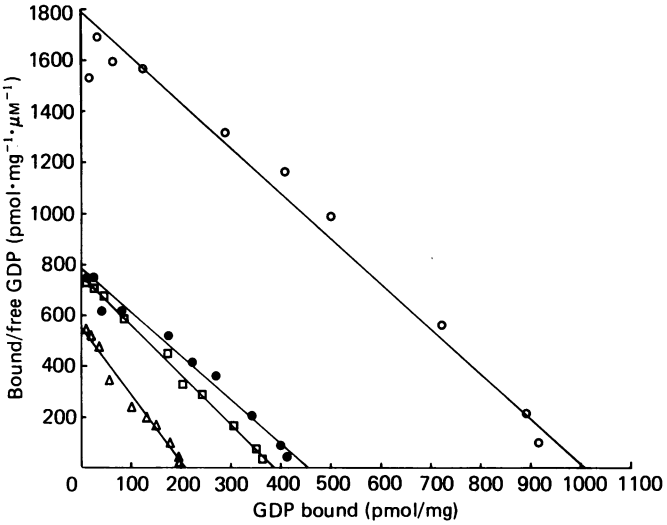

Fig. 1. Scatchard plot of specific [^{3}H]GDP binding to BAT mitochondria of rats housed at 4 °C for 7 days (○) and reacclimated to 27 °C for 3 h (●), 24 h (□) or 5 days (△)

transferred from 4 to 27 °C (Table 2). The molar binding ratio decreased from 0.3 to 0.16 in the initial 3 h period, and remained close to this value for up to 8 days after the rats were returned to 27 °C.

Scatchard analysis of GDP binding was performed on pooled samples of BAT mitochondria from both the cold-exposed and warm-re-exposed rats described above. All plots fitted best to single-site models. A sample plot is shown in Fig. 1, and K_d and $B_{max.}$ for all groups are given in Table 3. These data indicated that both the acute and the chronic increases in GDP binding on cold-acclimation and the acute and chronic decreases in GDP binding on rewarming reflected changes in the number of binding sites ($B_{max.}$) rather than any changes in affinity (K_d). In order to relate changes in GDP binding to the function of the proton conductance pathway, mitochondrial swelling and GDP binding were assayed on BAT mitochondria prepared from rats housed at 22 °C or at 4 °C for either 3 h or 7 days. Mitochondrial swelling, in the presence of potassium acetate and valinomycin, is an indication of the proton permeability of the mitochondrial inner membrane (Nicholls & Lindberg, 1973; Rial & Nicholls, 1984). Fig. 2 shows the close correlation between mitochondrial GDP binding and the GDP-inhibitable rate of swelling in these three groups of rats.

In the third experiment, the acute response of BAT mitochondria of lean and obese Zucker rats to noradrenaline was investigated. The specific [^{3}H]GDP binding to BAT mitochondria was less in the obese (*fa/fa*) Zucker rat than in the normal animal, but both lean and obese rats responded to noradrenaline treatment by increasing GDP binding (Table 4). A more pronounced response was observed in obese (approx. 90%) than in lean rats (approx. 40%). A two-way ANOVA indicated that the concentration of uncoupling protein was significantly lower in the obese than in the lean rats, but noradrenaline treatment did not result in any significant change in these values in either group (Table 4). The molar binding ratio was not significantly decreased in obese rats, but was significantly increased by noradrenaline treatment in both lean and obese animals.

Table 3. Scatchard analysis of BAT mitochondrial GDP binding in cold-acclimated and warm-reacclimated rats

Values represent data from single Scatchard analyses from pooled mitochondrial samples of rats used in experiments described in Tables 1 and 2. All data were fitted to one- and two-site models, but all plots gave best fits to a one-site model.

	$B_{max.}$ (pmol/mg)	K_d (μM)
(*a*) Time of cold exposure (h)		
0	429±32	0.58±0.07
3	560±13	0.55±0.12
24	668±34	0.62±0.09
120	1076±39	0.73±0.05
(*b*) Time of warm-reacclimation (h)		
0	1000±23	0.56±0.03
3	453±12	0.58±0.03
24	386±5	0.51±0.01
120	207±12	0.38±0.03

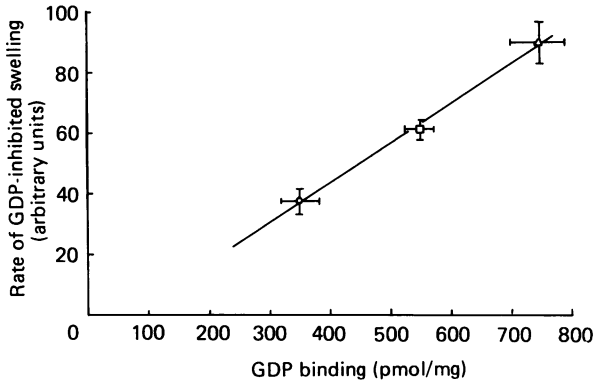

Fig. 2. Relationship of mitochondrial GDP binding to the rate of GDP-inhibitable swelling of mitochondria

BAT mitochondria were prepared either from rats housed at 22 °C (○) or from rats housed at 4 °C for 3 h (□) or 7 days (△). Specific [^{3}H]GDP binding and rate of swelling are those that were inhibited by 200 μM-GDP (unlabelled).

DISCUSSION

Previous studies on the adaptation of BAT mitochondrial thermogenesis in animals subjected to changing environmental temperature have relied predominantly on either ligand binding or conductance measurement to assay the functional capacity of the uncoupling protein, and on SDS/polyacrylamide-gel electrophoresis and densitometric traces to assay the quantity of the M_r-32000 uncoupling protein (Desautels *et al.*, 1978; Hogan & Himms-Hagen, 1980; Gribskov *et al.*, 1986; Rial & Nicholls, 1984; Ricquier & Kader, 1976). From these studies it has been concluded that in the chronically cold-acclimated rat and guinea pig the increase in mitochondrial proton conductance is associated with an increase in the amount of uncoupling protein in the mitochondrial inner membrane as well as a proliferation in the mitochondrial population of BAT. Such conclusions have been supported by the demonstration that

Table 4. Effect of noradrenaline on GDP binding and concentration of uncoupling protein (measured by enzyme-linked immunosorbent assay) in lean and obese Zucker rats

Values represent means ± S.E.M. for the numbers of rats shown in parentheses. Rats were injected with saline (0.9%, w/v) or noradrenaline (500 μg/kg) intraperitoneally and killed 30 min later. $*P < 0.05$, $**P < 0.01$ compared with lean controls; $††P < 0.01$, $†††P < 0.001$ compared with obese controls; N.S., not significant.

	Treatment	GDP binding (pmol/mg of protein)	Uncoupling protein (μg/mg of protein)	GDP bound (mol/mol of uncoupling protein)
Lean	Saline	392 ± 20.1 (4)	42 ± 1.15 (4)	0.30 ± 0.01 (4)
Obese	Saline	215.5 ± 17.8 (4)**	37.3 ± 3.4 (4)	0.19 ± 0.03 (4)*
Lean	Noradrenaline	543.5 ± 36.7 (4)*	44.0 ± 1.0 (3)	0.38 ± 0.03 (3)*
Obese	Noradrenaline	403.4 ± 22.1 (4)†††	30.8 ± 3.1 (4)*	0.45 ± 0.06 (4)*††
ANOVA	Genotype	$F(1,12)$ 38.1 $P < 0.01$	$F(1,11)$ 14.1 $P < 0.01$	$F(1,11)$ 0.61 N.S.
	Noradrenaline	$F(1,12)$ 50.6 $P < 0.01$	$F(1,11)$ 0.8 N.S.	$F(1,11)$ 23.7 $P < 0.01$
	Interaction	$F(1,12)$ 1.1 N.S.	$F(1,11)$ 3.1 N.S.	$F(1.11)$ 4.84 $P < 0.05$

protein-synthesis inhibitors prevent the chronic adaptation (Desautels & Himms-Hagen, 1979), by the demonstration that the synthesis of mRNA encoding for the uncoupling protein is increased within 15 min of stimulation of the brown adipocytes (Ricquier *et al.*, 1986), by the observations that chronic noradrenaline infusions mimic the effects of cold-acclimation on BAT function (Mory *et al.*, 1984) and by the confirmations, using specific immunoassays, that the uncoupling-protein concentration of interscapular BAT mitochondria of mice may be raised by 8-fold, compared with animals housed at thermoneutrality, after housing at −2 °C for several weeks (Ashwell *et al.*, 1983). The results in the present paper concur with these conclusions. Both mitochondrial GDP binding and uncoupling-protein concentration rose in parallel, approx. 2-fold after 1 day and 3-fold after 5 days, when rats were housed at 4 °C, compared with the normal housing temperature of 22 °C, supporting the hypothesis (Rial & Nichols, 1984) that the increase in mitochondrial thermogenic function is dependent on the incorporation of extra uncoupling protein into existing or newly synthesized mitochondria.

However, this close association between GDP binding and uncoupling-protein concentration was not always evident. In our studies changes in GDP binding were not associated with parallel changes in uncoupling protein concentration in a number of situations, i.e. during the acute phase of the responses to cold exposure (0–4 h) and exogenous noradrenaline (0–30 min) and after restoration of cold-acclimated rats to a 22 °C environment. This disassociation between GDP binding and uncoupling-protein concentration, as reflected in the changing molar binding ratios, would support the suggestion that the nucleotide-binding site of the uncoupling protein may exist in a masked or unmasked form. The close relationship demonstrated between mitochondrial swelling and GDP binding in these studies suggests that GDP binding is a good index of mitochondrial proton conductance in the rat, as it is in the guinea pig (Rial & Nicholls, 1984). These results thus support the suggestion that the uncoupling protein may exist in the mitochondria in either a non-functional (masked) or functional (unmasked) form. The existence of an unmasking–remasking process was first suggested from studies of the acute response to cold, when no change in the M_r-32000-band protein could be detected on SDS/polyacrylamide gels during the initial 24 h of cold exposure (Desautels *et al.*, 1978). Subsequent investigations using specific immunoassays for the uncoupling protein on the acute responses to refeeding after starvation for 48 h have also demonstrated this acute unmasking of GDP-binding sites (Trayhurn & Jennings, 1986), as have our previous studies on young obese rodents (Ashwell *et al.*, 1985). The unmasking–remasking process appears to be rapid and reversible in response to changes in environmental temperature, and may represent the acute physiological adaptation of thermogenic activity necessary to maintain body temperature during the longer period necessary for synthesis and incorporation of new uncoupling protein into mitochondria. Uncoupling-protein concentration appears to be increasing within 4 h of cold exposure, and was significantly enhanced by 24 h. The increasing concentration of uncoupling protein between 1 and 5 days was associated with a decrease in the GDP molar binding ratio back to and finally slightly below the original value observed before cold exposure. As GDP binding appears to reflect the functional activity of the uncoupling protein, these data suggest that the physiological response to cold provides thermogenic capacity in excess of requirements. Such a response would allow the animal to survive any additional cold stress by again unmasking uncoupling protein. This sequence of unmasking, increased uncoupling protein concentration and remasking may explain why *ob/ob* mice can withstand severe cold if they are initially exposed to moderate cold, but not if they are placed directly there from normal laboratory temperatures (22–24 °C) (Hogan & Himms-Hagen, 1980). Further, the rapid restoration of normal GDP molar binding ratios 24 h after initial cold exposure would explain why longer-term studies on the responses to cold and diet have been unable to demonstrate this apparent unmasking event (Ashwell *et al.*, 1983; Trayhurn *et al.*, 1983; Nedergaard *et al.*, 1984; Desautels, 1985). After returning cold-acclimated rats to a thermoneutral environment (27 °C),

there was a rapid decrease in mitochondrial GDP binding, but no decrease in mitochondrial uncoupling-protein concentration was observed in the initial 48 h period. The initial response, a marked decrease in GDP molar binding ratio, was maintained throughout the 8-day period of the experiment, since even by this time uncoupling-protein concentration had not fallen to the values observed in rats housed at 22–24 °C. This again suggests that, in the presence of high concentrations of uncoupling protein in the mitochondria, thermogenic activity was decreased by masking uncoupling protein. Scatchard analysis indicated that the acute increase and decrease in GDP binding on cold-exposure and re-warming respectively involved changes in the number of available binding sites rather than any changes in affinity of sites for GDP.

Noradrenaline, released from sympathetic nerve endings, is thought to be the major physiological regulator of BAT function. Sympathetic activation of the tissue is enhanced in situations where BAT thermogenesis is stimulated, and vice versa (Young *et al.*, 1982; Rothwell & Stock, 1983; York *et al.*, 1985). The demonstration that the acute response to exogenous noradrenaline was associated with an increase in GDP binding, without any change in uncoupling-protein concentrations in both lean and obese Zucker rats, supports the suggestion that unmasking of uncoupling protein is an acute physiological response to sympathetic stimulation of the tissue. The decrease in GDP binding in the obese rats may be related to the lack of BAT sympathetic stimulation that has been demonstrated in these animals (York *et al.*, 1985). At 7 weeks of age this decrease in GDP binding reflected a fall in uncoupling-protein concentration in the mitochondria, although in young animals a masking of binding sites has been observed (Ashwell *et al.*, 1985).

The molar binding ratios are indicative of the degree of unmasking of GDP-binding sites on the uncoupling protein. Lin and Klingenberg have postulated, from hydrodynamic studies (Lin *et al.*, 1980) and from GDP binding to the isolated uncoupling protein (Lin & Klingenberg, 1980), that the functional form of uncoupling protein is a dimer which possesses half-site reactivity. This would mean that a ratio of 0.5 mol of GDP/mol of monomer would represent the maximum binding capacity of the uncoupling protein. We do obtain ratios below 0.5, but, on acute cold exposure, ratios in excess of 0.8 were reached. This suggests that half-site reactivity is not the case, and all binding sites are capable of binding GDP when unmasked. Trayhurn *et al.* (1983) observed molar binding ratios in excess of 0.5 when hamsters were chronically cold-adapted, but in mice the ratios were always below 0.5 (Ashwell *et al.*, 1983). The results of French *et al.* (1985), who demonstrated both high- and low-affinity binding sites on the isolated uncoupling protein, also suggest that the number of binding sites per dimer may be greater than one.

Our results provide no insight into the mechanism of the unmasking process, but suggestions include a conformational change in the protein within the membrane, dimerization of pre-existing monomers, translocation and insertion of uncoupling protein into the inner membrane from a store within the matrix of the mitochondria or covalent modification. Nedergaard & Cannon (1987) have suggested that unmasking of GDP-binding sites could result from mitochondrial swelling, as indicated by changes in the volume of the mitochondrial matrix. However, Swick & Swick (1986) were unable to demonstrate any changes in GDP binding associated with mitochondrial swelling.

To summarize, it appears that the unmasking process of the uncoupling protein operates in response to cold exposure and that this is mediated via noradrenaline. Once the 'spare' GDP-binding sites have become functional, further stimulation results in an increase in the amount of uncoupling protein within the mitochondrial inner membrane.

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