The regulation of the proton conductance of brown fat mitochondria

Identification of functional and non-functional nucleotide-binding sites

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The binding of purine nucleotides to intact brown fat mitochondria is re-examined. In addition to the previously reported high affinity binding site, a low-affinity site is found, which requires several minutes to saturate. Only the high affinity site is functional in regulating the proton and halide permeabilities of the mitochondria. The low affinity site can introduce errors in the use of nucleotide binding to quantitate the M_r 32000 uncoupling protein unique to these mitochondria.

Brown fat *Mitochondria* *Purine nucleotide* *Proton conductance* *Swelling* *Binding*

1. INTRODUCTION

A M_r 32000 uncoupling protein [1] exists in the inner membrane of mitochondria from thermogenically active brown fat (for review see [2,3]). The existence of a specific protein was first indicated by the correlation between the binding of purine nucleotides to the mitochondria and the inhibition of proton conductance [4], while the use of a photoaffinity nucleotide analogue enabled the site to be identified as a polypeptide of M_r 32000 [1]. Since a transport protein loses its function when removed from the membrane, the subsequent purification of the M_r 32000 protein [5,6] has relied on the specific activity of purine nucleotide-binding to the protein fractions. Although immunoassays have been developed [7–9], the binding of a purine nucleotide such as GDP has been the most widely employed method for the quantitation of the protein [10–31].

The accuracy of these approaches would be compromised by the presence of additional binding sites on intact mitochondria accessible to the added nucleotide. Two findings suggest that such sites exist; firstly in our original report [4] we observed an initial rapid association of nucleotide, followed by a slow increase which continued for at least 5 min, even though the inhibition of conductance was maximal within 30 s (e.g., fig.5 in [32]). Secondly, experiments with the photoaffinity analogue 8-azido-ATP [1] indicated, in addition to the M_r 32000 protein and the adenine nucleotide translocator, additional binding to high-M_r components in the membrane.

Since current hypotheses for the role of brown adipose tissue in dietary and thermal regulation are largely dependent upon an accurate assay of this protein (for review see [3]) we have re-examined the nature of the nucleotide-binding to the mitochondria. We conclude that two major binding sites are present, but that only the higher affinity, rapidly equilibrated, site is involved in conductance regulation. While this finding does not invalidate Scatchard analyses of nucleotide-binding, it throws doubt upon the validity of using

Abbreviation: Tes, 2[2-hydroxy-1,1-bis[hydroxymethyl]-ethyl)amine]-ethane sulphonate

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a single nucleotide concentration to quantitate the M_r 32000 protein in intact mitochondria.

2. METHODS

Mitochondria were prepared from the pooled thoracic, interscapular and dorsal brown adipose tissue of adult Syrian hamsters which had been cold-adapted at 4°C for at least 10 days prior to sacrifice. The method was as in [4], except that 80 μM albumin was present in all the preparation media. The mitochondria were finally resuspended in 250 mM sucrose, 5 mM Tes (Na-salt), 80 μM albumin, pH 7.0. Protein was determined by the biuret method with albumin as standard.

Nucleotide binding was determined by a modification of the technique in [4], in media identical (except for the absence of ionophore) to those subsequently used for swelling studies. Thus, mitochondria (0.3 mg protein/ml incubation) were incubated at pH 7.0 and 30°C in media containing either 100 mM acetate (K salt) or 100 mM KCl, together with 5 mM Tes (Na salt), 2 μM rotenone and 0.1 μCi [^{14}C]sucrose per ml incubation. For the GDP-binding experiments varying concentrations of [^{3}H]GDP (0.3 μCi/ml incubation) were added. For ATP-binding the incubation medium contained varying concentrations of [^{3}H]ATP (0.3 μCi/ml incubation), with the additions of 2 mM $MgCl_2$ and 30 μM carboxyatractylate. After incubation for the time specified 250 μl aliquots were centrifuged for 60 s in an Eppendorf microcentrifuge. Supernatants were removed by aspiration and the pellets, after dissolution in 20 μl of 5% (w/v) sodium dodecylsulphate were transferred into vials for counting.

Proton and chloride permeabilities were estimated from the rate of light-scattering decrease in media in which Cl^- or H^+ permeation was rate limiting, essentially as in [33]. For the proton permeability, mitochondria were incubated in the acetate medium described above (in the absence of isotopes), while the chloride medium was used to estimate chloride permeability. Conditions are defined in the legends. Light-scattering was determined in an Eppendorf Fluorimeter with a primary wavelength of 546 nm, as in [33]. Swelling was initiated by the addition of 0.5 μM valinomycin or 0.5 μM nigericin 2.5 min after addition of mitochondria.

All radioisotopes were obtained from the Radiochemical Centre (Amersham). Carboxyatractylate was from Boehringer (Mannheim). Other reagents were from Sigma (Poole).

3. RESULTS AND DISCUSSION

We have previously reported a single class of GDP-binding sites on the inner membrane of intact hamster brown adipose tissue mitochondria [4]. In order to correlate with the time-course of conductance inhibition, this analysis was performed after a preincubation of only 30 s. However, as the dissociation constants obtained from a Scatchard analysis are by definition equilibrium parameters, we have re-examined the binding of GDP to the mitochondria using a 5 min preincubation to approach equilibrium more closely (fig.1). Under these conditions a non-linear plot is obtained. Computer analysis using the Rothamsted Maximum Likelihood Programme [34] reveals an acceptable fit with a two-site model with a high affinity site with a capacity of 0.74 nmol GDP/mg protein and a K_a of 1.9 μM, and a

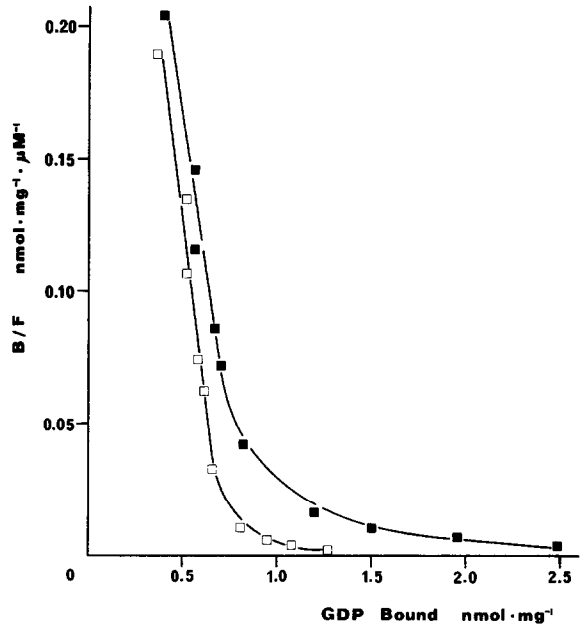

Fig.1. Scatchard plot of the binding of [^{3}H]GDP to mitochondria suspended in chloride medium (see section 2). Effect of incubation time. (□) 30 s incubation; (■) 5 min incubation. B/F, bound/free.

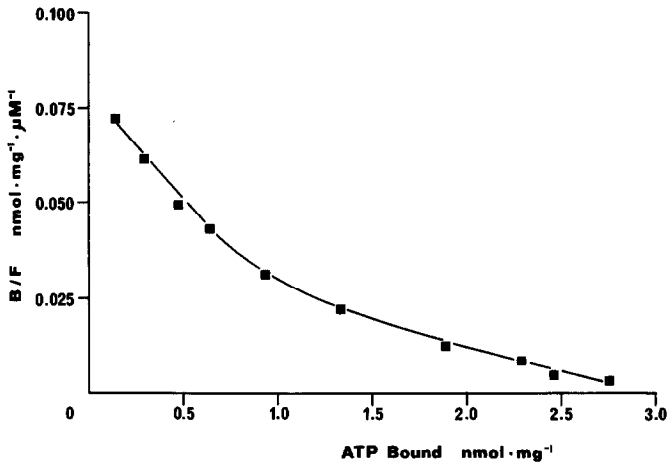

Fig.2. Scatchard plot of the binding of [^{3}H]ATP to mitochondria suspended in acetate medium in the presence of 2 mM $MgCl_2$ and 30 μM carboxyatractylate; 5 min incubation.

low affinity site with a capacity of 3.5 nmol/mg and a K_a of about 0.5 mM.

There may be two reasons why the low affinity site has not been detected previously. Firstly, centrifugation, rather than filtration, results in a decreased contamination with incubation medium, and thus allows for a greater accuracy at high nucleotide concentrations. Secondly the low affinity site may have a slow 'on' rate constant. This is confirmed in the Scatchard analysis after only 30 s incubation (fig.1). Equilibration of the high affinity site is almost complete (the apparent capacity being 0.67 nmol GDP/mg protein), whereas the low affinity site is much less prominent.

In the intact cell the M_r 32 000 protein will be exposed to a cytosol containing Mg–ATP as the predominant purine nucleotide. The affinity of the M_r 32 000 protein for ATP appears to be lower than for GDP as judged both from its ability to displace the latter nucleotide [1,4] and from the higher concentrations required to inhibit proton conductance [35]. In the presence of carboxyatractylate, to prevent the adenine nucleotide carrier from binding or translocating ATP, two binding sites are seen (fig.2). However, in comparison with GDP, the K_d for the first site is increased to 10 μM, while the capacity, 0.6 nmol/mg, is not significantly different. Part of this decrease in affinity is due to the presence of Mg, since a K_d of 4.8 μM was obtained for the high affinity site for GDP in the presence of Mg (not shown).

In contrast, the parameters for the low affinity site for ATP are not significantly different from those obtained with GDP, indicating that the low-affinity site does not discriminate between the two nucleotides.

Having established the presence of two binding sites on the intact mitochondria, it is necessary to examine whether both are functional in regulating ion permeation. The M_r 32 000 protein conducts both protons and Cl^- [33,36] and the permeability to these ions may conveniently be monitored by following the rate of light scattering decrease when non-respiring mitochondria are suspended in media where the permeation of the relevant ion is rate-limiting.

Fig.3 shows the inhibition of proton and Cl^- permeation as a function of increasing nucleotide concentration. The results confirm that GDP is more effective in inhibiting proton conductance than is Mg–ATP. The apparent K_i-values for the inhibition of swelling in acetate medium for the two nucleotides are, respectively, 1.75 μM and 7.5 μM, extremely close to the K_d-values calculated for binding to the high affinity site. It should be emphasized that this is the first study in which binding and permeation have been determined under essentially identical conditions. The apparent K_i for the inhibition by Mg–ATP of swelling in KCl is somewhat higher, at 30 μM.

In fig.4, the inhibition of proton or chloride permeability is replotted from fig.3 as a function of the saturation of the high affinity nucleotide-binding site. Since the permeabilities decrease pro-

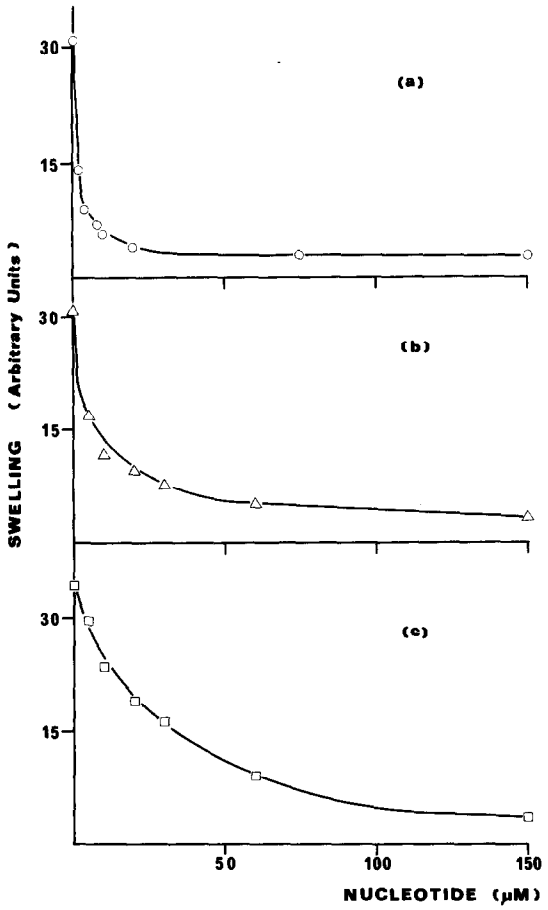

Fig.3. Swelling of mitochondria in acetate medium (a, b) or chloride medium (c) as a function of GDP (a) or ATP (b,c) concentration. The ATP incubations also contained 30 μM carboxyatractylate and 2 mM $MgCl_2$. Swelling was initiated by the addition of 0.5 μM valinomycin.

portionately to the occupancy of the high affinity site by either nucleotide, this indicates that the low affinity site does not participate in the regulation of ion permeability.

4. CONCLUSIONS

The results indicate that the use of purine nucleotide-binding to quantitate the M_r 32 000 protein in the inner membrane of brown fat mitochondria is subject to unnecessary error, unless Scatchard analysis is performed to take account of additional nucleotide-binding sites which do not affect proton permeability. As an example, under the

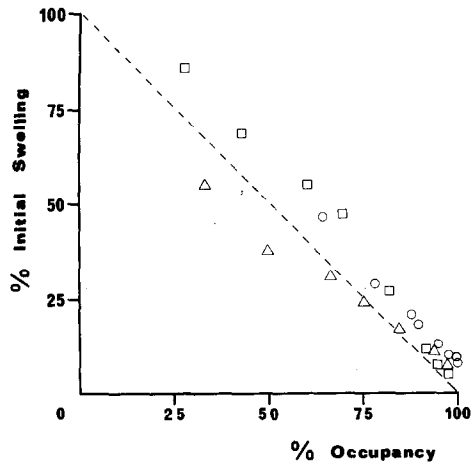

Fig.4. Correlation between occupancy of the high affinity site by nucleotide and rate of swelling in acetate medium plus GDP (○), acetate medium plus ATP (△), or in chloride medium plus ATP (□); data from fig.1–3. and 3.

present conditions only 56% of the nucleotide bound to mitochondria equilibrated with 100 μM GDP in the absence of Mg is associated with the M_r 32 000 protein.

The nature of the non-functional nucleotide-binding site is unclear. It is unlikely to represent nucleotide slowly translocated into the matrix, since we have shown [4] that exchange across the inner membrane is far too slow to account for the time-dependent increase in total binding. Similarly, the low-affinity site is unlikely to be located on the second subunit of the M_r 64 000 functional dimer of the protein [37] since there is not a 1:1 relationship in the capacity of the sites, and since the purified protein gives no indication of a second binding site [5]. Removal of the source of error now opens the way towards a more precise investigation of the kinetics of the M_r 32 000 protein. For example (fig.4) the proportionality between the inhibition of permeability and the occupancy of the nucleotide-binding site on the M_r 32 000 protein by either GDP or ATP only becomes apparent when non-functional binding is allowed for.

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