A study of GDP binding to purified thermogenin protein from brown adipose tissue

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1. The binding of GDP to purified thermogenin protein was studied by using fluorescence spectroscopy and equilibrium dialysis. 2. GDP binding to thermogenin diminished fluorescence emission in a concentration-dependent manner that exhibited saturation. 3. K_d values for binding of nucleoside di- and tri-phosphates were lower than those for nucleoside monophosphates. 4. The GDP-induced fluorescence quenching was decreased by increasing pH, but the apparent K_d was unaltered by pH changes. 5. Equilibrium dialysis showed a K_d change from 3 to 6 μM when the pH was increased from 6.6 to 8.5. 6. The apparent pK of the fluorescence changes induced by pH (8.3) was identical with the apparent pK of the GDP-binding response. 7. The data are consistent with the existence of protonated and non-protonated forms of thermogenin protein that both bind GDP.

INTRODUCTION

A unique 32000- M_r protein is present in the inner membrane of brown-adipose-tissue mitochondria (Heaton *et al.*, 1978). This protein, which has been termed thermogenin (Cannon *et al.*, 1982), is thought to be responsible for the proton-conductance pathway that allows the uncoupling of respiration from ATP synthesis during brown-adipose-tissue thermogenesis (for a review see Nicholls & Locke, 1984). Isolated brown-adipose-tissue mitochondria are largely uncoupled, but respiratory control may be restored by the addition of purine nucleotides, which bind to a site on thermogenin and inhibit proton translocation. The binding of [^3H]GDP to isolated brown-adipose-tissue mitochondria has been used frequently to assess the number of proton-conductance channels and thus the thermogenic state of the animal (Himms-Hagen & Desautels, 1978; Thurlby & Trayhurn, 1979; Brooks *et al.*, 1980; Holt & York, 1983).

Thermogenin has been isolated and purified from brown-adipose-tissue mitochondria by Lin & Klingenberg (1982), and [^3H]GDP binding to the isolated protein has been demonstrated both by equilibrium dialysis (Lin & Klingenberg, 1982) and in this laboratory by using both equilibrium dialysis and a flow-dialysis technique (French *et al.*, 1985). These studies have yielded valuable information concerning the interaction between GDP and thermogenin. However, both procedures are time-consuming, as they require prolonged exposure of the protein to incubation media. We have investigated the possibility that fluorimetric techniques could provide a rapid method for the investigation of the interaction of thermogenin with nucleotides. Like other proteins, thermogenin contains aromatic amino acid residues, including two tryptophan residues (Lin & Klingenberg, 1982), which fluoresce. In the present paper we describe experiments showing that GDP binding to thermogenin is associated with a decrease in the intensity of this fluorescence emission.

MATERIALS AND METHODS

Solubilization and purification of thermogenin from brown-adipose-tissue mitochondria

Thermogenin was solubilized and purified from brown-adipose-tissue mitochondria prepared from cold-adapted (3 weeks at 4 °C) 10-week-old lean (*Fa/?*) Zucker rats. The method of Lin & Klingenberg (1982) was followed, with the omission of the final sucrose-density-gradient centrifugation. The purified protein was concentrated to 1–2 mg/ml before storage in liquid N_2 . The purity of the protein was confirmed by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970), with the use of 12.5–16% gradient gels that were subsequently stained for protein with Coomassie Blue. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

Fluorimetric titrations

Fluorimetric titrations were performed on a Perkin-Elmer 650S fluorescence detector. An excitation wavelength of 290 ± 2 nm was used to excite protein fluorescence, with was observed at 360 ± 2 nm. The initial fluorescence intensity was set to 100 in each experiment. Nucleotide titration experiments were performed at 15 °C in a quartz cuvette, with 100 μg of thermogenin protein in 20 mM- Na_2SO_4 /1 mM-EDTA/20 mM-Mops/NaOH buffer, pH 6.7 (final volume 1 ml), unless stated otherwise. Additions of nucleotides were made from a 0.5 mM stock solution with a Hamilton syringe. Portions of nucleotide were added to the cuvette to give concentration increments of 1–10 μM as required. Thus a series of measurements were obtained with a single 100 μg amount of purified protein.

For experiments performed at a higher concentration of thermogenin (700 $\mu\text{g}/\text{ml}$) it was necessary to lower the concentration of Triton X-100 in the sample. This was done by passing the sample through a column of Bio-

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Fig. 1. Typical fluorescence changes obtained on addition of GDP to thermogenin

Curve A, quenching of protein fluorescence on addition of GDP to a solution of thermogenin (100 $\mu\text{g/ml}$). A value for 'non-specific' fluorescence changes was determined from the post-saturation linear portion of the curve. Curve B, fluorescence changes due to the specific interaction of GDP with thermogenin. The value for 'non-specific' fluorescence changes was subtracted from curve A to obtain this saturation curve for specific interactions. The inset shows Klotz-plot analysis of these data (α , fractional saturation; x_e , molarity of binding sites; n , molarity of ligand). Curve C, quenching of protein fluorescence on addition of GDP to a sample of thermogenin (100 $\mu\text{g/ml}$) previously denatured by heating at 50 $^{\circ}\text{C}$ for 5 min.

Beads SM2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as described by Horigome & Sugano (1983).

$[^3\text{H}]\text{GDP}$ binding to isolated thermogenin

$[^3\text{H}]\text{GDP}$ binding to isolated thermogenin was determined by using a standard equilibrium-dialysis procedure. A 100 μg portion of purified protein in 20 mM- Na_2SO_4 /1 mM-EDTA/250 mM-Mops/NaOH buffer, pH 6.7 or 8.5, containing 0.5–10 μM - $[^3\text{H}]\text{GDP}$ was dialysed against 1.5 ml of the same solution (with protein omitted) for 6 h at 4 $^{\circ}\text{C}$ with gentle shaking. Samples of the internal and external solutions were then counted for radioactivity.

RESULTS AND DISCUSSION

Electrophoresis on an SDS/12.5–16%-polyacrylamide gel showed that the protein preparations used in these studies were purified to homogeneity. The typical fluorescence changes obtained on addition of GDP to a solution of the thermogenin are illustrated in Fig. 1 (curve A). Thermogenin emits fluorescence over the range 300–370 nm (maximum 350 nm), which is typical for proteins containing tryptophan residues. The addition of GDP resulted in a quenching of this protein fluorescence indicative of an interaction between the nucleotide and the protein. The hyperbolic shape of the quenching curve appeared to describe a simple saturation process. After saturation of the putative GDP-binding sites the rate of change of fluorescence with increasing GDP concentration was minimal and reflected either non-specific interactions or an inner-filter effect. This interpretation is corroborated by the results of a number of control experiments. Firstly, the addition of equivalent portions of buffer alone caused no or little fluorescence

Fig. 2. Scatchard plots of $[^3\text{H}]\text{GDP}$ binding to isolated thermogenin

$[^3\text{H}]\text{GDP}$ binding to isolated thermogenin was determined by equilibrium dialysis at pH 6.8 ($\circ$) and pH 8.4 ($\bullet$) as described in the Materials and methods section.

change (results not shown). Secondly, when the protein was denatured by heating at 40 $^{\circ}\text{C}$ (curve C in Fig. 1) the slope of the fluorescence change obtained on GDP addition was very similar to the slope of the post-saturation linear portion of the GDP-binding curve to the intact protein (curve A in Fig. 1). Thirdly, addition of GDP to a solution of tryptophan caused no specific changes in fluorescence, showing no direct effect of GDP on tryptophan fluorescence (results not shown).

To obtain a saturation curve representing the fluorescence change due to the specific interactions of added nucleotide with the protein (curve B in Fig. 1) a value for 'non-specific' changes in fluorescence, as estimated from the post-saturation linear portion of the curve (15–25 μM region), was subtracted. The inset to Fig. 1 shows a plot (Stinson & Holbrook, 1973) of the saturation curve for GDP binding. Holbrook (1972) has shown that the data derived from experiments using protein fluorescence to monitor ligand binding can only be analysed by this technique if there is no more than one ligand-binding site per protein molecule (or oligomer). The slope of the line gives a value for the dissociation constant (K_d) for the GDP-thermogenin interaction of 2 μM , and its linearity suggests that the fluorescence change is mediated by the saturation of one type of binding site. The average value from ten determinations was $3.1 \pm 0.4 \mu\text{M}$. This K_d is similar to the values previously observed for binding to intact mitochondrial preparations (Nicholls, 1976; Holt & York, 1983) and to purified thermogenin (Lin & Klingenberg, 1982). The intercept of the plot suggests that the fluorescence change is mediated by the binding of one molecule of GDP per molecule of protein. To confirm this, fluorescence experiments were performed at higher concentrations of thermogenin (18 μM) (where the molarity of the binding sites is approx. 6 times the value of the K_d), and these showed that the decrease in fluorescence intensity no longer occurs after 20 μM of GDP had been bound (results not shown), again suggesting that there is probably one binding site per molecule of protomer. This is supported by the results of our equilibrium-dialysis experiments (Fig. 2). The Scatchard plots of GDP binding indicate a maximum binding value of 0.85 mol of GDP/mol of protein. Thus

Fig. 3. Saturation curves of fluorescence changes obtained on interaction of various nucleotides with thermogenin

The quenching of protein fluorescence on addition of various nucleotides to a solution of thermogenin (100 $\mu\text{g}/\text{ml}$) was determined and saturation curves were obtained as described in Fig. 1 legend. Curve A, GDP (●); curve B, GTP (○); curve C, IMP (□); curve D, GMP (■) was added to a concentration of 15 μM (marked by arrow), then subsequent additions were of GDP. The fluorescence changes are expressed as percentages of the maximum fluorescence change obtained with GDP.

Table 1. K_d values for the interaction of various nucleotides with thermogenin

K_d values for the interaction of various nucleotides with thermogenin were determined from saturation plots (see Fig. 2). The K_d values are given as means $\pm$ s.e.m. with the numbers of determinations in parentheses. Abbreviation: p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate.

Nucleotide	K_d (μM)
GDP	3.1 ± 0.4 (10)
GTP	4.0 ± 0.4 (4)
p[NH]ppG	3.8 ± 0.5 (3)
ADP	4.5 ± 0.4 (3)
IMP	> 20 (3)
GMP	> 20 (2)

there is close agreement on the number of binding sites between the fluorescence data and the equilibrium dialysis. Our data do not support a previous report utilizing equilibrium dialysis which showed the binding of only a 0.5 mol equivalent of GDP/mol of protein. Indeed, such values are exceeded in whole mitochondria when parallel measurements of nucleotide binding and thermogenin content have been measured (Ashwell *et al.*, 1985; Trayhurn *et al.*, 1987).

Fig. 3 shows examples of saturation plots obtained with various nucleotides, and the values of K_d for a wider selection of nucleotides are shown in Table 1. The fluorescence changes in the presence of GMP and IMP were very small compared with those observed on addition of the guanine and adenine nucleoside di- and tri-phosphates, and indicated that the affinity of thermo-

Fig. 4. Effects of pH on the fluorescence changes associated with GDP binding to thermogenin

(a) Saturation curves of the fluorescence changes obtained on the interaction of GDP with thermogenin were obtained at pH 6.7 (●), pH 7.4 (○), pH 8.4 (■) and pH 9.6 (□). The fluorescence changes are expressed as percentages of the maximum fluorescence change obtained at pH 6.7. (b) ●, The pH of a solution of thermogenin (10 $\mu\text{g}/\text{ml}$) was increased incrementally from 6.7 to 9.6 by the addition of portions of NaOH and the fluorescence intensity was recorded at each pH; ○, the magnitudes of the maximum fluorescence change obtained on GDP addition to thermogenin determined at various pH values (as in a) were plotted against pH.

genin for GMP and IMP was very low. These results concur with binding affinities measured previously by Lin & Klingenberg (1982) using equilibrium-dialysis techniques. Curve D in Fig. 3 illustrates the results of a control experiment that clearly demonstrates that GDP has a specific effect on the protein. In this experiment portions of GMP were added until a concentration of 15 μM was reached. This had little effect on the protein fluorescence. Subsequently the nucleotide added was changed to GDP and the titration continued. The shape of the curve describing changes in protein fluorescence immediately changed and followed closely that of curve A in Fig. 3. These observations strongly imply that the diminished fluorescence change exhibited in the presence of GMP reflects the low affinity of the protein for GMP and is not due to this nucleotide successfully binding to the protein but failing to cause spectral perturbation.

The effects of pH on the fluorescence changes

Table 2. pH-dependence of K_d for GDP binding

K_d values for the interaction of GDP with thermogenin were determined either by equilibrium dialysis (see Fig. 2) or from fluorescence changes (see Fig. 4a). The K_d values are means of duplicate determinations except where shown by the number of determinations in parentheses.

pH	K_d (μ M)	
	Equilibrium dialysis	Fluorescence changes
6.7		4.0
6.8	3.3	
7.4		3.1 ± 0.4 (10)
8.4	7.1	5.0
9.6		4.0

associated with GDP binding to thermogenin are described in Fig. 4 and tabulated in Table 2. The data show that a change in pH had two effects on the saturation curve. Firstly, the final amplitude of the observed fluorescence change decreased with increasing pH (Fig. 4a); however, the shapes of the saturation curves and hence the K_d values were little changed and similar to those obtained by the equilibrium-dialysis method (Table 2). These data suggest that pH had no detectable effect on the interaction that leads to changes in protein fluorescence. Secondly, the original fluorescence of the protein decreased with increasing pH (Fig. 4b). This led us to examine the possibility that the fluorescence changes induced either by formation of a GDP-thermogenin complex or by pH change were due to a common process involving the loss of a proton(s) from an amino acid residue; this directly or indirectly results in perturbation of the fluorescence characteristics of the protein. If the protein fluorescence was diminished by loss of a proton when the pH increased from 6.5 to 9.5 then the formation of a GDP-thermogenin complex may still be possible, although this would not be detected fluorimetrically. Our observations can therefore be explained by the model described in Scheme 1, where an equilibrium exists between high-fluorescence protein (T^{HF} , pH 6.5) and low-fluorescence protein (T^{LF} , pH 9.5, or in the GDP binary complex). At pH 6.5 the equilibrium favours the high-fluorescence T^{HF} , and at pH 9.5 the low-fluorescence T^{LF} predominates. As the pH of the solution is increased the concentration of T^{HF} would decrease, with a consequent attenuation of the maximal fluorescence change that could be induced by GDP binding, as indeed we observed (Fig. 4). Thus at pH 8.3 the species T^{LF} is 50% of the total thermogenin (Fig. 4) and therefore the maximum change in fluorescence on addition of GDP would also be 50% of that given at pH 6.5.

The above hypothesis is supported by the observation that the pK of the pH-induced spectral change was indeed identical with the pK of the GDP-induced fluorescence decrease at pH 8.3 (Fig. 4b), suggesting the possibility that a lysine residue is involved in these conformational changes. It has previously been suggested that a lysine residue may participate in the binding of GDP to thermogenin (Lin & Klingenberg, 1982). The model described above would require that the two species T^{LF} and T^{HF} have a similar affinity for GDP.

The interaction measured by this fluorescence technique for the formation of the binary complex would always reflect the binding between GDP and the protonated form of thermogenin at any pH. Further support for the proposed model is provided by the observation (results not given) that increasing pH had no effect on the fluorescence of thermogenin previously saturated with GDP at pH 6.7. The possible relevance of these interconversions to the proton-translocating function of thermogenin awaits further investigation.

The apparent pH-independence of the K_d for GDP binding as determined by the fluorescence technique is in marked contrast with the observations made by Lin & Klingenberg (1982), who reported a 10-fold change in K_d per unit of pH change in their experiments using equilibrium dialysis. Our data from equilibrium dialysis (see Fig. 2) showed only a 2-fold change in K_d between pH 6.8 and 8.4. This change was not, however, detected by the fluorescence technique, possibly because of the small change in fluorescence at the higher pH.

However, GDP-binding experiments using fluorescence spectroscopy can be performed rapidly after thawing the stored protein, minimizing the denaturation during the experiment, which takes only a few minutes to complete compared with the several hours needed to perform equilibrium dialysis. The transition of thermogenin from the high-fluorescence protonated form to the low-fluorescence non-protonated form that is induced by an increase in pH demonstrates a limitation of this method for monitoring GDP binding at higher pH values. However, the work reported in the present paper clearly establishes that this technique may provide a simple method for investigation of the interactions of nucleotides and other ligands, e.g. fatty acyl-CoA esters, with the thermogenin protein.

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