# Chemical modification of the brown fat mitochondrial uncoupling protein with tetranitromethane

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Tetranitromethane reacts with the uncoupling protein of intact brown fat mitochondria. The chloride permeability in the absence of the inhibitory nucleotide GDP is not affected, but the affinity with which GDP binds is decreased, and the coupling between binding of nucleotide and inhibition of chloride permeation is broken.

*(Brown fat) Chemical modification Mitochondria Purine nucleotide Tetranitromethane Uncoupling protein*

## 1. INTRODUCTION

The uncoupling protein enables brown adipose tissue mitochondria to uncouple respiration from ATP synthesis under thermogenic conditions by catalyzing re-entry of protons extruded by the respiratory chain [1]. The protein possesses a tight binding site for purine nucleotide [2], and in vitro removal of the nucleotide induces a permeability not only to $H^+$ but also to $Cl^-$ and $Br^-$ [3]. Due to the high concentration of nucleotides in the cytoplasm, the nucleotide binding site is always likely to be occupied in vivo. Instead, the $H^+$ conductance is modulated physiologically by the fatty acids liberated during noradrenaline-stimulated lipolysis [4–6]. However, the conductance changes induced by nucleotide removal provide a useful means of investigating structure-function relationships within the protein.

Although the primary sequence of the protein, and predictions for its secondary structure, have been published [7], there is still very little known about the nucleotide binding site or the molecular pathways for ion movement. Photo-oxidation and the lysine specific reagent 2,4,6-trinitrobenzenesulphonic acid decrease the GDP binding capacity of the purified uncoupling protein [8]. Dicyclohexylcarbodiimide (DCCD) binds to the protein [9] but the claim that the reagent inhibits $Cl^-$ conductance has been disputed by us [10].

Here we report that tetranitromethane (TNM) modifies the uncoupling protein of intact brown adipose tissue mitochondria. TNM has been widely used as a chemical modifier [11,12]; it possesses a high specificity towards tyrosyl residues, although it can also react with sulphydryl groups and to a lesser extent with methionine, histidine and tryptophan [13]. Under our conditions, TNM lowers the affinity of the nucleotide binding site and disturbs the coupling between binding and inhibition of chloride permeation. At higher concentrations of TNM nucleotide binding is prevented.

## 2. MATERIALS AND METHODS

Mitochondria were prepared as in [14] from the thoracic, interscapular and dorsal brown adipose tissue of Syrian hamsters that had been cold-adapted at 4°C for at least two weeks. The final spin and resuspension was in 250 mM sucrose, 5 mM Tes (Na salt), pH 7.4. Protein was determined by the Biuret method.

5–15 $\mu$l of a fresh ethanolic solution of TNM was added to 6 mg of mitochondria in 1 ml of 250 mM sucrose, 10 mM Tris, pH 9.0, at 2°C. The

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same volume of ethanol was added to the controls. The incubation was stirred continuously for 15 min. Control experiments following release of nitroformate at 350 nm [11] showed that reaction was complete within 10 min. The incubations of modified and control mitochondria were diluted with 4 ml of 250 mM sucrose, 5 mM Tes (Na salt), pH 7.4, and centrifuged for 10 min at 8500 × g. The pellets were resuspended in a further 5 ml of this medium and recentrifuged. The final pellets were resuspended in 250 mM sucrose, 80 μM albumin, 5 mM Tes (Na salt), pH 7.4.

Binding of [$^3$H]GDP (0.25–8 μM) and the statistical analysis were as described [14]. $Cl^-$ permeability was determined from the rate of light scattering decrease in 100 mM KCl, 5 mM Tes (Na salt) [15]. Mitochondrial swelling was initiated by the addition of 0.5 μM valinomycin [3].

Radioisotopes were obtained from the Radiochemical Centre (Amersham, England). TNM was from Aldrich (Gillingham, Dorset, England). Other reagents were from Sigma (Poole, Dorset, England).

## 3. RESULTS AND DISCUSSION

Mitochondrial swelling in KCl is a valid measure of $Cl^-$ transport through the uncoupling protein as long as the chemical modifiers do not induce a non-specific permeability to the anion. A second, technical, problem occurs if the reagent introduces a $K^+$ permeability which allows the mitochondria to begin swelling as soon as they are suspended in the KCl medium. This would prevent the initial rate from being determined since GDP requires a finite time to achieve equilibrium binding [2]. Additionally, estimation of the initial light scattering is required to confirm that the modified mitochondria have not swollen during the preparation procedure. Thus, we have found [10] that the reported ability of DCCD to inhibit $Cl^-$ permeation through the uncoupling protein [9] is an artifact due to the modified mitochondria having preswollen almost to their maximal extent prior to the addition of valinomycin.

Under our conditions TNM does not induce a non-specific anion permeability, since control ex-

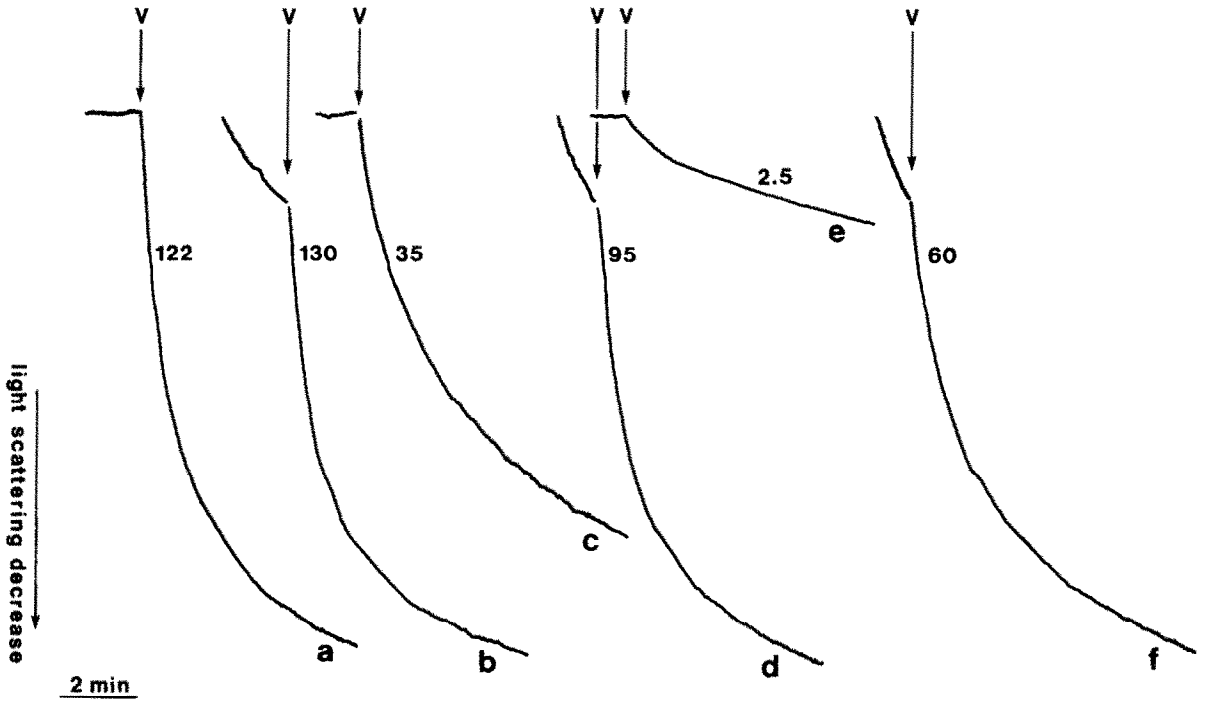


Fig.1. Effect of 150 μM TNM on mitochondrial swelling in 100 mM KCl. (a) Control mitochondria, (b) TNM-treated mitochondria, (c) control mitochondria plus 10 μM GDP, (d) TNM-treated mitochondria plus 10 μM GDP, (e) control mitochondria plus 300 μM GDP, (f) TNM-treated mitochondria plus 300 μM GDP. Valinomycin (0.5 μM) was added when indicated ('V'). The numbers are swelling rates in arbitrary units.

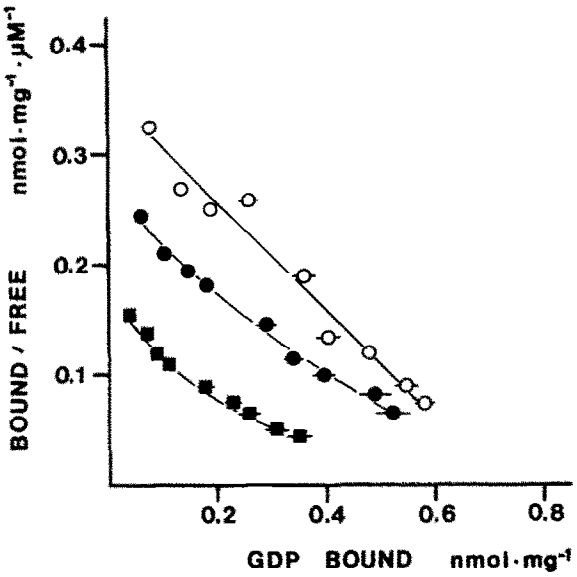


Fig.2. Scatchard plot of the binding of GDP to mitochondria. Effect of tetranitromethane. (○) Control mitochondria, (●) mitochondria treated with 150 μM TNM, (■) mitochondria treated with 250 μM TNM. Horizontal bars represent SE of bound nucleotide from 4–8 determinations. Where bars are absent, the SE was less than the dimension of the symbol.

periments in $K_2SO_4$ or $K^+$-Tes gave no light scattering decrease following the addition of valinomycin. Secondly the rate of swelling in KCl plus valinomycin in the absence of GDP was unchanged for mitochondria modified by 150 μM TNM (121 ± 17.5 and 119 ± 18.5 arbitrary units per min for the control and TNM-modified mitochondria, respectively ($n$ = 3)) (see also fig.1). These results indicate that under our conditions TNM does not interfere with the ability of the protein to transport $Cl^-$.

Fig.1, trace b shows that even when the reaction is carried out on ice with low concentrations of TNM, mitochondria do become slightly permeable to $K^+$, swelling slowly in KCl prior to the addition of valinomycin. This limits the concentration of TNM which can be employed to give reliable results. Our limiting criterion was the ability both to obtain a reliable initial light scattering and a linear initial swelling rate following valinomycin. Mitochondria reacted with TNM at room temperature (not shown) are damaged too extensively and swell rapidly when transferred to KCl.

10 μM GDP inhibits swelling by 70% in control mitochondria but only by 27% after TNM modification (fig.1c,d). A high concentration of GDP (300 μM) causes a 98% inhibition of the control, but only reduces swelling by 54% in the modified condition (fig.1e,f). The failure of 300 μM GDP to block $Cl^-$ permeation could be either because its binding is abolished, or because binding occurs without affecting the $Cl^-$ permeation.

The binding of GDP to control and TNM-modified mitochondria is shown in the Scatchard analyses in fig.2. In an earlier paper [15] we reported the presence of two binding sites for nucleotides in control mitochondria, and concluded that the high affinity site regulated the $H^+$ and $Cl^-$ permeability in vitro, whereas the low affinity site had no role in the regulation of ion permeability. The range of nucleotide concentrations used in this study (0.25–8 μM) does not reveal this low affinity site in control mitochondria. The high affinity, functional site has a capacity of 0.72 nmol/mg protein and a $K_d$ of 2 μM (table 1).

Mitochondria modified with 150 μM TNM show a biphasic Scatchard plot (fig.2) indicating the

Table 1

Statistical analysis of GDP binding to control and TNM-modified mitochondria

| | Unmodified | | Modified | |
|---|---|---|---|---|
| | $K_d$ | $B_{max}$ | $K_d$ | $B_{max}$ |
| Control | 2.0 ± 0.2 | 0.72 ± 0.03 | – | – |
| TNM (150 μM) | 1.3 ± 0.4 | 0.25 ± 0.05 | 7.7 ± 3.5 | 0.61 ± 0.15 |
| TNM (250 μM) | 1.5 ± 0.2 | 0.18 ± 0.03 | 9.2 ± 2.6 | 0.43 ± 0.05 |

Computer analysis of the Scatchard plots from fig.2 was performed using the Rothamsted Maximum Likelihood Program [19] to fit experimental results to a model with two independent binding sites [15]. $K_d$, affinity constant; $B_{max}$, capacity

presence of two sites with different affinities. The higher affinity site has a $K_d$ (table 1) which is not significantly different from that in the control, and presumably represents residual unmodified centres. The lower affinity site is evidently a product of TNM modification. At higher TNM concentrations (250 $\mu$M) there is a further decrease in the residual unmodified binding, but now a loss even of the lower affinity site becomes apparent (table 1), suggesting that TNM can react further with the protein to prevent nucleotide binding completely.

Table 1 reports the computer analysis of the binding constants for the modified and unmodified nucleotide sites. Due to the closeness of their affinities, there is some uncertainty in the analysis. However, we conclude that the residual higher affinity sites retain the $K_d$ of around 2 $\mu$M shown in the control plot, whereas those modified by TNM show a 4-fold decrease in affinity.

We have shown previously that the inhibition of $Cl^-$-dependent swelling correlates linearly with the occupancy of the high affinity binding site of unmodified mitochondria [15]. Fig.1 showed that 300 $\mu$M GDP could only decrease $Cl^-$ transport through the modified protein by about 50%. However, from the binding data of table 1 we can deduce that this concentration is sufficient to saturate both the 0.25 nmol/mg of unmodified and the 0.6 nmol/mg of modified sites. We conclude that the binding of GDP to the TNM-modified site has little or no effect on $Cl^-$ permeation.

The nature of the residue or residues modified by TNM cannot be established from the present data. While TNM reacts most readily with tyrosine at pH > 8 [12], cysteine [13,16] and even methionine, histidine or tryptophan [13] may also be modified. The formation of the by-product of modification, nitroformate, can be followed at 350 nm, but this gives no information on the modified amino acid, although this has been claimed [17].

## 4. CONCLUSIONS

In a previous study [18] we have shown that a large range of nucleotides and derivatives all inhibit the $Cl^-$ permeability of the uncoupling protein in parallel to their binding. Thus no 'antagonists', capable of competing for the binding site without influencing the permeability, have been found. The chemical modification described in this paper enables GDP to bind to the protein without inhibiting the permeability, thus showing for the first time that this linkage between binding and inhibition can be broken.

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