Direct thyroid hormone signalling via ADP-ribosylation controls mitochondrial nucleotide transport and membrane leakiness by changing the conformation of the adenine nucleotide transporter

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Abstract Addition of triiodothyronine at 10 pM in vitro to hypothyroid rat liver mitochondria doubles the rate of the adenine nucleotide transporter at low ADP concentrations. Nicotinamide abolishes this effect in parallel with its inhibition of the ADP-ribosylation of an inner membrane protein identical in size to the transporter. Nicotinamide also renders euthyroid preparations indistinguishable from hypothyroid ones. A mechanism is offered to explain these findings in which it is proposed that the adenine nucleotide transporter is a true allosteric protein and that its covalent modification by ADP-ribosylation increases the stability of the less favoured externally-facing C-conformation and thus increases the proportion of transporters in this orientation: although the C-conformation is significantly more leaky to cations than the tight matrix-facing M-conformation, this enhances ADP import. This model is shown to offer an explanation not only for the transport effects of T_3 but also for those of oxidative stress and ADP-ribosylation inhibitors on Ca^{2+}, H^+ and K^+ transfer across the mitochondrial inner membrane. Ca^{2+} at 30 nM appears to stabilize the M-conformation of the transporter by a mechanism other than ADP-ribosylation.

Key words: Adenine nucleotide transport; ADP-ribosylation; C/M-conformation of transporter; Calcium; H^+/K^+ leak; Nicotinamide; Signal amplification; Triiodothyronine

1. Introduction

Although early work proposing a direct interaction of thyroid hormones with mitochondria is now regarded as uncertain or unphysiological, in recent years evidence has begun to accumulate that triiodothyronine (T_3), in addition to longer-term effects exerted through transcription and translation, can indeed elicit a very rapid response from mitochondria. For example, mitochondria very rapidly and preferentially acquire T_3 administered in vivo [1,2] and saturable T_3 tight-binding sites have been reported in inner mitochondrial membranes only from tissues which respond to thyroid hormone [3]. To complement this evidence of a putative receptor, rapid effects of hormone on an energy-driven mitochondrial process such as respiratory control, uncoupler sensitivity [2] and calcium transport [4] have been observed. In our laboratory we have found that liver mitochondria from thyroidectomized rats show a number of altered responses to ADP stimulation that hormone addition at near-physiological concentrations both in vivo and in vitro can restore to normal within 15 min [1,5–8].

A striking feature of the assay of mitochondria in relatively low protein medium is that they responded to T_3 at concentrations as low as 10^{-13} M [8]. The possibility that some signal amplification mechanism involving covalent modification of mitochondrial proteins could be present was therefore examined. While no change in protein phosphorylation could be detected, nicotinamide, an inhibitor of mitochondrial mono-ADP ribosylation, abolished the T_3 effect on the phosphorylation of ADP by hypothyroid liver mitochondria [9]. Moreover, incubation with nicotinamide mimicked the effects of thyroid ablation in euthyroid liver mitochondria and mitoplasts; and these could be reversed by washing out the nicotinamide followed by T_3 addition [9].

We have shown that, in parallel with activity changes, T_3 in vitro rapidly stimulates the incorporation of radioactivity from extra-mitochondrial [^{14}C-adenine]-NAD^+ into inner membrane proteins in intact mitoplasts and in mitochondria and that this stimulation too is abolished by nicotinamide [10]. On polyacrylamide gel electrophoresis under acid conditions we reported without presenting any data that the incorporated radioactivity migrates as a single band with an apparent molecular mass of about 30 kDa. This was consistent with the reports from other laboratories of ADP-ribosylation of a 30 kDa inner membrane protein in liver sub-mitochondrial particles [11,12] although somewhat strangely this is proposed to result from NAD^+ hydrolysis and then very selective non-specific labelling by the ADP-ribosyl moiety [13,12]. An inner membrane protein of this size, the adenine nucleotide transporter (ANT) [14], has long been suggested as a mitochondrial target for thyroid hormones [15] and we had reported previously that the velocity of the ANT was halved in hypothyroid mitochondria because of a 2.2-fold increase in the apparent K_m and subject to restoration within 15 min by hormone in vivo [5]. However, we had found that in vitro incorporation of labelled NAD^+ into the 30 kDa species was abolished by T_3 [10] which we initially interpreted as providing no support for this protein as a target for T_3 signalling involving ADP-ribosylation. Here we reconsider this conclusion and demonstrate that the velocity of the ANT can be reduced by more than half by nicotinamide addition to isolated mitochondria: further, addition of T_3 at 10 pM restored the rate to normal and this was abolished by concomitant addition of nicotinamide. We go on to propose a model based on alteration of the relative proportions of the two distinct conformations of the ANT (see [16]) by ADP-ribosylation which may offer a molecular mechanism not only for the transport effects of T_3 but also for those of oxidative stress and ADP-ribosylation inhibitors on Ca^{2+} [17–20], H^+ [21,22], and K^+ [22–24] transfer across the mitochondrial inner membrane.

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2. Materials and methods

2.1. Animals and mitochondria

Male rats (130–150 g) of a Sprague–Dawley strain bred in the College were rendered hypothyroid either by thyroidectomy and then maintained on a calcium lactate supplemented diet as in [1] or supplied with water containing 6-n-propyl-2-thiouracil as in [10] and used after 6 weeks when their weight had stabilised at about 200 g. Liver mitochondria were prepared as in [10] and mitoplasts prepared from these by removing the outer membrane as detailed in [9]. Hormone was administered in vivo via the tongue veins 15 min before sacrifice [1] and in vitro as set out in [8]. Protein was measured as in [10].

2.2. Oxygen electrode and adenine nucleotide transport measurements

Oxygen electrode measurements were carried out in 130 mM KCl/2 mM $MgCl_2$/2 mM EGTA/5 mM Tris-HCl buffer at 37°C as detailed in [10]. Adenine nucleotide transport was measured using the integral form of the transport equations as set out in [5], except that the buffer used was the same as for the oxygen electrode experiments. In some experiments $CaCl_2$ was added to give a final buffered concentration of 30 nM (see [8]).

2.3. Incubation with labelled NAD^+ and LDS/PAGE

Incubations with [U-^{14}C]adenine-labelled NAD^+ were carried out exactly as detailed in [10]. After incubation the mitoplasts were pelleted in an Eppendorf microfuge and resuspended at 2 mg/ml in 8 M urea/0.25 M citric acid/0.031 M phosphoric acid/5% (w/v) LDS/4% (v/v) 2-mercaptoethanol (pH 4.0). The pelleted mitoplasts were separated into membrane and matrix fractions by disrupting them by freezing and thawing followed by recentrifugation to pellet the membranes. The resultant supernatant (matrix) fraction was denatured by addition of the urea/LDS buffer to give a final protein concentration of 2 mg/ml. A 10% acrylamide gel at pH 4 was prepared by the method of Jones et al. [27] and run for 14 h at 20 V in 93.8 mM citric acid/12.4 mM H_3PO_4/1% LDS (pH 4.0) buffer. The gel was stained in 42% methanol/16% acetic acid/0.12% (w/v) Kenacid Blue and destained in 10% methanol/2% acetic acid before measurement of radioactive incorporation.

2.4. Materials

Nicotinamide-[U-^{14}C]adenine dinucleotide ammonium salt (270 Ci/mol) and [8-^{14}C]ADP (52 and 57.1 Ci/mol) were purchased from ICN and NEN respectively. All other materials obtained as described in [8–10].

3. Results and discussion

3.1. The effect of thyroid hormone and Ca^{2+} ions on the rate of the adenine nucleotide transporter

We have shown that the rate of exchange catalysed by an obligatory antiport could be described by an integral form of the rate equations which dispenses with the need to maintain near-constant external substrate concentrations [5]. It is thus possible to measure transport rates at low substrate concentrations without the need to have large volumes of suspending fluid. We showed that thyroid deficiency led to an increase in the apparent K_m of the ANT of rat liver mitochondria from the euthyroid value of 2.8 μM to 6.3 μM and that this was returned to normal 15 min after a near-physiological intravenous dose of T_3 [5]. Increases in the ANT transport rate at longer times after thyroid hormone administration reported by others [25,26] probably reflect an increased number of transporters in the membranes. The data shown in Table 1 demonstrate that, in agreement with these earlier findings, the highly significant decrease in the transport rate in hypothyroid preparations observed at a concentration of ADP near to the K_m is not detectable at a much higher concentration. The administration of T_3 in vivo confirms the previous finding [5] that this is a rapidly reversible effect of thyroid hormone depletion.

Table 2 shows that the in vivo effect of T_3 on the transport of 2.5 μM ADP by the ANT can be reproduced by the in vitro addition of 10 pM hormone to isolated liver mitochondria prepared from hypothyroid animals. This doubling of the ANT transport rate in response to T_3 was abolished in the presence of nicotinamide: furthermore, the addition of nicotinamide led not only to a significant decrease in the transport rate in all conditions, it also rendered euthyroid preparations indistinguishable from hypothyroid ones. If this action of ni-

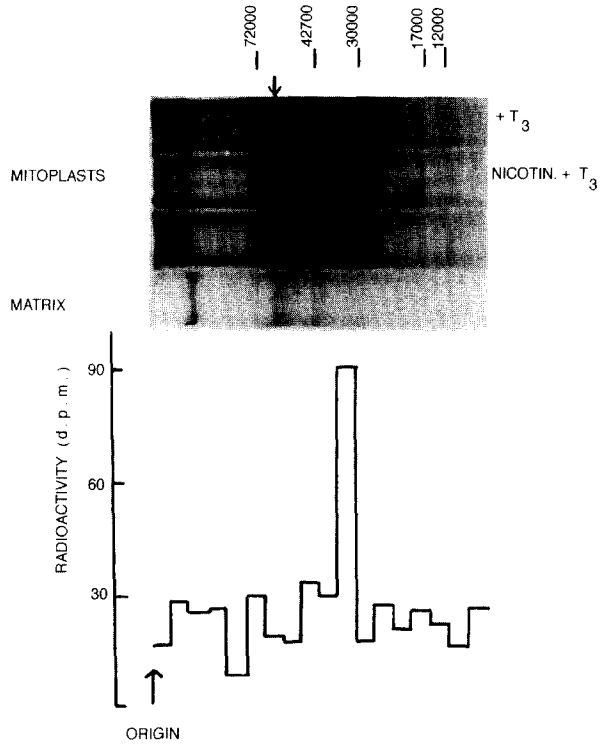

Fig. 1. LDS/PAGE of mitoplast and matrix proteins. The upper panel shows a photograph of the gels stained for protein after incubation with [U-^{14}C]adenine-NAD^+ in the absence or presence of 1 nM T_3 or 1 nM T_3 plus 20 mM nicotinamide as indicated. The lower panel shows the incorporation of radioactivity into each 5-mm section of a gel incubated in the absence of either nicotinamide or T_3. The distances of molecular-mass standard proteins are shown and the arrow indicates where a 55-kDa protein would be expected to run.

Table 1
The effects of thyroidectomy and medium ADP concentration on the rate of the adenine nucleotide transporter

Condition	n	Transport rate (nmol·min^{-1}·mg^{-1})	
		at 2 μM ADP	at 20 μM ADP
Normal	5	1.83 ± 0.02	3.44 ± 0.46
Thyroidectomized	5	0.97 ± 0.08*	3.22 ± 0.14
Thyroidectomized-T_3 injected	4	1.91 ± 0.25	3.57 ± 0.63

The values are means ± S.E.M. of n mitochondrial preparations. The rates were derived from the best straight lines fitted to plots of $Ln(R_{max}-R_m)$ vs. time (see Section 2 [5]) for initial ADP concentrations of either 2 μM (3.5 Ci/mol) or 20 μM (0.35 Ci/mol). T_3 was administered 15 min prior to sacrifice via the tongue veins (at 1 nmol/100 g body weight) to rats thyroidectomized 6 weeks previously. *$P < 0.005$ vs. normals.

Table 2
The effects of hypothyroidism, T_3 addition in vitro, and the presence of medium Ca^{2+} and nicotinamide on the rate of the adenine nucleotide transporter

Additions	Transport rate ($nmol \cdot min^{-1} \cdot mg^{-1}$)					
	(*n*)	Normals	(*n*)	Hypothyroid	(*n*)	Hypothyroid+T_3
none	(7)	2.15 ± 0.16	(6)	1.12 ± 0.07^{h}	(6)	1.97 ± 0.18
+nic.	(7)	0.86 ± 0.09^{af}	(6)	0.81 ± 0.09^{df}	(6)	0.93 ± 0.13^{bg}
$+Ca^{2+}$	(10)	1.26 ± 0.13^{ae}	(8)	0.76 ± 0.07^{bf}	(8)	1.21 ± 0.12^{bf}
$+Ca^{2+}$+nic.	(10)	0.51 ± 0.04^{a}	(8)	0.46 ± 0.04^{a}	(8)	0.62 ± 0.08^{a}

The rates, derived as in Table 1, are means ± S.E.M. of *n* mitochondrial preparations. The initial medium ADP concentration was 2.46 μM (10–30 Ci/mol). When present, the medium concentration of (buffered) Ca^{2+} was 30 nM, of nicotinamide (nic.) 10 mM and of T_3, 10 pM. Hypothyroidism was induced by propylthiouracil administration (see Section 2). Significance testing: medium additions vs. none: $P < 0.0005^{a}$; $< 0.005^{b}$; $< 0.01^{c}$; $< 0.025^{d}$; vs. Ca^{2+}+nicotinamide: $P < 0.0005^{e}$; $< 0.005^{f}$; $< 0.05^{g}$; hypothyroid vs. normal: $P < 0.0005^{h}$.

cotinamide is related to its inhibition of ADP-ribosylation of an inner membrane protein (see below), the finding that nicotinamide decreased further the rate in the hypothyroid preparations (Table 2) implies that there still remains some covalently modified protein in propylthiouracil-treated preparations.

This impairment of normal mitochondria by nicotinamide parallels that reported earlier [9] for measurements of the apparent ADP/O ratio at low protein concentrations. In these earlier studies [8,9], we had shown that in order to observe the maximum decrease in ADP/O ratio in hypothyroid preparations, the free Ca^{2+} ion concentration in the extramitochondrial medium had to be above 25 nM; Ca^{2+} ion concentrations as low as this had no effect on euthyroid preparations [8]. We thus measured the effect on the ANT of adding Ca^{2+} buffered at 30 nM. Table 2 shows that Ca^{2+} even at this low concentration decreases the ANT transport rate by 40%. The mechanism of the calcium inhibition may, however, be separate from that by which nicotinamide acts since, when mitochondria in all three conditions were rendered equivalent by nicotinamide addition, Ca^{2+} still produced a further 40% inhibition (Table 2). The concentration of free Mg^{2+} ions, which have been found to increase the apparent K_m of the ANT [28,5] since the transporter substrates are the free nucleotides, only increases by 1.25% from 1.74 mM on the addition of Ca^{2+} ions and thus seems unlikely to provide an explanation for the inhibitory effect.

3.2. *ADP-ribosylation of inner membrane proteins and the effect of nicotinamide and T_3*

Mitoplasts shown to be coupled by an oxygen electrode experiment, and therefore intact, were pre-incubated for 10 min at 37°C with [U-^{14}C]adenine-labelled NAD^+ and then for a further 8 min in the presence or absence of T_3 or T_3 plus nicotinamide as detailed in Section 2. After removal of the incubation buffer, the membrane proteins and matrix proteins were separated on lithium dodecyl sulfate polyacrylamide gel electrophoresis, stained and 5-mm slices of the gels assayed for radioactivity (see Section 2 for details).

Fig. 1 shows a photograph of the stained gels from such an experiment together with the radioactivity profile obtained in the absence of T_3 or nicotinamide. The only ADP-ribosylated proteins migrate with an apparent molecular mass of around 30 kDa in agreement with the findings reported from two other labs [11,13]. Although, as might have been expected, the labelling in this band was abolished by adding the ADP-ribosylation inhibitor nicotinamide, it was also unexpectedly abolished by adding T_3 alone (radioactivity profiles not shown). This not unreasonably caused us initially to discard this as evidence for T_3 stimulated ADP-ribosylation. However, an alternative interpretation can be offered which implies the opposite of this original conclusion. In the labelling experiment, the radioactive NAD^+ was added externally to the intact mitoplasts and NAD^+ has long been known to cross the mitochondrial membrane very slowly [29]. That there was no significant entry of the label during the course of the experiment is supported by our finding no evidence of labelling of the soluble matrix protein of around 50 kDa (see arrow, Fig. 1), which Hilz et al. [13] found in broken mitochondrial preparations, although there are unlabelled matrix proteins migrating in this region (Fig. 1). Thus the label incorporated into the 30 kDa membrane protein would have been from the outside of the inner membrane. In a similar experiment, Richter et al. [11] used sonicated mitoplasts and, although there is no indication what proportion of their vesicles were closed, it may be presumed that their labelling of the 30 kDa protein would have been mainly at the inside face of the inner membrane. Moreover, they demonstrated in a dual labelling experiment [11] that there was rapid turnover of the covalent label attached to the protein. ADP-ribosylation sites accessible from both sides of the membrane would be consistent with the double dimeric or tetrameric organisation of the ANT [16]. A T_3-stimulated ADP-ribosyl transfer using as substrate unlabelled matrix NAD^+ (as it was in our experiments) might be expected to alter the transporter conformation and thus the accessibility of external sites to ADP-ribosylation. Consonant with the matrix being the source of the NAD^+ are the observations that in hypothyroidism the mitochondrial content of both nicotinamide nucleotides (NAD^+ Kinase is a mitochondrial enzyme) have been found

Table 3
Factors influencing the two stable conformations of the mitochondrial adenine nucleotide tranporter

	Properties	Promoted by
M-conformation	NEM-sensitive	ADP; Bonkrekate
	low Ca^{2+} leak	ADP-ribosylation inhibitors
	low H^+/K^+ leak	hypothyroidism
	slow ADP transport	low external [Ca^{2+}]
C-conformation	NEM-insensitive	ATP; Atractylate
	increased Ca^{2+} leak	ADP-ribosylation
	increased H^+/K^+ leak	triiodothyronine
	increased ADP transport	high matrix [NAD^+]

See text Section 3.3 for discussion and references.

to increase significantly and conversely to decrease in hyperthyroidism [6,30,31]. In this view T_3 stimulation along with rapid turnover would lead to the apparent inhibition of incorporation from external labelled-NAD^+.

3.3. *A covalently modified two-state model of the adenine nucleotide transporter and its consequences*

It is well accepted that the mitochondrial ANT can exist in two distinct protein conformations which are differentially sensitive to thiol inhibitors [32,18] and to proteases [33]. They can be interconverted by the use of inhibitors [34]; atractylate induces the C-confirmation in which the nucleotide binding site faces the external mitochondrial environment and Bonkrekate induces the M-conformation in which the site faces the matrix.

Although these stable conformations are interconverted by binding ADP^{3-} and ATP^{4-} (see [16]), if it is presumed that the ANT is a true example of the Monod-Wyman-Changeux allosteric theory [35] then, like the nicotinic acetylcholine receptor [36], it would be expected to show clear switching between conformational states with or without ligand binding. Our hypothesis is that (1) the M-conformation is the preferred or more stable conformation which, as a tighter or less leaky state (see below) of the transmembrane protein, better protects the intramitochondrial environment: it is, however, less advantageous in transporting ADP from the intermembrane space which requires the C-conformation.

(2) Covalent modification of the ANT by ADP-ribosylation increases the stability of the C-conformation and hence the proportion of transporters and their residence time in that state: although this increases membrane leakiness, especially in the absence of substrate, it has clear advantages for ADP import.

Thus in the findings reported here, the hypothyroid preparations have a smaller fraction of the ANT in the externally oriented state and so show impaired ADP import which can be overcome by using higher concentrations of substrate so that the increased generation and export of ATP augments the number of transporters in the C-conformation.

(3) The mechanism proposed for the action of T_3 is that it activates an ADP-ribosyl transferase (possibly by increasing its affinity for free matrix NAD^+; see below) which, by covalently modifying the ANT, increases the number of transporters in the C-conformation. Nicotinamide reverses this covalent modification and so leads to fewer externally available transporter sites. This model accords with findings from a number of different mitochondrial studies.

In support of the proposal that hypothyroidism leads to a decreased proportion of ANT in the C-conformation is the finding that investigation of the kinetics of liver mitochondrial ANT found that thyroidectomy led to half maximal inhibition of the transporter at lower concentrations of atractylate (C-conformation inhibitor) and higher concentrations of bongkrekate (M-conformation inhibitor) than in controls [37]. Also hypothyroid mitochondria show lower H^+ [21] and K^+ [38] permeability, whereas the C-conformation of the ANT has been found to promote increased permeability of the inner membrane to K^+ and H^+ [22,23]; moreover, the decreased permeability in hypothyroid preparations can be rapidly reversed by a single dose of T_3 [21] which points to a mechanism separate from alteration of membrane lipids [39]. Conversion of ANT to the C-conformation has also been shown to promote rapid loss of Ca^{2+} from pre-loaded mitochondria [18] and to increase 1 μM Ca^{2+} uptake [24]. Of relevance in this context is the observation that T_3 induces calcium influx into liver cells within minutes and increases respiration and gluconeogenesis with the same kinetics leading to the proposal that, as with glucagon, the signalling involves increased mitochondrial uptake of cytosolic Ca^{2+} [40].

Several studies also support the link between ADP-ribosylation, the conformation of the ANT and ion leakage across the mitochondrial membrane. Hydroperoxides and quinones which cause rapid and extensive oxidation of mitochondrial pyridine nucleotides have been observed to cause Ca^{2+} release from rat liver mitochondria [17,20]. This efflux was accompanied by the hydrolysis of NAD^+ to release nicotinamide and could be prevented by added nicotinamide in one study [17] and by the more specific ADP-ribosylation inhibitor m-iodobenzylguanidine in the other [19,20]. In the context of the model proposed above, these suggest that the extensive increase in matrix NAD^+ concentrations promotes ADP-ribosylation of ANT and its conversion to the leaky C-conformation. In agreement with this, extensive oxidation of mitochondrial NAD^+ by incubation with acetoacetate which led to increased Ca^{2+} leakiness was also shown to increase the proportion of ANT in the C-conformation [18].

The nature of the observed ADP-ribosyl transfer to the inner membrane 30 kDa protein is somewhat controversial since two groups [13,12] have provided evidence that this appears to be a very specific non-enzymic covalent modification by ADP-ribose released by an NAD^+ glycohydrolase. However, a more recent study has suggested that all NAD^+ glycohydrolase activity in rat liver mitochondria is absent from the matrix and associated with the outer membrane [41]. Thus it may be that the intramitochondrial enzyme readily reverses the covalent modification and exchanges the ADP-ribosyl entity with free ADP-ribose.

Finally our observation that adding Ca^{2+} ions at the lower end of the resting physiological range impairs 2 μM ADP transport whether or not nicotinamide is present appears to suggest that these too may promote the M-conformation though not by altering ADP-ribosylation.

These findings and their proposed action in influencing the two conformations of ANT are summarized in Table 3. It remains to be shown by direct experiment that the 30 kDa protein ADP-ribosylated in response to T_3 is indeed identical to the adenine nucleotide transporter: and that T_3 stimulates this ADP-ribosylation using the matrix pool of NAD^+ as substrate.

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