The rapid response of isolated mitochondrial particles to 0.1 nM-tri-iodothyronine correlates with the ADP-ribosylation of a single inner-membrane protein

D. Louise HARDY and John MOWBRAY*

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Under defined conditions liver mitochondria from hypothyroid rats show an apparent lowering of the ADP/O ratio, which can be corrected by addition *in vitro* of 0.1 nM-tri-iodothyronine (T_3). Nicotinamide prevents this restoration by hormone, lowers the ADP/O ratio of euthyroid-rat mitochondria to hypothyroid-rat values and induces T_3 -sensitivity in euthyroid-rat mitoplasts indistinguishable from that found with hypothyroid-rat preparations. Incorporation into the trichloroacetic-acid insoluble fraction of mitoplasts and hypothyroid-rat mitochondria of radiolabel from [adenine- 14 C]-NAD $^+$ was stimulated by T_3 : this stimulation was abolished by nicotinamide. The findings strongly suggest that this incorporation occurs external to the matrix. Confirming the work of others, PAGE of radiolabelled mitoplasts shows alkali-labile modification of a major species of approx. 30 kDa: both nicotinamide and T_3 abolish this modification. By contrast, T_3 promotes incorporation of label into a single major 11 kDa species: this incorporated label is somewhat acid-labile, and the incorporation is abolished by nicotinamide. Comparative electrophoresis of purified sub-mitoplast fractions show that the 11 kDa species is in the inner membrane and absent from the matrix. The findings are consistent with a receptor-mediated ADP-ribosylation mechanism for the rapid action of T_3 on mitochondria.

INTRODUCTION

In addition to binding to chromatin-associated sites in the nuclei of target cells, tri-iodothyronine (T_3) is also rapidly acquired by mitochondria at very short times after administration in physiological amounts [1–3]. Saturable high-affinity binding sites ($K_s = 10^{-11}$ M $^{-1}$) have been reported on mitochondrial membranes from a number of tissues which respond to T_3 [4–6] and to be absent from those which are unresponsive [4]: partial purification of such a component from bovine heart mitochondria has been described [7].

To complement these studies of a putative mitochondrial receptor, rapid effects of thyroid hormone on a number of energy-driven mitochondrial processes have also been described both with isolated mitochondrial preparations [1,2,8–16] and with intact cells or tissues [17–22]. There is, however, some lack of correspondence between observations made in different laboratories. For example, although some report an apparently lowered ADP/O ratio with no evidence of uncoupling in mitochondria from hypothyroid animals [2,9,10,12,14], others do not [1,21,23]. More recently we [15] have offered one possible explanation for this discrepancy by showing that, in order to observe these lowered ADP/O ratios, a free external [Ca $^{2+}$] of at least 25 nM is required in the medium, and that the ratio is restored to normal within 2 min by the addition of T_3 . The mechanism for these effects remains obscure. Hafner & Brand [24] offered an alternative explanation by showing that, under their conditions, the ADP/O ratio decreased as the concentration of mitochondria in their assay was decreased. They proposed that the mechanism involved increased activation of adenylate kinase as the preparation was diluted, though this would seem unlikely in itself to give a lowered ADP/O ratio, and does not explain how T_3 would reverse this (see [15]). Moreover, the lowered ADP/O ratio can be observed with liver mitoplasts

[intact inner-membrane vesicles from which the outer membrane and intermembrane-space enzymes (such as adenylate kinase) have been removed by mild digitonin treatment] and is restored to normal by 0.1 nM- T_3 [16].

We have shown that, in mitochondrial preparations from hypothyroid animals, the apparent K_m of the adenine nucleotide translocator is increased [11] and there is increased energy-linked transhydrogenase activity [12]. Although both of these effects are rapidly reversed by T_3 administration, neither appears sufficient to account for any significant lowering of the apparent ADP/O ratio (see [11,12]): indeed, the flux control coefficient for the translocator is lower after thyroidectomy and is not rapidly increased by hormone [13].

A striking feature of the assay of mitochondria in the absence of serum albumin from the medium is that they respond to T_3 at concentrations as low as 0.1 nM [15]. The possibility that some signal-amplification mechanism involving covalent modification of mitochondrial proteins could be present (see [15]) was therefore examined. No change in protein phosphorylation in response to T_3 could be detected; by contrast, nicotinamide, an inhibitor of mono-ADP-ribosylation shown to take place in mitochondria [25–28], abolished the T_3 effect on the ADP/O ratio of hypothyroid liver mitochondria [16]. Moreover, incubation with nicotinamide produced an apparent lowering of the ADP/O ratio both in euthyroid-rat liver mitochondria and in mitoplasts prepared from these: washing out the nicotinamide restored the ratio in mitochondria, whereas the mitoplasts required T_3 addition [16].

Here we report experiments which show that, in parallel with raising the ADP/O ratio in both liver mitochondria from hypothyroid rats and mitoplasts from euthyroid rats, T_3 *in vitro* rapidly stimulates the incorporation of radioactivity from [adenine- 14 C]NAD $^+$ into trichloroacetic acid-precipitable material in both preparations. This stimulation is abolished, as is the raising

Abbreviations used: T_3 , tri-iodothyronine; PTU, 6-n-propyl-2-thiouracil; LDS, lithium dodecyl sulphate.

* To whom correspondence should be addressed.

of ADP/O ratio, by nicotinamide. The T_3 -stimulated radioactivity is found to migrate as a single band with material staining as protein on one-dimensional PAGE.

EXPERIMENTAL

Materials

Nicotinamide- $[U-^{14}C]$ adenine dinucleotide ($[^{14}C]NAD^+$) ammonium salt (270 Ci/mol) was bought from Amersham International. 6-n-Propyl-2-thiouracil (PTU) and lithium dodecyl sulphate (LDS) were bought from Sigma. Acrylamide, NN' -methylenebisacrylamide and SDS were bought from BDH.

The purity of ADP was monitored, and all other materials were obtained as described in [15].

Animals and mitochondria

Male rats (130–150 g) of a Sprague–Dawley strain bred in the Department were maintained on a normal diet, except that their drinking water contained 0.01 % (w/v) PTU and 2 % (v/v) ethanol. The PTU stock solution was made up in ethanol, and the water bottles were changed each second day. The animals were used after 6 weeks on this regime, when their weight was constant at around 200 g. Rats were killed by cervical dislocation, and liver mitochondria were prepared by differential centrifugation in 0.25 M-mannitol/1 mM-EDTA/25 mM-triethanolamine, pH 7.4, and resuspended in 0.25 M-mannitol. Mitoplasts were prepared from these as detailed in [16] and resuspended in 0.25 M-mannitol.

Oxygen-electrode experiments

The ADP/O ratios were estimated by using 0.35 mg of mitochondrial protein/ml in oxygen-electrode buffer (130 mM-KCl/2 mM-MgCl₂/2 mM-EGTA/5 mM-Tris/HCl, pH 7.2 at 37 °C) in a Clark-type electrode; 33 μ M-CaCl₂ was added to give a total free $[Ca^{2+}]$ of about 25 nM (see [15]) with 2 mM-phosphate, 3.3 mM-succinate as substrates in the presence of 3.3 μ g of rotenone/ml and 0.2 μ mol of ADP. Neither the ADP/O ratio nor State-4 respiration changed significantly with three successive additions of ADP to the same incubation.

Incubation of mitochondria/mitoplasts with labelled NAD^+

Mitochondria or mitoplasts from a preparation shown to be coupled by an oxygen-electrode experiment were suspended at 0.5 mg/ml in 5.8 ml of oxygen-electrode buffer and incubated for 10 min at 37 °C with $[U-^{14}C]$ adenine-labelled NAD^+ at concentrations between 0.75 and 2 μ M. After this, 200 μ mol of succinate and 120 μ mol of nicotinamide (where appropriate) were added, giving a final volume of 6 ml; the incubation was then continued for 3 min before addition of 60 μ l of 0.1 μ M- $L-T_3$ (where appropriate). After a further 5 min incubation, the reaction was stopped by addition of trichloroacetic acid (10 %, w/v)/methanol (25 %, v/v) solution at -10 °C. The resulting precipitate was washed once with trichloroacetic acid, once with acetone and twice with diethyl ether before being dried under nitrogen gas. The final pellet was weighed, dissolved in tissue solubilizer overnight before addition of scintillant and radioactivity counting.

Preparation of sample for PAGE

Sample suspension (0.5 ml) was removed after the final 5 min incubation period, spun down (2000 g for 3 min), and the pelleted proteins were resuspended in the appropriate denaturing buffer at a concentration of 2 mg/ml.

Samples to be separated on the low-pH gels by the method of Jones *et al.* [29] were resuspended in denaturing buffer, pH 4,

containing 8 M-urea, 0.25 M-citric acid, 0.031 M-phosphoric acid, 5 % (w/v) LDS and 4 % (v/v) 2-mercaptoethanol. Samples to be separated on high-pH gels by the method of Laemmli [30] were resuspended in denaturing buffer, pH 6.9, composed of 62.8 mM-Tris/HCl, 10 % (w/v) glycerol, 2 % (w/v) SDS and 5 % 2-mercaptoethanol.

PAGE

Alkali/SDS/PAGE was performed essentially by the method of Laemmli [30], by using a 16 %-acrylamide gel at pH 8.8 in the presence of 0.1 % SDS. Typically the running time was 14 h at 5 mA. The gel was then stained and destained [stain: 7.5 % (v/v) acetic acid/50 % (v/v) methanol/0.25 % (w/v) Coomassie Brilliant Blue R250; destain: 7 % acetic acid/10 % methanol] before measurement of radioactivity incorporation from $[U-^{14}C]$ adenine-labelled NAD^+ . The incorporation was determined by cutting the individual lanes into 5 mm segments, which were dissolved overnight at 75 °C in 1 ml of 15 % (w/v) H₂O₂ before addition of liquid scintillant and liquid-scintillation counting of radioactivity.

Acid/LDS/PAGE was carried out by a modification of the method of Jones *et al.* [29]. A 10 %-acrylamide gel at pH 4 was prepared by this method, whereas the running buffer contained 93.8 mM-citric acid, 12.4 mM-H₃PO₄ and 1 % LDS at pH 4; typically, running time was 14 h at 20 V. The gel was then stained/destained [stain: 42 % methanol/16 % acetic acid/0.12 % (w/v) Kenacid Blue; destain: 10 % methanol/2 % acetic acid] before measurement of radioactivity incorporation.

Other methods

Protein was measured by the method of Markwell *et al.* [31], and other methods were as previously described [15].

RESULTS AND DISCUSSION

Effect of nicotinamide on ADP/O ratios

Fig. 1 shows the result of adding nicotinamide at concentrations between 1 and 10 mM on the ADP/O ratios of liver mitochondria prepared from both euthyroid and hypothyroid rats. The findings are shown for both total oxygen consumption (State 4 oxygen uptake is assumed to cease completely on ADP addition) and for net oxygen consumption (State 4 uptake continues unchanged after ADP addition); the true value lies somewhere between these two, depending on the intactness of the preparation. As we have reported previously (see the Introduction), thyroidectomy results in an apparently lowered ADP/O ratio when tested under these specific conditions. Nicotinamide has a greater effect on the euthyroid-rat than on the hypothyroid-rat preparations, lowering the ratio to near the hypothyroid-rat value by 10 mM. Although it has no significant effect on the respiratory control ratio, nicotinamide also lowers the rate of oxygen consumption in both States 3 and 4 (results not shown). The somewhat different profiles for the ratios derived from total and net oxygen consumption (Fig. 1) suggest that the effect on oxygen consumption rates and on the ADP/O ratio may not comprise a single mechanism of action, but may involve components with differential sensitivities to the inhibitor. To avoid such problems, all subsequent experiments have used 10 mM-nicotinamide.

Table 1 presents the ADP/O ratios (net oxygen uptake) found with liver mitochondria and mitoplasts (intact inner-membrane vesicles devoid of outer-membrane and intermembrane-space enzymes) from normal rats and mitochondria from rats rendered hypothyroid by PTU administration. Just as we reported previously [15] for mitochondria from thyroid/parathyroid-ecto-

Fig. 1. Effect of nicotinamide concentration on the ADP/O ratio

The values determined in the presence of succinate and rotenone are the means of three preparations (S.E. 5% or less) from the livers of euthyroid (○, ●) rats or of rats thyroid-/parathyroid-ectomized [15] 6 weeks previously (□, ■). The values are plotted as both the total oxygen consumed (○, □) after addition of 200 nmol of ADP in State 4 until State 4 is regained and the net oxygen consumed (●, ■) after addition of ADP, assuming State 4 respiration continues unchanged. The mean respiratory control ratios for euthyroid and hypothyroid preparations were 4.1 and 3.8 respectively; they were not altered by nicotinamide. See the Experimental section for additional details.

mized animals, the PTU-treated preparation shows no lowering of the ADP/O ratio in the absence of free Ca^{2+} in the medium but a significant lowering in the presence of 25 nM free Ca^{2+} . Further, this lowered ratio is restored to normal by the addition *in vitro* of 0.05 nM- T_3 , an effect which can be countered by adding nicotinamide. The ratio with normal mitochondria is not altered by Ca^{2+} addition, but is lowered to a value indistinguishable from that of the PTU preparation by nicotinamide (Table 1). By contrast, removing the outer membrane of normal mitochondria by mild detergent treatment produces a preparation which mirrors the hypothyroid-rat one in its responses to Ca^{2+} , T_3 and

nicotinamide (see Table 1), suggesting that endogenous hormone is lost during mitoplast preparation.

T_3 , present *in vivo* in constant amount bound to serum proteins, is thought to be taken into hepatocytes via binding to specific plasma-membrane sites [32]. Studies of the subcellular distribution of T_3 in liver from short times after intravenous administration of physiological doses to hypothyroid rats have shown that most of the hormone remains cytoplasmic for up to 4 h [2]. There it is possibly bound to the recently described specific 58 kDa cytoplasmic binding protein [33], which some immunological studies suggest may be related to thyroxine-binding prealbumin [34] and which can also be found associated with mitochondrial outer membrane [34]. For kidney mitochondria [35], the specific T_3 -binding capacity of the outer membrane is substantially higher than that of the inner membrane, and binding of T_3 to mitochondria may involve prior binding to the cytoplasmic carrier protein to the outer membrane [36]. Thus it may be that cytoplasm and outer membrane constitute a pool of T_3 available to inner-membrane sites which is lost in the preparation of mitochondria and subsequently of mitoplasts, rendering the latter responsive to added hormone.

One possible interpretation of the inhibitory effect of nicotinamide on the action of T_3 is that the hormone mechanism involves ADP-ribosylation [16], whether via a specific ADP-ribosyltransferase or an NAD^+ glycohydrolase (see below). This possibility was tested directly in parallel experiments to those for measuring ADP/O ratios in which the preparation contained additionally NAD^+ radiolabelled in the adenine moiety.

Incorporation of radiolabel from [adenine- ^{14}C]NAD $^+$ into the trichloroacetic acid-precipitable fraction of mitochondria and mitoplasts

Preincubation of mitochondria or mitoplasts for 10 min with [^{14}C]NAD $^+$ (270 Ci/mol) before addition of succinate and ADP resulted in the incorporation of radioactivity into the fraction precipitated by trichloroacetic acid 8 min later: this radioactivity was stable to repeated acid washing. The amount of radioactivity incorporated was linear with NAD^+ concentrations tested from 0.75 to 2 μM (results not shown). Fig. 2 shows that this linearity also held for different preparations of mitoplasts: the same was true for mitochondria from PTU-treated animals, although the amount of radioactivity was about 10% of that in mitoplasts (results not shown).

Mitochondria (1 mg of protein) contain between 1.2 and 2.4 nmol of NAD^+ , depending on their respiratory state and thyroid states [12,37]. Mitoplasts, with 10% less protein per

Table 1. Effects of Ca^{2+} , nicotinamide and T_3 on the apparent ADP/O ratio

Mitochondria and mitoplasts from euthyroid rats and mitochondria from rats administered PTU were incubated as in the Experimental section either in oxygen-electrode buffer (OEB) alone or with additionally 25 nM free Ca^{2+} , 10 mM-nicotinamide or 0.05 nM- T_3 present. The mean respiratory control ratios were 3.5, 2.1 and 3.1 respectively for normal mitochondria, mitoplasts and PTU-treated-rat mitochondria respectively and were not significantly changed by assay conditions. The values given are means $\pm$ S.E.M. (including Bessel's Correction for small samples) of the number (n) of preparations used. Comparisons with OEB by Student's t test are * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$, **** $P < 0.0005$. T_3 significantly raises the lowered ratios in mitoplasts ($P < 0.01$) and PTU-treated-rat mitochondria ($P < 0.05$).

Conditions	ADP/O ratio		
	Normal mitochondria (3)	Mitoplasts (3)	PTU-treated-rat mitochondria (3)
OEB	1.88 $\pm$ 0.06	1.95 $\pm$ 0.01	1.80 $\pm$ 0.02
OEB + Ca^{2+}	1.86 $\pm$ 0.07	1.55 $\pm$ 0.02****	1.50 $\pm$ 0.08**
OEB + Ca^{2+} + T_3	—	1.91 $\pm$ 0.07	1.78 $\pm$ 0.07
OEB + Ca^{2+} + nicotinamide	1.50 $\pm$ 0.07***	—	—
OEB + Ca^{2+} + nicotinamide + T_3	—	1.64 $\pm$ 0.08**	1.55 $\pm$ 0.08*

Fig. 2. Incorporation of acid-stable radioactivity into trichloroacetic acid-insoluble material in mitoplasts as a function of NAD^+ precursor concentration

The mitoplasts were incubated with radioactive NAD^+ of constant specific radioactivity (270 Ci/mol) with NAD^+ concentrations in the medium between 0.75 and 2 μM : the values are corrected for slight variations in the quantity of mitoplasts added and expressed as $\mu\text{Ci/mg}$ of mitoplast protein. Each point is derived from a different mitoplast preparation incubated with NAD^+ alone (●) or additionally with 1 nM- T_3 (○). The least-squares best-fit straight lines are drawn.

vesicle than mitochondria (E. D. Saggerson, unpublished work), presumably contain a little more per mg of protein. Thus the mitoplasts added 0.6–1.2 nmol of NAD^+ to dilute the 0.5–2 nmol of labelled NAD^+ /ml in the medium. The linear incorporation of label from NAD^+ in a short time irrespective of the quantity of mitoplasts added implies either a rapid equilibration of the NAD^+ precursor specific radioactivities across the mitochondrial membrane or that incorporation is on the external surface of the inner membrane. NAD^+ has long been known to cross the inner membrane very slowly; Purvis & Lowenstein [38] estimated that, in rat liver, transfer from the cytosol could account for about 1% of the mitochondrial pool/min. It therefore seems highly unlikely that specific radioactivity equilibration occurs in 18 min, and hence that covalent modification by ADP-ribosylation is external to the inner membrane. This would be consistent with the much higher proportion of extramitochondrial nicotinamide nucleotide in the oxidized (precursor) form (see [37]).

Fig. 2 also shows that the addition of T_3 to the incubation 3 min after the substrate and ADP increased the quantity of trichloroacetic acid-precipitable radioactivity without altering the linear dependence on precursor concentration. These data are included in Table 2, which shows the effects on incorporation of T_3 and nicotinamide in both mitoplasts and mitochondria from PTU-treated rats as a percentage of that in the presence of

$[^{14}\text{C}]\text{NAD}^+$ alone. Hormone causes a significant increase of about 40% in the precipitable radioactivity, which is abolished by the administration of nicotinamide with substrate and ADP. Nicotinamide addition itself along with substrate and ADP decreases the level of incorporation, although not to a statistically significant degree with mitoplasts. Conversely, nicotinamide added 2 min before the labelled NAD^+ caused a significant increase in control incorporation, suggesting that some of the conjugates formed *in vivo* are readily reversed by nicotinamide, leaving free sites for labelled ADP-ribosylation.

These findings are consistent with the proposal that the effect of T_3 on the apparent ADP/O ratio may be via an ADP-ribosylation which can be prevented or diminished by nicotinamide addition. Although an alternative explanation of these findings as being due to increased transfer of precursor across the inner membrane cannot be eliminated, this is less likely given the evidence (see above) which suggests that the incorporation is external to the mitochondrial inner membrane. In order to test this further, proteins from similar experiments were examined by PAGE.

PAGE of acid-precipitable material from mitoplasts covalently labelled by incubation with $[^{14}\text{C}]\text{NAD}^+$ and the effects of T_3 and nicotinamide

When a mitoplast preparation after incubation under iso-osmotic conditions with [adenine- $^{14}\text{C}]\text{NAD}^+$ was subjected to PAGE in acid conditions (pH 4; see the Experimental section), virtually all of the incorporated radioactivity appears in a single band which migrates with an apparent molecular mass of 30–36 kDa (results not shown). This finding may be consistent with previous reports in which ADP-ribosylation of rat liver mitochondria and mitoplasts incubated with radiolabelled NAD^+ under hypo-osmotic conditions was found in two bands, a major one of 50–55 kDa and a minor one at around 30 kDa [37]. Others had shown ADP-ribosylation of a 50–55 kDa matrix protein in rat liver [26] and of a 30 kDa membrane protein in liver sub-mitochondrial particles [25,28]. The labelling of the 30 kDa membrane protein appears to result from NAD^+ hydrolysis and subsequent binding of the ADP-ribose moiety [26,28]. In our experiments, addition of nicotinamide, a good inhibitor of NAD glycohydrolase at 10 mM (see [27]), abolished labelling of the 30–36 kDa species (results not shown). The absence of label in the 50–55 kDa matrix protein tends to confirm the conclusion (see above) that no significant amount of labelled NAD^+ crossed the inner membrane during the 10 min incubation. Somewhat surprisingly, T_3 also abolished the incorporation into the 30 kDa species, in contrast with its ability to stimulate radioactivity

Table 2. Incorporation of radioactivity from [adenine- $^{14}\text{C}]\text{NAD}^+$ into the washed trichloroacetic acid-precipitate fraction of PTU-treated-rat mitochondria and mitoplasts

The values are expressed as percentages of the incorporation in the presence of a range of $[\text{NAD}^+]$ incubated as detailed in the Experimental section: control incorporation ranged from 3900 to 8800 d.p.m./mg of precipitate. Nicotinamide and T_3 were added to final concentrations of 10 mM and 1 M respectively. The nicotinamide preincubation involved adding 10 mM-nicotinamide 2 min before the labelled- NAD^+ assay as in control conditions was carried out. The significance of any difference from control values as determined by Student's *t* test is given; NS means $P > 0.05$.

	Incorporation (% of control)			
	+ T_3	Nicotinamide + T_3	Nicotinamide	Nicotinamide preincubation
Mitoplasts	136 ± 7 (5) ($P < 0.0025$)	99 ± 9 (5) (NS)	78 ± 11 (4) (NS)	173 ± 5 (3) ($P < 0.0005$)
PTU-treated-rat mitochondria	145 ± 9 (5) ($P < 0.0025$)	87 ± 9 (5) (NS)	74 ± 5 ($P < 0.01$)	143 ± 13 (5) ($P < 0.05$)

Fig. 3. SDS/PAGE of mitoplast proteins

(a) Incorporation of radioactivity into the proteins of mitoplasts incubated with [^{14}C]adenine-labelled NAD^+ . The proteins of these mitoplasts were separated by SDS/PAGE on a 16% acrylamide gel under alkaline conditions. The incorporation of radioactivity into each 5 mm section of the gel is shown against its distance from the origin. The relative positions of the molecular-mass standards (kDa) are also shown. The pattern of incorporation is shown for samples incubated with 1 nM- T_3 (ii) and for those incubated with 20 mM-nicotinamide and 1 nM- T_3 (i). (b) SDS/PAGE of mitoplasts and mitochondrial-inner-membrane samples. The samples were run on SDS/PAGE (16%-acrylamide gel) under alkaline conditions as described in the text. Lanes: 1, the incubated mitoplasts sample; 2, the mitochondrial-inner-membrane sample; 3, the molecular-mass standards (kDa).

incorporation into the trichloroacetic acid-insoluble fraction (Table 2; Fig. 2), suggesting that the covalent modification stimulated by T_3 was unstable under the prolonged acidic conditions of these gels.

This view was supported by the findings when the denatured proteins were separated under alkaline conditions (pH 8.8; see the Experimental section). Here all of the radioactivity in the presence of NAD^+ alone ran as low-molecular-mass species at the solvent front: that no label ran in the 30 kDa range is in agreement with the reports that this species is alkali-labile [28]. Addition of T_3 to the incubation gave rise to a single band which

migrated with an apparent molecular mass of 11 kDa (Fig. 3a, ii): furthermore, addition of nicotinamide with the T_3 abolished this incorporation (Fig. 3a, i), just as it abolished the hormone's effect on the ADP/O ratio (Table 1) and stimulation of incorporation into the acid-insoluble fraction (Table 2). Although these findings do not eliminate the possibility that the covalent modification may be adenylation (AMP addition) from hydrolysis products of the NAD^+ converted by the mitochondria into ATP, the relative acid-lability of the incorporated moiety, and especially the sensitivity to added nicotinamide, suggest ADP-ribosylation.

Submitochondrial fractions (generously provided by E. D. Saggerson) were run on the alkaline gel system and stained for protein. Only the inner-membrane fraction appears to have a species which migrates in the same place as that radiolabelled after T_3 (Fig. 3b). Attempts to purify and identify this small membrane protein and to characterize the covalent modification are necessary.

Conclusion

The findings are consistent with the proposal that the rapid effects of T_3 on mitochondria may be mediated, at least in part, by a receptor-mediated mechanism involving ADP-ribosylation of a small externally oriented inner-membrane protein.

We are indebted to Professor E. D. Saggerson of this Department for providing samples of liver mitochondrial inner membrane and matrix. We gratefully acknowledge project grant support from the Wellcome Trust.

REFERENCES

- Hoch, F. L. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 506–512
- Palacios-Romero, R. & Mowbray, J. (1979) *Biochem. J.* **184**, 527–528
- Sterling, K., Campbell, G. A., Taliadouros, G. S. & Nunez, E. A. (1984) *Cell Tissue Res.* **236**, 321–325
- Sterling, K., Lazarus, J. H., Milch, P. O., Sakwada, T. & Brenner, M. A. (1978) *Science* **201**, 1126–1129
- Goglia, F., Torresani, J., Bugli, P., Bareletti, A. & Liverini, G. (1981) *Pflugers Arch.* **390**, 120–124
- Hashizuma, K. & Ichibaura, K. (1982) *Biochem. Biophys. Res. Commun.* **106**, 920–926
- Sterling, K. (1986) *Endocrinology (Baltimore)* **119**, 292–295
- Herd, P. A. (1978) *Arch. Biochem. Biophys.* **188**, 220–225
- Sterling, K., Milch, P. O., Brenner, M. A. & Lazarus, J. H. (1977) *Science* **197**, 996–999
- Sterling, K., Brenner, M. A. & Sakwada, T. (1980) *Science* **210**, 340–342
- Mowbray, J. & Corrigall, J. (1984) *Eur. J. Biochem.* **139**, 96–99
- Corrigall, J., Tselentis, B. S. & Mowbray, J. (1984) *Eur. J. Biochem.* **141**, 435–440
- Holness, M., Crespo-Armas, A. & Mowbray, J. (1984) *FEBS Lett.* **177**, 231–235
- Crespo-Armas, A. & Mowbray, J. (1987) *Biochem. J.* **241**, 657–661
- Thomas, W. E., Crespo-Armas, A. & Mowbray, J. (1987) *Biochem. J.* **247**, 315–320
- Thomas, W. E. & Mowbray, J. (1987) *FEBS Lett.* **223**, 279–283
- Kaminski, T. (1980) *Acta Endocrinol. (Copenhagen) Suppl.* **234**, 15
- Muller, M. J. & Seitz, H. J. (1980) *Life Sci.* **27**, 827–835
- Muller, M. J. & Seitz, H. J. (1981) *Life Sci.* **28**, 2243–2249
- Seymour, A.-M. L., Keogh, J. M. & Radda, G. K. (1983) *Biochem. Soc. Trans.* **11**, 376–377
- Seitz, H. J., Muller, M. J. & Soboll, S. (1985) *Biochem. J.* **227**, 149–153
- Sterling, K., Tsuboyama, G. & Brenner, M. A. (1988) *Endocr. Res.* **14**, 109–116
- Verhoeven, A. J., Kamer, P., Groen, A. K. & Tager, J. M. (1985) *Biochem. J.* **226**, 183–192
- Hafner, R. P. & Brand, M. D. (1988) *Biochem. J.* **250**, 477–484
- Richter, C., Winterhalter, K. H., Baumhuter, S., Lotscher, H.-R. & Moser, B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3188–3192
- Hilz, H., Koch, R., Ranick, W., Klapporth, K. & Adamier, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3929–3933

27. Masmoudi, A. & Mandel, P. (1987) *Biochemistry* **26**, 1965–1969
28. Frei, B. & Richter, C. (1988) *Biochemistry* **27**, 529–535
29. Jones, G. D., Wilson, M. T. & Darley-Usmar, V. M. (1981) *Biochem. J.* **193**, 1013–1015
30. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
31. Markwell, M. A. K., Haas, S. M., Bicker, L. L. & Tolberg, N. E. (1978) *Anal. Biochem.* **87**, 206–210
32. Blondeau, J.-P. (1986) *FEBS Lett.* **204**, 41–46
33. Hashizume, K., Suzuki, S., Ichikawa, K. & Takeda, T. (1991) *Biochem. Biophys. Res. Commun.* **174**, 1084–1089
34. Azimova, S. S., Umarova, G. D., Petrova, O. S., Tukhtauer, K. R. & Abdugarimov, A. (1984) *Biokhimiya* **49**, 1478–1485
35. Hashizuma, K., Ichikawa, K. & Kobayashi, M. (1984) *Endocrinol. Jpn.* **31**, 311–320
36. Hashizume, K., Kobayashi, M., Miyamoto, T. & Yamauchi, K. (1986) *Endocrinology (Baltimore)* **119**, 1063–1070
37. Sies, H. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 205–231, Academic Press, London
38. Purvis, J. L. & Lowenstein, J. M. (1961) *J. Biol. Chem.* **236**, 2794–2803

Received 13 September 1991/25 November 1991; accepted 5 December 1991