Biochem. J. (1985) **226**, 183–192
Printed in Great Britain

Effects of thyroid hormone on mitochondrial oxidative phosphorylation

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(*Received 13 August 1984/Accepted 24 October 1984*)

In order to locate sites of action of thyroid hormone on mitochondrial oxidative phosphorylation we have used an experimental application of control analysis as previously described [Groen, Wanders, Westerhoff, Van der Meer & Tager (1982) *J. Biol. Chem.* **257**, 2754–2757]. Rat-liver mitochondria were isolated from hypothyroid rats or from hypothyroid rats 24h after treatment with a single dose of 3,3′,5-tri-iodothyronine (T_3). The amount of control exerted by four different steps on State-3 respiration with succinate as respiratory substrate was quantified by using specific inhibitors. The hormone treatment resulted in an increase in the flux control coefficient of the adenine nucleotide translocator, the dicarboxylate carrier and cytochrome *c* oxidase and a decrease in the flux control coefficient of the bc_1-complex. The results of this analysis indicate that thyroid hormone treatment results in an activation of the bc_1-complex and of at least one other enzyme, possibly succinate dehydrogenase. Measurement of the extramitochondrial ATP/ADP ratio at different rates of respiration (induced by addition of different amounts of hexokinase in the presence of glucose and ATP) showed that the adenine nucleotide translocator operates at a higher $(ATP/ADP)_{out}$ after T_3 treatment, which supports previous reports on stimulation of this step by thyroid hormone.

Several investigators have shown that pretreatment of rats with hormones leads to stimulation of State-3 respiration of isolated liver mitochondria with succinate as the respiratory substrate. Such hormones include thyroid hormone (Tata *et al.*, 1962; Bronk, 1963; Shears & Bronk, 1979; Maddaiah *et al.*, 1981), glucagon (Yamazaki, 1975; Titheradge & Coore, 1976; Halestrap, 1978), adrenaline (Titheradge & Coore, 1976) and dexamethasone (Allan *et al.*, 1983). The site of action of these hormones is not clear. In the case of thyroid hormone and glucagon, the stimulation has been attributed to a change in the activity of succinate dehydrogenase (Maddaiah *et al.*, 1981; Siess & Wieland, 1978; Titheradge & Haynes, 1979), the adenine nucleotide translocator (Babior *et al.*, 1973; Bryla *et al.*, 1977), or the respiratory chain (Bronk, 1966; Halestrap, 1982). The short-term effects of thyroid hormone appear not to be related to the synthesis of mitochondrial proteins (Bronk, 1966; Chen & Hoch, 1977) which occurs only after prolonged exposure to the hormone (Roodyn *et al.*, 1965; Jakovcic *et al.*, 1978).

In order to be able to locate sites of action of thyroid hormone on mitochondrial respiration, we have used control analysis based on the principles developed by Kacser & Burns (1973) and Heinrich & Rapoport (1974*b*), as recently applied by Groen *et al.* (1982) in a study of the control of respiration in isolated rat-liver mitochondria. Groen *et al.* (1982) used specific inhibitors to measure the flux control coefficient of various enzymes. The flux control coefficient of an enzyme is defined as the fractional change in pathway flux brought about by a fractional change in the activity of that enzyme. They found that control of respiration with succinate as respiratory substrate in the presence of excess ADP is distributed among several steps, including the adenine nucleotide translocator, the dicarboxylate carrier and cytochrome *c* oxidase. Since the stimulation of respiration by thyroid hormone must be mediated by activation of one or more (controlling) enzymes, it may be expected that treatment with thyroid hormone

Abbreviations used: T_3, L-3,3′,5-tri-iodothyronine; T_4, L-3,3′,5,5′-tetraiodothyronine (thyroxine); PTU, 6-n-propylthiouracil; TPMP, trimethylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

will lead to a change in the distribution of control. The advantage of this approach is that the enzymes are studied in intact mitochondria without disturbing the structure of the mitochondrial inner membrane which may play an important role in the expression of hormone effects (Hulbert *et al.*, 1976; Armston *et al.*, 1982). The results of the experiments described in the present paper show that thyroid hormone stimulates mitochondrial oxidative phosphorylation by simultaneously activating the bc_1-complex, the adenine nucleotide translocator and, possibly, succinate dehydrogenase.

Experimental

Hormonal treatment of rats

Fed male Wistar rats (200–250g) were used with or without pretreatment with PTU [added to the drinking water at 0.05% (w/v) for 15–20 days] to induce hypothyroidism (Laker & Mayer, 1981). The treatment with PTU resulted in a decrease in the growth-rate from 3.78 ± 0.37 g/day (mean ± S.E.M. for four untreated rats) to 0.73 ± 0.09 g/day ($n = 8$), and to a decrease in levels of circulating T_3 and T_4 from 1.33 ± 0.07 pmol/ml ($n = 4$) to 0.40 ± 0.06 pmol/ml ($n = 2$) in the case of T_3, and from 54.5 ± 1.4 pmol/ml ($n = 4$) to 3.5 ± 0.5 pmol/ml ($n = 2$) in the case of T_4.

For treatment of euthyroid rats with thyroid hormone, a single dose of T_4 (0.8mg/100g body wt.) was injected intraperitoneally. Control animals received vehicle (0.01M-NaOH) only.

For thyroid hormone treatment of rats pretreated with PTU, a single dose of T_3 (40μg/100g body wt.) was injected intraperitoneally. Control animals received vehicle (0.01M-NaOH) only. After 24h, the rats were decapitated and mitochondria were isolated from the livers.

Isolation and incubation of mitochondria

Liver mitochondria were isolated essentially as described by Hoogeboom (1955) using an isolation medium containing 250mM-mannitol, 2mM-Mops and 1mM-EGTA. The pH of the medium was adjusted to pH7.0 with Tris base. Mitochondria were isolated from liver homogenates (about 20%, w/v) by centrifugation at 800*g* for 5min and subsequent centrifugation of the supernatant at 6000*g* for 10min. The pellet of the first spin was homogenized and centrifuged again to improve the yield. The mitochondria were washed once by suspension in isolation medium and re-centrifugation at 10000*g* for 10min. The pellets were taken up in 6–8ml of isolation medium after removal of the fluffy layer, and stored on ice. In our experiments, no decrease in the activity of the mitochondria was found upon storage on ice for up to 8h, in contrast to reports of some other investigators (Siess & Wieland, 1978; Hoch, 1982).

Mitochondria (0.7–1.0mg of protein/ml) were incubated at 26°C in an oxygraph vessel equipped with a Clark-type electrode in a standard incubation medium containing 100mM-KCl, 25mM-Mops, 10mM-$MgCl_2$, 1mM-EGTA, 10mM-potassium phosphate, 20mM-succinate, 2mM-malate, 20mM-glucose, 1mM-ATP and 0.5μg of rotenone/ml. The final pH was adjusted to 7.0 with Tris base.

After an equilibration period of 1min, respiration was stimulated by the addition of hexokinase (dialysed against 10mM-potassium phosphate, pH7.0) or ADP (0.5mM). Inhibitors were added when the rate of oxygen uptake was constant (60–90s after the addition of hexokinase). The concentration of oxygen in the medium and the rate of removal of oxygen from the medium were recorded simultaneously. Rates of oxygen uptake were calculated by using the oxygen solubility data described by Hinkle & Yu (1979).

Determination of flux control coefficients of different steps in mitochondrial oxidative phosphorylation

The procedure used for the calculation of the flux control coefficients was that described previously by Groen *et al.* (1982). Carboxyatractyloside, phenylsuccinate, antimycin A and azide were used as specific inhibitors of the adenine nucleotide translocator, the dicarboxylate carrier, the bc_1-complex and cytochrome *c* oxidase, respectively. Since the incubations were carried out at pH7.0 instead of 7.4, the value used for the apparent K_i for total azide was 40μM (cf. Groen *et al.*, 1982). In the calculation of the flux control coefficient of the bc_1-complex it was assumed that antimycin A acts as an irreversible inhibitor (Berden & Slater, 1972).

Enzyme assays

Glutamate dehydrogenase, malate dehydrogenase and succinate dehydrogenase were measured in mitochondrial suspensions after freeze–thawing. Glutamate dehydrogenase activity and malate dehydrogenase activity were measured in the presence of detergent as described by Schmidt (1974) and Martin & Denton (1970), respectively. Succinate dehydrogenase activity was determined after repeated freeze–thawing according to the method of Arrigoni & Singer (1962) in a medium containing 50mM-potassium phosphate, 1.6mM-phenazine methosulphate, 75μM-dichloroindophenol, 1mM-KCN, 0.1mM-EDTA and 20mM-succinate at pH7.4. Cytochrome *c* oxidase activity in freshly isolated mitochondria was estimated by measuring the rate of oxygen uptake at pH7.4 in the presence of 0.25% (w/v) Tween-80, 20μM-cytochrome *c*, 10mM-ascorbate, 50mM-Tris/HCl

and 1 mM-EDTA. The assay was started by the addition of 1 mM-*NNN'N'*-tetramethyl-*p*-phenylenediamine from a freshly prepared stock solution (pH about 5) kept in the dark. Autoxidation of tetramethylphenylenediamine accounted for less than 2% of the observed rates.

Determinations

Determination of the extramitochondrial ATP/ADP ratio was carried out as described by Wanders *et al.* (1984). In the experiments in which the extramitochondrial ATP/ADP ratio was measured, the concentration of ATP added to the incubation medium was increased to 2 mM.

Determination of the membrane potential in isolated rat-liver mitochondria was carried out by using the lipophilic cation $TPMP^+$ (Rottenberg, 1979). In short, mitochondria (about 2 mg/ml) were incubated under a gas atmosphere of 100% O_2 in the presence of different concentrations of hexokinase in the standard incubation medium from which glucose had been omitted. After 1 min of incubation, glucose (final concn. 20 mM) was added from a concentrated stock solution. After a further 2 min of incubation, 0.14 μM-[^{3}H]TPMP (0.5 μCi/ml) was added. The distribution of TPMP was determined after 2 min of incubation in the presence of [^{3}H]TPMP by centrifugation of the mitochondria through silicone oil into a layer of 14% (w/v) $HClO_4$. In a parallel incubation, the amount of adhering water was determined by using [^{14}C]sucrose (0.25 μCi/ml) and ^3H_2O (5 μCi/ml). Radioactivity in the samples was corrected for cross-over and quenching.

Protein was determined by a biuret method (Cleland & Slater, 1953), using egg albumin as standard.

Materials

Mannitol was supplied by Merck (Darmstadt, Federal Republic of Germany). T_3, PTU, carboxyatractyloside and azide were from Sigma (St. Louis, MO, U.S.A.). L-Thyroxine (T_4) was supplied by Koch-Light Laboratories (Colnbrook, Bucks., U.K.) and phenylsuccinate and duroquinol by K&K Laboratories (Plainview, NY, U.S.A.). Antimycin A was from the Nutritional Biochemicals Corp. (Cleveland, OH, U.S.A.), FCCP was a gift from Dr. P. G. Heytler (E. I. du Pont de Nemours, Wilmington, DE, U.S.A.). Oligomycin, nucleotides and enzymes were purchased from Boehringer (Mannheim, Federal Republic of Germany). [^{3}H]TPMP was from New England Nuclear (Boston, MA, U.S.A.) and ^3H_2O and [^{14}C]sucrose were from The Radiochemical Centre (Amersham, Bucks., U.K.). All other reagents were of analytical grade.

Results

Effect of thyroid hormone treatment on State-3 respiration in isolated rat-liver mitochondria

In preliminary experiments, we used the protocol of Shears & Bronk (1979) to study the effect of thyroid hormone on mitochondrial respiration. Treatment of euthyroid rats with a single, high dose of T_4 (0.8 mg/100 g body wt.) resulted in a stimulation of State-3 respiration of about 20% in liver mitochondria isolated 24 h after injection (Table 1, lines 1 and 2).

Since this response was small and rather variable, the experiments were repeated with rats which had been made hypothyroid by adding PTU to the drinking water (Laker & Mayer, 1981). In the mitochondria from these rats the rate of State-3

Table 1. *Effects of thyroid hormone treatment on respiration in isolated rat-liver mitochondria*

Treatment of rats, isolation of liver mitochondria and the conditions for measurement of oxygen consumption are described in the Experimental section. State-3 respiration refers to the rate of oxygen uptake in the presence of added ADP (0.5 mM), State-4 respiration refers to the rate of oxygen uptake in the absence of added ADP. Values given are means ± S.E.M. with the number of observations in parentheses. The statistical significance of the results obtained with mitochondria from hormone-treated rats and control rats calculated by using Student's *t*-test are: *$P<0.05$; **$P<0.025$; ***$P<0.0005$.

Status of donor rats	Respiration (ng-atom of O/min per mg of protein) State-3	State-4
Euthyroid	305 ± 10 (6)	53 ± 3 (6)
Euthyroid + T_4 (24 h)	360 ± 18 (6)**	62 ± 4 (6)*
Hypothyroid	227 ± 4 (12)	33 ± 1 (12)
Hypothyroid + T_3 (24 h)	338 ± 7 (12)***	46 ± 2 (12)***

Table 2. *Enzyme activities in isolated rat-liver mitochondria from hypothyroid rats*
Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and measurement of mitochondrial marker enzymes are described in the Experimental section. Values given are means ± S.E.M. with the number of observations in parentheses. The statistical significance of the results calculated using Student's *t*-test were all $P > 0.05$.

Enzyme	Activity in mitochondria from: Hypothyroid rats	T_3-treated rats
Glutamate dehydrogenase (μmol/min per mg of protein)	1.61 ± 0.06 (9)	1.69 ± 0.07 (9)
Malate dehydrogenase (μmol/min per mg of protein)	8.67 ± 0.45 (9)	9.01 ± 0.47 (9)
Succinate dehydrogenase (μmol/min per mg of protein)	0.178 ± 0.016 (6)	0.183 ± 0.006 (5)
Cytochrome *c* oxidase (ng-atom of O/min per mg of protein)	615 ± 17 (6)	661 ± 26 (5)

Table 3. *Effect of thyroid hormone treatment on the distribution of control during State-3 respiration in isolated rat-liver mitochondria*
Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and determinations of the flux control coefficients of the different enzymes are described in the Experimental section. Values given are means ± S.E.M. with the number of observations in parentheses. The statistical significance of the differences between mitochondria from T_3-treated and untreated rats calculated by using Student's *t*-test for paired data are: *$P < 0.025$; **$P < 0.05$.

Step	Flux control coefficient in mitochondria from: Hypothyroid rats	T_3-treated rats
Adenine nucleotide translocator	0.18 ± 0.04 (3)	0.27 ± 0.05 (3)*
Dicarboxylate carrier	0.21 ± 0.02 (4)	0.34 ± 0.04 (4)*
bc_1-Complex	0.21 ± 0.02 (4)	0.14 ± 0.02 (4)*
Cytochrome *c* oxidase	0.14 ± 0.003 (3)	0.18 ± 0.005 (3)*
Sum of the measured flux control coefficients	0.74 ± 0.02 (3)	0.93 ± 0.08 (3)**

respiration was substantially lowered compared with that in mitochondria from euthyroid rats of the same age (Table 1, lines 3 and 1). Treatment with thyroid hormone for 24h reversed this effect completely (Table 1, lines 3 and 4). In these experiments, T_3 was used instead of T_4 in order to avoid possible interference of PTU with the conversion of T_4 into T_3 (Frumess & Larsen, 1975). The marked stimulation of State-3 respiration after T_3 treatment was not accompanied by an appreciable change in the levels of glutamate dehydrogenase, malate dehydrogenase, cytochrome *c* oxidase and succinate dehydrogenase (Table 2).

Effect of T_3 treatment on the distribution of control of mitochondrial respiration

In order to quantify the amount of control exerted by different steps on mitochondrial respiration we determined the flux control coefficients of these steps. This was achieved by carrying out inhibitor titrations. In control analysis the term flux control coefficient is now used instead of 'control strength' or 'sensitivity' (for a review of the new nomenclature see Westerhoff *et al.*, 1984). The effect of thyroid hormone treatment on the distribution of control during State-3 respiration in rat-liver mitochondria is shown in Table 3. Treatment with T_3 resulted in an increase in the flux control coefficients of the adenine nucleotide translocator, the dicarboxylate carrier and cytochrome *c* oxidase. In contrast, the amount of control exerted by the bc_1-complex decreased after thyroid hormone treatment, indicating that this step must have been activated. The values found for the bc_1-complex are somewhat higher than those found by Groen *et al.* (1982). The reason for this difference is as yet unclear. The activation within complex III of the respiratory chain was accompanied by an increase in the number of antimycin-binding sites (Fig. 1), as determined from the minimal amount of inhibitor required to obtain maximal inhibition of oxygen uptake. Treatment with T_3 increased the number of antimycin-binding sites from 27 ± 2 to 42 ± 4 pmol/mg of protein (means ± S.E.M. for four mitochondrial preparations in each experimental group). It also

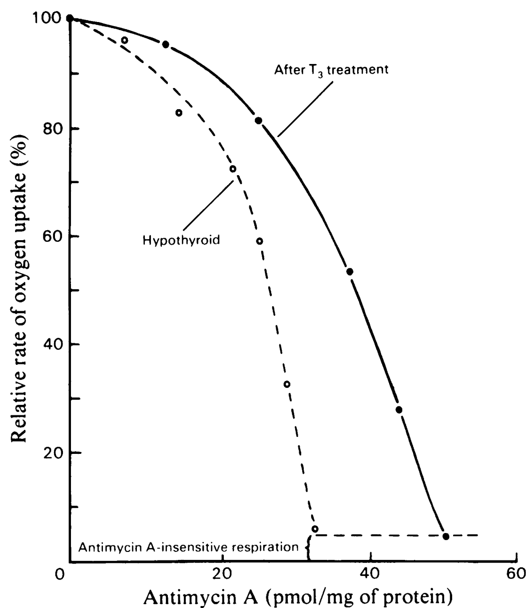

Fig. 1. *Effect of thyroid hormone treatment on the inhibition by antimycin A of State-3 respiration in rat-liver mitochondria*

Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and conditions for incubation of the mitochondria are described in the Experimental section. State-3 respiration was initiated by adding a saturating amount of hexokinase. Different amounts of antimycin A (indicated in the Figure) were added 60–90 s after the addition of hexokinase. Rates of oxygen uptake in the presence of inhibitor were expressed as a percentage of the rate in the absence of inhibitor. The experiment shown is representative of four different experiments. ○, mitochondria from hypothyroid rats; ●, mitochondria from hypothyroid rats isolated 24h after treatment with T_3.

caused a marked stimulation of State-3 respiration with duroquinol, which donates its reducing equivalents directly to complex III (Guerrieri & Nelson, 1975), as respiratory substrate [from 198 ± 18 to 382 ± 6 ng-atom of O/min per mg of protein ($n = 3$)].

The results shown in Table 3 indicate that activation of the bc_1-complex was not the only effect of the hormone treatment. The sum of the flux control coefficients of the enzymes measured was significantly higher after T_3 treatment (0.74 ± 0.02 compared with 0.93 ± 0.08). This indicates a decrease in the flux control coefficient of at least one enzyme which was not measured in these experiments, since the sum of all flux control coefficients in a linear pathway is equal to 1.0 (Kacser & Burns, 1973; Heinrich & Rapoport, 1974*a*). A possible candidate is succinate dehydrogenase. Although the $V_{max.}$ of succinate dehydrogenase (measured as the rate of reduction of dichloroindophenol mediated by phenazine methosulphate in freeze-thawed extracts) did not change after treatment for 24h with T_3 (Table 2), it is possible that in intact mitochondria the activity of the enzyme or the affinity towards succinate was increased. This has been observed in submitochondrial particles after treatment of hypophysectomized rats with T_4 for 7 days (Maddaiah *et al.*, 1981).

Additional effects of T_3 treatment on mitochondrial oxidative phosphorylation

The data shown in Table 3 were obtained from experiments in which mitochondria were incubated under State-3 conditions. Since the distribution of control of mitochondrial respiration is strongly dependent on the rate of respiration (Groen *et al.*, 1982), we also investigated the effect of the hormone at lower rates of respiration. In agreement with other reports (Nishiki *et al.*, 1978; Shears & Bronk, 1979), it was found that also State-4 respiration was very much dependent on the thyroid status of the rats (Table 1). Since under State-4 conditions almost all control of mitochondrial respiration is exerted by the passive permeability of the mitochondrial inner membrane to protons, it can be expected that the stimulation of State-4 respiration by thyroid hormone is caused by changes in this parameter, as already pointed out by Shears (1980). The passive permeability of the mitochondrial inner membrane to protons, i.e. the proton leakage pathway, can be quantified by carrying out a titration with uncoupler as described previously by Groen *et al.* (1982). The activity of the proton leakage pathway, expressed as an amount of uncoupler, increased by about 30% after treatment of hypothyroid rats with T_3 (Fig. 2), almost fully accounting for the stimulation of State-4 respiration. From these titrations no conclusions can be drawn about the process responsible for proton leakage that is affected by the hormone (cf. Shears & Bronk, 1981). It is not due to a higher adhering ATPase activity in the mitochondria from the rats treated with T_3, since oligomycin was present in these experiments.

The effect of thyroid hormone was also investigated at intermediate rates of respiration, obtained by adding limiting amounts of hexokinase in the presence of glucose (Fig. 3). Respiration was stimulated by a constant increment on going from State 4 up to 80% of State-3 respiration, so that the percentage of stimulation by thyroid hormone treatment was much smaller at intermediate rates of respiration than under State-4 or State-3 conditions. For instance, at about 70% of maximal

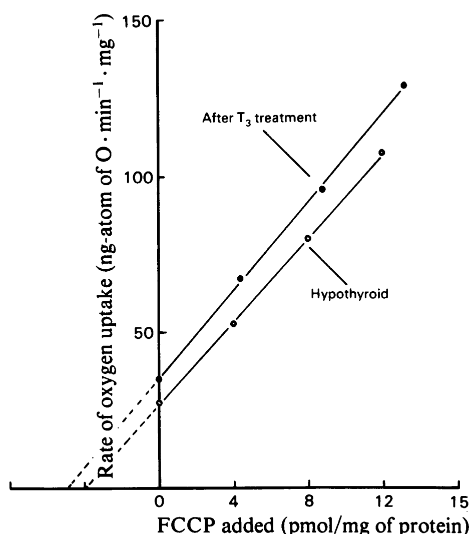

Fig. 2. *Effect of thyroid hormone treatment on the passive permeability to protons of the mitochondrial inner membrane*
Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and conditions for incubation of the mitochondria are described in the Experimental section. Oligomycin (1 μg/ml) was added to the incubation medium. Respiration in mitochondria from hypothyroid rats (○) and from hypothyroid rats treated with T_3 (●) was stimulated by titrating with FCCP. The intercept on the abscissa gives the amount of FCCP corresponding to the passive permeability to protons. Results of a typical experiment are shown. In four different experiments this amount of FCCP was 4.48 ± 0.36 pmol/mg of protein for mitochondria from hypothyroid rats and 5.76 ± 0.54 pmol/mg of protein for mitochondria from hypothyroid rats treated with T_3 ($P < 0.005$ by Student's *t*-test).

respiration in mitochondria from hypothyroid rats, the simulation by thyroid hormone treatment was about 10%. This can be explained by the high control on mitochondrial respiration exerted by hexokinase at intermediate rates of respiration (Groen *et al.*, 1982). Stimulation of other steps then has little effect on the flux.

At first sight, the results of the experiment depicted in Fig. 3 suggest that the effect of thyroid hormone on mitochondrial oxidative phosphorylation is not physiologically relevant, since in the intact tissue the mitochondria operate under conditions between State 4 and State 3. However, measurement of the extramitochondrial ATP/ADP ratio at different rates of respiration indicates that the effect of thyroid hormone treatment may yet prove to be important for the physiological situation. After T_3 treatment the curve relating oxygen uptake and the extramito-

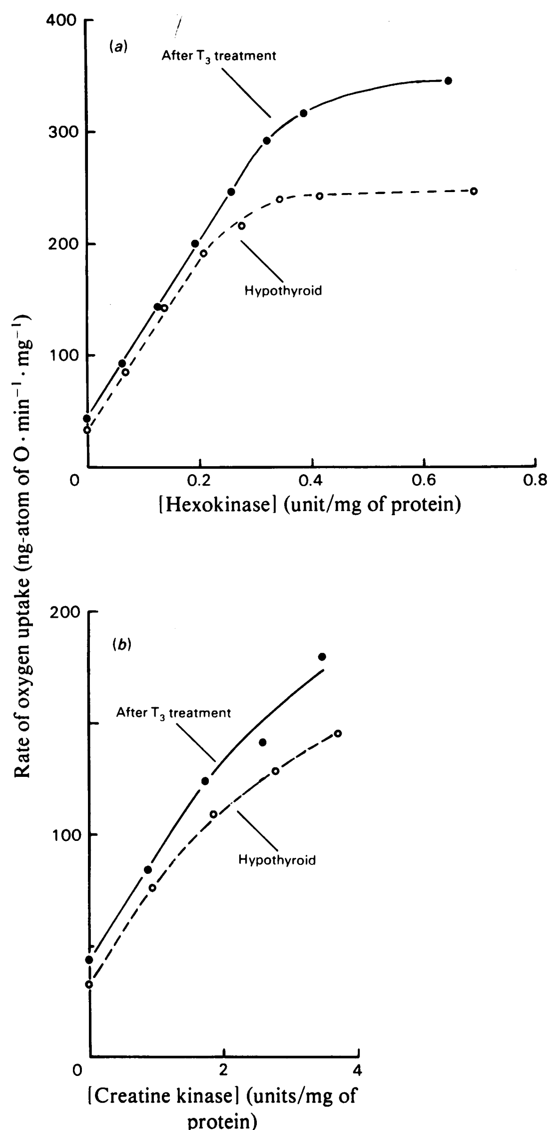

Fig. 3. *Effect of thyroid hormone treatment on mitochondrial respiration in the presence of (a) glucose plus hexokinase or (b) creatine plus creatine kinase*
Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and conditions for incubation of the mitochondria are described in the Experimental section. (*a*) Rates of oxygen uptake in mitochondria from hypothyroid rats (○) and from hypothyroid rats treated with T_3 (●) in the presence of 20 mM-glucose, 2 mM-ATP and different amounts of hexokinase as indicated in the Figure. (*b*) Rates of oxygen uptake in mitochondria from hypothyroid rats (○) and from hypothyroid rats treated with T_3 (●) in the presence of 20 mM-creatine, 0.5 mM-ATP and different amounts of creatine kinase as indicated in the Figure. The experiment shown is representative of three different experiments. Enzyme units are μmol/min.

chondrial ATP/ADP ratio was shifted to higher values of $(ATP/ADP)_{out}$ (Fig. 4). The same phenomenon is observed when the rate of phosphorylation, measured as the rate of glucose 6-phosphate formation, is plotted against the extramitochondrial ATP/ADP ratio, since the P/O ratio was not affected by thyroid hormone treatment (results not shown). This is in agreement with the results of several investigators (Bronk, 1963; Tata *et al.*, 1963; Chen & Hoch, 1977; Nishiki *et al.*, 1978) but in contrast to reports from Mowbray and coworkers (Palacios-Romero & Mowbray, 1979; Corrigall *et al.*, 1984). The increase in the extramitochondrial ATP/ADP ratio does not, however, result in a substantial increase in the flux, since under the experimental conditions used hexokinase is relatively insensitive to changes in the concentrations of ATP and ADP (Wanders *et al.*, 1984). When creatine kinase, which is much more sensitive to changes in the ATP/ADP ratio (Wanders *et al.*, 1984), plus creatine was used as the ADP-regenerating system, stimulation of mitochondrial respiration by the hormone treatment was about 20%, even at intermediate rates of respiration (Fig. 3).

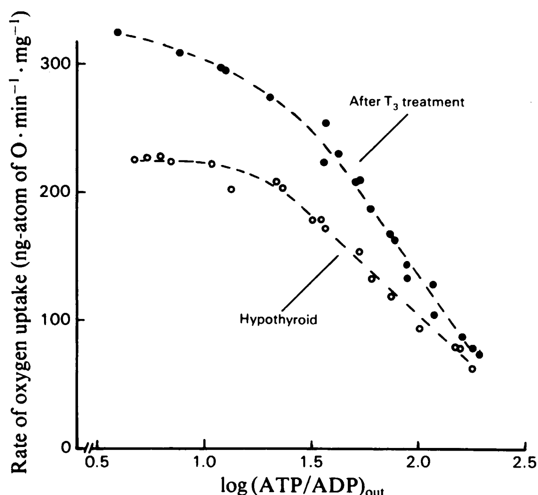

Fig. 4. *Effect of thyroid hormone treatment on the extramitochondrial ATP/ADP ratio at different rates of respiration*

The experimental conditions were as described in the legend to Fig. 3(*a*). After 2min of incubation in the presence of different amounts of hexokinase, a sample of the incubation mixture was quenched with an equal volume of ice-cold $HClO_4$ (final concn. 3.5%, w/v). Further handling of the samples is described in the Experimental section. Results with mitochondria from hypothyroid rats (○) and from hypothyroid rats treated with T_3 (●) were taken from three experiments.

Rate of mitochondrial oxygen uptake and the extramitochondrial ATP/ADP ratio

It can be postulated that the changed shape of the curve relating oxygen uptake and the extramitochondrial ATP/ADP ratio after hormone treatment is a consequence of the same activating effects on enzymes in this pathway that caused the increase in State-3 respiration, i.e., the activating effects on the bc_1-complex and, possibly, succinate dehydrogenase. The difference cannot be due to the increase in proton leakage observed after T_3 treatment (Fig. 2); when proton leakage in mitochondria from hypothyroid rats was increased by adding FCCP in the presence of hexokinase plus glucose, oxygen uptake was stimulated with a concomitant decrease in the extramitochondrial ATP/ADP ratio (results not shown).

The increase in the extramitochondrial ATP/ADP ratio at a particular rate of respiration could in principle be a reflection of an increased membrane potential, since the transport of adenine nucleotides across the mitochondrial inner membrane is electrogenic (LaNoue *et al.*, 1978). Shears & Bronk (1979) measured the membrane potential in liver mitochondria 24h after treatment of euthyroid rats with T_4. They observed an increase under State-4 conditions with no apparent difference under State-3 conditions. Fig. 5 shows that in our experiments at intermediate rates of

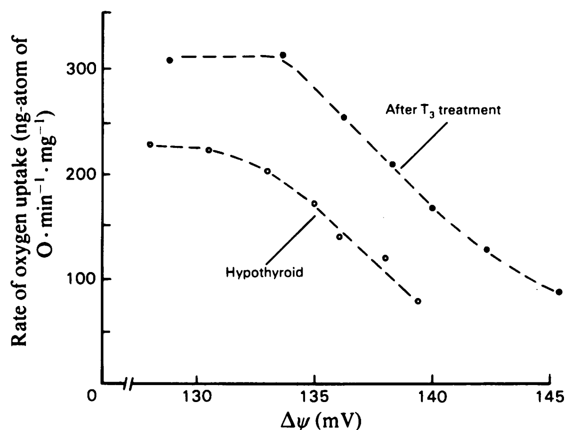

Fig. 5. *Effect of thyroid hormone treatment on the membrane potential in mitochondria at different rates of respiration*

Hormonal treatment of hypothyroid rats, isolation of liver mitochondria and determination of the membrane potential are described in the Experimental section. Rates of oxygen uptake in mitochondria from hypothyroid rats (○) and from hypothyroid rats treated with T_3 (●) in the presence of different amounts of hexokinase were measured in parallel incubations.

respiration the membrane potential, estimated from the distribution of $TPMP^+$ (Rottenberg, 1979), was about 5mV higher after thyroid hormone treatment. However, especially at relatively high rates of respiration, the difference in the extramitochondrial ATP/ADP ratio would predict a difference of about 11mV in the membrane potential. This can be calculated using the following formula:

$$\Delta G_T = F \cdot \Delta\psi + 2.3 \log \frac{[ATP]_{out}/[ADP]_{out}}{[ATP]_{in}/[ADP]_{in}} \quad (1)$$

in which ΔG_T is the free-energy difference of adenine nucleotide translocation. In this calculation it was assumed that the hormone treatment had not affected the activity of the adenine nucleotide translocator (and thereby ΔG_T; see also the Discussion section). An increase in the intramitochondrial ATP/ADP ratio of 15% induced by the hormone (results not shown) was also taken into account.

If, as postulated above, the effect of thyroid hormone on the relationship between oxygen uptake and the extramitochondrial ATP/ADP ratio is due only to the activating effects within the respiratory chain, it should be possible to reverse the change brought about by the hormone treatment *in vitro* by decreasing the activity of the respiratory chain. For this purpose we used phenylsuccinate to decrease the intramitochondrial concentration of the respiratory substrate, succinate. The concentration of phenylsuccinate used was sufficient to reverse the hormone-induced stimulation of mitochondrial respiration measured in the presence of 0.3 μM-FCCP (results not shown). This concentration of phenylsuccinate partially reversed the effect of the hormone treatment on the curve relating oxygen uptake and extramitochondrial ATP/ADP ratio (Fig. 6). In particular, the change in the slope of the curve at intermediate rates of respiration was reversed only partially. Partial inhibition of succinate oxidation therefore seems insufficient to reverse the effect of the hormone treatment *in vitro*.

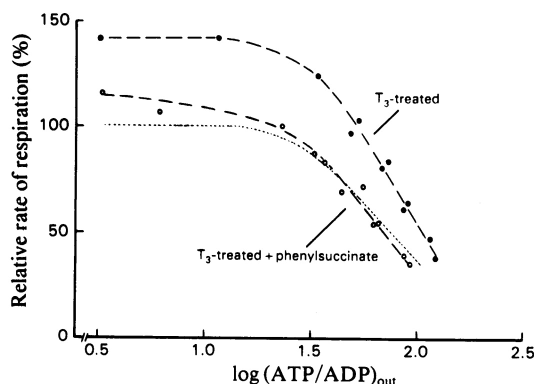

Fig. 6. *Effect of phenylsuccinate on the extramitochondrial ATP/ADP ratio at different rates of respiration in mitochondria isolated 24h after treatment with thyroid hormone*

The experimental conditions were as described in the legend to Fig. 4, except that with mitochondria from hypothyroid rats treated with T_3 the extramitochondrial ATP/ADP ratio was determined in the presence (○) and absence (●) of 14mM-phenylsuccinate. The dotted line indicates the curve relating oxygen uptake and the extramitochondrial ATP/ADP ratio in mitochondria from untreated hypothyroid rats (experimental points are omitted for the sake of clarity). Results were taken from two separate experiments.

Discussion

This paper describes the application of control analysis to locate the effects exerted by a hormone on mitochondrial oxidative phosphorylation. It was observed that the effect of thyroid hormone treatment was much more pronounced in hypothyroid rats than in euthyroid rats. Control analysis applied to experiments with liver mitochondria incubated under State-3 conditions clearly indicates that thyroid hormone activated at least two steps: the antimycin-sensitive step in the bc_1-complex and at least one other enzyme, possibly succinate dehydrogenase.

It has been shown in several studies that the activity of the adenine nucleotide translocator is stimulated after thyroid hormone treatment (Babior *et al.*, 1973; Hoch, 1977; Mak *et al.*, 1983). Activation of the adenine nucleotide translocator would result in an increase in the difference between the ATP/ADP ratio inside and outside the mitochondria, since the adenine nucleotide translocator is out of equilibrium already at 30% of State-3 respiration (Wanders *et al.*, 1981). Such an activation would result in a decrease in the ΔG of adenine nucleotide translocation and could thus explain the apparent discrepancy between the hormone-induced changes in the membrane potential and the extramitochondrial ATP/ADP ratio [see eqn. (1) in the Results section]. Furthermore, our experiments show that in mitochondria from rats treated with T_3, the adenine nucleotide translocator operates at a higher extramitochondrial ATP/ADP ratio when the same flux through the translocator is induced by the addition of hexokinase. This indicates that there is an increase in the affinity of the adenine nucleotide translocator towards extramitochondrial ADP. The increase in the flux control coefficient of the adenine nucleotide translocator observed after thyroid

hormone treatment (Table 3) cannot be taken as evidence against activation of the adenine nucleotide translocator, since the flux control coefficient of an enzyme is dependent not only on the properties of the enzyme in question, but also on the properties of the other enzymes in the pathway.

It has been proposed that changes in the structure of the mitochondrial inner membrane play a key role in the effects of thyroid hormone on mitochondrial oxidative phosphorylation (Hulbert *et al.*, 1976; Hoch, 1977; Hulbert, 1978). In this respect, it is of interest to note that State-3 respiration in mitochondria from hypothyroid rats with duroquinol as respiratory substrate was lower than when succinate was used. In contrast, in mitochondria from rats treated with T_3, State-3 respiration with duroquinol was higher than with succinate. These results suggest that thyroid hormone treatment leads to an increase in the permeability of the mitochondrial inner membrane for duroquinol.

In conclusion, the results of our experiments show that thyroid hormone stimulates mitochondrial respiration by affecting several steps in the pathway. Indeed, since the control of mitochondrial respiration is distributed among a number of steps, effective stimulation of this pathway by thyroid hormone treatment can only occur if several enzymes are activated simultaneously. It would be of interest to know whether this conclusion also holds for the effect of other hormones.

We thank Mr. T. Hendrix and Ms. J. Bouma for expert handling of the animals, Mr. J. Boot for carrying out some of the preliminary experiments, Drs. E. Enders and R. Hartongh of the Department of Endocrinology, University of Amsterdam, for carrying out the analysis of circulating levels of T_3 and T_4, Drs. A. J. Meijer and P. J. A. M. Plomp for critical comments on the manuscript, Dr. J. A. Berden and Professor E. C. Slater for helpful discussions, and Ms. G. E. E. van Noppen for her help in preparing the manuscript. This study was supported by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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