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# Thyroid-hormone control of state-3 respiration in isolated rat liver mitochondria

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Oxidative phosphorylation can be treated as two groups of reactions; those that generate protonmotive force (dicarboxylate carrier, succinate dehydrogenase and the respiratory chain) and those that consume protonmotive force (adenine nucleotide and phosphate carriers, ATP synthase and proton leak). Mitochondria from hypothyroid rats have lower rates of respiration in the presence of ADP (state 3) than euthyroid controls. We show that the kinetics of the protonmotive-force generators are unchanged in mitochondria from hypothyroid animals, but the kinetics of the protonmotive-force consumers are altered, supporting proposals that the important effects of thyroid hormone on state 3 are on the ATP synthase or the adenine nucleotide translocator.

## INTRODUCTION

Liver mitochondria from hypothyroid rats respire at a slower rate than mitochondria from euthyroid rats in both the presence and the absence of ADP (reviewed by Brand & Murphy, 1987). We have previously shown that, in the presence of oligomycin to prevent phosphorylation of ADP, the decreased respiration rate of mitochondria isolated from hypothyroid rats (compared with euthyroid rats) is due to a decreased proton conductance of the mitochondrial inner membrane (Hafner *et al.*, 1988, 1989). However, how thyroid hormone controls the rate of state-3 respiration (nomenclature of Chance & Williams, 1955) remains uncertain. Various workers have reported altered kinetics of the adenine nucleotide translocator (Babior *et al.*, 1973; Portnay *et al.*, 1973; Hoch, 1977; Mak *et al.*, 1983; Verhoeven *et al.*, 1985), changes in $Mg^{2+}$-dependent ATPase activity of isolated mitochondria (Chen & Hoch, 1977; Maddiah *et al.*, 1981; Clot & Baudry, 1982) and changes in the mitochondrial respiratory chain and dehydrogenases (Bronk, 1966; Maddiah *et al.*, 1981; Clot & Baudry, 1982; Verhoeven *et al.*, 1985; Horrum *et al.*, 1985, 1986) under different thyroid states. It has yet to be demonstrated which of the reported changes in the mitochondrial respiratory chain and phosphorylation system are responsible for the changes in state-3 respiration rate under different thyroid states.

In the present paper we use a kinetic method to determine the site of thyroid hormone action on mitochondria. We measure the kinetics of the sum of the reactions that generate $\Delta p$ and the kinetics of the sum of the reactions that consume $\Delta p$. We show that the kinetics of the $\Delta p$ generators are unchanged, but the consumers are inhibited in mitochondria from hypothyroid rats, compared with mitochondria from euthyroid rats.

## EXPERIMENTAL

Hypothyroid rats were prepared by parathyroidthyroidectomy as described in Hafner *et al.* (1988). Preparation of mitochondria from fed hypothyroid and euthyroid sham-operated littermate rats followed the method of Chappell & Hansford (1972) as described in Hafner *et al.* (1988). Mitochondrial protein was measured by the biuret method (Gornall *et al.*, 1949). Mitochondrial membrane potential was determined from the distribution of the lipophilic cation $Ph_3MeP^+$ by using a $Ph_3MeP^+$ electrode as described in Brown & Brand (1985). Respiration rate was determined simultaneously with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) by using the arrangement described in Hafner *et al.* (1988). A $Ph_3MeP^+$ binding correction was determined as in Brown & Brand (1988). The value obtained was 0.54 for mitochondria from both hypothyroid and euthyroid rats.This may have led to a slight underestimate of the membrane potential in mitochondria from hypothyroid rats by about 5 mV (cf. Hafner *et al.*, 1988). Mitochondrial volume and $\Delta$pH were determined from the distributions of $^3H_2O$ and [$^{14}$C]sucrose and of [$^3$H]acetate and [$^{14}$C]sucrose respectively as described in Brown & Brand (1985). Air-saturated medium was assumed to contain 475 nmol of O/ml at 25 °C (Reynafarje *et al.*, 1985). Experimental design and the sources of materials were as indicated in Hafner *et al.* (1988). In addition, ADP [di(monocyclohexylammonium) salt] and hexokinase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

## THEORY

We can consider state-3 respiration to consist of two component systems, those that generate $\Delta p$ (the respiratory chain, succinate dehydrogenase and the dicarboxylate carrier) and those that consume $\Delta p$ (the ATP synthase, adenine nucleotide translocator, phosphate transporter and proton leak). The two component systems interact via the protonmotive force, which we assume to be entirely delocalized. Stimulation of a step in

Abbreviations used: ADP/O, number of molecules of ADP phosphorylated per oxygen atom reduced; $\Delta\psi$, difference in electrical potential across the mitochondrial inner membrane; $\Delta p$, protonmotive force across the mitochondrial inner membrane; $\Delta$pH, pH gradient across the mitochondrial inner membrane; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $Ph_3MeP$, methyltriphenylphosphonium; $Me_4N$, tetramethylammonium.

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the system can only increase respiration rate if that step has a significant flux control coefficient over mitochondrial respiration rate. Under state-3 conditions, the control of mitochondrial respiration rate is shared between the respiratory chain (and associated reactions) and the phosphorylation system; the control by the proton leak is negligible (Groen *et al.*, 1982; Hafner *et al.*, 1990). Thyroid hormone could therefore potentially stimulate state-3 respiration by three mechanisms.

(*a*) Thyroid hormone could primarily increase the activity of the $\Delta p$ generators. It could do this by direct activation, or indirectly, by increasing their relative concentration. This would lead to a higher $\Delta p$ under state-3 conditions, causing secondary stimulation of the $\Delta p$ consumers.

(*b*) Thyroid hormone could primarily increase the activity of the $\Delta p$ consumers, either by direct activation or by increasing their relative concentration. This would lower the $\Delta p$ under state-3 conditions, causing a secondary activation of the $\Delta p$ generators.

(*c*) Thyroid hormone could increase the activity of both the $\Delta p$ generators and the $\Delta p$ consumers directly or indirectly. This could lead to an increase, a decrease or no change in $\Delta p$, depending on the relative size of the activation of each group.

These three possibilities can be distinguished by examining the relationship between respiration rate and $\Delta p$ under different thyroid states. The overall kinetic response of the $\Delta p$ generators to $\Delta p$ can be obtained from an uncoupler titration of respiration rate versus $\Delta p$, and the overall kinetic response of the $\Delta p$ consumers to $\Delta p$ can be obtained from an inhibitor titration of respiration rate versus $\Delta p$. This procedure has been used previously by us (Murphy & Brand, 1987; Brand *et al.*, 1988; Hafner *et al.*, 1990) and others (Westerhoff & Van Dam, 1987).

Thus we can investigate if thyroid hormone activates the overall reaction catalysed by the $\Delta p$ generators by comparing uncoupler titrations of mitochondria isolated from rats of different thyroid status. If thyroid hormone activates the $\Delta p$ generators, then a plot of mitochondrial respiration rate against $\Delta p$ obtained from an uncoupler titration of euthyroid-rat mitochondria will lie above the plot obtained with mitochondria from hypothyroid rats, such that at any given $\Delta p$ the respiration rate will be higher in mitochondria obtained from euthyroid rats than at the same $\Delta p$ in mitochondria from hypothyroid rats. Similarly we can investigate if thyroid hormone stimulates the $\Delta p$ consumers, by comparing inhibitor titrations of state-3 respiration. If thyroid hormone acts to stimulate the $\Delta p$ consumers, then a plot of respiration rate against $\Delta p$ obtained from an inhibitor titration of state-3 respiration of mitochondria from euthyroid rats will lie below the plot obtained for hypothyroid rats. If thyroid hormone does not stimulate the $\Delta p$ consumers, the two plots will be superimposable over most of their range, but the plot for mitochondria from euthyroid rats will extend further (towards higher respiration rates and $\Delta p$) than the plot for mitochondria from hypothyroid rats.

## RESULTS

Fig. 1(*a*) shows a plot of respiration rate against $\Delta\psi$ during a malonate titration of state-3 respiration for mitochondria from hypothyroid and euthyroid rats.

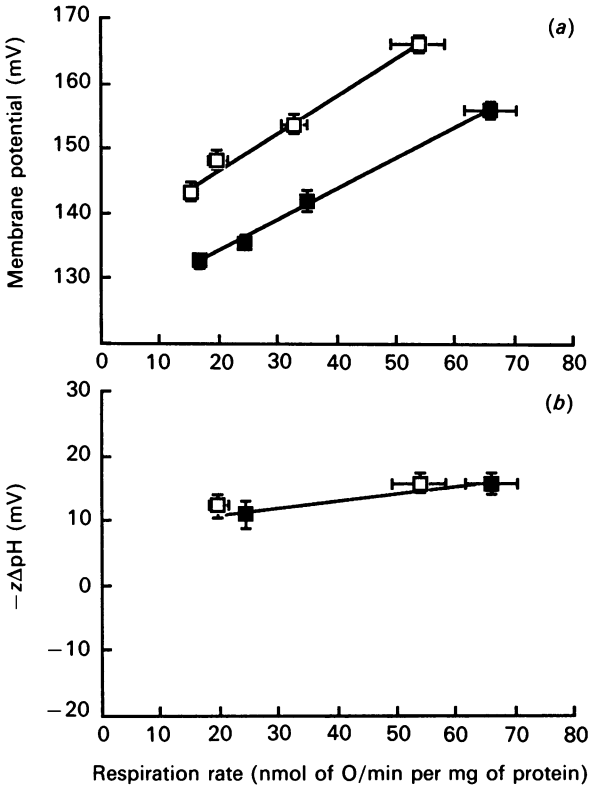


**Fig. 1. Malonate titration of state-3 respiration for mitochondria from hypothyroid (□) and euthyroid (■) rats**

(*a*) Relationship between respiration rate and $\Delta\psi$. Mitochondria (1 mg of protein/ml) were incubated in 5 ml of 100 mM-KCl/20 mM-glucose/20 mM-sucrose/10 mM-$P_i$/5 mM-Hepes/2 mM-$MgCl_2$/1 mM-EGTA/100 $\mu$M-acetate, pH 7.1 (with $Me_4NOH$) at 25 °C. The incubation medium was supplemented with 100 $\mu$M-ADP, 5 $\mu$M-rotenone and 12.5 units of hexokinase. Mitochondria were incubated in the medium for 4 min before addition of 4 $\mu$M-$Ph_3MePBr$ to calibrate the $Ph_3MeP^+$ electrode. Respiration was then initiated by addition of 5 mM-succinate (pH 7.1 with KOH). Respiration was titrated with sequential 0.25 mM additions of malonate (pH 7.1 with KOH). Volumes were constant at about 0.9 $\mu$l/mg of protein and not different for mitochondria from hypothyroid and euthyroid rats; $-z\Delta$pH was between 10 and 15 mV and not different between mitochondria from hypothyroid and euthyroid rats across the range of the malonate titration (*b*). Error bars represent S.E.M.for three independent experiments.

Under these conditions $-z\Delta$pH was between 10 and 15 mV and did not differ between mitochondria from hypothyroid and euthyroid rats (Fig. 1*b*). Thus $\Delta\psi$ is a good approximation to $\Delta p$. Fig. 1 shows that, at any given value of $\Delta\psi$ during a malonate titration of state-3 respiration, mitochondria from euthyroid rats have a higher respiration rate than do mitochondria from hypothyroid rats. The graph therefore demonstrates that thyroid hormone stimulates the $\Delta p$ consumers.

Stimulation of the $\Delta p$ consumers does not exclude simultaneous activation of the $\Delta p$ producers by thyroid hormone. The effect of thyroid hormone on the $\Delta p$ producers is examined in Fig. 2, which shows a plot of respiration rate against $\Delta\psi$ during an uncoupler titration for mitochondria from hypothyroid and euthyroid rats; $-z\Delta$pH varied between 0 and 10 mV and was not different in mitochondria from hypothyroid and euthy-

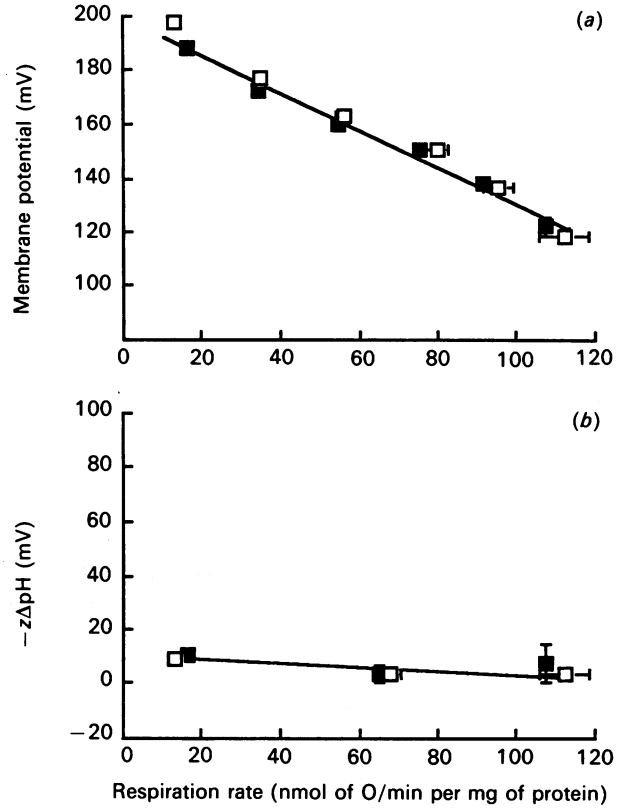


**Fig. 2. FCCP titration of mitochondria from hypothyroid (□) and euthyroid (■) rats**

(*a*) Relationship between respiration rate and $\Delta\psi$. Conditions were as for Fig. 1. In addition, of the medium was supplemented with 1 $\mu$g of oligomycin/ml. Respiration was titrated by sequential additions of 100 pmol of FCCP. Volume was constant at about 0.95 $\mu$l/mg of protein and not different between mitochondria from hypothyroid and euthyroid rats; $-z\Delta$pH fell from 10 mV to 3 mV across the range of the uncoupler titration in both sets of mitochondria (*b*). Error bars represent S.E.M. for three independent experiments.

roid rats (Fig. 2*b*). Fig. 2 shows that the uncoupler titrations for mitochondria from hypothyroid and euthyroid rats are superimposable, demonstrating that thyroid hormone does not significantly stimulate the overall reaction catalysed by the $\Delta p$ producers at rates of respiration between non-phosphorylating and state 3.

Any difference in uncoupled rates between mitochondria from hypothyroid and euthyroid rats must be due to changes in the kinetics of the $\Delta p$ generators, since there is no other reaction taking place in the system. Any such difference does not appear to be important under state-3 conditions.

## DISCUSSION

Our results show that thyroid hormone stimulates the state-3 respiration rate of isolated liver mitochondria by stimulating the $\Delta p$ consumers. Its small activation of the $\Delta p$ generators is not a significant factor.

Of the $\Delta p$ consumers (ATP synthase, adenine nucleotide translocator, phosphate transporter and proton leak), which is the most likely target of thyroid-hormone action? We have previously reported that mitochondria from hypothyroid rats have 3-fold less proton leak than do mitochondria from euthyroid rats. Under identical conditions the proton flux through the proton leak in euthyroid mitochondria in state 3 is balanced by a respiration rate of $3.7 \pm 0.5$ (S.E.M.) nmol of O/min per mg of protein (Hafner *et al.*, 1990). If we assume that hypothyroid-rat mitochondria have 3-fold less leak than this at the same value of $\Delta p$ (Hafner *et al.*, 1988, 1989), then the maximum difference in respiration rate at the same membrane potential, owing to the leak, is 2.5 nmol of O/min per mg. Under state-3 conditions Fig. 1 shows that mitochondria from euthyroid rats respire at 66 nmol of O/min per mg of protein. At the same membrane potential, mitochondria from hypothyroid rats respire at 34 nmol of O/min per mg of protein. Thus the difference in leak contributes approx. 2.5 out of a total difference in respiration rate of 32 nmol of O/min per mg of protein. An additional argument against a change in the proton flux through the leak being responsible for the change in kinetics of the $\Delta p$ consumers under state-3 conditions is that this would cause mitochondria from hypothyroid and euthyroid rats to have different ADP/O ratios. We have previously shown that such mitochondria have the same ADP/O ratios under state-3 conditions (Hafner & Brand, 1988).

This leaves the adenine nucleotide translocator, ATP synthase and phosphate transporter as potential candidates for thyroid-hormone action. The phosphate transporter has a very small flux control coefficient over the rate of state-3 respiration in isolated mitochondria (Kunz *et al.*, 1988), so this is an unlikely site of thyroid-hormone action. The kinetics of the adenine nucleotide translocator and the total amount of $Mg^{2+}$-dependent ATPase per mg of mitochondrial protein have both been reported to be controlled by thyroid hormone (Babior *et al.*, 1973; Portnay *et al.*, 1973; Chen & Hoch, 1977; Hoch, 1977; Maddiah *et al.*, 1981; Clot & Baudry, 1982; Mak *et al.*, 1983; Verhoeven *et al.*, 1985). In addition, changes in the kinetics of the adenine nucleotide translocator and ATP synthase could be secondary to changes in the matrix adenine nucleotide content.

We (Brand & Murphy, 1987; Hafner, 1987) have previously argued against the adenine nucleotide translocator as a site of thyroid-hormone action, on the grounds that its flux control coefficient falls in hypothyroidism (Holness *et al.*, 1984; Verhoeven *et al.*, 1985). We now realize that changes in the flux control coefficient of a step cannot be used to make statements about the site of hormone action, because the change in elasticities to $\Delta p$ of all the other system components is unknown (see also Crabtree & Newsholme, 1987). One observation that argues strongly against changes in the kinetics of the adenine nucleotide translocator being responsible for the difference in respiration rate of mitochondria from hypothyroid and euthyroid rats is that the kinetics of this step can be restored to euthyroid values in mitochondria from hypothyroid rats after tri-iodothyronine treatment without stimulating the respiration rate (Mowbray & Corrigall, 1984).

Preparation of submitochondrial particles should allow effects of thyroid hormone on the ATP synthase to be differentiated from effects on the adenine nucleotide translocator or adenine nucleotide content. However, there is no consensus view as to the effect of thyroid hormone on respiration rate in submitochondrial par-

ticles (cf. Chen & Hoch, 1977; Maddiah *et al.*, 1981).

Changes in adenine nucleotide content under different thyroid states could lead to secondary changes in the kinetics of the ATP synthase and adenine nucleotide translocator. The effect of thyroid hormone on mitochondrial adenine nucleotide content is uncertain (Hoch, 1977; Palacios-Romero & Mowbray, 1979; Seitz *et al.*, 1985).

Changes in the mitochondrial respiratory chain and dehydrogenases have also been reported in different thyroid states (Bronk, 1966; Maddiah *et al.*, 1981; Clot & Baudry, 1982; Verhoeven *et al.*, 1985; Horrum *et al.*, 1985, 1986), although the total activity of substrate dehydrogenases does not appear to change when assayed in broken mitochondria (Lee & Lardy, 1965; Verhoeven *et al.*, 1985). The data presented here demonstrate that changes in the dehydrogenase/chain intermediates do not contribute to the difference in state-3 respiration rate. This may be because the respiratory-chain components have insignificant flux control coefficients over state-3 respiration rate. Alternatively, the changes in the chain may be secondary to changes in $\Delta p$ inhibiting the cytochrome $bc_1$ complex more than cytochrome *c* oxidase (Brown & Brand, 1985; Murphy & Brand, 1987).

Shears & Bronk (1979) reported that mitochondria from 24 h-thyroxine-treated rats and euthyroid rats had the same $\Delta p$ under state-3 conditions, although they suggested that the effective $\Delta p$ required for ATP synthesis was lower in mitochondria from 24 h-thyroxine-treated rats. The measurement of $\Delta p$ by these workers has been discussed previously (Hafner *et al.*, 1988). Verhoeven *et al.* (1985) reported that mitochondria from hypothyroid and 24 h-tri-iodothyronine-treated hypothyroid rats had the same $\Delta\psi$ under state-3 conditions. These workers measured $\Delta\psi$ from the distribution of $[^3H]Ph_3MeP^+$ in the absence of carrier $Ph_3MeP^+$, which may be responsible for their different findings from ours. Both of these groups examined 24 h effects of thyroid hormone, whereas we have investigated longer-term effects. It remains possible that the actions of thyroid hormone at 24 h are different from its longer-term effects.

## Conclusion

In summary, this work has demonstrated that thyroid hormone stimulates the rate of state-3 respiration by stimulating the kinetics of the phosphorylation machinery rather than the respiratory chain. The work is consistent with reports in the literature that the adenine nucleotide translocator and the mitochondrial ATP synthase are sites of thyroid-hormone action, and indicates that actions of thyroid hormone on succinate dehydrogenase and the respiratory chain do not contribute to the stimulation of state-3 respiration under these conditions.

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