Effect of 3,5-di-iodo-L-thyronine on the mitochondrial energy-transduction apparatus

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We examined the effect of a single injection of 3,5-di-iodo-L-thyronine (3,5-T2) (150 μg/100 g body weight) on the rat liver mitochondrial energy-transduction apparatus. We applied 'top-down' elasticity analysis, which allows identification of the site of action of an effector within a metabolic pathway. This kinetic approach considers oxidative phosphorylation as two blocks of reactions: those generating the mitochondrial inner-membrane potential ($\Delta\Psi$; 'substrate oxidation') and those 'consuming' it ('proton leak' and 'phosphorylating system'). The results show that 1 h after the injection of 3,5-T2, state 4 (respiratory state in which there is no ATP synthesis and the exogenous ADP added has been exhausted) and state 3 (respiratory state in which ATP synthesis is at maximal rate) of mitochondrial respiration were significantly increased (by approx. 30%). 'Top-down' elasticity analysis revealed that these increases were due to the stimulation of reactions involved in substrate oxidation; neither 'proton leak' nor the 'phosphorylating system' was influenced by 3,5-T2. Using the same approach we divided the respiratory chain into two blocks of reactions: cytochrome *c* reducers and cytochrome *c* oxidizers. We found that both cytochrome *c* reducers and cytochrome *c* oxidizers are targets for 3,5-T2. The rapidity with which 3,5-T2 acts in stimulating the mitochondrial respiration rate suggests to us that di-iodo-L-thyronine may play an important role in the physiological conditions in which rapid energy utilization is required, such as cold exposure or overfeeding.

INTRODUCTION

The control of mitochondrial functions by thyroid hormones has been the subject of several studies. Thyroid hormones induce an increase in both oxygen consumption and heat production in many mammalian tissues, and this, in turn, results in an increase in the metabolic rate. Liver mitochondria isolated from hyperthyroid rats show faster state 4 (respiratory state in which there is no ATP synthesis and the exogenous ADP added has been exhausted) and state 3 (respiratory state in which ATP synthesis is at maximal rate) respiration rates, and liver mitochondria from hypothyroid rats show slower state 4 and state 3 respiration rates than mitochondria isolated from euthyroid rats [1,2]. These effects may be the consequence of the action of 3′,3,5-tri-iodo-L-thyronine on several cellular mechanisms, including: (a) an increase in the concentration of some components of the respiratory chain [3,4], (b) a stimulation of processes involved in the phosphorylation of ADP and export of ATP [5–9], which would affect mainly state 3 respiration [10], and (c) a stimulation of 'proton leak' across the mitochondrial inner membrane [11,12], which would affect mainly the state 4 respiration [10].

It has recently been shown that di-iodothyronines such as 3,3′-di-iodo-L-thyronine and 3,5-di-iodo-L-thyronine (3,5-T2) (together referred to as T2s) are able to rapidly stimulate states 4 and 3 of mitochondrial respiration and cytochrome *c* oxidase activity when injected into hypothyroid rats [13,14]. The finding that chronic treatment of hypothyroid rats with T2s does not induce an increase in mitochondrial protein content [13], together with the ability of 3,5-T2 to stimulate respiration rate in the presence of an inhibitor of protein synthesis [15], led us to hypothesize a direct action of T2s at the mitochondrial level. The existence of specific binding sites for T2s in mitochondria supports our hypothesis [16,17].

More recently, we have shown that the administration of T2s to hypothyroid rats both induces an increase in their resting metabolism [18] and improves their cold tolerance [19]. In view of the earlier results, we thought it interesting to investigate the cellular mechanism by which T2s stimulate both mitochondrial respiration and the metabolic rate of a rat.

In this paper we report the effect of a single injection of 3,5-T2 on oxidative phosphorylation in rat liver mitochondria and we apply 'top-down' elasticity analysis (for review see [20]), which allows the identification of the site(s) of action of an effector, such as 3,5-T2, within a metabolic pathway.

To identify where 3,5-T2 acts at the mitochondrial level, we studied (i) the kinetics of the sum of the reactions that generate the mitochondrial proton motive force (measured as membrane potential, $\Delta\Psi$) and the kinetics of the sum of the reactions that consume $\Delta\Psi$; (ii) the kinetics of the sum of the reactions able to reduce cytochrome *c* ('*c*-reducers') and those of the sum of the reactions able to oxidize cytochrome *c* ('*c*-oxidizers'). This was done in euthyroid control rats and euthyroid rats injected with a single dose of 150 μg/100 g body weight (b.w.) of 3,5-T2 (the latter group being referred to as the 3,5-T2 group). In addition, the flux control coefficients, which quantify the control exerted by each step over the flux in a pathway, were determined for '*c*-reducers' and '*c*-oxidizers' using mitochondria from euthyroid controls and from euthyroid rats injected with 3,5-T2.

EXPERIMENTAL

Hormonal treatment of rats and preparation of mitochondria

Male Wistar rats (Charles-Rivers, Lecco, Italy) weighing 270–300 g were used in this study. A commercial mash (Charles-Rivers, Lecco, Italy) was available *ad libitum* and the animals had free access to water. All experiments were performed in

Abbreviation used: 3,5-T2, 3,5-di-iodo-L-thyronine; T2s, 3,5-di-iodo-L-thyronine and 3,3′-di-iodo-L-thyronine; b.w., body weight.

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accordance with local and national guidelines covering animal experiments. Rats had received a single intraperitoneal injection of 150 μg/100 g b.w. of 3,5-T2 (3,5-T2 group) and were killed 1 h after the injection. Control rats were injected with vehicle (10 mM NaOH). Rats were anaesthetized by intraperitoneal administration of chloral hydrate (40 mg/100 g b.w.) and killed by decapitation. The livers were immediately removed and processed, liver mitochondria being prepared as described by Lanni et al. [13]. The kinetic responses of 'substrate oxidation', 'proton leak' and 'phosphorylating system' to a change in $\Delta\Psi$ was evaluated using fresh mitochondria. Other aliquots of mitochondria were stored at -20 °C. On the day of their use, the frozen mitochondria were thawed at room temperature, then kept on ice and used to evaluate the kinetic responses of '*c*-oxidizers' and '*c*-reducers' to the cytochrome *c* redox state. Protein concentration in the mitochondria was determined by the method of Hartree [21].

Measurement of protonmotive force and respiration rate

Protonmotive force is the sum of $\Delta\Psi$ and the pH gradient across the mitochondrial membrane. To simplify measurements, the pH gradient was abolished by the addition of nigericin so that the mitochondria increased $\Delta\Psi$ to compensate, and the whole of the protonmotive force was expressed as $\Delta\Psi$. $\Delta\Psi$ was determined from the distribution of the lipophilic cation triphenylmethylphosphonium (Ph_3MeP^+), which was measured using a Ph_3MeP^+-sensitive electrode, as described by Brown and Brand [22]. A Ph_3MeP^+-binding correction of 0.4 was applied for mitochondria from both the control and 3,5-T2 groups. Respiration rate was determined simultaneously using a Clark-type oxygen electrode. Mitochondrial respiration rate and membrane potential were measured in a mitochondrial suspension (0.5 mg/ml) at 30 °C in standard incubation medium A [80 mM KCl/50 mM Hepes (pH 7)/1 mM EGTA/5 mM K_2HPO_4/20 mM glucose/5 mM $MgCl_2$/4 μM rotenone/100 μM ADP/nigericin (80 ng/ml)/0.1 % (v/v) fatty-acid-free BSA] using a saturating amount of succinate as substrate (5 mM). Hexokinase and different inhibitors were added as described in the figure legends.

Measurement of cytochrome *c* redox state

A different standard incubation medium (B) was used for determination of the values for mitochondrial cytochrome *c* redox state and mitochondrial respiration rate that were used to evaluate the kinetic responses of '*c*-reducers' and '*c*-oxidizers' to the cytochrome *c* redox state. This medium contained 80 mM KCl, 50 mM Hepes, 1 mM EGTA, 5 mM K_2HPO_4, 4 μM rotenone, 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 5 mM succinate (pH 7).

The endogenous cytochrome *c* redox state was measured spectrophotometrically at 550–540 nm. The influence of membrane potential on the cytochrome *c* redox state was completely abolished by the freezing and subsequent thawing of the mitochondria and by the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the medium. The absorbance obtained when succinate was absent from the incubation medium was considered to represent the complete oxidation of cytochrome *c*; the absorbance value obtained in the presence of succinate and an excess of KCN (8 mM) was considered to represent its complete reduction. To permit the system to reach steady state, each incubation lasted 3 min; throughout this period the mitochondrial suspension (2 mg/ml) was vigorously stirred and its temperature was maintained at 30 °C.

Statistical analysis

Data were analysed using Student's *t* test. A P value of less than 0.05 was considered statistically significant.

Materials

3,5-T2 was from Henning Berlin (Berlin, Germany); all the other products used were from Sigma–Aldrich S.r.l.

THEORY

The multi-enzyme complex of reactions constituting a 'system' can be conceptually divided into blocks of reactions connected by a common intermediate: one block contains the reactions that produce the intermediate and the other block the reactions that consume it. The overall kinetic response of each block to the concentration of the intermediate is measured both in the presence and in the absence of the effector. The blocks, having a changed kinetic response to the concentration of the intermediate, contain the site(s) of action of any effector that induces a significant change in flux through the intermediate.

Using this kinetic approach, we have considered oxidative phosphorylation as blocks of reactions connected by the mitochondrial inner membrane potential ($\Delta\Psi$). The 'substrate oxidation' block (consisting of the dicarboxylate carrier, succinate dehydrogenase and the respiratory chain) 'produces' $\Delta\Psi$, and the 'proton leak' block (representing the cation cycles and the leak of protons across the inner membrane) together with the 'phosphorylating system' (consisting of adenine nucleotide and phosphate carriers, and ATP synthase) 'consume' $\Delta\Psi$ (Figure 1).

The 'substrate oxidation' block can itself be divided into blocks of reactions. In fact, it can be considered to comprise two blocks of reactions: those reactions able to reduce cytochrome *c* (referred to as '*c*-reducers') and those reactions able to oxidize it

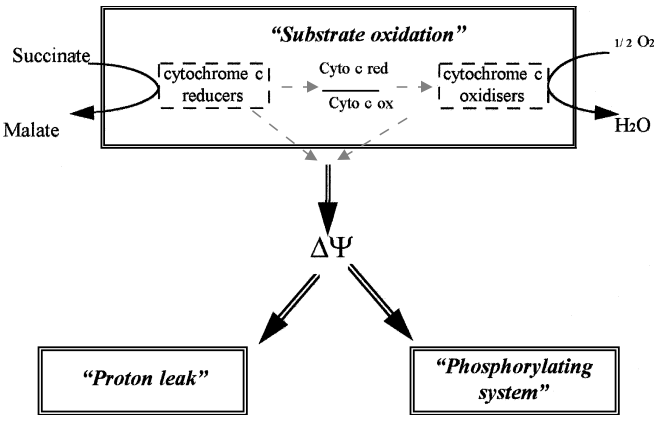

Figure 1 Schematic representation of the system under consideration

$\Delta\Psi$ is produced by the 'respiratory chain' (which consists of all the steps from external succinate to $\Delta\Psi$), and 'consumed' by both the phosphorylating system (which includes all the steps from $\Delta\Psi$ to glucose 6-phosphate), and by 'proton leak' (which includes the proton leak and any cation cycling). The substrate oxidation block can be considered to comprise two blocks of reactions: those that reduce cytochrome *c*, including all the steps from succinate to cytochrome *c*, and those that oxidize it, including all the steps from cytochrome *c* to oxygen. In the presence of uncouplers the cytochrome *c* redox state is the only intermediate between cytochrome *c* reducers and cytochrome *c* oxidizers.

(referred to as '*c*-oxidizers'). In the presence of uncouplers, these two blocks have the cytochrome *c* redox state as their only intermediate.

3,5-T2 could stimulate the mitochondrial respiration rate by three mechanisms:

(1) 3,5-T2 could primarily increase the activity of the reactions that consume $\Delta\Psi$. This would lower the $\Delta\Psi$ causing a secondary activation of $\Delta\Psi$ producers;

(2) 3,5-T2 could primarily increase the activity of the $\Delta\Psi$ producers. This would lead to a higher $\Delta\Psi$ causing a secondary stimulation of $\Delta\Psi$ consumers. 3,5-T2 could increase the activity of the '$\Delta\Psi$ producers' either by a direct interaction or by increasing the relative concentration of the (i) *c*-reducers, (ii) *c*-oxidizers or (iii) both *c*-reducers and *c*-oxidizers;

(3) 3,5-T2 could increase the activity of both the $\Delta\Psi$ producers and the $\Delta\Psi$ consumers directly or indirectly. Depending on the activation of each group, this could led to an increase, a decrease or no change in $\Delta\Psi$.

These three possibilities can be evaluated by examining the relationship between respiration rate and $\Delta\Psi$ in mitochondria from control group and 3,5-T2 group.

If 3,5-T2 activates the $\Delta\Psi$ consumers (proton leak and phosphorylating system) the plots of respiration rate against $\Delta\Psi$, when $\Delta\Psi$ is changed by inhibition of substrate oxidation, will not be superimposable: the plot from 3,5-T2 group will lie below the plots obtained from control rats. In effect, at any given $\Delta\Psi$, the respiration rate will be higher in the 3,5-T2 group than in the control. If 3,5-T2 does not stimulate the $\Delta\Psi$ consumers, then the two plots will be superimposable over most of their range; however, the plot obtained from 3,5-T2 group will extend further towards higher respiration rate and $\Delta\Psi$ than that for mitochondria from control rats. The relationship between $\Delta\Psi$ and respiration rate for proton leak can be obtained by malonate titration of oligomycin-inhibited respiration rate. The same relationship for the 'phosphorylating system' can be obtained by titrating the state 3 respiration rate with malonate and subtracting, at any given $\Delta\Psi$, the value of the respiration rate for the 'proton leak'.

If 3,5-T2 activates the '$\Delta\Psi$ producers' then the plots of mitochondrial respiration rate against $\Delta\Psi$ (obtained by titrating the respiration rate with hexokinase) from control and 3,5-T2 groups will not be superimposable. At any given $\Delta\Psi$ the respiration rate will be higher in mitochondria from 3,5-T2-treated rats than in those from the control.

To determine if a possible activation of $\Delta\Psi$ producers is due to stimulation of the cytochrome *c* reducers or the cytochrome *c* oxidizers, one needs to evaluate overall kinetic response of each block to a change in redox state of cytochrome *c*. If 3,5-T2 stimulates the cytochrome *c* reducers, then the plot of mitochondrial respiration rate against redox state of cytochrome *c* (obtained by KCN titration of uncoupled respiration) from 3,5-T2-treated rats will lie above the plot obtained from control rats. In contrast, if 3,5-T2 stimulates the cytochrome *c* oxidizers, the plot of mitochondrial respiration rate against redox state of cytochrome *c* (obtained by malonate titration of uncoupled respiration) from 3,5-T2 rats will lie below the plot obtained from control rats.

The control of flux through biochemical pathways is shared unequally between all of the participating steps; some steps exert little control whereas others exert more control. The control of *c*-oxidizers and *c*-reducers over the flux ($C^J_{c\text{-oxidizers}}$ and $C^J_{c\text{-reducers}}$, which represent the flux control coefficient over respiration rate for cytochrome *c* reducers and cytochrome *c* oxidizers respectively) can be calculated as reported by Brand et al. [23] using the following equation

$$C^J_v = \frac{\delta J}{\delta v}\cdot\frac{v}{J}$$

where v represents the enzyme rate of the block and J the corresponding system flux. The above equation states that the flux control coefficient is the ratio of the effects of a parameter change on the system flux (when system variables relax to a new value) and on the local rate (when system variables do not relax to a new value). To calculate $C^J_{c\text{-oxidizers}}$ and $C^J_{c\text{-reducers}}$ with the cytochrome *c* pool as the only intermediate, there must be no control over the flux by substrate transport or protonmotive force. Freezing, thawing and uncoupling of the mitochondria was used to ensure that this was so.

The flux control coefficients for the *c*-reducers and *c*-oxidizers can be obtained by measuring the effect of a small concentration of a flux inhibitor under two different conditions: when the system is permitted to relax after inhibition (to obtain δJ) and when it is not able to do this (to measure the effect on local enzyme rate δv). δJ can be obtained from the effect of inhibition on the rate when the redox state of cytochrome *c* is allowed to alter, and δv can be found from the effect of the same concentration of the inhibitor on the rate when cytochrome *c* redox state is returned to the original value by a different experimental intervention.

To measure the value of $C^J_{c\text{-oxidizers}}$ (Figure 4A, see later) the system is inhibited by using KCN and allowed to relax to a new steady state. It is then brought back to the original cytochrome *c* redox state by titration with malonate. The change in oxygen consumption (percentage of the standard state) and the change in the cytochrome *c* redox state for each point due to KCN or to KCN + malonate can be used to calculate δJ and δv (Figure 4B). δJ is the effect on flux when the *c*-oxidizers are inhibited with cyanide and the system is allowed to relax to a new steady state (in Figure 4B it corresponds to the difference between points *S* and *h* for euthyroid control rats and between *S* and *h*′ for the 3,5-T2 treated group). δv is the effect on flux when the *c*-oxidizers are inhibited with cyanide and the flux is measured when the cytochrome *c* redox state is brought back to its original value (in Figure 5B δv corresponds to the difference between points *S* and *k* in euthyroid control rats and between *S* and *k*′ in the 3,5-T2-treated group).

The $C^J_{c\text{-reducers}}$ can be calculated in a similar fashion. In this case, the *c*-reducers are inhibited by malonate, then the system is brought back to the original value for the cytochrome *c* redox state by using KCN (Figure 5A, see later). In Figure 5(B), the value of δJ is represented by the difference between the points *S* and *h* or *S* and *h*′; δv is represented by the difference between *S* and k or *S* and *k*′. In the calculation of either of the flux control coefficients , the ratio v/J has the value 1 because in the standard state both the enzyme rate and the flux rate are considered to be 100%.

RESULTS

Respiratory parameters in controls and 3,5-T2-treated rats

In euthyroid control rats, mitochondrial oxygen consumption (measured as nmol of O/min·mg of protein) was 36 ± 2 and 276 ± 19 for states 4 and 3 respectively (average ± S.E.M. from seven different mitochondrial preparations). Administration of 3,5-T2 significantly stimulated both state 4 and state 3 (each by approx. 30%, reaching values of 47 ± 3 and 360 ± 25 respectively) and did not induce a significant change in the mitochondrial RCR (ratio between state 3 and state 4 of mitochondrial respiration rate using succinate as substrate: 7 ± 0.2 in euthyroid animals and 6.9 ± 0.2 in the 3,5-T2-treated group).

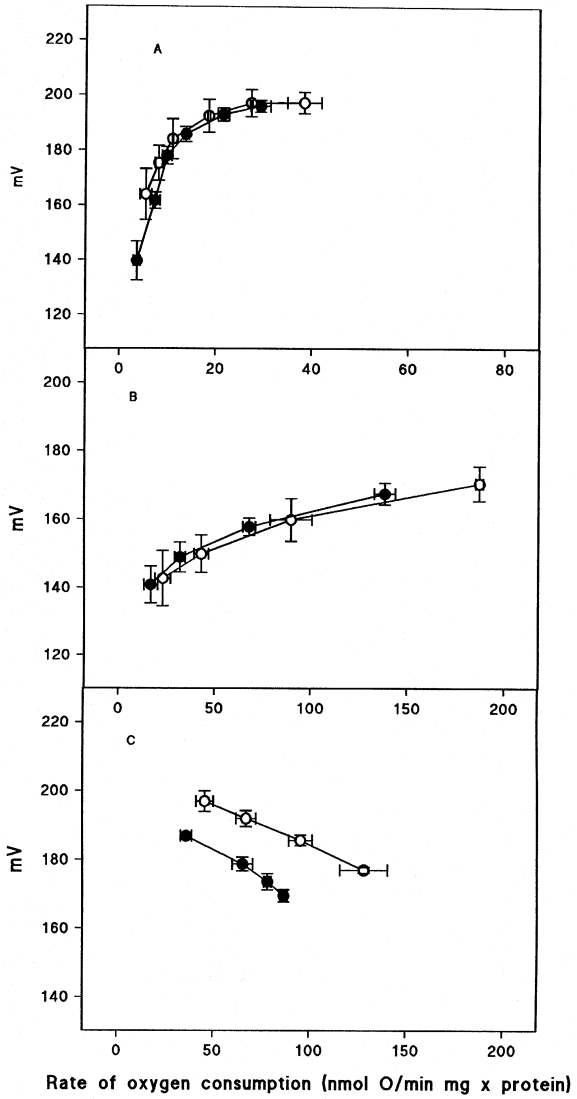

Figure 2 Kinetic responses of proton leak (A), phosphorylation system (B) and substrate oxidation (C) to a change in $\Delta\Psi$ in mitochondria: comparison between euthyroid and 3,5-T2 -injected rats

Mitochondria were incubated in medium A for three min before the addition of 4 μM Ph_3MeP^+ to calibrate the electrode. Respiration was then initiated by addition of 5 mM succinate. (**A**) For the evaluation of the kinetic response of mitochondrial proton leak to a change in $\Delta\Psi$, the incubation medium was supplemented with oligomycin (1 μg/ml) and hexokinase (0.6 units). The respiration rate was inhibited by sequential addition of malonate (up to 2.5 mM). (**B**) For the evaluation of the kinetic response of the phosphorylating system to a change in $\Delta\Psi$, the incubation medium was supplemented with 0.6 units of hexokinase. The state 3 respiration rate was inhibited by sequential addition of malonate (up to 250 μM) and, at any given $\Delta\Psi$, the value of the respiration rate for the proton leak was subtracted. (**C**) For the evaluation of the kinetic response of substrate oxidation to a change in $\Delta\Psi$, the respiration rate was stimulated by sequential addition of hexokinase (up to 0.6 units). (●) Control group; (○) 3,5-T2 group. Data are means ± S.E.M. of triplicate determinations in four different mitochondrial preparations.

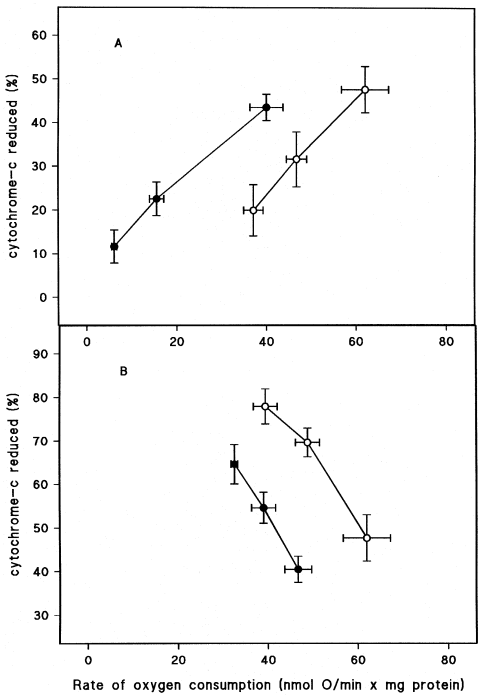

Figure 3 Kinetic responses of *c*-oxidizers and *c*-reducers to changes in the cytochrome *c* redox state in mitochondria: comparison between euthyroid and 3,5-T2-injected rats

Mitochondria were incubated in medium B for 3 min to permit the system to reach the steady state. (**A**) The kinetic response of the *c*-oxidizers to the changes in the redox state of cytochrome *c* was measured by titrating the respiration rate of uncoupled frozen-then-thawed mitochondria with malonate. This was obtained by supplementing the incubation medium with increasing concentrations of malonate (up to 1 μM for control group and 40 nM for the 3,5-T2 group). (**B**) The kinetic response of the *c*-reducers to changes in the redox state of cytochrome *c* was determined by titrating the respiration rate with KCN. This was done by supplementing the incubation medium with increasing concentrations of KCN (up to 36 μM). (●) Control group; (○) 3,5-T2 group. Data are means ± S.E.M. of triplicate determinations in three different mitochondrial preparations.

Top-down elasticity analysis of the kinetic responses of 'substrate oxidation', 'proton leak' and 'phosphorylation system' to changes in $\Delta\Psi$

To identify the subsystems that are possible targets for 3,5-T2 and therefore responsible for the increase in the mitochondrial respiration rate described above, we measured the kinetic response to changes in $\Delta\Psi$ shown by the blocks of reactions that produce $\Delta\Psi$ and by those that consume it (Figure 1).

Figure 2 illustrates the kinetic response of 'proton leak' (A), 'phosphorylating system' (B) and 'substrate oxidation' (C) to changes in $\Delta\Psi$. It is evident that a stimulation of the kinetics of substrate oxidation occurred in the 3,5-T2 group by comparison with the euthyroid control group. In other words, at any given mitochondrial-membrane potential, mitochondria from the 3,5-T2 group showed a higher respiration rate. The kinetic response of 'proton leak' to changes in $\Delta\Psi$ seems not to be primarily affected by 3,5-T2. The plots from control and 3,5-T2 rats were superimposable over most of their range and the plot for 3,5-T2 rats extends further towards higher respiration rate and $\Delta\Psi$ (Figure 2A). Similar behaviour is shown by the phosphorylating system, indicating that 3,5-T2 does not affect its kinetic response to $\Delta\Psi$.

Kinetic responses of *c*-reducers and *c*-oxidizers to changes in the cytochrome *c* redox state and their control coefficients over the flux

Having identified the 'substrate oxidation' block as containing the only significant site of action of 3,5-T2, we applied the top-down elasticity analysis to the respiratory chain. This can be

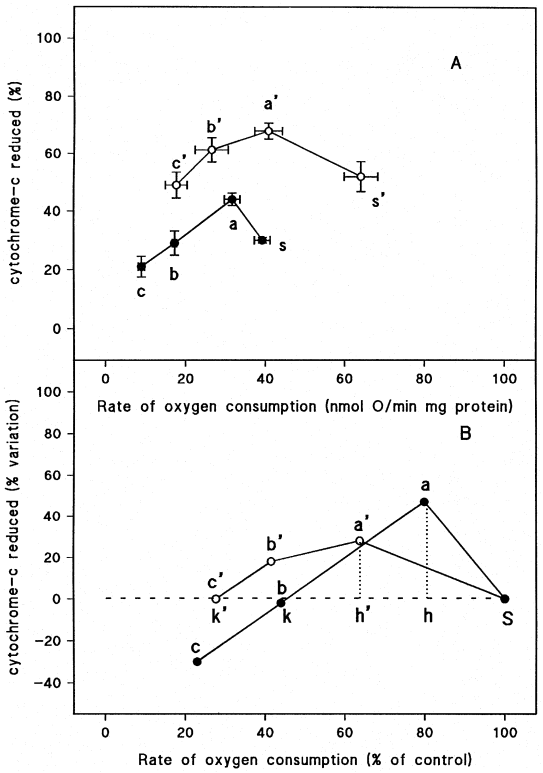

Figure 4 Determination of the flux control coefficient of cytochrome *c* oxidizers over respiration in uncoupled, frozen-then-thawed rat-liver mitochondria from euthyroid and euthyroid 3,5-T2-injected rats

Panel (**A**) illustrates the effect of KCN (4 μM for control, point a, and 12 μM for 3,5-T2 group, point a′) and KCN plus increasing concentrations of malonate (up to 0.5 μM for control, points b and c, and up to 20 nM for 3,5-T2, points b′,c′) on the steady state (s,s′). Data are means $\pm$ S.E.M. of triplicate determinations in four different mitochondrial preparations. Panel (**B**) illustrates the variations in respiration rate and cytochrome *c* redox state due to KCN (a, a′) or KCN plus malonate (b,b′ and c,c′) vs. the steady state (S). The values were calculated from the mean data in (**A**). The change in respiration rate due to KCN (difference between points S and h or S and h′) represents the effect on flux when the system is allowed to relax to a new steady state (δJ). The change in respiration rate corresponding to no variation in the redox state of cytochrome *c*, after the altered system has been permitted to relax to its initial value, represents the effect on flux when the system is not allowed to relax to a new steady state (δv) (difference between points S and k or S and k′).The control coefficient is then calculated as $\delta J/\delta v$. (●) Control group; (○) 3,5-T2 group.

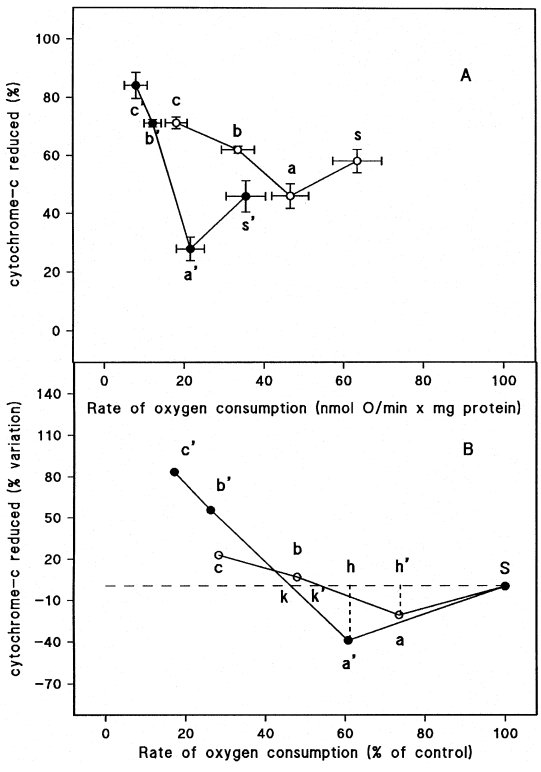

Figure 5 Determination of the flux control coefficient of cytochrome *c* reducers over respiration in uncoupled, frozen-then-thawed rat-liver mitochondria from euthyroid and euthyroid 3,5-T2-injected rats

Panel (**A**) illustrates the effect of malonate (0.5 mM for control group, point a, and 20 nM for 3,5-T2 group, point a′) or malonate plus increasing concentrations of KCN (up to 30 nM, points b,c and b′,c′) on the steady state (s,s′). Data are means $\pm$ S.E.M. of triplicate determinations in four or three different mitochondrial preparations for euthyroid and 3,5-T2 groups respectively. Panel (**B**) illustrates the variations in respiration rate and cytochrome *c* redox state due to malonate (a,a′) or malonate plus KCN (b,b′ and c,c′) vs. the steady state S. The values were calculated from the data in (**A**). The variation in respiration rate due to malonate (difference between points S and h or S and h′) represents the effect on flux when the system is allowed to relax to a new steady state (δJ). The variation in respiration rate corresponding to no variation in the redox state of cytochrome *c*, after the altered system has been permitted to relax to its initial value, represents the effect on flux when the system is not allowed to relax to a new steady state (δv) (difference between points S and k or S and k′). The control coefficient is then calculated as $\delta J/\delta v$. (●) Control group; (○) 3,5-T2 group.

considered to comprise *c*-reducers and *c*-oxidizers connected by the redox state of cytochrome *c* (Figure 1, small boxes bounded by broken lines). The kinetic responses of *c*-oxidizers and *c*-reducers to variations in the cytochrome *c* redox state (expressed as the percentage of cytochrome *c* reduced) are illustrated in Figure 3. Under steady state conditions (corresponding to frozen-then-thawed uncoupled mitochondria respiring with succinate as substrate), the respiration rate and the percentage of cytochrome *c* reduced were 40.3 ± 3.4 nmol of O/min·mg of protein and $43.5 \pm 4.0\%$ respectively for the euthyroid controls and 62.0 ± 5.2 nmol of O/min·mg of protein and $47.6 \pm 5.3\%$ for the 3,5-T2 group. These values suggest that freezing then thawing damaged the mitochondria, probably with a partial loss of cytochrome *c*. However, if the same kind of damage can be assumed to have occurred in both control and 3,5-T2-treated mitochondria, then this should not affect our conclusion. From the data shown in Figure 3, it is evident that the kinetics of both *c*-oxidizers and *c*-reducers are affected by 3,5-T2 treatment. Figure 3(A) shows that the injection of 3,5-T2 rapidly stimulated the kinetics of the *c*-oxidizers. In other words, at any redox state of cytochrome *c*, there is a greater respiration rate in mitochondria from 3,5-T2 animals than in those from the controls.

Treatment with 3,5-T2 also induced a change in the kinetics of the *c*-reducers (Figure 3B). The plots of the respiration rate against the redox state of cytochrome *c* from the control and 3,5-T2 groups did not overlap. A stimulation of '*c*-reducers' block is evident: at any given cytochrome *c* redox state the respiration rate was greater in mitochondria from the 3,5-T2 group than in the controls.

The control over the flux in uncoupled mitochondria from euthyroid rats was shared between *c*-oxidizers and *c*-reducers unequally; most of the control was exerted by the *c*-reducers. In the 3,5-T2 group the control over the flux by *c*-reducers and *c*-oxidizers was almost the same.

The $C^J_{c\text{-oxidizers}}$ and $C^J_{c\text{-reducers}}$ were calculated using data from Figures 4 and 5 respectively.

The $C^J_{c\text{-oxidizers}}$ values were 0.35 ± 0.03 and 0.48 ± 0.04 in the control and 3,5-T2 groups respectively and the $C^J_{c\text{-reducers}}$ values

were 0.74 ± 0.03 in the euthyroid control group and 0.58 ± 0.06 in the 3,5-T2 group. Similar results were obtained using flux-control summation and connectivity theorems [24,25] (results not shown). The sum of $C^J_{c\text{-oxidizers}}$ and $C^J_{c\text{-reducers}}$ in the control and in the 3,5-T2 group was very close to the theoretical value of unity [24] (1.09 and 1.06 respectively). The slight difference could be due to the fact that we added means together and that experimentally there were relatively large finite changes in flux instead of the infinitesimal changes demanded by the theory.

The values we have obtained for the control coefficient are similar to those obtained in a slightly different way by Ainscow and Brand [26].

DISCUSSION

In the present paper, we have investigated the mechanism of action of 3,5-T2 at the mitochondrial level in an attempt to evaluate how it might increase the activity of these organelles. Our results show that the effects of 3,5-T2 on mitochondria are not due to a change in the kinetics of the proton leak across the mitochondrial inner membrane nor to a change in the reactions that form and export ATP. Rather, they involve the reactions that generate the protonmotive force. The administration of 3,5-T2 to rats leads to the stimulation of these reactions; the stimulation is very rapid and is already evident 1 h after the injection (Figure 2).

Our analysis of the overall kinetic response of the two blocks of reactions that constitute the electron-transport chain to a change in the cytochrome *c* redox state show that 3,5-T2 induces both the *c*-reducers and the *c*-oxidizers. The short-term effect of 3,5-T2, together with its ability to stimulate mitochondrial respiration rate and rat metabolic rate, even in the presence of inhibitors of protein synthesis [15,27], leads us to speculate that its effect on the *c*-reducers and the *c*-oxidizers is not due to an enhanced concentration of the molecular components of these blocks, but rather to an alteration in their kinetic properties.

The effect exerted by 3,5-T2 on the kinetics of the *c*-oxidizers fits well with our previous results showing a direct interaction between 3,5-T2 and the cytochrome oxidase complex, the interaction probably being with its nuclear-encoded subunits [28].

The observed increase in the activity of *c*-reducers may be due to different factors (e.g. altered activity of mitochondrial dehydrogenases, increased activity of substrate transport, increased activities of complexes II and III). As we used succinate as substrate, we have no information about any possible action of 3,5-T2 on complex I.

Under our experimental conditions, the concentration of malonate necessary for the titration of the respiration rate of uncoupled mitochondria from euthyroid rats (respiring with succinate as substrate) was approx. 20-fold higher than that necessary for the titration of the respiration of mitochondria from 3,5-T2-treated rats. This could indicate altered kinetics of succinate dehydrogenase or succinate transport in which their affinity for substrate and its competitive inhibitors were increased.

The results presented in this paper may help to answer another question about the physiological meaning of the effect exerted by 3,5-T2. An increased activity of the *c*-reducer and *c*-oxidizer blocks causes the increase in the mitochondrial respiration rate observed in the 3,5-T2 group. In the whole animal, this phenomenon may result in an increased metabolic rate. Because of this, the effect of 3,5-T2 could be important in certain physiological conditions in which rapid energy utilization is required (such as cold exposure, overfeeding, etc.). The effect of 3,5-T2 in improving the cold tolerance of hypothyroid rats supports this hypothesis [19].

3,5-T2, originating from precursor molecules (thyroxine and 3′,3,5-tri-iodo-L-thyronine) through peripheral metabolism, may be a cellular mediator for the short-term effect of thyroid hormones on cellular energetic metabolism, an effect that is independent of protein synthesis [15,27]. The presence of specific binding sites for 3,5-T2 in rat liver mitochondria would seem to support this hypothesis [16].

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