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The rapid alteration by tri-iodo-L-thyronine *in vivo* of both the ADP/O ratio and the apparent H^+/O ratio in hypothyroid-rat liver mitochondria

Adelaida CRESPO-ARMAS and John MOWBRAY
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

Mitochondria from the livers of thyroidectomized rats have a lowered ADP/O ratio, which can be restored to normal within 15 min after intravenous injection of a near-physiological dose of tri-iodothyronine. Thyroidectomy lowered the measured ΔpH, which appears to be compensated by a rise (not statistically significant) in the membrane potential, so that the protonmotive force is unaltered. A simple simulation technique is described for use in estimating H^+/O ratios by the oxygen-pulse technique, which circumvents the problem that this ratio can be seriously underestimated because of re-uptake of protons from the bulk phase by the mitochondria before their expulsion is complete. By this procedure the H^+/O ratio of hypothyroid mitochondria is shown to be lowered by the same factor as the ADP/O ratio, and both these ratios are very rapidly restored in parallel by hormone administration. Although these findings could be consistent with a proposal that tri-iodothyronine rapidly modulates by some mechanism the efficiency of the respiratory-chain-linked proton pumps, the kinetic properties of the proton exchange suggest that the bulk-phase protons measured may not reflect faithfully those that drive the ATP synthetase.

INTRODUCTION

Thyroid hormone has long been proposed to regulate mitochondrial metabolism (see [1]) and much research has concentrated on studying a nuclear mechanism (e.g. [2]). Some early work proposed a direct interaction with mitochondria, but this is now regarded as uncertain or unphysiological. However, more recently evidence has begun to accumulate that tri-iodothyronine (T_3) can indeed elicit a very rapid direct response from mitochondria.

Both we [3] and Hoch [4] have shown that mitochondria acquire T_3 preferentially at very short times after administration to thyroidectomized animals. Three laboratories [5–7] have reported the presence of specific tight-binding receptors for T_3 in mitochondrial membranes, though these observations have to be interpreted with caution in the absence as yet of any functional assay for these receptors. More importantly, rapid effects of hormone on energy-driven mitochondrial processes such as respiratory control and uncoupler sensitivity [4], phosphorylation in submitochondrial vesicles [1,8] and Ca^{2+} transport [9] have been observed. In our laboratory we have developed a preparation of hypothyroid-rat liver mitochondria which shows ADP/O ratios around 60% of normal, with no evidence of uncoupling or loss of respiratory control [3,10–13]. Further, intravenous injection of a near-physiological dose of T_3 15 min before preparing the mitochondria restores the ADP/O ratio to normal, although the rate of O_2 uptake remains significantly low.

These studies on the decreased phosphorylating ability of hypothyroid-rat mitochondria are complemented by ^{31}P n.m.r. measurements by the saturation transfer method of ATP synthetase compared with O_2 uptake in perfused hearts from hypothyroid rats [14], and by measurements of the effects of T_3 on adenine nucleotide concentrations in freeze-clamped [3] and perfused [15,16] hypothyroid-rat livers.

In attempting to explain the mechanism whereby mitochondria can show decreased phosphorylation efficiency without loss of ADP respiratory control, we have investigated the H^+-pumping capacity of hypothyroid-rat mitochondria and the response of this to a near-physiological dose of T_3 given 15 min before preparation of the mitochondria. A preliminary account of this work has been presented at a Biochemical Society Meeting [17].

MATERIALS AND METHODS

Animals and mitochondria

Male rats (130–150 g, bred in the Department) were thyroidectomized and maintained on normal diet, except that their drinking water contained 0.5 μM-calcium lactate. Animals were used after at least 6 weeks, when their weight was constant at 180–220 g (weight of the normal controls by the same time about 400 g). Where required, animals were given 3 nmol of T_3/100 g body wt. intravenously 15 min before death. Rats were killed by cervical dislocation, and liver mitochondria were prepared as previously described [3].

Oxygen-electrode experiments

The ADP/O ratios were estimated in oxygen-electrode buffer (0.13 M-KCl/2 mM-$MgCl_2$/2 mM-EGTA/5 mM-Tris/HCl/2% defatted albumin, pH 7.2) at 37 °C in a Clark-type electrode with 3.3 mM-succinate as substrate in the presence of 1 μM-rotenone; 0.4 mg of mitochondrial protein was added to 3 ml of buffer, and State 3 was induced with 0.1 μmol of ADP. Neither the ADP/O

Abbreviations used: T_3, tri-iodo-L-thyronine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

ratio nor State-4 respiration changed significantly with three successive additions of ADP to the same incubation.

Determination of the H^+/O quotient

H^+/O quotients were measured by the oxygen-pulse technique [18], under the same buffer conditions as in the ADP/O-ratio determinations, with a final volume of 1 ml containing about 5 mg of mitochondrial protein, 200 ng of valinomycin, 5 μg of oligomycin and 0.15 mM-*N*-ethylmaleimide. All solutions were freed of air by sparging with O_2-free N_2 for 30 min. O_2 was added as a pulse of 50 μl of air-saturated 120 mM-KCl at 37 °C (equivalent to 22 ng-atoms of O).

The standard solution was obtained by equilibrating a 5 mm-deep layer of KCl solution by gentle shaking at 37 °C for 3 h in a stoppered flask equipped with a centre cup containing 2 M-KOH and a filter-paper wick.

Measurement of the protonmotive force (Δp)

This involves the separate measurement of the mitochondrial membrane potential ($\Delta\psi$) and the proton gradient across the membrane (ΔpH). $\Delta\psi$ was measured with a tetraphenylphosphonium (TPP^+) electrode [19] in 1 ml of oxygen-electrode buffer containing 1 mg of mitochondrial protein, 10 μM-TPP^+ and 0.09 mM-ADP; 3.3 mM-succinate was used as oxidizable substrate in the presence of 1 μM-rotenone. ΔpH was determined in the same medium by the anion-distribution method, with 1 μCi of [U-^{14}C]lactate and 4.5 mg of mitochondrial protein [20]. The mitochondrial matrix volume was estimated by using ^3H_2O as a total water marker and [U-^{14}C]sucrose as extramitochondrial-space marker.

Calculation of H^+ generated in response to an oxygen pulse

The original application of this technique [21] relied on the premise that the rate of proton expulsion by mitochondria is so much greater than the rate of re-uptake that, provided the response of the pH electrode is very fast and the pulse of oxygen small enough to prevent ΔpH attaining a saturating value, the quantity of protons expelled could be estimated from the meeting point of the lines produced by extrapolating the increasing and decreasing pH traces. This will give an underestimate, since, however fast the expulsion, some protons will leak back before the burst of expulsion is completed. This can become a very significant error where the rate of the re-uptake process is substantially increased, as is the case after thyroidectomy (see below). To overcome this problem, we have devised a simple mathematical model and used computer simulation to fit the experimental pH-electrode traces. This gives values for the rate constants and for the total amount of H^+ generated by the burst of respiration in response to an oxygen pulse.

Model

$$H^+_{\text{mitochondria}} \xrightarrow[\text{expulsion}]{k_1} H^+_{\text{medium}} \xrightarrow[\text{reuptake}]{k_2}$$

where $H^+_{\text{mitochondria}}$ represents the quantity of H^+ produced by the oxygen pulse, H^+_{medium} represents the quantity of H^+ detected by the pH electrode, and k_1 and k_2 are the rate constants for the expulsion and re-uptake processes respectively.

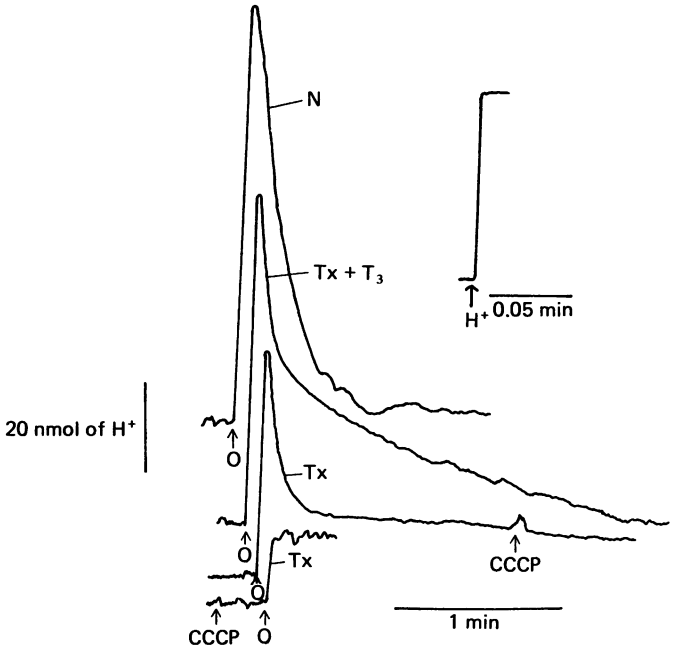

Fig. 1. Change in pH of the medium containing anaerobic mitochondria after an O_2 pulse

The Figure shows the recorder traces from a meter registering the output of a screened glass pH microelectrode monitoring stirred mitochondrial suspensions incubated as detailed in the Materials and methods section. The electrode was calibrated by the addition of 20–100 nmol of HCl and had a maximum speed of response at least 10 times greater than the observed expulsion of mitochondrial protons. The preparations were from euthyroid animals (N), from thyrodectomized animals (Tx) or from thyroidectomized animals which had been given T_3 intravenously 15 min previously (Tx + T_3). The inset shows the very rapid response of the electrode to proton addition to incubation buffer in the absence of mitochondria (note the altered time scale).

Hence:

$$\frac{d(H^+_{\text{medium}})}{dt} = k_1 H^+_{\text{mitochondria}} - k_2 H^+_{\text{medium}} \quad (1)$$

Assuming that $H^+_{\text{mitochondria}}$ can be described thus (see [22]):

$$H^+_{\text{mitochondria}} = H^+_{\text{generated}} \cdot e^{-k_1 t} \quad (2)$$

where $H^+_{\text{generated}}$ represents the total quantity of H^+ produced in response to a small oxygen pulse, then:

$$\frac{d(H^+_{\text{medium}})}{dt} + k_2 H^+_{\text{medium}} = k_1 H^+_{\text{generated}} \cdot e^{-k_1 t} \quad (3)$$

whence:

$$H^+_{\text{medium}} = \frac{k_1}{k_2 - k_1} \cdot H^+_{\text{generated}} \cdot (e^{-k_1 t} - e^{-k_2 t}) \quad (4)$$

Materials

Tetraphenylphosphonium chloride was purchased from Fluka A.G. (Buchs, Switzerland), and oligomycin, valinomycin, *N*-ethylmaleimide and CCCP were from Sigma. ^3H_2O, [U-^{14}C]sucrose and [U-^{14}C]lactate were from Amersham International. The sources and handling

of other chemicals, as well as all other techniques used, were as previously described [3], except for protein determination, which was carried out by a modification [23] of the method of Lowry *et al.*

RESULTS AND DISCUSSION

Effect of thyroidectomy on the H^+/O ratio

Using the oxygen-pulse technique of Mitchell & Moyle [21], we have measured H^+/O ratios in mitochondria prepared from euthyroid and hypothyroid animals. Fig. 1 gives examples of the pH changes seen when anaerobic mitochondrial suspensions were given a 22 ng-atom pulse of O_2. The euthyroid preparation responds in the expected [18] manner, with the very rapid expulsion to the medium of protons by the respiratory-chain-linked pump, followed by a slower re-uptake process which restores the pH of the medium to its initial value by about 1 min or so after the pulse. By contrast, with the hypothyroid preparation the re-uptake process appears considerably faster than normal, and, since it is presumably taking place before the expulsion of protons has ceased, will contribute to the obviously lower peak height attained by these preparations. In order to attempt to circumvent the underestimate of the H^+/O ratio which this would provide if the original method [18] were followed, we have employed a simple model for the proton movements (see the Materials and methods section) and have fitted this by computer simulation to points taken from these experimental traces.

A second notable difference between the responses of normal and hypothyroid preparations was that, despite the increased rate of H^+ re-uptake, the pH did not immediately return to its initial value, but settled at a higher $[H^+]$ about 0.5 min after the O_2 pulse (Fig. 1), and declined slowly from there so that it finally returned to the original baseline at 8–10 min (results not shown) after the pulse. Addition of a proton ionophore 1.5 min after the O_2 did not lower the medium $[H^+]$ (Fig. 1): moreover, addition of CCCP before O_2 addition abolished the H^+ peak, but not this baseline shift. The true source of these very slowly removed protons is unknown. In practice we subtracted the baseline shift in fitting the model and have calculated H^+/O both including and ignoring these 'shift protons'.

The response of the hypothyroid-rat mitochondria to T_3 replacement therapy 15 min before removal of the liver is also shown in Fig. 1. The maximum $[H^+]$ in the medium is increased, although the re-uptake process remains faster than normal. In addition, the decay of the baseline shift is substantially faster than in the untreated preparation, and in most cases the shift was somewhat smaller than with the hypothyroid-rat mitochondria.

Mean values at selected time points after O_2 addition taken from experimental traces given by our different mitochondrial preparations in each condition are shown in Fig. 2. Also shown are the computed values of H^+ in the medium as a function of time after the O_2 pulse calculated by inserting in eqn. (4) mean values for the rate constants and H^+ generated derived from fitting each individual set of experimental results. Table 1 sets out the values of these constants. For euthyroid-rat preparations it proved possible to find a satisfactory fit to the experimental values both with $k_1 > k_2$ and with $k_2 > k_1$. In the latter case, where the re-uptake exceeds the expulsion rate, it is necessary to supply a very high value for H^+ generated in order to provide appreciable amounts of H^+ in the medium. Values derived from one such fitting are given in Table 1: the consequent value of H^+/O derived (18) is unsupportably high.

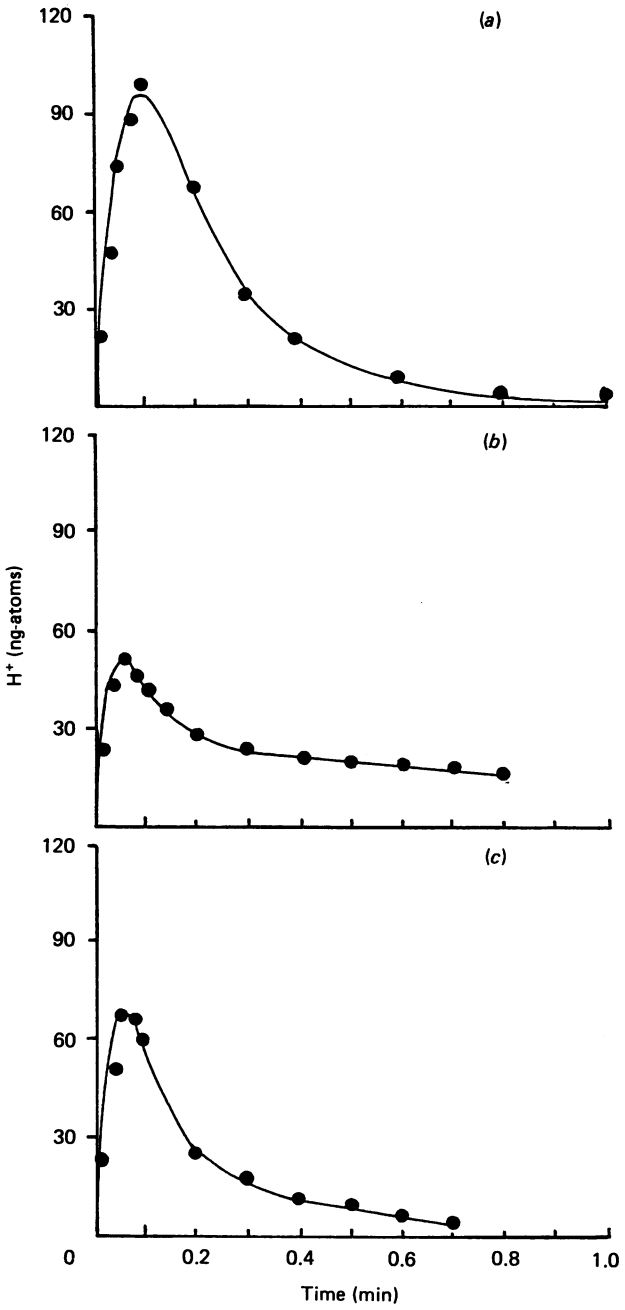

Fig. 2. Fitting of the model for H^+ expulsion and re-uptake to experimental results

The points are values taken at selected time points from experimental traces such as shown in Fig. 1 and are the means of four different mitochondrial preparations from animals in each of the three thyroid conditions. The lines show H^+_{medium} as a function of time when the mean values (see Table 1) of k_1, k_2 and $H^+_{generated}$ are inserted in eqn. (4) (see the Materials and methods section).

Calculated by the extrapolation method [18], the value for euthyroid-rat mitochondria oxidizing succinate is close to 4. By using the fit to eqn. (4), the values are very

Table 1. H^+/O ratios and constants computed from proton expulsion and re-uptake

Values for the change in $[H^+]$ in the medium induced by addition of 22 ng-atoms of O were taken from experimental traces and plotted by using a simple graph-plotting routine on a BBC-B microcomputer. The value for H^+_{medium} given by eqn. (4) (see the Materials and methods section) was also plotted, and the values of k_1, k_2 and $H^+_{generated}$ were adjusted until the computed function closely followed the experimental points (see, e.g., Fig. 2). Where more than one set of values provided an equally good fit, e.g. k_1, k_2, $H^+_{generated}$ = 14, 35, 170 or 16, 30, 150, then the values taken were the mean ones, i.e. 15, 32.5, 160. The constants given are the mean values (±S.E.M.) derived from four different mitochondrial preparations from animals in each of three thyroid conditions. the 'shift H^+' (see the text) are zero in euthyroid preparations. *$P < 0.001$, †$P < 0.001$, ††$P < 0.0025$, †††$P < 0.0125$ versus normals.

Condition	k_1 (min^{-1})	k_2 (min^{-1})	$H^+_{generated}$ (ng-atoms)		H^+/O	
			−shift H^+	+shift H^+	−shift H^+	+shift H^+
Normal	17±2	6±1	167±4		7.57±0.22	—
	7	15	400		18	
Thyroidectomized	14±5	23±6	98±10††	117±14†††	4.43±0.80*	5.33±0.53†
Thyroidectomized/ T_3-injected	17±3	23±11	152±12	168±10	6.91±0.50	7.62±0.39

Table 2. Effect of thyroid hormone on ADP phosphorylation and the protonmotive force (Δp) in mitochondria

The details of the techniques used are described in the Materials and methods section, and the values are means±S.E.M. ($n = 4$). The ADP/O and H^+/O ratios were measured in the same preparations of mitochondria. The H^+/O ratios quoted do not include the 'shift H^+'. *$P < 0.005$, **$P < 0.0025$, †$P < 0.001$, ††$P < 0.0005$ versus normal.

Condition	$\Delta\psi$ (mV)	−61.3ΔpH (mV)	Δp (mV)	ADP/O	Respiratory control ratio	State-3 respiration (ng-atoms of O/min per mg)	H^+/O
Normal	166±5	25.5±2.5	192±20	1.89±0.08	3.61±0.19	187±10	7.57±0.22
Thyroidectomized	179±5	11.8±1.7*	191±28	1.15±0.06††	3.30±0.10	95±10**	4.43±0.80†
Thyroidectomized/ T_3-injected	180±4	18.7±3.5	199±37	1.75±0.06	3.92±0.20	108±20†	6.91±0.50

close to those reported for rat liver mitochondria oxidizing succinate in the presence of rotenone and *N*-ethylmaleimide by Lehninger and co-workers (see [2]), who have also devised procedures to correct for back-flow of H^+ from the very beginning of H^+ ejection. The H^+/O ratios found for mitochondria from thyroidectomized animals were significantly lower whether or not the 'shift protons' were taken into account: this low ratio was restored to normal in animals that had been given a near-physiological dose of T_3 15 min before preparation of mitochondria (Table 1).

The protonmotive force and comparison of ADP/O and H^+/O ratios in liver mitochondria from rats in different thyroid conditions

Shears & Bronk [24] have suggested, on the basis of experiments with liver mitochondria from hyperthyroid animals, that thyroid hormone might act by altering the protonmotive force (Δp). Table 2 presents the results of experiments aimed at measuring Δp in State 3 in liver mitochondria from normal, thyroidectomized and thyroidectomized/hormone-treated animals. No significant changes were found in the membrane potential ($\Delta\psi$) or in Δp, although the measured ΔpH was lowered by thyroidectomy and restored within 15 min by intravenous administration of T_3. The difference in ΔpH could be consistent with the lowered H^+/O ratios found with these mitochondria, whereas the unaltered Δp when the ADP/O ratio is lowered would support experiments which question [25–32] whether a simple relationship between measured Δp and energy-driven processes in mitochondria is adequate.

The lowered ADP/O ratios shown by hypothyroid-rat mitochondria incubated under the conditions detailed in the Materials and methods section are clear from the results shown in Table 2. The State-4 respiration remains constant after three successive additions of ADP to induce State-3 respiration and is significantly lower after thyroidectomy. The low ADP/O ratio shown by hypothyroid-rat preparations is corrected by intravenous injection of a near-physiological amount of T_3 15 min before preparation of the mitochondria: by contrast, the O_2 uptake per mg of mitochondrial protein is unchanged by hormone addition. These findings are in accord with those reported previously [3,11–13].

Comparing the ADP/O and H^+/O ratios measured on the same preparations of mitochondria shows that the former is decreased to 61% and the latter to 58% of normal by thyroidectomy. At 15 min after intravenous hormone injection, the mitochondria show ADP/O and H^+/O ratios of 93% and 91% of the euthyroid value.

General discussion

The implication in these findings is that the lowered ADP/O ratio is the result of a lowered yield of protons per electron transported in the respiratory-chain-linked proton pumps and that, by some mechanism which remains unclear, thyroid hormone can very rapidly alter the apparent stoicheiometry of these pumps. The problem with this interpretation is the kinetic behaviour of the H^+ pool in the medium. Not only is the ejection rate (and rate constant k_1) unaltered by thyroidectomy, but the apparent leak-back of H^+ and k_2 have actually substantially increased (Fig. 1 and Table 1). This is in direct contrast with the lower rates of respiration in both State 3 and State 4 (Table 2) shown by preparations from thyroidectomized animals. The State-4 rates in all three preparations were actually lowered slightly by valinomycin and *N*-ethymaleimide and substantially lowered by oligomycin added to the oxygen electrode at the concentrations used in the H^+/O-ratio experiments (results not shown). These findings suggest that the changes in bulk-phase $[H^+]$ measured by the pH electrode, although presumably in some way the result of stimulating respiration, may not reflect faithfully the behaviour of protons which are driving ATP synthesis.

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