J Membrane Biol (2007) 215:135–145
DOI 10.1007/s00232-007-9013-4

Thyroid Hormone Treatments Differentially Affect the Temperature Kinetics Properties of FoF_1 ATPase and Succinate Oxidase as well as the Lipid/Phospholipid Profiles of Rat Kidney Mitochondria: A Correlative Study

Hiren R. Modi · Samir P. Patel · Surendra S. Katyare · Minal Patel

Received: 15 September 2006 / Accepted: 8 March 2007 / Published online: 14 June 2007

Abstract Effect of thyroidectomy (Tx) and subsequent treatment with 3,5,3′-triiodo-L-thyronine (T_3) or replacement therapy (T_R) with T_3 + L-thyroxine (T_4) on the temperature kinetics properties of FoF_1 adenosine triphosphatase (ATPase, ATP synthase, H^+-translocating ATP synthase EC 3.6.3.14) and succinate oxidase (SO) and on the lipid/phospholipid makeup of rat kidney mitochondria were examined. Tx lowered ATPase activity, which T_3 treatment restored. SO activity was unchanged in Tx but decreased further by T_3 treatment. T_R restored both activities. The energies of ATPase activation in the high and low temperature ranges (E_H and E_L) increased in the Tx and T_3 animals with decrease in phase transition temperature (Tt). T_R restored E_H and E_L but not Tt to euthyroid levels. E_H and E_L of SO decreased in Tx animals. T_3 and T_R restored E_H whereas E_L was restored only in the T_R group; Tt increased in both groups. Total phospholipid and cholesterol contents decreased significantly in Tx and T_3-treated animals. In Tx animals, sphingomyelin (SPM) and phosphatidylcholine (PC) components decreased, while phosphatidylserine (PS) and diphosphatidylglycerol components increased. T_3 and T_R treatments caused decreases in SPM, phosphatidylinositol and PS. PC and phosphatidylethanolamine (PE) increased in the T_3 group. T_R resulted in increased lysophospolipids and PE. Changes in kinetic parameters of the two enzymes were differently correlated with specific phospholipid components. Both T_3 and T_R regimens were unable to restore normal membrane structure-function relationships.

Keywords Thyroidectomy · Thyroid hormone · FoF_1 ATPase · Succinate oxidase · Temperature kinetics · Lipid/phospholipid profile

H. R. Modi (✉) · S. P. Patel · S. S. Katyare · M. Patel
Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390 002, India
e-mail: modi_hiren@yahoo.co.in

Introduction

The role of thyroid hormones in the regulation of basal metabolic rate (BMR), cell growth and differentiation and the metabolism of carbohydrates, proteins and lipids is well recognized (Tata, 1964, 1966). At the subcellular level, thyroid hormones significantly influence the respiratory parameters of the mitochondria in the thyroid hormone-responsive tissues, i.e., skeletal muscle, liver, kidney and heart (Gustafsson et al., 1965; Katyare et al., 1977; Parmar et al., 1995; Rajwade et al., 1975; Satav & Katyare, 1991; Tata et al., 1963; Tata, 1964, 1966). Thus, deficiency of thyroid hormones results in decreased respiratory activity, and treatment of hypothyroid animals with a single dose of L-thyroxine (T_4) or of 3,5,3′-triiodo-L-thyronine (T_3) (15-25 μg/100 g body weight) brings about stimulation of the respiratory activity of mitochondria in these tissues (Gustafsson et al., 1965; Katyare et al., 1977; Satav & Katyare, 1991; Tata et al., 1963; Tata, 1964, 1966). The effect(s) of thyroid hormone treatment becomes discernible after a lag period of 24 h, reaches an optimum by 45–50 h and then wears off by 72 h, when thyroid hormone levels fall off (Gustafsson et al., 1965; Katyare et al., 1977; Satav & Katyare, 1991; Tata et al., 1963; Tata, 1964, 1966). The early effects of treatment with thyroid hormones include stimulation of microsomal protein and RNA synthesis. In particular, the increased synthesis of RNA polymerase II is

the primary event which leads to stimulation of mRNA synthesis (Tata & Widnell, 1966). This is turn leads to the synthesis of essential components of the electron transport chain (ETC), which include cytochromes and β-subunit of FoF_1 ATPase and adenine nucleotide translocase protein (ANT), besides uncoupling protein (UCP) (Wrutniak-Cabello, Casas & Cabello, 2001). The delay in the manifestation of thyroid hormone effects on mitochondrial functions is thus attributed to this fact (Tata & Widnell, 1966).

In earlier studies from our laboratory, we noted that the thyroid hormones significantly altered the lipid/phospholipid profiles and membrane fluidity of rat liver and brain mitochondria (Bangur, Howland & Katyare, 1995; Parmar et al., 1995). Thyroid status-dependent changes in lipid/phospholipid profiles of rat liver mitochondria have also been reported by other researchers (Cappello & Gnoni, 1994; Paradies, Ruggiero & Dinoi, 1991; Pasquini et al., 1980). However, we also noted that treatment with a single dose of T_3, which improved respiratory functions, was not able to restore the lipid/phospholipid profiles to normal; even long-term treatment with T_3 was ineffective in this contest (Parmar et al., 1995).

Dependence of growth and development of the kidneys on thyroid hormones has been demonstrated (Katz, Emmanouel & Lindhemer, 1975). Additionally, thyroid hormones play an important role in renal hemodynamics and salt and water metabolism (Vargas et al., 2006). Effects of thyroid hormones in regulating Na^+, K^+-ATPase activity in the kidney have been demonstrated (Del Compare et al., 2001; Fukuda et al., 1992; Horowitz et al., 1990). Earlier studies from our laboratory showed that in thyroidectomized (Tx) rats the respiratory activity of the kidney mitochondria decreased significantly and treatment with a single dose of T_3 was able to stimulate the respiratory activity in a substrate-specific manner (Katyare et al., 1977; Satav & Katyare, 1991). The aforementioned changes were accompanied by parallel changes in the contents of cytochromes. Thus, in Tx rats the contents of cytochromes aa_3, b and $c+c_1$ decreased significantly and treatment with a single dose of T_3 resulted in substantial increases in the contents of all the cytochromes (Katyare et al., 1977; Satav & Katyare, 1991). We also found that synchrony of turnover of kidney mitochondrial proteins was lost in Tx animals (Rajwade et al., 1975).

While treatment with a single dose of T_3 is able to elicit maximum stimulatory response in the respiratory activity of the mitochondria at the end of 48 h (Gustafsson et al., 1965; Katyare et al., 1977; Satav & Katyare, 1991; Tata et al., 1963; Tata, 1964, 1966), it has been reported that combined treatment with T_3 and T_4 (0.9 μg T_4 + 0.15 μg T_3/100 g body weight) for 18 consecutive days can practically restore the euthyroid status of all tissues (Escobar-Morreale et al., 1996). In light of the above it was of interest to find out if this treatment is able to restore mitochondrial membrane function to normal, i.e., the euthyroid state. These studies assume importance in view of the fact that hypothyroid patients are given combined treatment with T_3 and T_4.

To illustrate this point, we decided to determine the temperature kinetics properties of two representative marker enzymes of mitochondria, FoF_1 adenosine triphosphatases (ATPase, ATP synthase, H^+-translocating ATP synthase EC 3.6.3.14) and succinate oxidase (SO). The rationale for selecting these two enzymes was as follows. FoF_1 ATPase (complex V) is distributed in restricted microdomains in the inner mitochondrial membrane but is not a component of the ETC. On the other hand, SO comprises complexes II, III and IV of the ETC. The activity of both enzyme systems is known to be dependent on membrane lipids (Daum, 1985). We then tried to correlate the temperature kinetics parameters with lipid/phospholipid profiles of the kidney mitochondria as affected by the thyroid status of the animals. In parallel studies, we also examined the effect of treatment with a single dose of T_3 on these parameters (Katyare et al., 1977; Tata, 1964, 1966). The results of these investigations are summarized in the present communication.

Our results indicate that although the two treatment regimens had selective effects on stimulation of the two enzyme activities, the structure-function relationship was not restored to normal by either of the treatments. As far as we are aware, the effects of thyroid hormones on lipid/phospholipid profiles of kidney mitochondria have not been reported thus far.

Materials and Methods

Chemicals

Bovine serum albumin (BSA) fraction V, T_3, T_4, 1,6-diphenyl-1,3,5-hexatriene (DPH) and sodium salt of ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, MO). Sodium salt of succinic acid and ATP were purchased from SRL (Mumbai, India). Silica gel G was from E. Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade and purchased locally.

Animals and Treatment with Thyroid Hormones

Weanling male albino rats (3–4 weeks old, 30–35 g body weight) of Charles-Foster strain were surgically thyroidectomized and allowed to grow for 8–10 weeks. Only those animals showing considerable decrease in body weight (50–60%) were used for further studies (e.g., *see* Table 1).

Table 1 Effect of Tx and subsequent treatment with thyroid hormones on body and kidney weight

Animals	Body weight (g)	Kidney weight	
		Grams	% of body weight
Control (n = 8)	232.6 ± 9.7	1.63 ± 0.05	0.70 ± 0.02
Tx (n = 6)	102.3 ± 2.9**	0.73 ± 0.03**	0.71 ± 0.01
T_3 (n = 6)	92.9 ± 5.6**	0.76 ± 0.03**	0.83 ± 0.03*,†
T_R (n = 6)	186.7 ± 8.1*,‡	1.26 ± 0.04**,‡	0.68 ± 0.03

The results are given as mean ± standard error of the mean of the number of independent experiments indicated in parentheses

* $p < 0.01$ and $^{**}p < 0.001$ compared to euthyroid controls, $^{\dagger}p < 0.02$ and $^{\ddagger}p < 0.001$ compared to the Tx group

Controls were sham-operated. Tx rats were randomly divided in three groups. One group of Tx animals received a single dose of 25 μg T_3/100 g body weight subcutaneously (s.c.) (Satav & Katyare, 1991). The animals were killed after 48 h. This group is referred to as Tx + T_3. The second group of animals received replacement therapy with 0.9 μg T_4 + 0.15 μg T_3/100 g body weight s.c. for 18 consecutive days (Escobar-Morreale et al., 1996). This group is hereafter referred to as T_R. The third group received an equivalent volume of saline/NaOH vehicle in which thyroid hormone solutions were prepared. Thyroid hormone solutions were prepared freshly in 0.9% saline containing 5 mM NaOH. The sham-operated controls received an equivalent volume of saline/NaOH vehicle.

Isolation of Mitochondria

Isolation of mitochondria from kidney cortex was carried out according to the procedures described earlier using the isolation medium composed of 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (TRIS)-HCl buffer (pH 7.4) and 1 mM EDTA; 250 μg BSA/ml of isolation medium was included (Satav & Katyare, 1982, 1991).

Enzyme Assays

ATPase

ATPase activity was measured in the assay medium (total volume 0.1 ml) containing 50 mM Tris-HCl buffer (pH 7.4), 75 mM KCl, 0.4 mM EDTA, 6 mM $MgCl_2$ and 100 μM 2,4-dinitrophenol (DNP). After preincubating the mitochondrial protein (30–50 μg) in the assay medium for 1 min, the reaction was initiated by addition of ATP at a final concentration of 5 mM (Katyare et al., 1977). The reaction was terminated after 10–30 min (depending on the assay temperature) by adding 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution, and the amount of liberated inorganic phosphorus was estimated by the method of Katewa & Katyare (2003). The enzyme activity (v) is expressed as micromoles of inorganic phosphate (Pi) liberated per hour per milligram of protein.

SO

Measurement of SO activity was carried out polarographically using a Clarke-type oxygen electrode. The assay medium (total volume 1.6 ml) consisted of 50 mM potassium phosphate buffer (pH 7.4), containing 0.4 mM each of $CaCl_2$ and $AlCl_3$ (Katyare, Fatterpaker & Sreenivasan, 1971). After introducing the mitochondrial protein (0.1–2.5 mg protein depending on the temperature at which the assay was performed), respiration was initiated by addition of sodium succinate (final concentration 10 mM) and the linear rate of oxygen uptake recorded for up to 2–5 min, depending on the temperature of the assay. The enzyme activity (v) is expressed as nanomoles of O_2 per minute per milligram of protein.

Measurements of ATPase and SO activities were carried out over the temperature range 5–53°C, with an increment of 4°C at each step.

The data were analyzed for determination of energies of activation in the high and low temperature ranges (E_H and E_L, respectively) and phase transition temperature (Tt) according to the methods described previously (Dave, Syal & Katyare, 1999; Dave & Katyare, 2002; Dixon & Webb, 1979).

Analytical Methods

The extraction of mitochondrial lipids/phospholipids with freshly prepared chloroform:methanol (2:1vol/vol) was according to the procedure described previously (Folch, Lees & Sloane Stanley, 1957; Pandya, Dave & Kotyare, 2004).

Separation of phospholipid classes by thin-layer chromatography was according to the methods described (Skipski, Peterson & Barclay, 1964; Pandya et al., 2004). Estimation of cholesterol (CHL) and phospholipid phosphorus was according to the procedures detailed earlier (Bartlett, 1959; Zlatkis & Zak, 1969). Membrane fluidity measurements were carried out using DPH as the probe (Pandya et al., 2004).

The contents of individual phospholipid classes were calculated by multiplying the values of total phospholipid (TPL) by the percentage composition of the individual phospholipid classes (Pandya et al., 2004).

Estimation of protein was by the method of Lowry et al. (1951) using BSA as the standard.

Statistical evaluation of the data was by Students' t-test. Regression analysis across the groups was carried out using

Table 2 Effect of Tx and subsequent treatment with thyroid hormones on FoF_1 ATPase activity in rat kidney mitochondria

Animals	Activity (μmol Pi liberated/h/mg protein)		Activity ratio
	25°C	37°C	
Control (n = 8)	10.44 ± 0.22	23.89 ± 0.47	2.29 ± 0.06
Tx (n = 6)	3.37 ± 0.17**	8.15 ± 0.34**	2.44 ± 0.13
T_3 (n = 6)	3.55 ± 0.15**	9.70 ± 0.27**,†	2.73 ± 0.09*
T_R (n = 6)	11.60 ± 0.90‡	25.95 ± 1.99‡	2.24 ± 0.05

The results are given as mean ± standard error of the mean of the number of independent experiments in parentheses. Activity ratio = activity at 37°C/activity at 25°C

* $p < 0.002$ and $^{**}p < 0.001$ compared to euthyroid controls, † $p < 0.01$ and $^{\ddagger}p < 0.001$ compared to the Tx group

Table 3 Effect of Tx and subsequent treatment with thyroid hormones on SO activity in rat kidney mitochondria

Animals	Activity (nmole O_2 min^{-1} · mg $protein^{-1}$)		Activity ratio
	25°C	37°C	
Control (n = 6)	14.1 ± 0.41	61.8 ± 1.16	4.39 ± 0.14
Tx (n = 4)	13.6 ± 0.11	36.6 ± 0.90**	2.69 ± 0.09**
T_3 (n = 4)	9.0 ± 0.75**,‡	38.6 ± 0.40**	4.37 ± 0.31‡
T_R (n = 4)	13.4 ± 0.48	53.2 ± 2.30*,‡	3.98 ± 0.13‡

The results are given as mean ± standard error of the mean of the number of independent experiments indicated in parentheses. Activity ratio = activity at 37°C/activity at 25°C

* $p < 0.02$ and $^{**}p < 0.001$ compared to euthyroid controls, $^{\ddagger}p < 0.001$ compared to the Tx group

Jandel Sigmastat Statistical Software, version 2.0 (Jandel, San Rafael, CA).

Results

Data in Table 1 show that Tx resulted in a 56% decrease in body weight and that treatment with a single dose of T_3 had no restorative effect. By contrast, in the T_R group, body weight increased significantly by 83% compared to Tx animals but was still somewhat low (11% decrease) compared to euthyroid controls. Kidney weight registered a parallel decrease in Tx animals (55%) and did not show appreciable improvement after treatment with a single dose of T_3, whereas in the T_R group kidney weight improved and the increase in kidney weight paralleled that in body weight (Table 1).

Measurements of ATPase activity at 25°C revealed that Tx resulted in a substantial decrease (68%) and treatment with a single dose of T_3 had no restorative effect. The T_R regimen brought the activity back to euthyroid level. A similar trend was seen for measurements made at 37°C. As a consequence, the activity ratio (activity at 37°C/activity at 25°C) was more or less comparable in all the groups (Table 2). However, the picture for SO activity was entirely different. The measurement at 25°C revealed that Tx had no effect on SO activity. However, paradoxically, treatment with a single dose of T_3 resulted in a further significant lowering (36%) of activity. Activity was unchanged in the T_R group. When measurements were carried out at 37°C, in both the Tx and T_3 groups the activities were lowered by 38–40%. In the T_R group, activity increased, reaching 86% of the euthyroid value. The activity ratio was significantly low in Tx animals; these values were comparable to euthyroid animals in the other two groups (Table 3). These differential effects on the two enzyme systems suggested that the temperature kinetics properties of the two enzymes may be affected differently by the thyroid status of the animals. Hence, further studies were carried out to examine the temperature dependence of the activities of these two enzymes.

The results on the temperature dependence of ATPase are shown in Figure 1a-d. As can be noted, compared to the euthyroid controls, the plots of activity *vs.* temperature differed significantly in the Tx and thyroid hormone-treated Tx animals. Thus, at any given temperature, the activity was significantly low in Tx animals and treatment with a single dose of T_3 had only a marginal effect. In the T_R group, there was improvement in the activity profile. In Tx animals, the temperature optimum increased by 8-53°C. Treatment with T_3 alone lowered the optimum temperature to 49°C, whereas in the T_R group the optimum temperature was restored to the euthyroid value of 45°C.

The corresponding Arrhenius plots are shown in Figure 1e-h. For all groups, the Arrhenius plot pattern was biphasic and the values of E_H were low while those of E_L were high. However, the slopes and intersection points differed, suggesting that there were thyroid status-dependent changes in the energies of activation E_H, E_L and Tt. The values of E_H, E_L and Tt are given in Table 4. Thus, in euthyroid animals, the values of E_H and E_L were 56 and 80 KJ/mole, respectively, with phase transition occurring at 22°C. Tx or the T_R regimen had no effect on E_H, while treatment with a single dose of T_3 caused a small increase. By contrast, the values of E_L doubled in the Tx and T_3 groups; T_R was effective in lowering the value of E_L. Tt decreased by 2.4°C in Tx animals. Treatment with thyroid hormone regimens T_3 and T_R caused further decreases in Tt.

The plots of activity *vs.* temperature for SO are shown in Figure 2a-d. Consistent with the data in Table 3, the activities were generally low in the experimental groups. Also, thyroid status-dependent differences in the pattern of

activity *vs.* temperature became apparent in high temperature ranges. The optimum temperature (49°C) was unchanged in the Tx and T_R groups. However, treatment with a single dose of T_3 lowered the optimum temperature by 4°C.

The corresponding Arrhenius plots for SO are shown in Figure 2e-h. Although the plots were biphasic, they represented a reversed pattern compared to that noted for ATPase (Fig. 1e-h). Thus, the values of E_H were high, and the opposite was true for values of E_L. These results are consistent with our previously reported observation (Patel & Katyare, 2006). Tx caused significant but disproportionate reduction in the values of E_H and E_L (29% and 46% decrease, respectively) without affecting Tt. Treatment with a single dose of T_3 was able to correct only the value of E_H. Under this condition, Tt increased by 3.2°C. In the T_R group, E_H was corrected to euthyroid value while E_L increased beyond control and Tt increased by 3.5°C (Table 5).

In light of these differential effects, it was of interest to find out how thyroid status affected the lipid milieu and to seek its possible correlation with the temperature kinetics parameters of the two enzyme systems. The data on the TPL and CHL contents as influenced by thyroid status are given in Table 6. Tx resulted in a small but reproducible reduction in TPL content (15%), which could be corrected

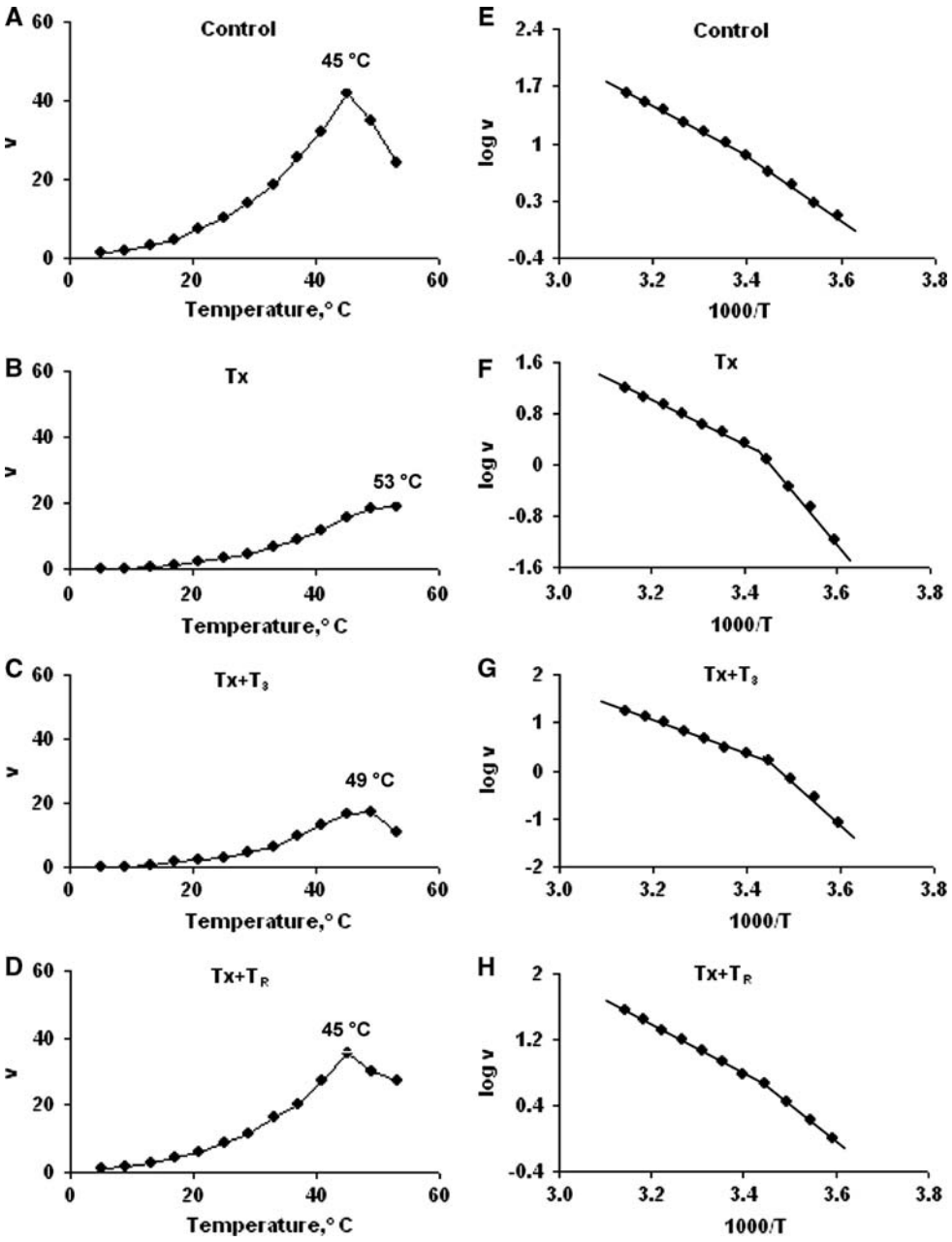

Fig. 1 Typical plots depicting dependence of enzyme activity on temperature and the corresponding Arrhenius plots for FoF_1 ATPase from rat kidney mitochondria. In the temperature curves, the enzyme activity (v, μmol Pi liberated/h/mg protein) on the ordinate is plotted *vs.* temperature (°C) on the abscissa. In Arrhenius plots, the log of enzyme activity (v) is plotted against 1,000/T, where v and T represent the activity at corresponding absolute temperature (T, in °C + 273.2). The plots are typical of six to eight independent experiments

Table 4 Effect of Tx and subsequent treatment with thyroid hormones on Arrhenius kinetics properties of rat kidney mitochondrial FoF_1 ATPase

Animals	Energy of activation (KJ/mol)		Phase transition temperature (Tt, °C)
	E_H	E_L	
Control (n = 8)	56.2 ± 0.88	79.7 ± 2.04	22.1 ± 0.80
Tx (n = 6)	56.9 ± 2.87	164.5 ± 10.69***	19.7 ± 0.63**
T_3 (n = 6)	62.8 ± 1.75*	167.5 ± 6.38***	16.8 ± 0.25***,‡
T_R (n = 6)	55.2 ± 1.28	91.8 ± 3.29*,‡	17.7 ± 0.40***,†

The results are given as mean ± standard error of the mean of the number of independent experiments indicated in parentheses

* $p < 0.01$, $^{**}p < 0.05$ and $^{***}p < 0.001$ compared to euthyroid controls; $^{\dagger}p < 0.02$ and $^{\ddagger}p < 0.001$ compared to the Tx group

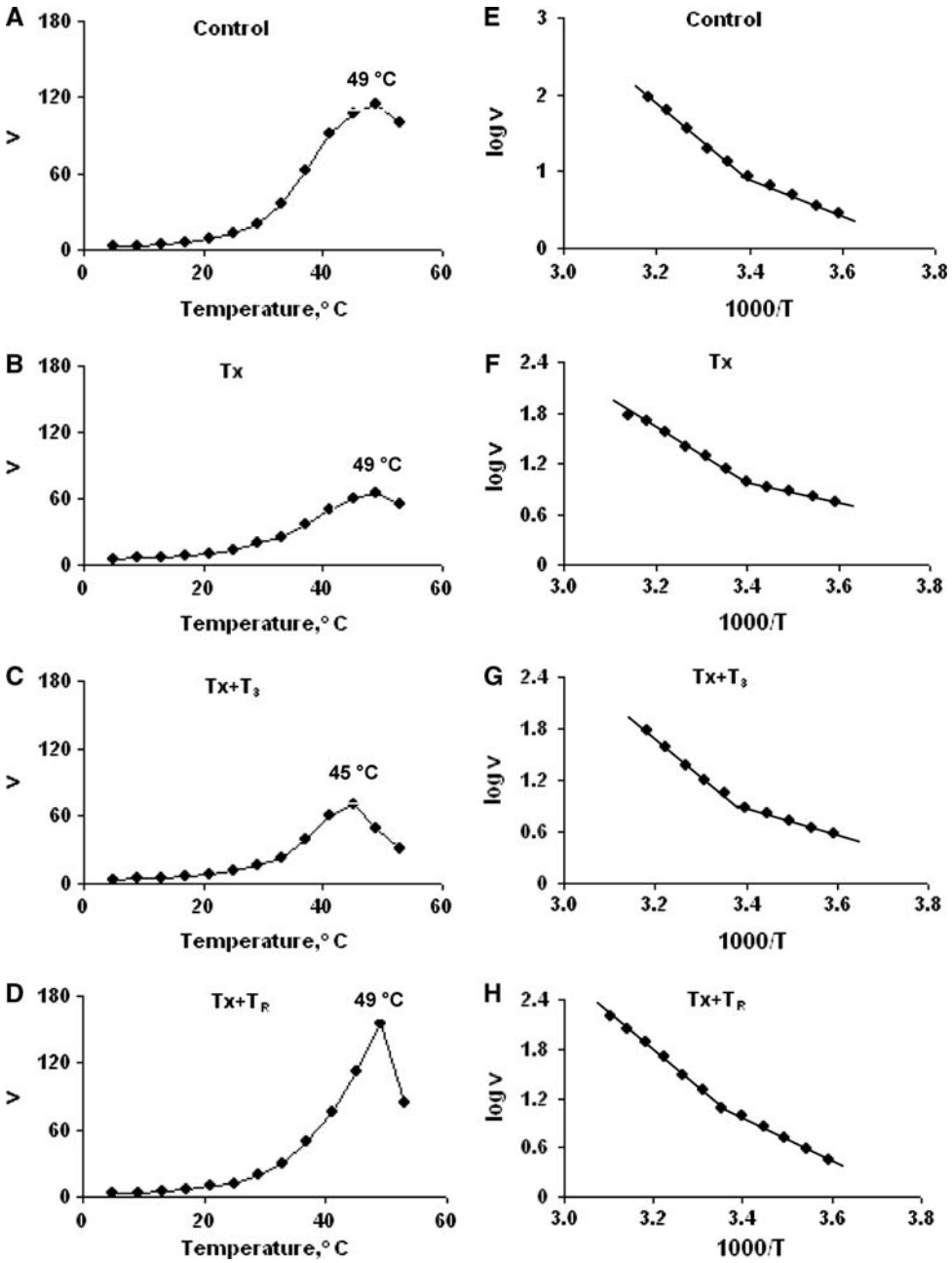

Fig. 2 Typical plots depicting dependence of enzyme activity on temperature and the corresponding Arrhenius plots for SO from rat kidney mitochondria. In the temperature curves, the enzyme activity (v, nmol O_2/min/mg protein) on the ordinate is plotted *vs.* temperature (°C) on the abscissa. In Arrhenius plots, the log of enzyme activity (v) is plotted against 1,000/T, where v and T represent the activity at corresponding absolute temperature (T, in °C + 273.2). The plots are typical of four to six independent experiments

Table 5 Effect of Tx and subsequent treatment with thyroid hormones on Arrhenius kinetics properties of rat kidney mitochondrial SO

Animals	Energy of activation (KJ/mol)		Phase transition temperature (Tt, °C)
	E_H	E_L	
Control (n = 6)	86.7 ± 1.45	42.5 ± 2.18	20.9 ± 0.31
Tx (n = 4)	61.4 ± 2.86***	22.9 ± 1.23***	20.9 ± 0.32
T_3 (n = 4)	91.8 ± 4.08‡	28.5 ± 1.86***,§	24.1 ± 0.61*,†
T_R (n = 4)	85.7 ± 2.10‡	52.1 ± 1.14*,‡	24.4 ± 1.38**,§

The results are given as mean ± standard error of the mean of the number of independent experiments indicated in parentheses
* $p < 0.01$, ** $p < 0.05$ and *** $p < 0.001$ compared to euthyroid controls; † $p < 0.01$, § $p < 0.05$ and ‡ $p < 0.001$ compared to the Tx group

Table 6 Effect of Tx and subsequent treatment with thyroid hormones on TPL, CHL, TPL/CHL ratio and fluorescence polarization in rat kidney mitochondria

Animals	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)	Fluorescence polarization
Control (n = 8)	197.3 ± 9.25	110.3 ± 2.80	0.89 ± 0.02	0.234 ± 0.001
Tx (n = 4)	167.6 ± 5.66*	81.0 ± 2.64***	1.04 ± 0.01***	0.176 ± 0.002***
T_3 (n = 4)	151.0 ± 9.62**	33.9 ± 1.98***,‡	2.26 ± 0.15***,‡	0.267 ± 0.001***,‡
T_R (n = 4)	242.6 ± 19.91†	63.2 ± 7.89***	1.96 ± 0.08***,‡	0.247 ± 0.001***,‡

The results are given as mean ± standard error of the mean of the number of independent experiments indicated in parentheses
* $p < 0.01$, ** $p < 0.05$ and *** $p < 0.001$ compared to euthyroid controls; † $p < 0.01$ and ‡ $p < 0.001$ compared to the Tx group

only by T_R. The CHL content decreased by 27% in Tx animals; treatment with a single dose of T_3 resulted in a further substantial (69%) decrease. Even in the T_R group the CHL content decreased by 43%. These changes were accompanied by increases in the ratio of TPL/CHL (mole:mole). Thus, compared to the euthyroid control value of 0.89, the ratio increased to 1.04 in the Tx group and more than doubled in the T_3 and T_R groups. Paradoxically, however, as judged from the fluorescence polarization parameter (p), the membrane seemed to be more fluidized in Tx animals.

The thyroid status-dependent changes in phospholipid composition are summarized in Table 7. As can be noted in the Tx animals, the sphingomyelin (SPM) and phosphatidylcholine (PC) components decreased, whereas the phosphatidylserine (PS) and diphosphatidylglycerol (DPG) components increased. Treatment with a single dose of T_3 caused a substantial decrease in the phosphatidylinositol (PI) and PS components, with a further decrease noted for the SPM component. Under these conditions, the PC and phosphatidylethanolamine (PE) components increased, whereas DPG was normalized. The T_R regimen resulted in increased proportion of lysophospholipids (Lyso). The PC and DPG components were normalized without any change being seen in the other components. The observed changes were also reflected in terms of the contents of the individual phospholipids (Table 8).

We then tried to correlate the observed changes in the lipid/phospholipid profiles (Tables 6–8) with the temperature kinetics parameters of the two enzyme systems. The data are given in Table 9. Thus, for ATPase, the activity correlated positively with TPL content, whereas E_L correlated negatively with CHL. Tt showed a positive correlation with PI and CHL. However, the ratio of TPL/CHL exerted a negative effect on Tt (Table 9).

For SO, the activity at 25°C correlated positively with PI, PS and CHL, whereas a strong negative correlation was obtained with PC, PE and TPL/CHL. For activity at 37°C, CHL was the only positive influencing factor. The E_H value showed positive and negative correlations, respectively, with PC and PS, whereas E_L showed a strong positive correlation with TPL. Tt correlated positively with Lyso and the TPL/CHL ratio but negatively with PI and DPG (Table 9). We also tried to correlate membrane fluidity with changes in the lipid composition. These data revealed that membrane fluidity correlated negatively with PS, total basic phospholipids and TPL/PI, TPL/PS and TPL/CHL, whereas a positive correlation with acidic phospholipids was evident (*data not shown*).

Discussion

From the data presented, it is clear that the activities of ATPase and SO were affected differently by the thyroid status. As is evident, ATPase activity decreased significantly in hypothyroid rats and treatment with a single dose of T_3 had no restorative effect. Only T_R was able to restore

Table 7 Effects of Tx and subsequent treatment with thyroid hormones on phospholipid composition in rat kidney mitochondria

Phospholipid class	Composition (% of total)			
	Control (n = 8)	Tx (n = 4)	T_3 (n = 4)	T_R (n = 4)
Lyso	3.19 ± 0.26	3.42 ± 0.20	3.57 ± 0.25	4.25 ± 0.09****,¶
SPM	12.42 ± 0.51	10.32 ± 0.29**	7.33 ± 0.07****,¶	10.25 ± 0.34**
PC	35.64 ± 0.43	33.99 ± 0.38*	39.54 ± 0.22****,¶	35.15 ± 0.10‡
PI	4.95 ± 0.22	4.30 ± 0.20	2.39 ± 0.05****,¶	3.41 ± 0.16****,†
PS	4.48 ± 0.23	6.71 ± 0.21****	2.36 ± 0.01****,¶	2.50 ± 0.04****,¶
PE	26.23 ± 0.40	26.05 ± 0.41	32.31 ± 0.25****,¶	30.88 ± 0.44****,¶
DPG	13.10 ± 0.38	15.20 ± 0.30***	12.50 ± 0.08¶	13.56 ± 0.15§

The results are given as mean ± standard error of the mean of the independent observations indicated in parentheses

$^{*}p < 0.02$, $^{**}p < 0.01$, $^{***}p < 0.002$ and $^{****}p < 0.001$ compared to euthyroid controls; $^{\dagger}p < 0.02$, $^{\ddagger}p < 0.05$, $^{\S}p < 0.002$ and $^{\P}p < 0.001$ compared to the Tx group

Table 8 Effects of Tx and subsequent treatment with thyroid hormones on phospholipid content in rat kidney mitochondria

Phospholipid class	Content (μg/mg mitochondrial protein)			
	Control (n = 8)	Tx (n = 4)	T_3 (n = 4)	T_R (n = 4)
Lyso	6.3 ± 0.56	5.7 ± 0.44	5.3 ± 0.41	10.3 ± 0.69*****,‡
SPM	24.4 ± 1.40	17.3 ± 0.94*****	10.7 ± 0.72*****,‡	24.9 ± 2.50§
PC	70.5 ± 3.39	57.0 ± 2.55**	57.3 ± 3.18**	85.2 ± 6.88†
PI	9.9 ± 0.75	7.2 ± 0.43**	3.4 ± 0.20*****,‡	8.2 ± 0.53
PS	9.0 ± 0.55	11.2 ± 0.23***	3.4 ± 0.21*****,‡	6.0 ± 0.49*****,‡
PE	51.5 ± 2.74	43.7 ± 1.22*	46.9 ± 3.03	75.0 ± 6.34****,†
DPG	25.5 ± 1.67	25.5 ± 0.68	18.1 ± 1.12***,‡	33.0 ± 3.00§

The results are given as mean ± standard error of the mean of the independent observations indicated in parentheses

$^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.01$, $^{****}p < 0.002$ and $^{*****}p < 0.001$ compared to euthyroid controls; $^{\dagger}p < 0.01$; $^{\S}p < 0.05$ and $^{\ddagger}p < 0.001$ compared to the Tx group

the activity to normal (Table 2). It has been reported that thyroid deficiency results in decreased synthesis of the β-subunit of ATPase in the liver (Izquierdo & Cuezva, 1993). Our result suggests that a similar situation may exist even in the kidney mitochondria. The results also demonstrate that sustained treatment with $T_3 + T_4$ (Escobar-Morreale et al., 1996) is only effective in restoring ATPase activity, which implies possible stimulation of synthesis of the β-subunit. However, this possibility needs to be verified experimentally. Additionally, our results suggest that changes in TPL content may modulate the enzyme activity (e.g., *see* Table 9).

In case of SO, the decrease in activity in hypothyroid rats was manifest only at 37°C but not at 25°C (Table 3). Paradoxically, however, following treatment with a single dose of T_3, the activity even at 25°C registered a further significant decrease. The reason for this remains unclear at this stage. By contrast, the T_R regimen was able to restore the activities at 25°C as well as 37°C to near normal. Earlier, we reported that while the contents of cytochromes in the kidney mitochondria from Tx animals decreased, the succinate dehydrogenase activity in the kidney was unaffected by Tx (Rajwade et al., 1975; Satav & Katyare, 1991). Treatment with a single dose of T_3 brought about selective increase in the content of cytochromes but had only a marginal effect on succinate dehydrogenase activity (Satav & Katyare, 1991). In view of the above, it may be suggested that the observed changes in SO activity may relate to the compositional changes in the ETC components rather than to changes in succinate dehydrogenase activity. Thyroid status-dependent differential changes in the lipid/phospholipid profiles (Tables 7 and 8) may be an additional regulatory factor since SO has a known requirement for bulk phospholipids (Daum, 1985).

It has been reported that the half-life ($t_{1/2}$) of mitochondrial proteins is of the order of 3.8 days (Swick & Ip, 1974; Katyare & Shallom, 1988). Interestingly, however, the components of two classes of SPMs from rat brain showed nonsynchronous and heterogeneous rates of turnover (Freyzs, Lastennet & Mandel, 1976). Earlier studies

Table 9 Correlation of kinetics parameters of FoF_1 ATPase and SO with membrane lipid/phospholipid composition

Parameter	Correlation with phospholipid class			
	FoF_1 ATPase		SO	
	Positive	Negative	Positive	Negative
Activity at 25°C	TPL (+ 0.675)	—	PI (+ 0.614)	PC (– 0.789)
			PS (+ 0.592)	PE (– 0.745)
			CHL (+ 0.746)	TPL/CHL (– 0.699)
Activity at 37°C	TPL (+0.692)	—	CHL (+ 0.660)	—
E_H	—	—	PC (+ 0.625)	PS (– 0.845)
E_L	—	CHL (– 0.702)	TPL (+ 0.758)	—
Tt	PI (+ 0.781)	TPL/CHL (– 0.766)	Lyso (+ 0.648)	PI (– 0.737)
	CHL (+ 0.793)		TPL/CHL (+ 0.678)	DPG (–0.618)

The experimental details are given in the text. Values given in parentheses indicate the regression coefficient, *r*, which is based on four to eight independent experiments in each group

from our laboratories demonstrated that Tx resulted in loss of synchrony of turnover of rat liver and kidney mitochondrial proteins and DNA, implying that thyroid hormones may constitute a factor for maintaining the synchrony of turnover of mitochondria (Rajwade et al., 1975). Thus, it may be suggested that synchronous accretion and turnover of mitochondrial proteins may be an additional regulatory factor for maintaining proper membrane structure-function relationships. It is not clear at this stage whether the two thyroid hormone regimens, T_3 and T_R, which we employed in the present studies, are able to fulfill these conditions. However, from the data presented, it may be assumed that a single dose of T_3 may not be able to effectively replace or replenish the essential components of the ETC. The sustained combined treatment (T_R) is more likely to fulfill these requirements.

Thyroid status also significantly altered the lipid/phospholipid profile, and this aspect deserves some comment. Thus, in Tx animals, TPL and CHL content decreased, which is similar to our earlier reported observations for the liver mitochondria (Parmar et al., 1995). Paradoxically, however, treatment with T_3 had a further lowering effect on CHL content. By contrast, the T_R regimen restored the TPL content but could not bring the value of CHL to euthyroid level. Tx also produced a small but reproducible decrease in SPM and PC components and about a 50% increase in PS. Treatment with T_3 resulted in a further decrease in SPM with simultaneous reductions of PI, PS and DPG. By contrast, the two major basic phospholipids, PC and PE, showed small but reproducible increases. In the T_R group, practically similar results were obtained except for a significant increase in the Lyso component.

It is well recognized that DPG is synthesized by the mitochondria themselves, and the effect of thyroid hormones on DPG synthesis by liver mitochondria has been well documented (Hostetler, 1991; Porcellati & Arienti, 1983). However, since the DPG composition/content decreased only in the T_3 group, it may be suggested that under normal physiological conditions thyroid hormones may not play a significant role in controlling DPG synthesis by the kidney mitochondria. Similarly, we noted earlier that even in diabetic animals insulin has no regulatory role in DPG synthesis by the kidney mitochondria (Patel & Katyare, 2006). Also, the effects on other phospholipid classes in the kidney mitochondria were at variance from those observed in liver mitochondria (Parmar et al., 1995). The results of the present studies thus point out that thyroid hormones may have a tissue-specific differential effect on phospholipid metabolism and the phospholipid makeup of the mitochondria.

The data of regression analysis suggest the dependence of ATPase activity on TPL. The data also suggest that the observed decrease in SO activity at 25°C may be related to the strong negative influence of the major basic phospholipids (PC and PE) and the TPL/CHL ratio (Table 9).

The other kinetic parameters, e.g., E_H, E_L and Tt, were also differentially influenced by thyroid status. Thus, E_L for ATPase correlated negatively with CHL, whereas these values showed a strong positive correlation with TPL. E_H was not affected by lipid makeup except in the case of SO. PC and PS seemed to have opposite positive and negative influences, respectively. The value of Tt in ATPase was under the positive influence of PI and CHL but correlated negatively with TPL/CHL. For SO the picture was somewhat opposite in that TPL/CHL with Lyso seemed to be the positive modulator, whereas the acidic phospholipids, DPG and PI, showed a negative correlation.

The differential effects on ATPase and SO activity are not really surprising since ATPase is uniformly distributed in the inner membrane and has restricted microdomains.

Also, it has been reported that ATPase has a requirement for acidic phospholipids, in particular DPG (Daum, 1985). The DPG content of kidney mitochondria is sufficiently high, and thyroid status had only a minor influence on DPG composition or content (Tables 7 and 8). In other words, it would seem that in the experimental groups DPG is more than sufficient to meet the requirement of ATPase.

By contrast, SO spans a large segment of the ETC. It has been reported that SO has a requirement for bulk phospholipids for its activity (Daum, 1985). This indeed seems to be the case, as judged from regression analysis given in Table 9.

The role of lipids, proteins, lipid-protein interactions and protein-protein interactions in maintaining the proper membrane, structure integrity and structure-function relationship is very well documented (Balla, 2005; Booth & Curran, 1999; Maggio et al., 2005; Parmar et al., 1995). Based on these interactions, a membrane catalysis model has been proposed. More recently, it has been demonstrated that the mitochondrial outer membrane contains a multisubunit translocase (TOM), which is responsible for transport of precursor proteins into the internal compartments of this organelle and for the insertion of proteins in the outer membrane (Rapaport, 2005). Similarly it has been proposed that peripheral benzodiazepine receptor may have a role in the transport of CHL (Papadopoulo, 2003). As we have pointed out above, thyroid hormones not only differentially and selectively influence the compositional changes in the kidney mitochondrial ETC but also constitute a factor for maintaining the synchrony of turnover of mitochondria (Rajwade et al., 1975). Therefore, it may be suggested that because of these compositional changes the normal protein makeup may not be reconstituted to the euthyroid level by the two thyroid hormone regimens employed in the present studies. Likewise, thyroid status also differentially affected the CHL, TPL and phospholipid compositions. Therefore, one can anticipate that the proper lipid-protein and protein-protein interactions and formation of lipid rafts in the membrane milieu may not reach the euthyroid level. These changes would, in turn, ultimately affect the kinetic properties of the membrane-bound enzymes. As pointed out above, the two enzyme systems under study are localized in different membrane domains. This is consistent with our finding that the temperature kinetics properties of these two enzymes were affected differently by thyroid status.

The changes in the composition/content of the phospholipids may have additional implications. It has been shown that DPG and ceramide are responsible for release of cytochrome *c*, which may be responsible for apoptosis (McMillin & Dowhan, 2002; Iverson & Orrenius, 2004; Richter & Ghafourifar, 1999). Similarly, the role of inositol lipids as universal lipid regulators of protein signaling complexes is being increasingly recognized (Balla, 2005). Also, the role of SPM in ceramide-induced signaling has been demonstrated (Ohanian & Ohanian, 2001). The other lipid factor which can influence the function of membrane proteins is the acylation of proteins (Resh, 2004). The role of myristilation has been especially well demonstrated (Marsh et al., 2002). Thyroid status-dependent alterations in fatty acid composition of mitochondrial membrane lipids has been reported (Hoch, 1988). Viewed in this context, the changes in lipid composition which we observed here, especially the decrease in PS and PI following the T_3 and T_R regimens, the lowering of SPM in Tx and T_3 animals and the increase in Lyso in the T_R group, are noteworthy (Tables 7 and 8). These compositional changes not only will affect the membrane lipid-protein and protein-protein interactions and lipid rafts but may also affect signaling mechanisms in a thyroid status-dependent manner.

The present investigations were undertaken to find out if treatments with replacement combined therapy with T_3 and T_4 (Escobar-Morreale et al., 1996) could restore mitochondrial function to the euthyroid state. Our present results suggest that both treatment regimens were unable to restore to normal the temperature kinetics properties of kidney mitochondrial ATPase and SO, as well as the lipid/phospholipid profile makeup. Therefore, it may be suggested that while replacement therapy is able to restore the thyroid hormone status of a given tissue to the euthyroid state (Escobar-Morreale et al., 1996), a more complex and intricate mechanism may be involved in restoring the membrane composition, structure-function relationships and enzyme functions to euthyroid status.

References

Balla T (2005) Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. J Cell Sci 118:2093–2104

Bangur CS, Howland JL, Katyare SS (1995) Thyroid hormone treatment alters phospholipid composition and membrane fluidity of rat brain mitochondria. Biochem J 305:29–32

Bartlett GR (1959) Phosphorus assay in column chromatography. J Biol Chem 234:466–468

Booth PJ, Curran AR (1999) Membrane protein folding. Curr Opin Struct Biol 9:115–121

Cappello AR, Gnoni GV (1994) Influence of triiodothyronine and insulin on the synthesis of glycerolipids in rat hepatocytes. Biochem Mol Biol Int 34:101–107

Daum G (1985) Lipids of mitochondria. Biochim Biophys Acta 822:1–42

Dave KR, Katyare SS (2002) Effect of alloxan-induced diabetes on serum and cardiac butyrylcholinesterases in the rat. J Endocrinol 175:241–250

Dave KR, Syal AR, Katyare SS (1999) Tissue cholinesterases. A comparative study of their kinetic properties. Z Naturforsch 55c:100–108

Del Compare JA, Aguirre JA, Ibarra FR, Barontini M, Armando I (2001) Effects of thyroid hormone on the renal dopaminergic system. Endocrine 15:297–303

Dixon M, Webb EC (1979) In: Dixon M, Webb EC, Thorne C Jr, Tipton KF (eds), Enzymes Kinetics, 3rd ed. Longman, London, pp 47–206

Escobar-Morreale HF, del Rey FE, Obregon MJ, de Escobar GM (1996) Only the combined treatment with thyroxine and triiodothyronine ensures euthyroidism in all tissues of the thyroidectomized rat. Endocrinology 137:2490–2502

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Freyzs L, Lastennet A, Mandel P (1976) Metabolism of brain sphingomyelins: half-lives of sphingosine, fatty acids and phosphate from two types of rat brain sphingomyelins. J Neurochem 27:355–359

Fukuda Y, Eklof AC, Malmborg AS, Aperia A (1992) Calcium supplementation and thyroid hormone protect against gentamicin-induced inhibition of proximal tubular Na^+,K^+-ATPase activity and other renal functional changes. Acta Physiol Scand 145:93–98

Gustafsson R, Tata JR, Lindberg O, Ernster L (1965) The relationship between the structure and activity of rat skeletal muscle mitochondria after thyroidectomy and thyroid hormone treatment. J Cell Biol 26:553–578

Hoch FL (1988) Lipids and thyroid hormones. Prog Lipid Res 27:199–270

Horowitz B, Hensley CB, Quintero M, Azuma KK, Putnam D, McDonough AA (1990) Differential regulation of Na,K-ATPase alpha 1, alpha 2, and beta subunit mRNA and protein levels by thyroid hormone. J Biol Chem 265:14308–14314

Hostetler KY (1991) Effect of tyroxine on the activity of mitochondrial cardiolipin synthase in rat liver. Biochim Biophys Acta 1086:139–140

Iverson SL, Orrenius S (2004) The cardiolipin-cytochrome *c* interaction and the mitochondrial regulation of apoptosis. Arch Biochem Biophys 423:37–46

Izquierdo JM, Cuezva JM (1993) Thyroid hormones promote transcriptional activation of the nuclear gene coding for mitochondrial beta-F1-ATPase in rat liver. FEBS Lett 323:109–112

Katewa SD, Katyare SS (2003) A simplified method for inorganic phosphate determination and its application for phosphate analysis in enzyme assays. Anal Biochem 323:180–187

Katyare SS, Fatterpaker P, Sreenivasan A (1971) Effect of cytochrome *c* on succinate oxidation in rat liver mitochondria. Indian J Biochem Biophys 8:130–132

Katyare SS, Joshi MV, Fatterpaker P, Sreenivasan A (1977) Effect of thyroid deficiency on oxidative phosphorylation in rat liver, kidney, and brain mitochondria. Arch Biochem Biophys 182:155–163

Katyare SS, Shallom JM (1988) Altered cerebral protein turnover in rats following prolonged in vivo treatment with nicotine. J Neurochem 50:1356–1363

Katz AI, Emmanouel DS, Lindheimer MD (1975) Thyroid hormone and the kidney. Nephron 15:223–249

Lowry OH, Rosebrough NJ, Farr AL Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Maggio B, Rosetti CM, Borioli GA, Fanani ML, Del Boca M (2005) Protein-mediated surface structuring in Biomembranes. Braz J Med Biol Res 38:1735–1748

Marsh D, Horvath LI, Swamy MJ, Mantripragada S, Kleinschmidt JH (2002) Interaction of membrane-spanning proteins with peripheral and lipid-anchored membrane proteins: perspectives from protein-lipid interactions. Mol Membr Biol 19:247–255

McMillin JB, Dowhan W (2002) Cardiolipin and apoptosis. Biochim Biophys Acta 1585:97–107

Ohanian J, Ohanian V (2001) Sphingolipids in mammalian cell signaling. Cell Mol Life Sci 58:2053–2068

Pandya JD, Dave KR, Katyare SS (2004) Effect of long-term aluminum feeding on lipid/phospholipid profiles of rat brain myelin. Lipids Health Dis 3:13–18

Papadopoulo V (2003) Peripheral benzodiazepine receptor: structure and function in health and disease. Ann Pharm Fr 61:30–50

Paradies G, Ruggiero FM, Dinoi P (1991) The influence of hypothyroidism on the transport of phosphate and on the lipid composition in rat-liver mitochondria. Biochim Biophys Acta 1070:180–186

Parmar DV, Khandkar MA, Pareira L, Bangur CS, Katyare SS (1995) Thyroid hormones alter Arrhenius kinetics of succinate-2,6-dichloroindophenol reductase, and the lipid composition and membrane fluidity of rat liver mitochondria. Eur J Biochem 230:576–581

Pasquini JM, Faryra de Raveglia I, Capitman N, Soto EF (1980) Differential effect of L-thyroxine on phospholipid biosynthesis in mitochondria and microsomal fraction. Biochem J 186:127–133

Patel SP, Katyare SS (2006) Effect of alloxan-diabetes and subsequent treatment with insulin on kinetic properties of succinate oxidase activity from rat liver mitochondria. Z Naturforsch 61c:756–762

Porcellati G, Arienti G (1983) Metabolism of phosphoglycerides In: Lajtha A (ed), Handbook of Neurochemistry, 2nd ed, vol 3. Plenum Press, New York, pp 133–161

Rajwade MS, Katyare SS, Fatterpaker P, Sreenivasan A (1975) Regulation of mitochondrial protein turnover by thyroid hormone(s). Biochem J 152:379–387

Rapaport D (2005) How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane? J Cell Biol 171:419–423

Resh MD (2004) Membrane targeting of lipid modified signal transduction proteins. Subcell Biochem 37:217–232

Richter C, Ghafourifar P (1999) Ceramide induces cytochrome *c* release from isolated mitochondria. Biochem Soc Symp 66:27–31

Satav JG, Katyare SS (1982) Effect of experimental thyrotoxicosis on oxidative phosphorylation in rat liver, kidney and brain mitochondria. Mol Cell Endocrinol 28:173–189

Satav JG, Katyare SS (1991) Effect of thyroidectomy and subsequent treatment with triiodothyronine on kidney mitochondrial oxidative phosphorylation in the rat. J Biosci 16:81–89

Skipski VP, Peterson RF, Barclay M (1964) Quantitative analysis of phospholipids by thin-layer chromatography. Biochem J 90:374–378

Swick RW, Ip MM (1974) Measurement of protein turnover in rat liver with [^{14}C] carbonate. J Biol Chem 249:6836–6841

Tata JR (1964) Biological action of thyroid hormones at the cellular and molecular levels. In: Litwack G, Kritchevsky D (eds), Action of Hormones on Molecular Processes. Wiley, New York, pp 58–131

Tata JR (1966) The regulation of mitochondrial structure and function by thyroid hormones under physiological conditions. In: Tager JM, Papa S, Quagliariello E, Slater EC (eds), Regulation of Metabolic Processes in Mitochondria. Elsevier Biomedical Press, Amsterdam, pp 489–507

Tata JR, Ernster L, Lindberg O, Arrhenius E, Pedersen S, Hedman R (1963) The action of thyroid hormones at the cell level. Biochem J 86:408–428

Tata JR, Widnell CC (1966) Ribonucleic acid synthesis during the early action of thyroid hormones. Biochem J 98:604–620

Vargas F, Moreno JM, Rodriguez-Gomez I, Wangensteen R, Osuna A, Alvarez-Guerra M, Garcia-Estan J (2006) Vascular and renal function in experimental thyroid disorders. Eur J Endocrinol 154:197–212

Wrutniak-Cabello C, Casas F, Cabello G (2001) Thyroid hormone action in mitochondria. J Mol Endocrinol 26:67–77

Zlatkis A, Zak B (1969) Study of a new cholesterol reagent. Anal Biochem 29:143–148