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Thyroid Hormone-Induced Alterations in Membrane Structure-Function Relationships: Studies on Kinetic Properties of Rat Kidney Microsomal Na^+,K^+-ATPase and Lipid/Phospholipid Profiles

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Abstract The effects of thyroidectomy (Tx) and subsequent treatment with 3,5,3′-triiodothyronine (T_3) or combined replacement therapy (T_R) with T_3 and thyroxine (T_4) on the substrate and temperature kinetics properties of Na^+,K^+-ATPase and lipid/phospholipid makeup of rat kidney microsomes were examined. Enzyme activity was somewhat high in the hypothyroid (Tx) animals and increased significantly following T_3 treatment, while T_R treatment caused a decrease. In the Tx and T_3 groups enzyme activity resolved in two kinetic components, while in the T_R group the enzyme showed allosteric behavior up to 0.5 mM ATP concentration. The K_m and V_{max} values of both the components decreased in Tx animals without affecting the catalytic efficiency. T_3 treatment caused a significant increase in the V_{max} of both the components, with a significant increase in the catalytic efficiency, while the K_m values were not upregulated. The T_R regimen lowered the K_m and V_{max} of component II but improved the catalytic efficiency. Thyroid status-dependent changes were also noted in the temperature kinetics of the enzyme. Regression analysis revealed that changes in the substrate and temperature kinetics parameters correlated with specific phospholipid components.

Keywords Thyroid hormone · Triiodothyronine · Thyroxine · Microsomal Na^+,K^+-ATPase · Substrate and temperature kinetics · Lipid/phospholipid profile

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Introduction

Thyroid hormones affect the function of several tissues and organs, including the kidneys. Thus, it has been reported that in hypothyroid rats renal plasma flow, glomerular filtration rate and filtered Na^+ load decreased (Holmes & Di Scala, 1970; Katz & Lindheimer, 1973; Katz, Emmanouel & Lindheimer, 1975; Kinsella & Sacktor, 1985; Lo et al., 1976; Michael et al., 1972). These changes have been correlated with the decrease in levels of Na^+,K^+-ATPase in the kidneys (Lo et al., 1976). In animals made hypothyroid by feeding aminotriazole in the diet, the Na^+,K^+-ATPase activity in the proximal convoluted tubules decreased by 57%, which was corrected by simultaneous treatment with L-thyroxine (T_4) (Garg & Tisher, 1985). In surgically thyroidectomized (Tx) rats the Na^+,K^+-ATPase activity in the kidney cortex decreased by about 50% and treatment with 50 μg 3,3′,5-triiodo-L-thyronine (T_3) on 3 alternate days caused 67% stimulation of the enzyme activity over the hypothyroid value (Lo et al., 1976). The observed increase was correlated directly with the proportionate upregulation of the mRNA encoding the α- and β-subunits of Na^+,K^+-ATPase (Chaudhury et al., 1987; Gick, Ismail-Beigi & Edelman, 1988; McDonough et al., 1988). It is well documented that Na^+,K^+-ATPase in the kidneys represents a pure α_1 and β_1 combination (Blanco & Mercer, 1998).

While the Na^+,K^+-ATPase in the whole kidney cortex represents predominantly a pure α_1 and β_1 combination (Blanco & Mercer, 1998), recently it has been demonstrated that the microsomal enzyme in several tissues comprises a combination of α- and β_3-subunits (Arystarkhova & Sweadner, 1997). Also, the stoichiometry of α- and β_3-subunits is tissue-specific (Arystarkhova & Sweadner, 1997). Thus, in the kidney microsomal enzyme α- and β_3-subunits occur in proportion of about 3:1

(Arystarkhova & Sweadner, 1997). In earlier studies from our laboratory we demonstrated that the liver microsomal Na^+,K^+-ATPase shows significant differences from the plasma membrane Na^+,K^+-ATPase with respect to its K_m for ATP, Na^+ and K^+ (Katewa & Katyare, 2003a). Based on these observations, we suggested that the microsomal enzyme may be involved in the temperature homeostasis of the internal organs (Katewa & Katyare, 2003a).

The requirement of acidic phospholipids for Na^+,K^+-ATPase has been well documented (Robinson & Flashner, 1979), and the thyroid hormones are known to regulate lipid metabolism in responsive tissues (Hoch, 1988). That the microsomes are the major sites of lipid biosynthesis is also well recognized.

In light of the above, microsomal Na^+,K^+-ATPase offers an interesting and unique system to evaluate the regulation of the structure-function relationships of membrane-bound enzymes such as Na^+,K^+-ATPase by thyroid hormones.

Although treatment with T_3 is known to elicit maximum stimulatory response at the end of 48 h (Satav & Katyare, 1991; Tata, 1964), it has now been pointed out that tissue euthyroid status with respect to the contents of thyroid hormones is reached only after a 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 (Escobar-Morreale et al., 1996).

In view of the above, it was of interest to find out the effect of thyroid hormone deficiency and treatment with the two thyroid hormone regimens cited (Escobar-Morreale et al., 1996; Satav & Katyare, 1991; Tata, 1964) on kidney microsomal lipid/phospholipid profiles and how the possible changes could influence the microsomal structure-function relationships. More importantly, it was of interest to examine if the combined T_4 and T_3 treatment (Escobar-Morreale et al., 1996) was able to restore membrane structure-function relationships to the normal euthyroid state.

To illustrate this point, we determined the substrate and temperature kinetics properties of microsomal Na^+,K^+-ATPase together with the lipid/phospholipid profile of kidney microsomes and their correlation with the kinetics properties of Na^+,K^+-ATPase.

Materials and Methods

Chemicals

T_3, T_4, bovine serum albumin (BSA) fraction V and 1,6-diphenyl-1,3,5-hexatriene were purchased from Sigma (St. Louis, MO). ATP was 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 underwent Tx and were allowed to grow for 8–10 weeks (Katyare & Rajan, 2005). Only those animals showing considerable decrease in body weight (50–60%) were used for further studies. The controls were sham-operated. Tx rats were randomly divided in three groups. One group of Tx animals received 25 μg T_3/100 g of body weight subcutaneously (s.c.) and were killed after 48 h (Modi et al., 2007; Satav & Katyare, 1991; Tata, 1964). This group is referred to as "T_3." The second group of animals received a 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). The animals were killed on day 19. This group is hereafter referred to as "T_R." The third group (designated "Tx") received an equivalent volume of vehicle (0.9% saline containing 5 mM NaOH) in which thyroid hormone solutions were prepared. Thyroid hormone solutions were prepared freshly prior to use. Sham-operated controls received an equivalent volume of saline/NaOH vehicle.

The experimental protocol was approved by the Departmental Ethical Committee.

Isolation of Microsomes

The animals were killed by decapitation. The kidneys were removed quickly and kept in chilled (0–4°C) isolation medium (0.25 M sucrose containing 10 mM Tris-HCl buffer [pH 7.4] and 1 mM ethylenediaminetetraacetic acid [EDTA] and 25 μg BSA/ml) (Patel & Katyare, 2006a). After removing the kidney capsule, the medulla was carefully scraped off and the cortex homogenized using a Potter Elvehjem-type glass-Teflon homogenizer to obtain 10% (w/v) homogenate. After sedimenting mitochondria, the postmitochondrial supernatant was subjected to a further centrifugation at 12,000 x *g* for 10 min to sediment the light mitochondrial fraction. The resulting supernatant was then centrifuged at 100,000 x *g* for 1 h to sediment the microsomal fraction. The pellet was washed once by resuspending and resedimenting (Kaushal, Dave & Katyare, 1999; Patel & Katyare, 2006a).

ATPase Assay

After preincubating the microsomal protein (30–50 μg) in the assay medium containing 50 mM Tris-HCl buffer (pH 7.4), 120 mM NaCl, 10 mM KCl and 5 mM $MgCl_2$ at 37°C for 1 min, the reaction was initiated by addition of ATP at a final concentration of 5 mM (Katewa & Katyare, 2003a,

2003b). The reaction was terminated after 10 min by addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution, and the amount of liberated inorganic phosphate (Pi) was estimated by the method of Katewa & Katyare (2003b).

For the substrate kinetics studies, concentration of ATP was varied in the range 0.1–5 mM.

For temperature kinetics studies, experiments were carried out with a fixed ATP concentration (5 mM) and the temperature was varied from 5°C to 53°C with an increment of 4°C at each step.

Reaction velocity, v, is expressed as micromoles of Pi liberated per hour per milligram protein.

Lipid Analysis

Microsomal lipids/phospholipids were extracted with freshly prepared chloroform:methanol (2:1vol/vol) according to procedures described previously (Folch, Lees & Sloane Stanley, 1957; Pandya, Dave & Katyare, 2004).

Separation of phospholipid classes by thin layer chromatography (Pandya et al., 2004; Skipski, Peterson & Barclay, 1964), estimations of cholesterol (Zlatkis, Zak & Boyle, 1953) and phospholipid phosphorus (Bartlett, 1959) and determination of membrane fluidity were carried out as described (Pandya et al., 2004). These procedures have been described in detail (Modi et al., 2007; Pandya et al., 2004).

The content of individual phospholipid classes was calculated by multiplying the values of total phospholipid (TPL) with percent composition of the individual phospholipid classes (Pandya et al., 2004).

Data Analysis

The data for substrate kinetics were computer-analyzed using Sigma Plot version 6.1 (Jandel Corporation, San Rafael, California, USA) by three methods: Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots for the determination of K_m and V_{max} (Dixon & Webb, 1979; Patel et al., 2000). The values of K_m and V_{max} obtained by the three methods were in close agreement and were averaged for the final presentation of results.

The values of Kcat (turnover number) were calculated from the corresponding V_{max} values using the equation

$$\text{Kcat} = \frac{V_{max}\,(\text{moles}) \times N}{3600 \times 15.13 \times 10^4}$$

where N is Avogadro's number and 15.13×10^4 is the molecular weight of Na^+,K^+-ATPase (Blanco & Mercer, 1998; Wetzel, Arystarkhova & Sweadner, 1999). Since it is not possible to know the number of the ATPase molecules, the values of Kcat/K_m was normalized to ^{App}Kcat/K_m (Patel & Katyare, 2006b).

$$^{App}\text{Kcat}/K_m = \frac{\text{Kcat}}{K_m \times V(37^\circ C)}$$

where V (37°C) refers to the values given in Table 1.

The regression analysis across the groups was carried out using Jandel (Corte Madera, CA) Sigmastat Statistical Software, version 2.0. Estimation of protein was by the method of Lowry et al. (1951) using BSA as the standard. Statistical evaluation of the data was by Student's t-test.

Table 1 Effect of Tx and subsequent treatment with thyroid hormones on Na^+,K^+-ATPase activity in rat kidney microsomes

Animals	Activity (μmoles of Pi liberated/h/mg protein)		Activity ratio
	25°C	37°C	
Control (8)	9.50 ± 0.51	18.11 ± 0.73	1.92 ± 0.042
Tx (6)	12.36 ± 0.63[a]	22.60 ± 1.11[a]	1.83 ± 0.029
T_3 (6)	34.54 ± 0.92[c,*]	75.72 ± 2.15[c,*]	2.19 ± 0.056
T_R (6)	7.05 ± 0.27[b,*]	15.04 ± 0.59[a,*]	2.14 ± 0.070

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

[a] $p < 0.01$, [b] $p < 0.002$, [c] $p < 0.001$ compared to euthyroid control; *$p < 0.001$ compared to Tx

Results

In the preliminary experiments, we measured Na^+,K^+-ATPase activity at 25°C and 37°C. Measurements at 25°C revealed that Tx resulted in a 30% increase in the activity and treatment with a single dose of T_3 resulted in a further 3.6-fold increase compared to euthyroid controls. The T_R regimen, on the other hand, resulted in a 26% decrease. A similar trend was seen even for measurements made at 37°C. Thus, the activity ratios were more or less comparable for all groups (Table 1).

In the next set of experiments, we determined the dependence of the enzyme activity on substrate concentration. Typical substrate saturation curves and corresponding Eadie-Hofstee plots are shown in Figure 1. As can be noted in the control, Tx and T_3 groups, the enzymes displayed a normal pattern of substrate saturation kinetics (Fig. 1a–c), whereas in the T_R group up to the ATP concentration of 1 mM the enzyme displayed allosteric characteristics (e.g., *see* Fig. 1d, inset), beyond which the normal substrate saturation pattern was followed (Fig. 1d).

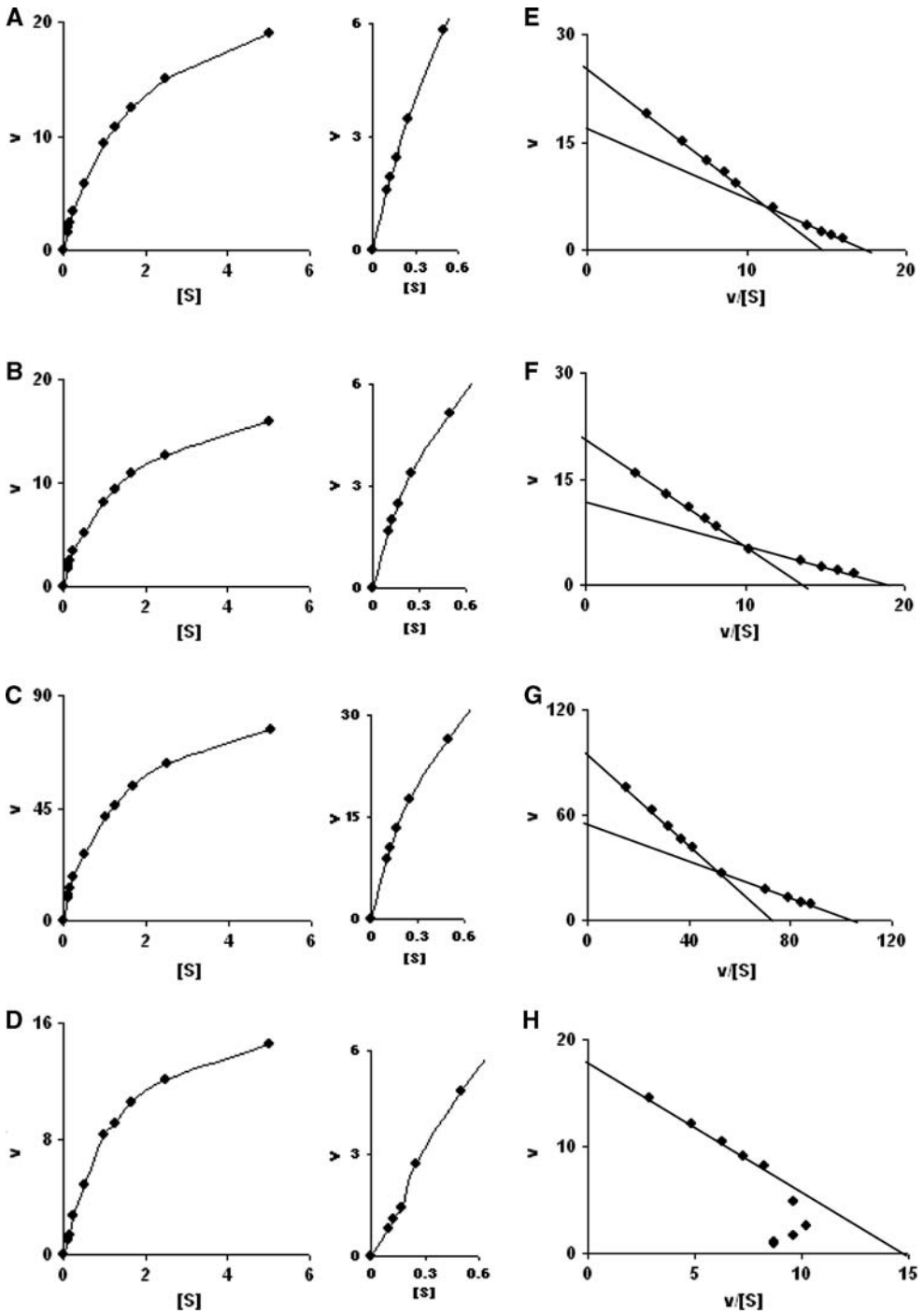

Fig. 1 Typical substrate saturation plots for rat kidney microsomal Na^+,K^+-ATPase. The enzyme activity, *v*, on the abscissa is plotted *vs.* substrate (ATP) concentration (S) on the ordinate. **a–d** Control, Tx, T_3 and T_R groups, respectively. *Insets* in **a–d** show magnified views of substrate saturation curve up to ATP concentration of 0.5 mM. Note especially the allosteric pattern in inset for **d**. Corresponding Eadie-Hofstee plots are shown in **e–h**. The enzyme activity, *v*, on the abscissa is plotted *vs.* v/S on the ordinate. The plots are typical of four to eight independent experiments, as indicated in Table 2

These differences were also illustrated when the data were transformed into Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots. For the sake of brevity, only Eadie-Hofstee plots are shown in Figure 1e–h. Thus, in the control, Tx and T_3 groups the enzyme activity resolved in two kinetically differentiable components (Fig. 1e–g); in the T_R animals only the low-affinity component II was present (Fig. 1h).

In the control group the K_m values for the two components were 0.90 and 1.90 mM, respectively, and the

Table 2 Effect of Tx and subsequent treatment with thyroid hormones on substrate kinetics properties of rat kidney microsomal Na^+,K^+-ATPase

Animals	Component I		Component II	
	K_m	V_{max}	K_m	V_{max}
Control (8)	0.90 ± 0.080	14.23 ± 0.87	1.90 ± 0.16	23.62 ± 1.00
Tx (6)	0.55 ± 0.018[c]	10.79 ± 0.44[b]	1.52 ± 0.11	21.39 ± 1.47
T_3 (6)	0.57 ± 0.022[c]	54.77 ± 2.10[d,**]	1.22 ± 0.04[d, *]	90.80 ± 2.98[d, **]
T_R (4)	— Allosteric —		1.42 ± 0.11[a]	19.04 ± 0.78[b]

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses. The kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate. K_m, mM; V_{max}, μmole of Pi liberated/h/mg protein.

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.002$, [d] $p < 0.001$ compared to euthyroid control; *$p < 0.05$, **$p < 0.001$ compared to Tx

Table 3 Effect of Tx and subsequent treatment with thyroid hormones on $^{App}Kcat/K_m$ values for the kinetic components of microsomal Na^+,K^+-ATPase in rat kidney microsomes

Animals	$^{App}Kcat/K_m \times 10^{11}$	
	Component I	Component II
Control (8)	9.92 ± 0.45	7.86 ± 0.47
Tx (6)	9.73 ± 0.46	7.03 ± 0.42
T_3 (6)	14.12 ± 0.47[b,**]	10.90 ± 0.46[b,**]
T_R (4)	-	10.58 ± 0.86[a,*]

Values of $Kcat/K_m$ were computed as described in the text. The results are given as mean ± SEM of the number indicated in parentheses.

[a] $p < 0.02$, [b] $p < 0.001$ compared to euthyroid control; *$p < 0.01$, **$p < 0.001$ compared to Tx

corresponding values for V_{max} were 14.23 and 23.62 units (μmole of Pi liberated/h/mg protein). In the Tx group the K_m of component I decreased appreciably. A similar tendency toward decrease was noted even for the K_m of component II. However, the decrease was not statistically significant. Even in T_3-treated animals, the K_m of component I remained low and comparable to that of Tx animals. However, the V_{max} increased significantly (3.35- and 5.03-fold increase compared to control or Tx group) (Table 2). The T_R regimen was unable to upregulate the K_m of component II, and the V_{max} decreased by 19%. Component I was not discernible in the T_R group.

In view of the observed changes in the K_m and V_{max} values under the different experimental conditions (Table 2), it was of interest to find out if the catalytic efficiency of the enzyme was influenced by thyroid hormone status. Catalytic efficiency was computed in terms of $^{App}Kcat/K_m$ values, as described above in "Materials and Methods." As is evident, the hypothyroid state had no effect on catalytic efficiency of the enzyme (Table 3). T_3 treatment, on the other hand, resulted in a 1.4-fold increase in the efficiency of components I and II. A similar 1.4-fold increased was seen even in the T_R group for component II (Table 3).

Table 4 Effect of Tx and subsequent treatment with thyroid hormones on Hill plot analysis on rat kidney microsomal Na^+,K^+-ATPase

Animals	Hill coefficient		Transition concentration (mM)
	n_1	n_2	
Control (8)	0.94 ± 0.013	1.33 ± 0.016	0.50 ± 0.031
Tx (6)	0.88 ± 0.014	1.35 ± 0.035	0.53 ± 0.050
T_3 (6)	0.89 ± 0.010	1.36 ± 0.009	0.55 ± 0.027
T_R (4)	–	1.33 ± 0.026	–

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

Hill plot analysis of the substrate kinetics data indicated that for the control, Tx and T_3 groups at up to 0.5–0.55 mM concentration of ATP one ATP molecule was bound, while beyond this concentration two molecules of ATP were bound (Table 4). By contrast, for the T_R group two ATP molecules were bound throughout the substrate concentration range. Typical Hill plots depicting these substrate binding characteristics are shown in Figure 2.

We then examined the influence of thyroid status on the temperature kinetics of the enzyme. The typical activity *vs.* temperature curves and the corresponding Arrhenius plots are shown in Figure 3. As can be noted, in the Tx, T_3 and T_R groups optimal temperature decreased to 45°C; in the control group the optimal temperature of the enzyme was 49°C (Fig. 3a–d).

The Arrhenius plots in general followed a biphasic pattern (Fig. 3) in the control, Tx and T_3 groups, and the values of energy of activation in the low temperature range (E_L) were higher than those of energy of activation in the high temperature range (E_H). However, the T_R group proved to be an exception, where the pattern was reversed (Fig. 3h); i.e., the value of E_H was high and that of E_L was substantially low.

The data on values of E_H, E_L and phase transition temperature (Tt) are summarized in Table 5. Thus, in the control group the values of E_H and E_L were 39.9 and 65.7 KJ/mole, respectively, with Tt occurring at 23.5°C. In the

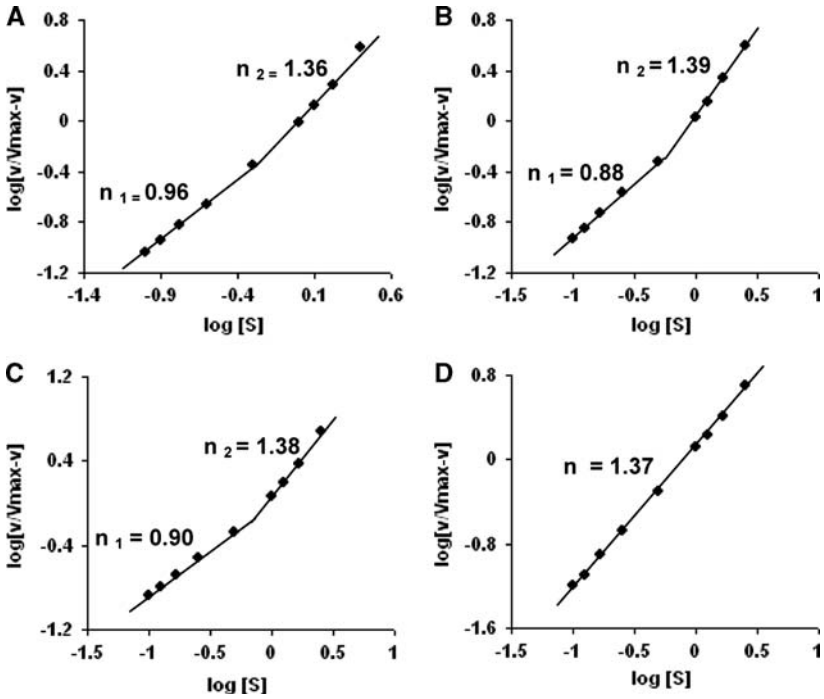

Fig. 2 Typical Hill plots for rat kidney microsomal Na^+,K^+-ATPase. Log (v/V_{max} - v) on the abscissa is plotted *vs.* log (S) on the ordinate. The Hill coefficients n_1 and n_2 represent the number of ATP molecules bound over the given concentration range of ATP. **a** Control, **b** Tx, **c** T_3 and **d** T_R. The plots are typical of four to eight independent experiments, as indicated in Table 4

Tx group the value of E_H increased by 44% while that of E_L increased by 14%; Tt decreased by 4°C. T_3 treatment resulted in further increases in the values of E_H and E_L (52% and 46%, respectively) while returning Tt to normal. The T_R group was characterized by a 27% increase in E_H but a 45% decrease in E_L; Tt was comparable to the euthyroid control (Table 5).

In light of these differential effects, it was of interest to find out how the thyroid status affected the lipid milieu and to seek its possible correlation with the substrate and temperature kinetics parameters. The data on the TPL and cholesterol (CHL) contents as influenced by thyroid status are given in Table 6; values of fluorescence polarization, p, are also included. Tx resulted in no appreciable change in TPL content but CHL content became almost half compared to the euthyroid control. This was also reflected in the doubling of the TPL/CHL (mole:mole) ratio in the Tx group. However, the membrane fluidity was unchanged. T_3 treatment increased TPL content without affecting CHL content. As a consequence, the TPL/CHL (mole:mole) ratio increased in the Tx group together with a decrease in membrane fluidity. The T_R regimen resulted in a significant increase in TPL content and tended to increase CHL to the euthyroid level. The TPL/CHL (mole:mole) ratio was still comparable to the Tx group. The T_R regimen also resulted in a significant decrease in membrane fluidity.

The effects of thyroid status on phospholipid composition are summarized in Table 7. As can be noted, Tx resulted in an almost two fold increase in the phosphatidylserine (PS) and phosphatidic acid (PA) components, while the phosphatidylethanolamine (PE) component decreased by 27%. The composition of other phospholipid components was not affected. Treatment with T_3 brought about a significant reduction in phosphatidylinositol (PI), but T_3 was unable to restore to normal PS and PA, which remained elevated. Likewise, PE remained low as in the Tx animals. The T_R regimen resulted in a two fold increase in the lysophospholipid (Lyso) component, while the effects on PI and PS were opposite. PI decreased compared to the control and Tx groups, and PS decreased compared to Tx animals, thus bringing its value close to that of the euthyroid control. PE was restored to normal, and PA became more or less comparable to the euthyroid value. The phosphatidylcholine (PC) component was unchanged under all experimental conditions. These changes were also reflected in terms of the content of individual phospholipid classes (Table 8).

Discussion

From the data presented it is clear that the microsomal enzyme differed in several respects from the predominant Na^+,K^+-ATPase in the kidneys, which comprises the pure α_1 and β_1 combination. Thus, the activity of the microsomal enzyme in hypothyroid animals increased beyond the value in euthyroid controls (Table 1). Hypothermia is a well-recognized feature of hypothyroidism (Tata, 1964). It may hence be suggested that the increase in enzyme activity may represent a compensatory mechanism to

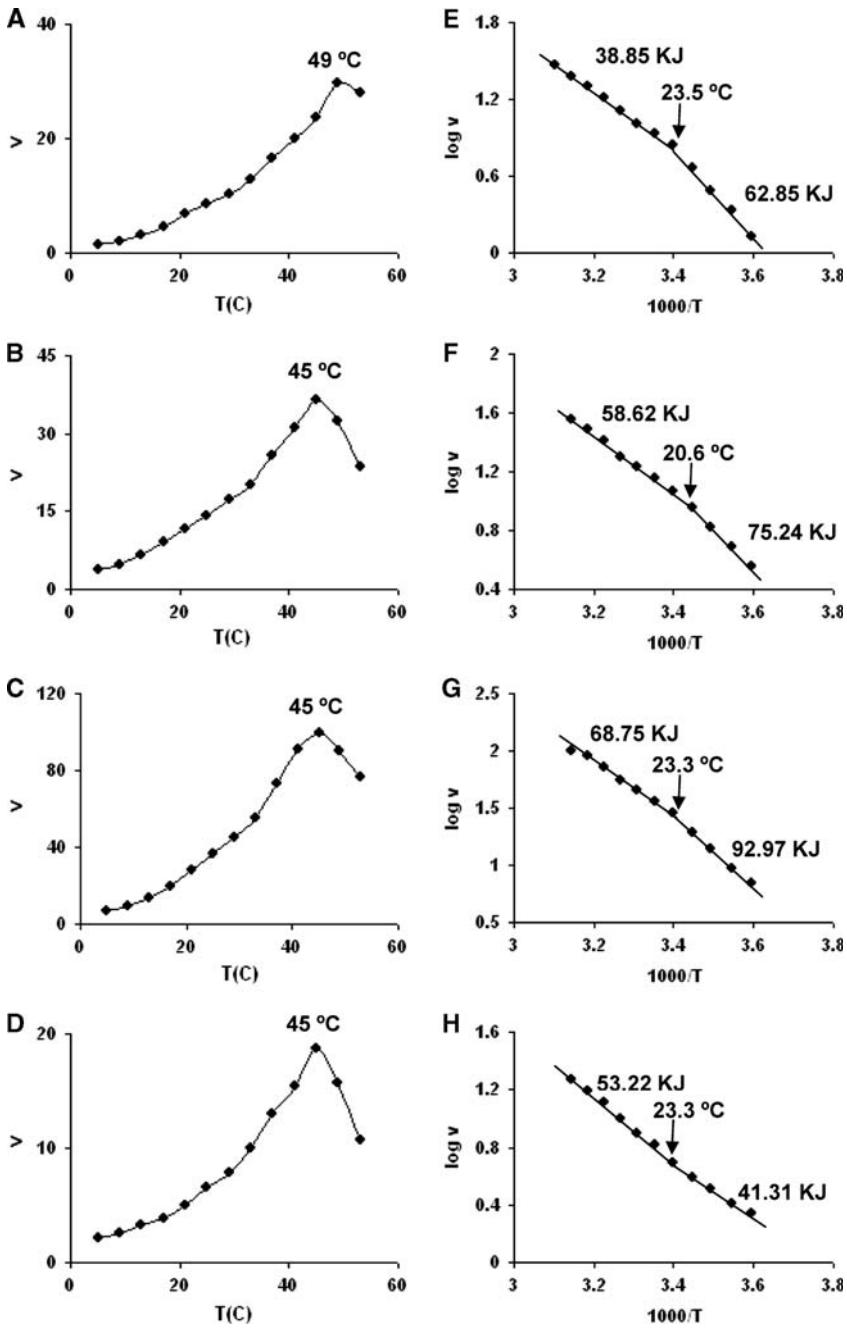

Fig. 3 Typical plots showing dependence of Na^+,K^+-ATPase activity on temperature and the corresponding Arrhenius plots. In temperature curves, enzyme activity, *v*, on the abscissa is plotted *vs.* temperature on the ordinate. **a** Control, **b** Tx, **c** T_3 and **d** T_R. The corresponding Arrhenius plots are shown in **e–h**. In Arrhenius plots log *v* on the abscissa is plotted *vs.* 1,000/T on the ordinate, where *v* and *T* represent, respectively, the activity at the corresponding absolute temperature (temperature in °C + 273.2). The plots are typical of six to eight independent experiments, as indicated in Table 5

combat hypothermia. Such an assumption would agree with our earlier suggestion that the microsomal enzyme may play a role in temperature homeostasis of the internal organs rather than in translocation of Na^+ and K^+ (Katewa & Katyare, 2003a, 2003b). The decreased K_m value especially of component I in the Tx animals (Table 2) may also represent an additional compensatory mechanism for combating hypothermia, which agrees well with the decreased energy potential of kidney mitochondria in hypothyroidism (Katyare et al., 1977; Satav & Katyare, 1991). The hypothyroid state, however, did not influence the catalytic efficiency ($^{App}Kcat/K_m$) of the enzyme (Table 3).

The microsomal enzyme also differed from the predominant $\alpha_1\beta_1$ Na^+,K^+-ATPase of the kidneys with respect to the exaggerated response to treatment with a single injection of T_3 (Table 1). It has been reported that the activity of $\alpha_1\beta_1$ Na^+,K^+-ATPase increased by about 70% above the hypothyroid value following treatment with T_3 on 3 alternate days; the dose of T_3 used in these studies was 50 μg/100 g body weight, i.e., two times higher than the single-dose regimen employed in our present studies (Lo

et al., 1976). These authors also reported that the K_m of the enzyme was unchanged by T_3 treatment. However, their studies were restricted by use of a substrate (ATP) concentration range of 0.25–1 mM (Lo et al., 1976). Against this we find that the K_m of both the kinetic components of the microsomal enzyme was significantly lowered. As is to be expected, the catalytic efficiency of both kinetic components increased significantly under these conditions (Table 3). The T_R regimen, on the other hand, presented a rather interesting feature. While the activity decreased below the euthyroid value (Table 1), the enzyme displayed allosteric characteristics up to the ATP concentration of 0.5 mM; the $S_{0.5}$ ($S_{0.5}$ is the concentration of substrate required for half-maximal activity) for the allosteric component was around 0.9 mM (*data not shown*). However, the T_R regimen was not able to upregulate either the activity or the V_{max} of component II. However, in animals receiving T_R treatment, the catalytic efficiency of component II increased significantly (Table 3).

It has been reported that increased activity of kidney cortex Na^+,K^+-ATPase following treatment with T_3 directly correlated with increased transcripts of α- and β-subunits and their respective mRNAs in equal proportion (Chaudhury et al., 1987; Gick et al., 1988; McDonough et al., 1988). Therefore, one wonders if a similar situation would prevail in the case of the microsomal enzyme which comprises the α_1,β_3 isoform.

It is well recognized that α is the catalytic subunit while β is the regulatory subunit of Na^+,K^+-ATPase (Blanco & Mercer, 1998; Wetzel et al., 1999). That the enzyme activity did not change in hypothyroid animals (Table 1) suggests that thyroid hormones may regulate the synthesis of the predominant $\alpha_1\beta_1$ isoform without affecting the microsomal enzyme, which may be a compensatory mechanism for combating hypothermia, as pointed out above. By the same consideration, exaggerated response to T_3 treatment suggests that this experimental condition may specifically stimulate the synthesis of the catalytic α_1-subunit without stimulating the synthesis of the regulatory β_3-subunit. It has been reported that in control rat kidney the stoichiometry of β_3:α_1 is about 1:3. Therefore, it is possible that a disproportionate increase in the α_1-subunit may lead to hyperstimulation of the microsomal enzyme. This interesting possibility, however, needs to be verified and confirmed by more direct experiments. By similar logic, it may be suggested that sustained long-term combined treatment with T_3 and T_4 might introduce another kind of disproportionate combination of $\alpha_1\beta_3$ isoforms, thereby lowering the activity. The results also suggest that the synthesis of α_1 and β_3 subunits may be differentially regulated by T_3 and T_4, respectively. Once again, this interesting possibility needs further confirmation by direct experimentation.

Thyroid status also introduced subtle changes in the temperature kinetics of the enzyme, which included lowering of the optimal temperature by 4°C in all the experimental groups. However, the most important change was an increase in the energies of activation in Tx animals, which was not corrected by T_3 treatment (Fig. 1, Table 5). The T_R group presented a reversed Arrhenius pattern, where the value of E_H increased and that of E_L became almost half. Clearly, the T_3 and T_R regimens did not restore the membrane characteristics to normal, although both treatments restored the value of Tt, which had decreased in the Tx animals.

Table 5 Effect of Tx and subsequent treatment with thyroid hormones on Arrhenius kinetics properties of rat kidney microsomal Na^+,K^+-ATPase

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt, °C)
	E_H	E_L	
Control (8)	39.9 ± 1.46	65.7 ± 2.02	23.5 ± 0.84
Tx (6)	57.6 ± 0.57[b]	75.0 ± 1.60[a]	19.4 ± 0.57[b]
T_3 (6)	64.5 ± 1.83[b,*]	96.0 ± 3.15[b,**]	24.4 ± 0.65**
T_R (4)	50.7 ± 1.54[b,*]	35.4 ± 3.28[b,**]	23.1 ± 0.96*

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.01$, [b] $p < 0.001$ compared to euthyroid control; *$p < 0.01$, **$p < 0.001$ compared to Tx

Table 6 Effect of Tx and subsequent treatment with thyroid hormones on total phospholipids (TPL), cholesterol (CHL) and fluorescence polarization (p) in rat kidney microsomes

Animals	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)	p
Control (8)	235.1 ± 6.72	204.2 ± 2.86	0.58 ± 0.02 0.	0.190 ± 0.001
Tx (4)	250.4 ± 7.94	97.3 ± 2.87[b]	1.29 ± 0.02[b]	0.192 ± 0.002
T_3 (4)	276.6 ± 5.66[b,*]	93.0 ± 6.27[b]	1.57 ± 0.09[b,***]	0.267 ± 0.001[b,***]
T_R (4)	396.5 ± 6.33[b,***]	166.5 ± 9.14[a,***]	1.21 ± 0.06[b,**]	0.247 ± 0.001[b,***]

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.01$, [b] $p < 0.001$ compared to euthyroid control; *$p < 0.05$; **$p < 0.02$, ***$p < 0.001$ compared to Tx

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

Phospholipid class	Composition (% of total)			
	Control (8)	Tx (4)	T_3 (4)	T_R (4)
Lyso	2.91 ± 0.14	2.86 ± 0.36	2.86 ± 0.15	6.62 ± 0.20^{c,****}
SPM	24.15 ± 0.36	23.60 ± 0.63	25.19 ± 0.35	24.00 ± 0.31
PC	36.22 ± 1.47	35.91 ± 1.05	38.07 ± 1.20	34.25 ± 1.19
PI	3.82 ± 0.12	3.25 ± 0.24	2.08 ± 0.13^{c,***}	2.71 ± 0.09^{c}
PS	4.91 ± 0.12	8.22 ± 0.27^{c}	8.00 ± 0.11^{c}	4.21 ± 0.14^{b,****}
PE	22.59 ± 1.52	16.58 ± 1.34^{a}	15.32 ± 1.02^{b,*}	22.12 ± 0.96*
PA	5.41 ± 0.11	9.59 ± 0.35^{c}	8.48 ± 0.10^{c,**}	6.10 ± 0.07^{c,****}

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.02$, [b] $p < 0.01$, [c] $p < 0.001$ compared to euthyroid control; *$p < 0.05$, **$p < 0.02$, ***$p < 0.002$, **** $p < 0.001$ compared to Tx

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

Phospholipid class	Content (μg /mg of microsomal protein)			
	Control (8)	Tx (4)	T_3 (4)	T_R (4)
Lyso	6.8 ± 0.38	7.1 ± 0.44	7.9 ± 0.28^{a}	26.3 ± 0.87^{d,****}
SPM	56.8 ± 1.95	58.8 ± 1.12	69.7 ± 1.98^{d,***}	95.1 ± 1.32^{d,***}
PC	85.4 ± 3.10	90.5 ± 5.39	105.3 ± 2.74^{d}	135.8 ± 3.45^{d,****}
PI	9.0 ± 0.45	8.2 ± 0.65	5.8 ± 0.24^{d,**}	10.8 ± 0.41^{c,**}
PS	11.6 ± 0.48	20.7 ± 1.27^{d}	22.1 ± 0.57^{d}	16.7 ± 0.52^{d,*}
PE	52.8 ± 2.53	41.3 ± 0.86^{b}	42.4 ± 1.85^{b}	87.9 ± 2.61^{d,****}
PA	12.8 ± 0.50	23.8 ± 2.33^{d}	23.5 ± 0.55^{c}	24.2 ± 0.37^{d}

The results are given as mean ± SEM of the number of independent experiments indicated in parentheses.

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.02$; [d] $p < 0.001$ compared to euthyroid control; *$p < 0.05$, **$p < 0.02$, ***$p < 0.002$, ****$p < 0.001$ compared to hypothyroid

The analysis of lipid/phospholipid profiles revealed thyroid status-dependent changes. Thus, both the T_3 and T_R regimens tended to increase TPL but were unable to bring back to euthyroid level the decreased CHL content. Thyroid status-dependent changes were also evident in the composition and contents of individual phospholipids. These changes, one may anticipate, could cause differences in charge distribution across the membranes, thereby influencing the substrate and temperature kinetics parameters of the enzyme. We explored this possibility by seeking correlation between compositional changes in the lipid/phospholipid components and various kinetic parameters. These data from regression analysis are given in Tables 9 and 10.

Thus, analysis across the groups revealed that K_m 1 correlated positively with PS, PE and CHL, while PI showed a negative correlation. K_m 2 was not influenced by any lipid component. V_{max} 1 and V_{max} 2 showed positive correlations, respectively, with TPL and PI, while both V_{max} 1 and V_{max} 2 correlated negatively with PS and PE (Table 9). This is somewhat paradoxical since kidney cortex Na^+,K^+-ATPase has been shown to be dependent on acidic phospholipid PS and PI for its activity (Robinson & Flashner, 1979). The results thus suggest that the

Table 9 Correlation of substrate kinetics parameters of microsomal ATPase with membrane lipid/phospholipid composition across all the groups

Parameter	Correlation	
	Positive	Negative
K_m 1	PS (+0.597)	PI (−0.711)
	PE (+0.635)	
	CHL (+0.728)	
V_{max} 1	TPL (+0.605)	PS (−0.760)
		PE (−0.587)
K_m 2	–	–
V_{max} 2	PI (+0.550)	PS (−0.641)
		PE (−0.630)

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, indicated in Tables 6 and 7

Table 10 Correlation of temperature kinetics parameters of microsomal ATPase with membrane lipid/phospholipid composition

Parameter	Control, Tx, T_3		Control, T_R		Tx, T_R	
	Positive	Negative	Positive	Negative	Positive	Negative
E_H	PI (+0.863)	PS (−0.742)	Lyso (+0.654)	PS (−0.721)	PI (+0.746)	Lyso (−.868)
	PA (+0.795)	PE (−0.858)	TPL (+0.665)	PI (−0.599)	PA (+0.698)	PE (−0.656)
		CHL (−0.917)		CHL (−0.783)	Fluidity (+0.761)	TPL (−0.773)
		Fluidity (−0.696)		Fluidity (−0.799)		CHL (−0.943)
E_L	PI (+0.633)	PS (−0.845)	PC (+0.681)	Lyso (−0.891)	PS (+0.604)	Lyso (−0.867)
	PA (+0.577)	PE (−0.694)	PS (+0.571)	PA (−0.706)	PI (+0.955)	PE (−0.911)
		CHL (−0.721)	CHL (+0.668)		PA (+0.939)	TPL (−0.977)
		Fluidity (−0.880)			Fluidity (+0.985)	CHL (−0.944)
Tt	-	-	SPM (+0.669)	PE (−0.601)	Lyso (+0.648)	PI (−0.859)
					PE (+0.803)	PA (−0.929)
					TPL (+0.932)	Fluidity (−0.896)
					CHL (+0.822)	

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, as indicated in Tables 6 and 7

microsomal $\alpha_1\beta_3$ isoform also differs from its $\alpha_1\beta_1$ counterpart with respect to the specific lipid/phospholipid requirements.

Correlation studies for temperature kinetics were found to be a bit more complex since the Arrhenius pattern reversed in the T_R group (Fig. 3, Table 5). Hence, we carried out the regression analysis for the control, Tx and T_3; for the control and T_R; and for the Tx and T_R groups. These data are given in Table 10.

In the control, Tx and T_3 groups both E_H and E_L correlated positively with PI and PA and negatively with PS, PE, CHL and membrane fluidity.

Analysis of the control and T_R groups revealed that Lyso and TPL were major positive modulators of E_H, whereas PS, PI, CHL and membrane fluidity showed a negative correlation. E_L correlated positively with PC, PS and CHL and negatively with Lyso and PA.

Correlation studies for the Tx and T_R group show that PI, PA and membrane fluidity correlated positively with E_H, while PS, PI, PA and fluidity correlated positively with E_L. Lyso, PE, TPL and CHL were negative modulators of E_H and E_L.

In the control and T_R groups, Lyso and PE were positive and PI and PA were negative modulators of Tt. For the Tx and T_R groups the correlation with Tt was more complex: Lyso, PE, TPL and CHL played a positive role and PI, PA and membrane fluidity had a negative influence.

The present investigations were undertaken to find out if treatments with combined replacement therapy with T_3 and T_4 (Escobar-Morreale et al., 1996) could restore the microsomal function to the euthyroid state. Our present results show that neither treatment was able to restore the kinetics properties of microsomal Na^+,K^+-ATPase or the lipid/phospholipid profile to the normal euthyroid state. 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), more complex and intricate mechanisms may be involved in restoring the membrane structure-function relationship and enzyme kinetics parameters to the euthyroid state.

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