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The effect of experimental hypothyroidism on phosphofructokinase activity and fructose 2,6-bisphosphate concentrations in rat heart

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Experimental hypothyroidism was induced in rats by the administration of $NaClO_4$. Hearts from normal and hypothyroid rats were homogenized, and the extracts were assayed for phosphofructokinase-1 and phosphofructokinase-2 activity and fructose 2,6-bisphosphate concentrations. Hypothyroidism was associated with a drastic loss of phosphofructokinase-1 activity. A hyperbolic relationship between plasma thyroxine concentrations and phosphofructokinase-1 activity was found. As treatment with $NaClO_4$ progressed, the decrease in blood thyroxine was faster than the decrease in enzyme activity. After prolonged hypothyroidism (a decrease in thyroxine of more than 10-fold), a 4-fold decrease in phosphofructokinase-1 activity was observed. In this metabolic condition 2-fold decreases in phosphofructokinase-2 activity and in fructose 2,6-bisphosphate were observed. A similar decrease in phosphofructokinase-1 activity in a partially purified preparation was found. The addition of L-thyroxine in the diet had little effect on phosphofructokinase-1 activity. However, exposure of minced pieces of hearts of hypothyroid rats to tri-iodothyronine for 5 h resulted in a clear increase in phosphofructokinase-1 activity, which was partially prevented by the simultaneous addition of cycloheximide. These results could account for the decrease in carbohydrate metabolism in heart from hypothyroid rats.

INTRODUCTION

It is currently accepted that 6-phosphofructokinase-1 (PFK-1) plays a major role in the control of glycolysis in nearly all types of cells (Stadtman, 1966; Mansour, 1972). Muscle PFK-1 activity, as in other tissues, is controlled by several metabolites (Garland *et al.*, 1963; Pogson & Randle, 1966; Sols *et al.*, 1981), including its most potent effector, fructose-2,6-bisphosphate (Fru-2,6-P_2) (Van Schaftingen *et al.*, 1980; Uyeda *et al.*, 1981). It has been found that this molecule relieves the inhibition by ATP and co-operates synergistically with AMP to maintain the enzyme in an active form in various tissues (Van Schaftingen *et al.*, 1981). The enzyme which catalyses its synthesis, 6-phosphofructokinase-2 (PFK-2), is also present in the heart (Rider & Hue, 1984).

Most studies on the effects of thyroid hormones on carbohydrate metabolism have been performed in the liver. Hypothyroidism results in decreased anaerobic glycolysis and a fall in glycogen synthesis in liver (Bargoni *et al.*, 1966). In addition, the activities of hepatic pyruvate kinase (Böttger *et al.*, 1970) and glucose-6-phosphate dehydrogenase (Novello *et al.*, 1969) are decreased after thyroidectomy. Cardiac modifications in hypothyroidism have long been recognized (De Visscher & Ingenbleek, 1980), and it has been suggested that the functional changes (e.g. decreased contractile force and a slow heart rate) are related to a decreased myosin ATPase activity (Suko, 1973).

The modifications of the activities of PFK-1 and PFK-2 from hearts of hypothyroid rats were previously unknown and are the subject of the present work. Since Fru-2,6-P_2 is present in heart (Hue *et al.*, 1982), the present study attempts to assess the role of Fru-2,6-P_2 in the hypothyroid state by examining its concentrations in the tissue, and its effect on cardiac PFK-1 and PFK-2 activities. The results indicate that hypothyroidism induces a decrease in the maximal activities of PFK-1 and PFK-2, and experiments *in vitro* using the protein-synthesis inhibitor cycloheximide suggest that this may be due to changes in the synthesis of the two enzymes. The inactivation, which is antagonized by tri-iodothyronine (T_3) *in vitro*, would account, at least in part, for the decreased carbohydrate metabolism in the heart in hypothyroidism.

EXPERIMENTAL

Animals and treatment

Male Wistar rats (180–200 g) were used in all experiments. Animals were fed with a standard diet and given water *ad libitum*. The rats were killed by stunning and cervical dislocation. Hearts were rapidly excised and freeze-clamped between aluminium blocks precooled in liquid N_2 (Wollenberger *et al.*, 1960). Blood was collected from hearts, transferred to paper filters and stored at 4 °C for radioassay of blood thyroxine (T_4).

Rats were made hypothyroid by treatment with 1% $NaClO_4$ administered in drinking water for different periods. Hypothyroidism was assessed by the method of Obregon *et al.* (1984). When the effect of T_4 was studied, the hormone at a concentration of 1 mg/ml was dissolved in the drinking water and given for 7 days. In

Abbreviations used: PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; T_3, tri-iodothyronine; T_4, thyroxine.

some experiments *in vitro* (Table 2), T_3 was added directly to the incubation medium containing cardiac tissue.

Materials

Chemicals were of analytical grade and obtained from Merck, Darmstadt, Germany. Biochemical reagents and enzymes were from Boehringer G.m.b.H., Mannheim, Germany, or Sigma Chemical Co., St. Louis, MO, U.S.A. T_4 was donated by Laboratories Leo (Sevilla, Spain). T_3 was from a kit of T_3 from Diagnostic Products Co. (Los Angeles, CA, U.S.A.). Samples of Fru-2,6-P_2 were kindly given by Dr. E. Van Schaftingen and Professor H. G. Hers (University of Louvain, Brussels, Belgium). The radioassay kit for T_4 was from Diagnostic Products Co.

Enzyme and metabolite assays

The activity of PFK-1 was measured in freeze-clamped tissue extracts or in partially purified preparations, as described by Rider & Hue (1985), except that the tissue was homogenized in 50 vol. (v/w) of 50 mM-Hepes/100 mM-KCl/50 mM-NaF, pH 7.4, at 0 °C. The activity is expressed as μmol of Fru-1,6-P_2 formed/min per μg of DNA in crude extracts, or per mg of protein in purified preparations. Assays of PFK-1 were carried out under conditions of maximal activity at pH 7.8 (i.e. in the presence of 5 mM-KH_2PO_4/5 mM-fructose 6-phosphate/1.5 mM-ATP/1.25 mM-AMP). For kinetic measurement, the assay was performed at pH 7.0 under sub-optimal conditions (i.e. 2.5 mM-ATP and 5 mM-KH_2PO_4) as well as substrates and effectors at the concentrations indicated in the Figures. The change in A_{340} was measured in a Kontron recording spectrophotometer (model Uvikon) at 25 °C. PFK-1 activity was partially purified by precipitation in $(NH_4)_2SO_4$ and heat treatment by the methods described previously (Mansour *et al.*, 1966; Tarui *et al.*, 1972). In brief, hearts from four or five rats were cut into small pieces and homogenized in 4 vol. (v/w) of 50 mM-Hepes/100 mM-NaF/15 mM-EGTA, pH 7.4, at 0 °C. The homogenate was centrifuged at 24000 *g* for 15 min. A solution of $(NH_4)_2SO_4$, saturated at room temperature, was added dropwise to the supernatant with continuous stirring to give a final concentration of 42% satn. The residue was sedimented at 24000 *g* for 15 min and discarded. $(NH_4)_2SO_4$ was added to the supernatant to give a concentration of 60% satn. (Mansour *et al.*, 1966). After centrifugation at the same speed, the precipitate was dissolved in a minimum volume of cold homogenization buffer plus 5 mM-mercaptoethanol, and the pH adjusted to 8 at 0 °C. The extract from the previous step was heated to 48 °C with constant stirring (Tarui *et al.*, 1972). After centrifugation at 24000 *g* for 15 min, the supernatant was assayed for PFK-1 activity.

PFK-2 activity was measured as described by Rider & Hue (1984). The activity is expressed as pmol of Fru-2,6-P_2 formed/min per mg of DNA. Fru-2,6-P_2 was measured as described previously (Sobrino & Gualberto, 1985). DNA was measured by the method of Kissane & Robins (1958). Relationships found between DNA and the weight of tissue were 1.82 ± 0.18 and 1.91 ± 0.11 mg of DNA/g of tissue in control and hypothyroid rats respectively.

Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as a standard.

Apparent K_m values for Fru-6-*P* and $K_{0.5}$ values for AMP and Fru-2,6-P_2 were determined mathematically by fitting the velocity and substrate (or effector) concentrations to straight lines. Activation constants ($K_{0.5}$) are defined as the concentration of positive effector which produce one-half of the maximum activation.

RESULTS

Characteristics of $NaClO_4$-induced hypothyroidism

The administration of 1% $NaClO_4$ in drinking water produced a progressive hypothyroidism (Alexander & Wolff, 1966; Ortiz-Caro *et al.*, 1983), which was characterized by low concentrations of T_4 in the blood of the rat (Fig. 1). A value of 4.8 μg/100 ml for the concentration of T_4 in blood from normal rats was found, in accordance with previous findings (Segal *et al.*, 1985; St. Germain & Galton, 1985). Hearts of hypothyroid animals weighed approximately half those of euthyroid rats. A 50% decrease in body weight was observed after 30 days of treatment with 1% $NaClO_4$. The hypothyroid state did not modify the relative content of DNA in hearts when compared with the weight of the tissue.

Correlation between T_4 concentration and PFK-1 activity

As indicated above, $NaClO_4$ treatment resulted in a pronounced hypothyroidism, defined by a drastic decrease in plasma T_4. The development of hypothyroidism caused a progressive decrease in cardiac PFK-1

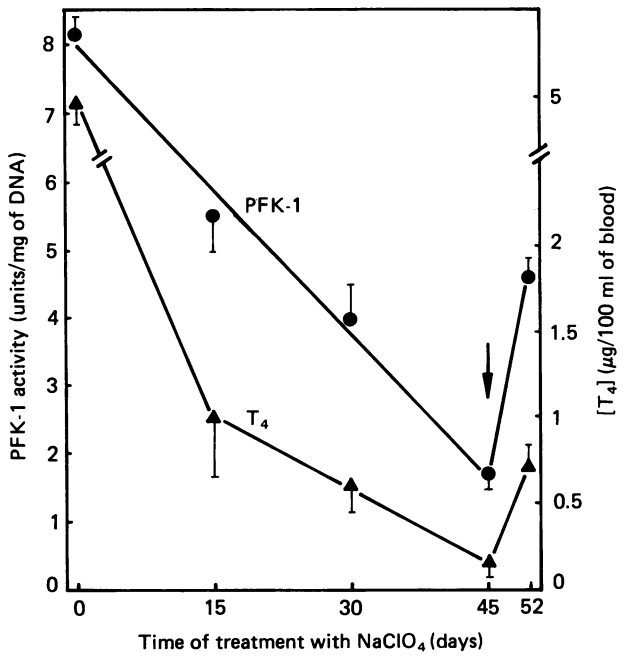

Fig. 1. Time course of $NaClO_4$ treatment on blood T_4 concentrations and cardiac PFK-1 activity

At the indicated times, hearts and samples of blood were isolated and assayed for PFK-1 activity under conditions of maximal activity at pH 7.8 (●) and for T_4 concentration (▲) as described in the Experimental section. Values represent means ± S.E.M. (vertical bars) for at least five samples. The arrow indicates the time at which the treatment with perchlorate was suppressed.

Table 1. Effect of simultaneous administration of $NaClO_4$ and T_4 on cardiac PFK-1 activity and blood T_4 concentrations

$NaClO_4$ (1%) and T_4 were administered in drinking water for 30 days. Rat hearts were freeze-clamped between tongs pre-cooled in liquid N_2. PFK-1 activity (optimal conditions) and T_4 concentrations were assayed as indicated in the Experimental section. The numbers of rats used in each condition are shown in parentheses.

Additions to drinking water	PFK-1 activity (units/mg of DNA)	T_4 (μg/100 ml of blood)
None (16)	8.03±0.20	4.80±0.1
$NaClO_4$ (5)	3.88±0.30	1.55±0.39
$NaClO_4$+T_4 (0.5 mg/l) (5)	8.12±0.11	5.01±0.1
$NaClO_4$+T_4 (1 mg/l) (5)	8.78±0.06	5.08±0.5

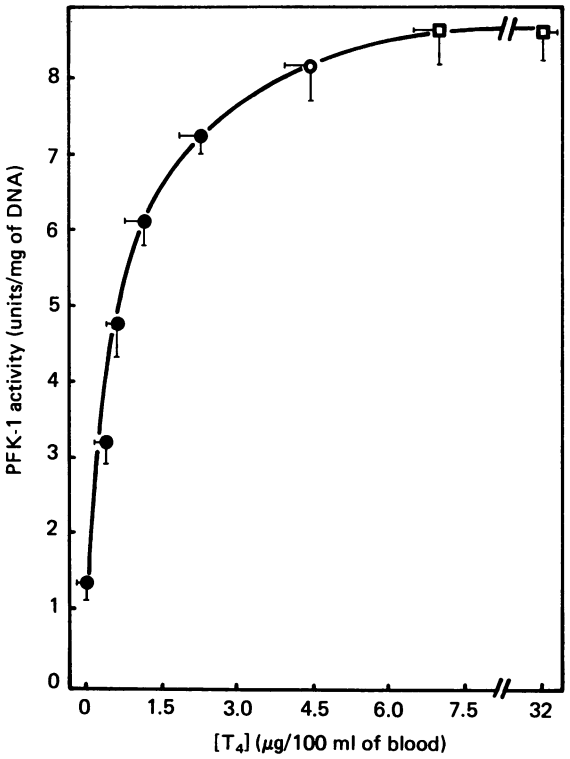

Fig. 2. Correlation between the cardiac PFK-1 activities and blood T_4 concentrations

The values of PFK-1 found in normal rats (○), hypothyroid rats (●) (taken from Fig. 1) and normal rats treated with T_4 (1 mg/ml) in the drinking water for 1 week (□) were plotted versus the corresponding values of T_4 in blood.

activity (Fig. 1). Treatment with $NaClO_4$ for periods less than 1 week was without effect on both parameters (results not shown).

After 45 days of $NaClO_4$ administration, blood T_4 concentrations were almost undetectable, and cardiac PFK-1 activity was about 25% of that found in euthyroid-rat hearts. PFK-1 activity measured in the hearts of normal rats is in agreement with values previously reported (for a review, see Newsholme & Leech, 1983). A fast reversibility of the effect was observed, since, 7 days after the salt was omitted from the diet, both T_4 concentrations and PFK-1 activity were markedly increased (Fig. 1). A direct effect of $NaClO_4$ on PFK-1 activity was ruled out, since, when the salt and L-T_4 were given simultaneously, cardiac PFK-1 activity was unchanged (Table 1). The relationship between PFK-1 activity and T_4 concentrations is shown in Fig. 2. Additional values were obtained from T_4-treated rats (hyperthyroid rats). A hyperbolic curve was found, and half-maximal inactivation for PFK-1 was achieved with approx. 0.50 μg of T_4/100 ml of blood.

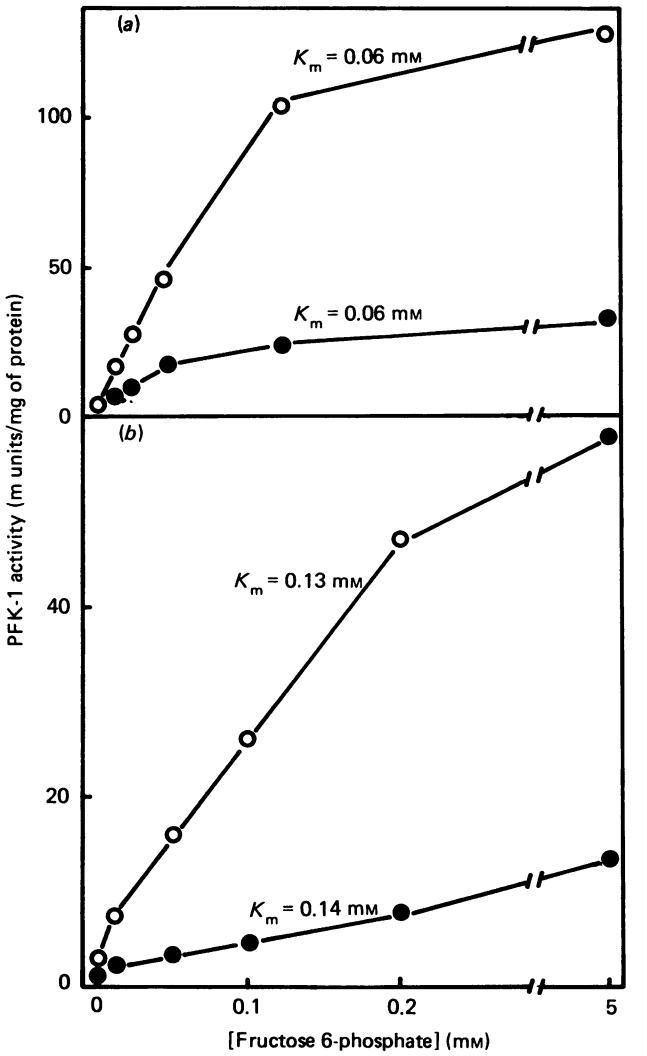

Fig. 3. Affinity of cardiac PFK-1 for Fru-6-*P* assayed under optimal (*a*) and sub-optimal (*b*) conditions

Purified enzyme from normal rats (○) and hypothyroid rats (●) was assayed for PFK-1 activity. Assays were performed in the presence of 1.5 mM-ATP/1.25 mM-AMP at pH 7.8 (*a*) or with 2.5 mM-ATP at pH 7.0 (*b*). Blood T_4 concentrations in hypothyroid rats were 0.52 μg/100 ml in (*a*) and 0.25 μg/100 ml in (*b*). Apparent K_m values are shown beside the curves.

Kinetic properties of PFK-1

Hearts extracts from normal and hypothyroid rats were incubated with increasing concentrations of fructose 6-phosphate (0.01–5 mM) under optimal assay condi-

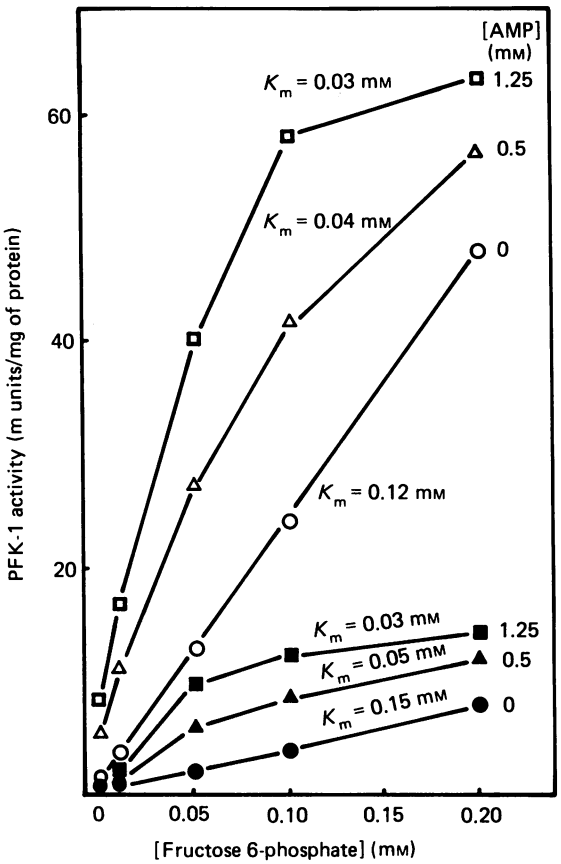

Fig. 4. Effect of AMP on the affinity of purified PFK-1 for Fru-6-*P*

Cardiac PFK-1 from normal (○, △, □) and hypothyroid (●, ▲, ■) rats was assayed in the presence of 2.5 mM-ATP at pH 7.0 (sub-optimal conditions). Blood T_4 concentration in hypothyroid rats was 0.2 μg/100 ml.

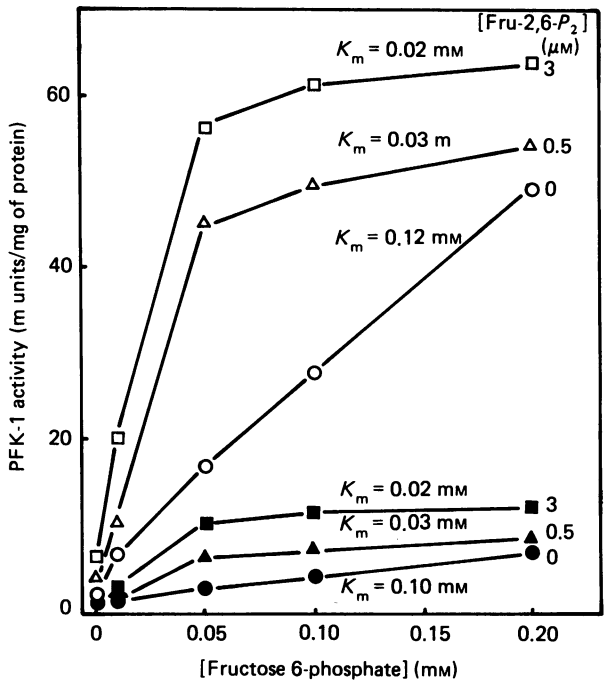

Fig. 5. Effect of Fru-2,6-P_2 on the affinity of purified PFK-1 for Fru-6-*P*

Symbols and assay conditions were as in Fig. 4, except that 0.1 mM-AMP was also present.

tions. Homogenates from hearts of hypothyroid rats showed a clear diminution of PFK-1 activity at all concentrations of sugar ester when compared with PFK-1 activity from the hearts of normal rats (results not shown). However, no changes in affinity for Fru-6-*P* were detected, with an apparent K_m of 0.05 mM in both metabolic states.

Fig. 3(*a*) illustrates similar results when partially purified PFK-1 was used, which ruled out that the decrease in the PFK-1 activity in the hypothyroid state could be caused by factor(s) present in the extract. It is possible that the optimal conditions used in the assay of PFK-1 might obscure changes in the kinetic properties of the enzyme. To test the possibility, experiments were performed under sub-optimal conditions. Fig. 3(*b*) shows that in the presence of 2.5 mM-ATP at pH 7.0 the apparent K_m values of the purified enzyme from hearts of normal and hypothyroid rats for Fru-6-*P* were not different (0.13 and 0.14 mM respectively).

These data demonstrate a marked decrease in cardiac PFK-1 activity without modifications in the apparent K_m for Fru-6-*P* when the plasma concentrations of T_4 are low. Therefore it was decided to ascertain whether the enzyme from hypothyroid rats maintains its capacity to respond to well-known activators of the normal enzyme, such as AMP and Fru-2,6-P_2.

The effects of Fru-6-*P* concentration on reaction velocity, under sub-optimal conditions of assay, in the presence of AMP and Fru-2,6-P_2, are shown in Fig. 4 (AMP) and Fig. 5 (Fru-2,6-P_2). The results show that the response was greatly dependent on the concentration of both effectors in normal and hypothyroid preparations. A greater sensitivity of PFK-1 from hearts of normal and hypothyroid rats was observed in the presence of increasing concentrations of AMP and Fru-2,6-P_2. It is noteworthy that the apparent K_m for Fru-6-*P* in the presence of these effectors was of the same order of magnitude in both metabolic conditions. Fig. 6 illustrates the effect of increasing concentrations of Fru-2,6-P_2 and AMP on PFK-1 activity. To obtain maximal stimulation, the concentration of Fru-6-*P* was fixed at 0.1 mM for the effect of Fru-2,6-P_2 and at 0.05 mM for the effect of AMP. Although the stimulatory effect of Fru-2,6-P_2 and AMP on PFK-1 activity from hypothyroid rats was less than that observed in normal rat hearts, the $K_{0.5}$ value for each effector was similar in both cases. The concentration-dependence of the activity of cardiac PFK-1 from hypothyroid animals for ATP displays an analogous kinetic profile to that for normal enzyme, and similar to that previously observed for liver enzyme (Van Schaftingen *et al.*, 1981) (results not shown). These results indicate that the decreased activity of PFK-1 in the hypothyroid state was not due to the loss of regulatory properties of the enzyme.

In order to obtain information as to whether the effect of hypothyroidism might be ascribed to inhibition of synthesis of the enzyme *de novo*, cycloheximide was used to inhibit protein synthesis. Table 2 shows that the incubation of cardiac fragments from hypothyroid rats (T_4 in blood was 0.1 μg/100 ml) with 0.125 μg of T_3/ml

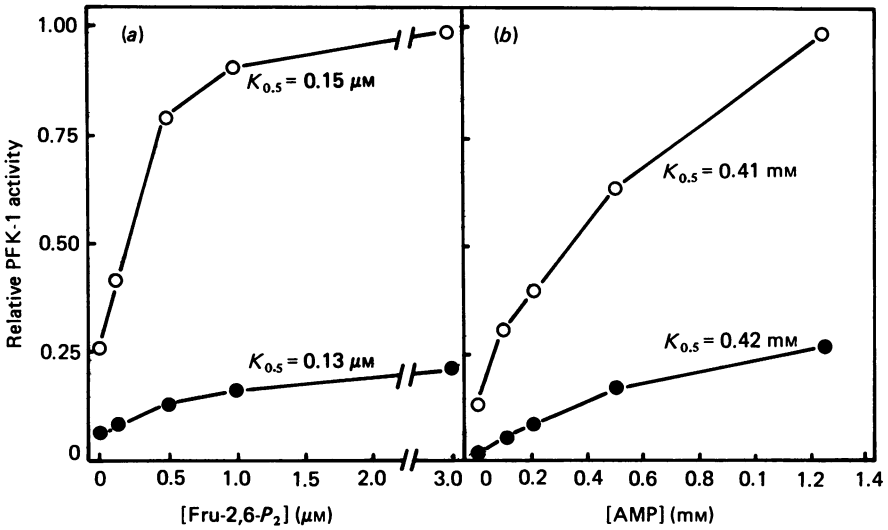

Fig. 6. Effect of Fru-2,6-P_2 (*a*) and AMP (*b*) on the activity of purified PFK-1 from normal (○) and hypothyroid (●) rats

All assays were performed in the presence of 2.5 mM-ATP at pH 7.0. In (*a*), Fru-6-*P* and AMP were each 0.1 mM; in (*b*), Fru-6-*P* was 0.05 mM. Other conditions were as in Fig. 4. Maximal activities found in (*a*) and (*b*) were 63.5 and 41.2 munits/mg of protein respectively.

Table 2. Effect of L-T_3 and cycloheximide on PFK-1 activity in hearts from hypothyroid rats *in vitro*

Hearts from hypothyroid rats (T_4 in blood = 0.2 μg/100 ml) were minced into small pieces (about 50–100 mg) and incubated in Krebs–Ringer solution buffered with 50 mM-triethanolamine at pH 7.8 for 5 h at 37 °C. The concentrations of T_3 and cycloheximide were 0.12 μg/ml and 35 μg/ml respectively. The glucose concentration was 20 mM in all conditions. Results shown are means ± S.E.M. for two preparations of heart pieces, with quadruple incubations. Value for a normal untreated control was also included.

Thyroid status	Additions	PFK-1 activity (units/mg of DNA)
Normal	(not incubated)	8.03 ± 0.20
Hypothyroid	None	1.62 ± 0.2
	Cycloheximide	1.59 ± 0.1
	T_3	4.38 ± 0.3
	T_3 + cycloheximide	2.81 ± 0.2

Table 3. PFK-2 activity and Fru-2,6-P_2 concentrations in hearts of normal and hypothyroid rats

Rat hearts were freeze-clamped between tongs pre-cooled in liquid N_2. Frozen powdered heart tissue was prepared for the assay of PFK-2 or Fru-2,6-P_2 content as indicated in the Experimental section. Blood T_4 concentrations in the two conditions were 4.9 ± 0.8 and 0.14 ± 0.4 μg/100 ml of blood in normal and hypothyroid rats respectively. The numbers of rats used in each condition are shown in parentheses. Results shown are means ± S.E.M.

Thyroid status	PFK-2 activity (units/mg of DNA)	Fru-2,6-P_2 (nmol/mg of DNA)
Normal (5)	0.27 ± 0.02	3.07 ± 0.68
Hypothyroid (7)	0.12 ± 0.02	1.52 ± 0.47

for 5 h resulted in a clear restoration of the PFK-1 activity, which was partially suppressed when 35 μg of cycloheximide/ml was added to the incubation medium. It was verified that cycloheximide alone had no effect on PFK-1 activity from incubated heart pieces.

Fructose-2,6-bisphosphate and PFK-2 activity

Table 3 summarizes our results on the concentration of Fru-2,6-P_2 and cardiac PFK-2 activity in hypothyroid rats. In this metabolic condition PFK-2 activity falls to about half of that found in normal heart. It is noteworthy that this decrease was less than that observed for PFK-1 activity at similar blood concentrations of T_4. A concomitant decrease in the concentration of Fru-2,6-P_2 in the hypothyroid heart was found.

DISCUSSION

The results presented in this study indicate that alterations in thyroid status have a significant effect on the activity of cardiac PFK-1 and PFK-2. In the most severe hypothyroidism studied (in which T_4 concentration was 0.1 μg/100 ml of blood), a 4-fold decrease in the maximal activity of PFK-1 and a 2-fold decrease in that of PFK-2 was found. In addition, hypothyroidism induced a concomitant decrease in Fru-2,6-P_2. The effect on PFK-1 activity was demonstrable at saturating Fru-6-*P* concentrations, indicating that the maximal velocity of the enzyme had been diminished. However, the substrate affinity remained unchanged under sub-optimal assay conditions (Figs. 4 and 5).

Regarding the mechanism responsible for the decreased PFK-1 activity in the hypothyroid state, several possibilities should be considered. First, the permissive effect of thyroid hormones on synthesis of protein is well established (for a review, see Oppenheimer, 1979). The recovery of PFK-1 activity observed after T_3 addition to pieces of hypothyroid heart incubated *in vitro* (Table 2)

suggests that the synthesis of cardiac PFK-1 is also controlled by thyroid hormones. Moreover, the inhibition of the restoration of the $V_{max.}$ of the enzyme exerted by cycloheximide, a protein inhibitor, agrees with this interpretation. However, it is noteworthy that when T_4 was added to the diet of normal rats the activity of PFK-1 was not affected (Fig. 2). Likewise, T_3 administration has no effect on pyruvate dehydrogenase activity in the heart (Holness *et al.*, 1985). A second possibility is that the decreased activity of PFK-1 could be caused by a decrease in the concentrations of some enzyme activators. This possibility seems unlikely, because the addition of Fru-2,6-P_2 and AMP activated the enzyme from hearts of hypothyroid rats, although the maximal activity did not reach that of PFK-1 from the hearts of normal rats (Figs. 4, 5 and 6). An alternative possibility is that changes in kinetic properties of PFK-1 are the result of modifications in the substrate (Fru-6-*P*) availability. However, the catalytic properties of cardiac PFK-1 from hypothyroid rats were broadly similar to those found from previous studies on purified PFK-1 from hearts of normal rats (Mansour *et al.*, 1966). Moreover, the intracellular concentration of Fru-6-*P* in the heart is about 0.10 mM (recalculated from the data of Newsholme & Randle, 1964), which represents a concentration above the value of the apparent K_m (optimal conditions) of PFK-1 found in hearts of normal and hypothyroid rats. Therefore the decrease in cardiac PFK-1 activity can be explained by a decrease in enzyme synthesis when the blood concentrations of thyroid hormones were experimentally decreased. It is noteworthy that the kinetics of the decreases in T_4 concentration and PFK-1 activity were different; the decrease in T_4 preceded the decrease in the enzyme activity. When the hormone concentration was about 0.5 μg/100 ml of blood (about 4.8 μg/100 ml is the concentration of T_4 found in normal rats), PFK-1 activity fell to half of its normal activity. The drastic decrease in T_4 required to cause a fall in PFK-1 activity could explain the only slight ability of moderate hypothyroid states to modify some carbohydrate and lipid pathways (Laker & Mayes, 1981).

In addition, this study shows that, in the hypothyroid state, the Fru-2,6-P_2 concentrations in rat heart are decreased to half those found in the hearts of normal rats (Table 3). Since the key role of this molecule in cardiac PFK-1 activation is well recognized, it is reasonable to assume that the low concentrations of Fru-2,6-P_2 may contribute to the decreased rate of glycolysis described in the hypothyroid animals (Burns & Reddy, 1975). It would be interesting to see whether other glycolytic enzymes also share this phenomenon.

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