Species and tissue distribution of the regulatory protein of glucokinase

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Rat liver is known to contain a regulatory protein that inhibits glucokinase (hexokinase IV or D) competitively versus glucose. This inhibition is greatly reinforced by the presence of fructose 6-phosphate and antagonized by fructose 1-phosphate and by KCl. This protein was now measured in various rat tissues and in the livers of various species by the inhibition it exerts on rat liver glucokinase. Rat, mouse, rabbit, guinea-pig and pig liver, all of which contain glucokinase, also contained between 60 and 200 units/g of tissue of a regulatory protein displaying the properties mentioned above. By contrast, this protein could not be detected in cat, goat, chicken or trout liver, or in rat brain, heart, skeletal muscle, kidney and spleen, all tissues from which glucokinase is missing. Fructose 1-phosphate stimulated glucokinase in extracts of human liver, indicating the presence of regulatory protein. In addition, antibodies raised against rat regulatory protein allowed the detection of an ~ 60 kDa polypeptide in rat, guinea pig, rabbit and human liver. The livers of the toad *Bufo marinus*, of *Xenopus laevis* and of the turtle *Pseudemys scripta elegans* contained a regulatory protein similar to that of the rat, with, however, the major difference that it was not sensitive to fructose 6-phosphate or fructose 1-phosphate. In rat liver, the regulatory protein was detectable 4 days before birth. Its concentration increased afterwards to reach the adult level at day 30 of extra-uterine life, whereas glucokinase only appeared after day 15. In the liver of the adult rat, starvation and streptozotocin-diabetes caused a 50–60% decrease in the concentration of regulatory protein after 7 days, whereas glucokinase activity fell to about 20% of its initial level. When 4-day-starved rats were refed, or when diabetic rats were treated with insulin, the concentration of regulatory protein slowly increased to reach about 85% of the control level after 3 days, whereas the glucokinase activity was normalized after the same delay. The fact that there appears to be no situation in which glucokinase is expressed without regulatory protein is in agreement with the notion that the regulatory protein forms a functional entity with this enzyme.

INTRODUCTION

Glucokinase (hexokinase IV or D) is a high-K_m hexokinase which is present in the liver of species such as rat, mouse, rabbit, man, dog and pig (Lauris and Cahill, 1966; Pilkis et al., 1968), but not of others such as cat, ruminants and birds (Lauris and Cahill, 1966; Pilkis et al., 1968; Ureta et al., 1973). It is also present in the liver of some reptiles and of some batrachians (Lauris and Cahill, 1966; Pilkis et al., 1968). In the rat, its expression is restricted to the liver and to the β-cells of pancreatic islets (Iynedjian et al., 1986), two cell types that display a metabolic response to changes in blood glucose concentration (for reviews see Weinhouse, 1976; Lenzen and Panten, 1988). Furthermore, its expression in hepatocytes is under developmental, metabolic and hormonal control. Glucokinase is absent from the liver of newborn rats, only appearing during week 3 of extra-uterine life (Walker, 1963; Iynedjian et al., 1987). In the adult liver, its concentration decreases during starvation as well as after induction of diabetes, and can be restored to the initial level by refeeding or by insulin treatment respectively (Sharma et al., 1963; Iynedjian et al., 1987, 1988).

Recent work from our laboratory has shown that rat liver contains a regulatory protein which, in the presence of fructose 6-phosphate, binds to glucokinase and causes its inhibition (Van Schaftingen, 1989; Vandercammen and Van Schaftingen, 1990). Fructose 1-phosphate prevents the association of the two proteins, thus antagonizing the inhibition exerted by fructose 6-phosphate. High concentrations of salts such as KCl also antagonize the inhibition (Vandercammen and Van Schaftingen, 1991). The regulatory protein is without effect on other hexokinases (Vandercammen and Van Schaftingen, 1991). It was of interest to know if the regulatory protein is expressed in the liver of other species that have, or do not have, glucokinase, and if the concentration of the regulatory protein in the liver of the rat was affected by the conditions known to alter the expression of this hexokinase. The purpose of the present work was to answer these questions.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol) 6000 was from UCB (Brussels, Belgium). DEAE-Sepharose Fast Flow was from Pharmacia–LKB Biotechnology (Uppsala, Sweden). Streptozotocin, antipain, leupeptin, fructose 6-phosphate, fructose 1-phosphate, sorbitol 6-phosphate and immunochemicals were from Sigma (St. Louis, MO, U.S.A.). Dithiothreitol was from Janssen Chimica (Beerse, Belgium). Hepes, auxiliary enzymes and other biochemicals were from Boehringer (Mannheim, Germany). Chemicals were from Merck and were of analytical grade. Insulin Actrapid MC and Lente MC were from Novo Nordisk (Bagsvaerd, Denmark).

Source and treatment of animals

Wistar rats, NMRI mice, guinea pigs and rabbits were from a local university husbandry. Turtles (*Pseudemys scripta elegans*) and toads (*Bufo marinus*) were from Kons Scientific Co. (Germantown, WI, U.S.A.). Specimens of *Xenopus laevis* were from Amrep (Breda, Netherlands). Goat and pig liver were obtained from a local slaughterhouse, and cat liver was from a

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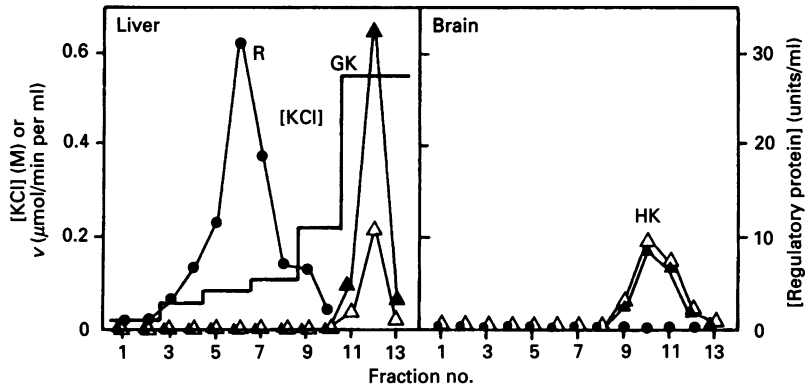

Figure 1 Elution profile of the regulatory protein and hexokinases from small (1 ml) DEAE-Sepharose columns

A poly(ethylene glycol) fraction prepared from 1 g of liver (**a**) or 1 g of brain (**b**) was chromatographed on DEAE-Sepharose columns. Protein was eluted with a stepwise KCl gradient as indicated; 1 ml fractions were collected. Glucokinase (GK) was assayed at 50 mM glucose (▲) and hexokinases (HK) were assayed at 1 mM glucose (△); R, regulatory protein (●).

veterinary service. Except for the pig and the turtle, which were starved, the animals were used in the fed state.

Unless otherwise indicated, the rats were fed *ad libitum* on standard laboratory diet. Diabetes was induced by intraperitoneal injection of 75 mg of streptozotocin/kg body wt. Animals were used 4–7 days after the injection and had a plasma glucose concentration of 23.6 ± 2.5 mM. Except for the experiment shown in Figure 6, only male rats (250–300 g) were used.

Purification of proteins

Rat liver glucokinase and regulatory protein were purified as described previously (Vandercammen and Van Schaftingen, 1990). Glucokinase and regulatory protein from turtle liver were purified by poly(ethylene glycol) precipitation and chromatography on DEAE-Sepharose as described previously for rat liver (Vandercammen and Van Schaftingen, 1990), except that antipain (10 μg/ml) and leupeptin (10 μg/ml) were included in all buffers.

For assay of the regulatory protein in different tissues, we have used on a small scale the first two steps of the purification protocol. The procedure was as follows: 1 g of freeze-clamped tissue was homogenized in 4 vol. of buffer A (25 mM Hepes, pH 7.1, 25 mM glucose, 1 mM dithiothreitol, 10 μg/ml antipain, 10 μg/ml leupeptin) containing 100 mM KCl. A 6–22% poly(ethylene glycol) fraction was prepared and applied to a DEAE-Sepharose column (bed volume ~ 1 ml) equilibrated in buffer A. The column was washed with 2 ml portions of buffer A containing successively 0, 50, 75, 100, 200 and 500 mM KCl; 1 ml fractions were collected. The regulatory protein was eluted at 75 mM KCl, and glucokinase at 200 mM KCl. The recovery of regulatory protein through this purification procedure was estimated at > 90% in experiments in which the purified regulatory protein was added to liver homogenates (110 units/5 ml).

Enzymic assays

Glucokinase was assayed by a glucose 6-phosphate dehydrogenase-coupled assay, using the enzyme from *Leuconostoc mesenteroides*. The assay mixture comprised, unless otherwise indicated, 25 mM Hepes, pH 7.1, 25 mM KCl, 2.5 mM $MgCl_2$, 0.6 mM NAD^+, 1 mM ATP-Mg, 50 mM glucose, 1 mM dithiothreitol and 10 μg/ml glucose-6-phosphate dehydrogenase from *L. mesenteroides*. One unit of glucokinase is the amount catalysing the formation of 1 μmol of glucose 6-phosphate/min in the presence of 50 mM glucose. Hexokinases were assayed similarly at 1 mM glucose.

The regulatory protein was assayed by its ability to inhibit glucokinase in the presence of 5 mM glucose and 100 μM sorbitol 6-phosphate. One unit of regulatory protein is defined as the amount causing half-maximal inhibition of glucokinase in the presence of 5 mM glucose and 100 μM sorbitol 6-phosphate or 200 μM fructose 6-phosphate, in a total volume of 1 ml (Van Schaftingen, 1989). It corresponds to 5 pmol in the case of the rat liver protein (Vandercammen et al., 1992).

Preparation of antibodies and Western blots

For preparation of antiserum, New Zealand rabbits were injected subcutaneously with 25 μg of homogeneous (Vandercammen and Van Schaftingen, 1991) rat liver regulatory protein in Freund's complete adjuvant. The injection was repeated twice at 3-week intervals, and blood was collected 2 weeks after the last injection. SDS/PAGE was carried out in 10% gels as described by Laemmli (1970). The proteins were transferred to nitrocellulose membranes (Hybond-C extra; Amersham) in a Pharmacia LKB Multiphor II apparatus, following the instructions of the manufacturer. The membrane was blocked and incubated with 1/1000-diluted antiserum (Towbin et al., 1979) and developed with an anti-rabbit IgG antibody coupled to alkaline phosphatase. The membrane was stained with Naphthol AS-TR phosphate/Fast Red C (Voller et al., 1976).

RESULTS

Assay method and expression in different tissues

We have previously mentioned that the regulatory protein cannot be accurately quantified in liver extracts by the inhibition it exerts on glucokinase, but that it can be assayed after separation from endogenous glucokinase by anion-exchange chromatography (Van Schaftingen, 1989). We therefore set up a simplified purification procedure, based on the precipitation of protein with poly(ethylene glycol) and subsequent chromatography on DEAE-Sepharose, using a stepwise gradient for elution of the retained protein. As illustrated in Figure 1, the procedure allowed the separation of the regulatory protein from glucokinase. From the amount recovered we calculated that 1 g of liver from a

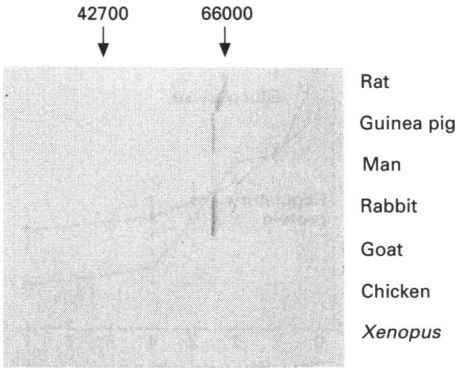

Figure 2 Immunological detection of the regulatory protein in liver from different species

Western blots were carried out with extracts of the indicated tissues (~ 125 μg of protein/lane). Positions of molecular-mass markers (Da) are indicated at the top.

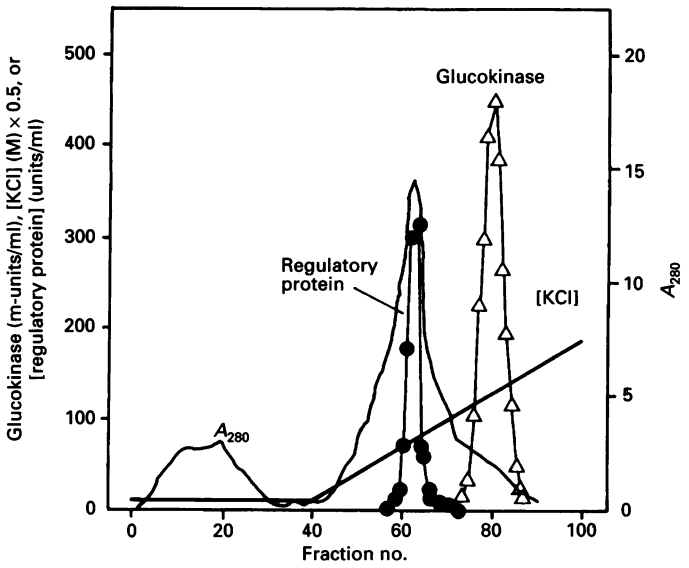

Figure 3 Separation of glucokinase and regulatory protein from turtle liver by DEAE-Sepharose chromatography

A poly(ethylene glycol) fraction obtained from 20 g of liver was applied to a DEAE-Sepharose column (1.6 cm × 15 cm), which was developed with a KCl gradient; 2 ml fractions were collected. The regulatory protein was assayed in the presence of 5 mM glucose, by using rat liver glucokinase.

fed rat contained 143 ± 19 units/g of liver, corresponding to 715 pmol/g. When the method was applied to other tissues, we could not detect an inhibitor of glucokinase in the fractions derived from brain (Figure 1), kidney, skeletal muscle, spleen and heart (results not shown).

We have also raised a polyclonal antibody against rat liver regulatory protein. When used in Western blots, this antibody allowed the detection of a major peptide of ~ 62 kDa in rat liver extracts (Figure 2), although not in brain, kidney, skeletal muscle, spleen or heart extracts (results not shown).

Expression in different animal species

We confirmed the presence of glucokinase in rat, mouse, rabbit, guinea-pig and pig liver, and in addition, found that the livers of these species also contained between 60 (guinea pig) and 200 (pig) units of regulatory protein/g of liver. In all cases, the inhibition was sensitive to fructose 6-phosphate and fructose 1-phosphate (results not shown). In contrast, goat, bovine, cat, chicken and trout liver contained neither glucokinase nor regulatory protein (< 10 units/g). Human liver was not tested by the method used for other species. We observed, however, that, in a human liver extract (final concentration in the assay 25 mg wet wt./ml), the rate of phosphorylation of 5 mM [^{14}C]glucose in the presence of 5 mM ATP-Mg, 1 mM glucose 6-phosphate and 0.25 mM fructose 6-phosphate was stimulated by about 50 % by 1 mM fructose 1-phosphate, indicating the presence of regulatory protein. Western-blot analysis confirmed the presence of regulatory protein in rabbit, guinea pig and human liver, but did not

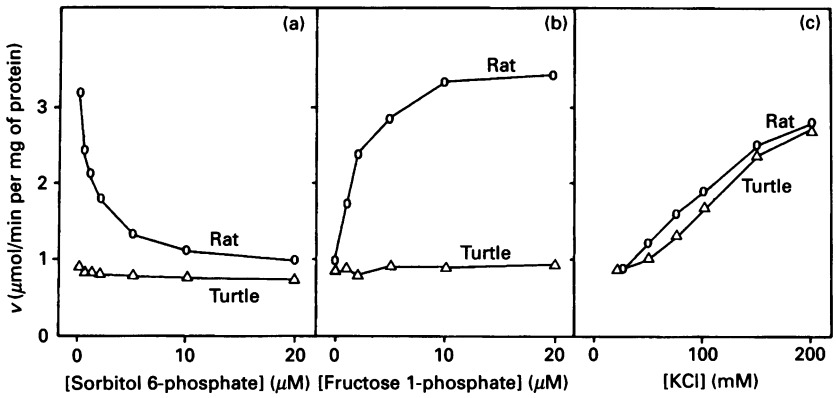

Figure 4 Effect of sorbitol 6-phosphate, fructose 1-phosphate and KCl on the activity of glucokinase measured in the presence of rat or turtle regulatory protein

Rat liver glucokinase was assayed in the presence of 5 mM glucose, 2 units/ml regulatory protein from rat or turtle liver, and the indicated concentrations of sorbitol 6-phosphate (**a**), 0 (turtle) or 100 μM (rat) sorbitol 6-phosphate (**b** and **c**) and variable concentrations of fructose 1-phosphate (**b**) or KCl (**c**). In the absence of regulatory protein, the activity was 3.4 μmol/min per mg of protein.

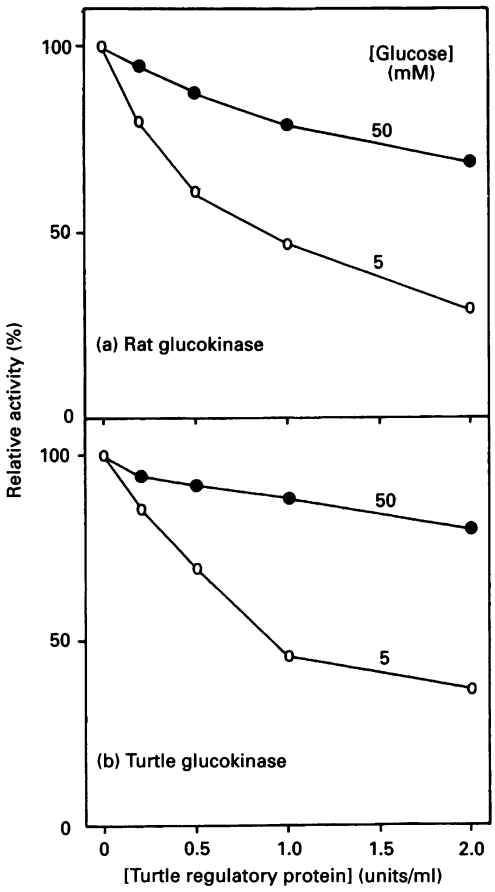

Figure 5 Effect of turtle regulatory protein on rat (a) or turtle (b) glucokinase measured in the presence of 5 or 50 mM glucose

Rat (**a**) or turtle (**b**) regulatory protein was assayed in the presence of 5 or 50 mM glucose and the indicated concentrations of turtle liver regulatory protein. In the absence of regulatory protein, the activity of rat liver glucokinase was 3.1 and 9.2 μmol/min per mg of protein at 5 and 50 mM glucose respectively, and that of turtle glucokinase was 0.13 and 0.72 μmol/min per mg of protein, at the same glucose concentrations.

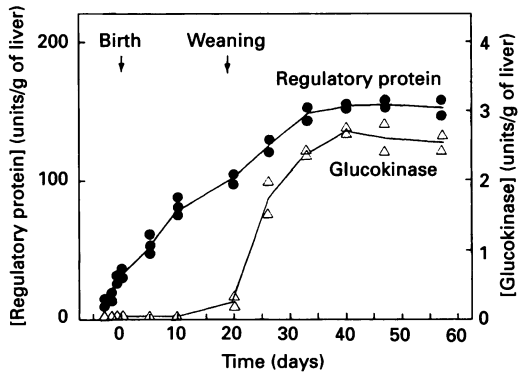

Figure 6 Concentration of regulatory protein and activity of glucokinase in rat liver during the perinatal period

Until day 5, each value was obtained with a pool of 5–10 livers. For older rats, each point represents the mean of at least two values obtained from the liver of one rat. Pups of both sexes were used.

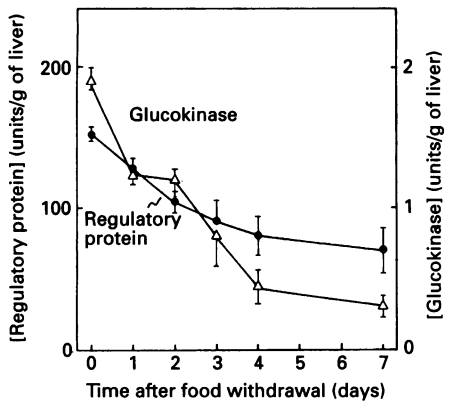

Figure 7 Concentration of regulatory protein and activity of glucokinase in rat liver during starvation

Each point represents the mean ± S.E.M. for $n = 19$ (0 days), 3 (7 days) or 4 (all others) values obtained from different animals. All values corresponding to fasted animals (from day 1 to day 7) are significantly different ($P < 0.02$, unpaired t test) from the fed control.

show a cross-reacting polypeptide in chicken or goat liver (Figure 2).

We detected glucokinase in turtle (*Pseudemys scripta elegans*) and toad (*Bufo marinus*) liver (0.3 and 0.2 unit/g respectively), in confirmation of previous work (Pilkis et al., 1968), and found that it was also present in *Xenopus laevis* liver (0.2 unit/g). Turtle (Figure 3), toad and *Xenopus* liver (results not shown) also contained an inhibitory protein that, like rat liver regulatory protein, was eluted from anion-exchangers at a lower salt concentration (~ 100 mM) than glucokinase. Gel filtration on Sephacryl S-200 indicated a molecular mass of 60 kDa for the proteins from *Xenopus* or toad, and of 75 kDa for the turtle protein (result not shown). The inhibition exerted by the regulatory protein of these amphibians or reptiles was released by high concentrations of KCl (Figure 4), as is known for the rat protein (Vandercammen and Van Schaftingen, 1991), and was more pronounced at low than at high concentrations of glucose (Figure 5). Glucokinase from turtle and from rat was similarly sensitive to inhibition by the regulatory protein from both species (Figure 5; results not shown). However, in contrast with rat liver regulatory protein, the protein found in turtle (Figure 4), *Xenopus* or toad liver (results not shown) exerted an inhibition that was unaffected by fructose 6-phosphate, sorbitol 6-phosphate and fructose 1-phosphate. In addition, we found that the effect of turtle regulatory protein was not modified by the following effectors of rat regulatory protein (Detheux et al., 1991): fructose 6-phosphate (5 mM), arabitol 5-phosphate (0.5 mM), psicose 1-phosphate (2 mM), gluconate 6-phosphate (2 mM), glycerate 3-phosphate (2 mM), glucosamine 6-phosphate (2 mM), ribose 5-phosphate (1 mM). We also observed that the regulatory protein from turtle and from toad did not bind radiolabelled sorbitol 6-phosphate or fructose 1-phosphate (results not shown).

No cross-reacting polypeptide was observed when *Xenopus* (Figure 2) or turtle (results not shown) liver extracts were analysed by Western blotting using the antiserum raised against rat regulatory protein.

Expression of the regulatory protein in the perinatal period

We confirmed that glucokinase appears in the liver between day 10 and day 20 of extra-uterine life and that its activity increased

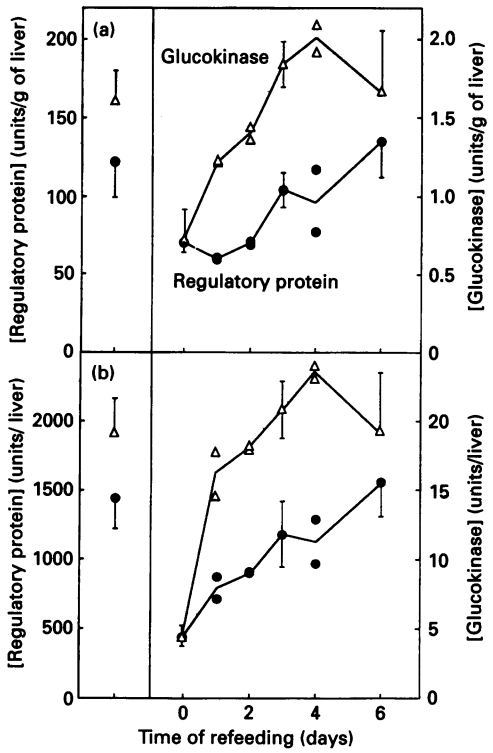

Figure 8 Effect of refeeding on the concentration of regulatory protein and on the activity of glucokinase in rat liver

Rats (290–300 g) were starved for 4 days and refed with standard laboratory chow for the indicated time. Each point represents individual values or means ± S.E.M. for $n = 4$ (control and fasted) or 3 (refed for 3 or 6 days). The results are expressed per g wet mass (**a**) or per liver (**b**). The values corresponding to the animals refed for 3 or 6 days were significantly different ($P < 0.01$, unpaired t test) from those of the fasted rats, although not from those of the fed rats ($P > 0.2$).

rapidly in the following days to reach the adult level at day 30 (Figure 6). Figure 6 also shows that the regulatory protein was detectable in the liver at day 18 of gestation. Its concentration increased afterwards almost linearly to reach the adult level 30 days after birth. At birth, this concentration amounted to 28 ± 5 units/g. It was verified that the inhibition exerted by the regulatory protein from 0- or 5-day-old pups was reinforced by fructose 6-phosphate and released by fructose 1-phosphate.

Effect of starvation and diabetes

Upon starvation, the hepatic concentration of regulatory protein progressively decreased to reach about 50% of the fed value after 7 days, whereas glucokinase activity fell to about 20% of its initial value (Figure 7). Upon refeeding, the concentration of regulatory protein underwent a transient decrease, followed by a progressive increase, to reach only about 85% of the fed level after 3 days and to be restored to the original level after 6 days (Figure 8a). No transient decrease was observed when the results were expressed as content (Figure 8b), as a consequence of the fact that the liver mass almost doubled (from 6 to 11 g) after 24 h of refeeding (results not shown). The changes in glucokinase were much more rapid, since the activity of this enzyme was restored to its original level after only between 2 and 3 days of refeeding.

Streptozotocin diabetes caused a 90% decrease in the activity of glucokinase and a 60% decrease in the concentration of regulatory protein in 7 days (Table 1). Treatment of diabetic rats with insulin significantly increased glucokinase activity after 7 h and restored it to the initial level after 3 days. The same treatment did not increase the concentration of regulatory protein during the first 14 h, although it allowed it to reach about 85% of the control value after 3 days.

Table 1 Effect of diabetes and of insulin on the concentration of glucokinase and of the regulatory protein in rat liver

Results are expressed as means ± S.E.M.: *significantly different from 4-day-diabetic ($P < 0.005$) and from 7-day-diabetic ($P < 0.02$); †significantly different from control ($P < 0.005$, unpaired t test).

	n	[Glucokinase] (units/g of liver)	[Regulatory protein] (units/g of liver)
Healthy control, fed	19	1.86 ± 0.25	142.9 ± 11.8
Diabetic: 4 days after streptozotocin injection	9	0.37 ± 0.14†	82.4 ± 12.1†
7 days after streptozotocin injection	3	0.15 ± 0.04†	66.3 ± 1.4†
Diabetic (4 days), insulin-treated: for 7 h‡	4	0.81 ± 0.27†	85.7 ± 4.4†
for 14 h‡	4	1.29 ± 0.38†	84.3 ± 10.9†
for 3 days§	5	2.25 ± 0.15*†	117.8 ± 20.4*†

‡ Actrapid insulin (1 U/100 g body weight) was injected intraperitoneally at times 0, 2 h, 4 h, 6 h, 9 h and 13 h.

§ Lente insulin (3 U/100 g body weight) was injected subcutaneously once daily.

DISCUSSION

The regulatory protein and glucokinase, a functional unit

The regulatory protein is present together with glucokinase in the liver of a series of species including some mammals, one reptile and two amphibians. Both proteins are absent from the liver of the cat, the goat, the chicken and the trout, as well as from several rat tissues other than the liver. It was previously concluded that the regulatory protein is present in pancreatic islets (Malaisse et al., 1990), on the basis that glucose phosphorylation was stimulated by fructose 1-phosphate in islet extracts, and also on the fact that D-glyceraldehyde, which causes the formation of fructose 1-phosphate in pancreatic islets, stimulated the detritiation of [5-^{3}H]glucose in suspensions of islets. To summarize these data, there appears to be no tissue in which glucokinase is expressed without regulatory protein; the converse proposal is true if one excepts the liver in the perinatal period.

We have previously reported that the regulatory protein has no effect on the activity of hexokinases other than glucokinase, and that the property of the latter enzyme to be affected by the regulatory protein is well conserved through evolution (Vandercammen and Van Schaftingen, 1991). We have also found that the regulatory protein did not affect the activity of glucose-6-phosphatase or that of other enzymes (A. Vandercammen and E. Van Schaftingen, unpublished work). These results, as well as those of the present study, indicate that glucokinase and its regulatory protein form a functional unit. In this respect the fact that the regulatory protein appears in the liver before glucokinase could mean that the regulatory protein is required for the proper functioning of glucokinase.

The regulatory protein from lower vertebrates

The regulatory protein from lower vertebrates was unaffected by fructose 6-phosphate, fructose 1-phosphate or other phosphate esters known to be effectors (Detheux et al., 1991) of rat regulatory protein. Despite these differences, these phosphate-ester-insensitive proteins shared several properties with the regulatory protein from rat: (1) they acted as competitive inhibitor versus glucose; (2) their inhibition was released by KCl; (3) their molecular mass was about 60–75 kDa; (4) they were retained on anion-exchangers, from which they are eluted at a salt concentration of 100 mM. Moreover, the structures conferring on the regulatory protein the property to bind glucokinase were remarkably conserved through evolution, since rat liver glucokinase was inhibited by the regulatory protein from such distant species as turtle or toad, and vice versa.

For these reasons, we propose that the regulatory protein found in lower vertebrates is a counterpart of the fructose 6-phosphate- and fructose 1-phosphate-sensitive protein found in mammals which is devoid of the phosphate-ester-binding site. Accordingly, regulatory protein from turtle and from toad did not bind radiolabelled sorbitol 6-phosphate (a fructose 6-phosphate analogue) or fructose 1-phosphate. The role of the regulatory protein in these species could be to transduce the effect of other intermediary metabolites that have not been tested in the present study. Alternatively, the regulatory protein of these species could be affected in its ability to inhibit glucokinase by covalent modification.

The fact that the regulatory protein from *Xenopus laevis* did not cross-react with an antiserum raised against rat protein is presumably due to the large genetic distance between these species and the relatively high rate of evolution of the protein. It is worth noting in this respect that frog glucokinase did not react with antibodies raised against rat liver glucokinase (Pilkis et al., 1968).

Regulation of the expression in rat liver

In the adult rat, the concentration of regulatory protein decreases during starvation or following induction of diabetes to a lesser extent than does glucokinase activity. It increases upon refeeding or after treatment with insulin, suggesting that the expression of the regulatory protein is under the control of this hormone, as is known for glucokinase (Sharma et al., 1963; Iynedjian et al., 1988). The fact that the changes in the regulatory protein content are more sluggish than those in glucokinase indicate a difference in turnover time.

The regulatory protein is present in the liver during the last few days of fetal life and during the first fortnight after birth. In contrast, glucokinase is not expressed until week 3 of life. At this time it starts to be synthesized as a result of transcriptional stimulation, due, at least partly, to an increase in the blood concentration of insulin (Iynedjian et al., 1987). The fact that the regulatory protein is present before glucokinase indicates that the mechanisms that control the expression of both proteins are not identical.

Assuming that pure glucokinase has a specific activity of 180 units/mg of protein, and that the molecular mass of this enzyme is 52 kDa (Andreone et al., 1989), it can be calculated that 1 unit of glucokinase corresponds to approx. 100 pmol. Thus the concentration of glucokinase in the liver of the adult rat amounts to 20–200 pmol/g of liver, depending on the conditions, whereas that of the regulatory protein is in the range approx. 300–700 pmol/g. These considerations indicate that the regulatory protein is always in excess compared with the enzyme that it regulates. The phosphorylation of glucose in rat liver is, however, incompletely inhibited even in the absence of fructose 1-phosphate (Davies et al., 1990; Van Schaftingen and Davies, 1991), presumably because effectors such as P_i antagonize the inhibition by lowering the affinity of the regulatory protein for glucokinase (Detheux et al., 1991).

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