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Hexokinase and not glycogen synthase controls the flux through the glycogen synthesis pathway in frog oocytes

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ABSTRACT

Here we set out to evaluate the role of hexokinase and glycogen synthase in the control of glycogen synthesis in vivo. We used metabolic control analysis (MCA) to determine the flux control coefficient for each of the enzymes involved in the pathway. Acute microinjection experiments in frog oocytes were specifically designed to change the endogenous activities of the enzymes, either by directly injecting increasing amounts of a given enzyme (HK, PGM and UGPase) or by microinjection of a positive allosteric effector (glc-6P for GS). Values of 0.61 ± 0.07, 0.19 ± 0.03, 0.13 ± 0.03, and −0.06 ± 0.08 were obtained for the flux control coefficients of hexokinase EC 2.7.1.1 (HK), phosphoglucomutase EC 5.4.2.1 (PGM), UDPglucose pyrophosphorylase EC 2.7.7.9 (UGPase) and glycogen synthase EC 2.4.1.11 (GS), respectively. These values satisfy the summation theorem since the sum of the control coefficients for all the enzymes of the pathway is 0.87. The results show that, in frog oocytes, glycogen synthesis through the direct pathway is under the control of hexokinase. Phosphoglucomutase and UDPG-pyrophosphorylase have a modest influence, while the control exerted by glycogen synthase is null.

1. Introduction

Almost 60 years elapsed since Michaelis and Menten published their results in enzyme kinetics for isolated enzymes in 1913 [1] and the appearance of Metabolic Control Analysis (MCA), developed in the early 1970s by Kacser and Burns [2] and Heinrich and Rapoport [3]. Despite increased interest to study flux control through metabolic pathways by means of MCA, the concept of rate-limiting enzymes is nevertheless widely accepted. This is the case for glycogen synthase, still viewed by many as the enzyme controlling the flux in glycogen formation, mainly because the enzyme is subjected to several regulatory mechanisms, such as sensitivity to allosteric effectors [4], covalent phosphorylation [5], modification by nucleo-cytoplasmic relocation [6], and specific proteolysis mediated by maline and laforine [7]. This rate-limiting concept for GS has been challenged by authors who have applied the MCA approach to the study of flux control in the pathway. *In vivo* studies performed by different groups suggest that the control of the flux is at the level of the glucose transporter and hexokinase [8,9] or is exerted by hexokinase [10]. Also, it has been shown that glucokinase exerts a high control of the flux in hepatocytes, which is dependent on compartmentation of the enzyme [11]. The major determinant of the high control coefficient of glucokinase would be the negative control coefficient of the glucokinase regulatory protein [12].

As pointed out by MCA, several enzymes might affect the flux in a pathway, sharing its control. Quantifying the response of the flux to a change in enzyme activity requires measurement of the flux and of enzyme activity. The magnitude of the effect is expressed in terms of the flux control coefficient for a given enzyme, and is defined as the ratio between the fractional changes in the enzyme and the flux [13]. Mathematically, it implies that the flux control coefficient may be obtained from the slope of the tangent on a plot of the logarithm of the flux versus the logarithm of enzyme activity. According to the summation theorem, the addition of the flux control coefficients for all the enzymes involved in the pathway is 1.0. If all the enzymes of the pathway affect the flux, then their flux control coefficients should be very low. A corollary of this statement is that if the flux control coefficient of an enzyme is 1.0, the other enzymes of the pathway will have flux control

Abbreviations: HK, hexokinase (EC 2.7.1.1); PGM, phosphoglucomutase (EC 5.4.2.1); UPGase, UDPglucose pyrophosphorylase (EC 2.7.7.9); GS, glycogen synthase (EC 2.4.1.11); MCA, metabolic control analysis

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[2] We report with great sadness that Dr. Ureta died on 9th June 2012. He had seen and approved a version of this paper that was essentially final apart from some minor details.

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coefficients equal to 0; only in this case this particular enzyme may be considered as the rate-limiting enzyme.

Metabolic flux measurements under in vivo conditions are relatively scarce because of experimental limitations. Control coefficients have been usually estimated by supplementing tissue homogenates with purified enzymes. This system has several disadvantages, such as dilution of homogenates compared to the intracellular medium and loss of cellular organization. The possibility then is to change the activity of a particular enzyme by overexpression, or by the use of knock out organisms or enzyme activity modifiers. Nevertheless, even when only a single gene has been introduced to a cell the expression of other proteins may show altered levels [14]. In early work, we have shown that amphibian oocytes are a powerful tool for metabolic studies in vivo. The advantages of this system have been described elsewhere for a review see [15]. Concerning the present research, measurement of the flux through the glycogen synthesis pathway and intracellular variation of the enzyme activities involved were easily performed in oocytes.

After radioactive glucose microinjection into *Caudiverbera caudiverbera* oocytes, around 95% of the label is incorporated into glycogen. The synthesis proceeds both by the classic direct and the indirect pathways. The latter implies glycolytic degradation of glucose to pyruvate or lactate followed by gluconeogenic synthesis of glucose-6-P with further metabolization to glycogen [16]. The preferential operation of one pathway over the other depends on glucose availability [17].

Our aim in this work was to determine the flux control coefficients in vivo for all the enzymes involved in glycogen synthesis through the direct pathway, and to elucidate if the flux was under the control of hexokinase or glycogen synthase. We concluded that, in frog oocytes, glycogen synthesis through the direct pathway is under the control of hexokinase. Phosphoglucomutase and UDPG-pyrophosphorylase have a modest influence, while the control exerted by glycogen synthase is null.

2. Materials and methods

2.1. Materials

HK (H 5625), UGPase (U 8501), Glc-6-P dehydrogenase (G 8404), and metabolites were from Sigma Chemical Co. UDP-[6-^{3}H]-glucose and U-^{14}C-glucose were obtained from Amersham. PGM (9001-81-4) was from Roche. Other reagents were mostly from Merck and Sigma–Aldrich, IL, USA.

2.2. Animals and cells

Female frogs *C. caudiverbera* were obtained from a local dealer, kept in tap water and fed once a week with chicken or bovine liver. An ovary fragment was removed from cold anaesthetized frogs by a small costal incision and placed in a beaker containing amphibian modified Barth saline [18]. Individual, same size stage VI oocytes [19] were manually released from surrounding tissue by blunt dissection with jeweler's forceps and kept in saline until used. Animal use and experimental procedures were approved by the Faculty of Sciences local Ethics Committee, in accordance with Conicyt (Comisión Nacional de Investigación Científica y Tecnológica).

2.3. Substrate microinjection

Oocytes were microinjected with calibrated glass needles in the equatorial zone under a Nikon 102 magnifying glass using a Narishige IM-200 automatic injector. Volume injection was around 50 nl. Before injection, radioactive substrates were dried by evaporation in a UniEquip vacuum centrifugal equipment, and further resuspended in saline plus unlabelled substrates to achieve the desired concentrations.

2.4. Increase of endogenous enzymatic activities in oocytes

Two strategies were followed: microinjection of commercial enzymes for HK, PGM and UGPase, and activation of endogenous GS by injection of Glc-6-P.

Yeast hexokinase was diluted in Barth saline to achieve the desired concentrations in a volume of 50 nl. For PGM, 150 µl of ammonium sulfate precipitated enzyme was centrifuged for 10 min at 15,982×*g*. The supernatant liquid was discarded and the precipitated enzyme was washed in 150 µl of saline. The enzyme was further concentrated to a final volume of 20 µl and finally diluted in saline. For UGPase, the homogenous enzyme obtained after chromatography on Sephacryl was concentrated and diluted in saline. In all 3 conditions, increasing amounts of enzyme were microinjected into the oocytes to obtain at least 4.5 times the endogenous activity. Unperturbed oocytes (not microinjected) and oocytes injected with Barth saline were used as controls. After enzyme microinjection, groups of 10 oocytes were left 1 h in Barth saline to allow intracellular diffusion of the enzyme. Then, the oocytes were reinjected with 50 nl of saline containing 6 nmoles of U-^{14}Cglucose (50,000–100,000 cpm). Groups of 5 oocytes were incubated during 20 min at 22° in 120 µl saline in sealed tubes with continuous O_2 flow. Labelled CO_2 was collected as described [10].

Since commercial homogenous GS was not available, the endogenous activity was increased by microinjecting the oocytes with different concentrations of Glc-6-P, a potent activator of oocyte GS [20]. After 8 min, the oocytes were reinjected with 6 nmoles of [U-^{14}C]glucose. Groups of 5 oocytes were further incubated for 12 min, and CO_2 evolution and glycogen were measured. Previous experiments to find the appropriate Glc-6-P concentration producing the highest enzyme activation during the times required were performed (Supplementary data).

2.5. Glycogen isolation and CO_2 measurement

As described by Ureta et al. [10], groups of 5 oocytes microinjected with enzyme and radioactive glucose were incubated in sealed tubes containing 120 l Barth during 20 min at 22° under 100% O_2. CO_2 released was collected in connected vials containing 600 l of 0.3 N NaOH and drops of Triton ×-100. After addition of 4 ml of scintillation mix and 300 l of water, the samples were counted in a 1600 TR Packard scintillation counter. For glycogen isolation, individual oocytes were transferred to Eppendorf tubes containing 200 l of 30% KOH and incubated for 30 min at 100°. Finally, samples were placed on small squares of ET31 Whatman filter paper. Air dried papers were left overnight in 66% ethanol at −20°. After, they were washed three times with continuous stirring during 30 min in 66% ethanol at room temperature, dried over paper towel under infrared light, and placed in vials with 4 ml of scintillation mixture. Glucose radioactivity incorporated into glycogen was measured in a 1600 TR Packard scintillation counter.

2.6. Protein determination

Protein concentration of the different enzymatic preparations was determined according to Bradford [21].

2.7. Enzymatic assays

Hexokinase. Enzyme activity was measured by the coupled Glc-6-P dehydrogenase method [22]. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 5 mM ATP,

5.1 mM glucose, 2.0 mM EDTA, 0.5 mM NAD^+ and 0.5 U of Glc-6-P dehydrogenase in a final volume of 1 mL. NADH formation was monitored at 340 nm in a Unicam UV2-100 spectrophotometer at 28°. Oocytes endogenous activity was determined in the supernatant fraction obtained after homogenizing 5 oocytes in 100 µl Barth saline and centrifuging at 15,982×g during 25 min. A unit of HK activity is defined as the amount of enzyme catalyzing the formation of 1 mol of Glc-6-P per min at 28°.

Phosphoglucomutase. Enzyme activity was determined in the supernatant fraction obtained after homogenizing 1 oocyte/9 µl of Barth saline and centrifuging 1 h at 15,982×g. Activity was measured by the coupled Glc-6-P dehydrogenase method [23]. Formation of NADH was monitored at 340 nm in a Hewlett Packard 8453 spectrophotometer at 24°. The reaction mixture contained in a final volume of 1 ml: 30 mM imidazole buffer (pH 7.5), 3.3 mM $MgCl_2$, 0.85 mM NAD^+, 0.9 mM EDTA, 0.015 mM Glc-1,6-bisP, 1.5 mM glc-1-P and 0.33 U of Glc-6-P dehydrogenase. One unit of PGM activity is defined as the amount of enzyme catalyzing the formation of 1 mol of Glc-6-P per min at 24°.

UDPG-pyrophosphorylase. The spectrophotometric method described by Martz et al. [24] was used with slight modifications. Oocytes were homogenized 1:2 (oocytes:buffer, w:v) in saline. The assay medium contained: 1 mM UDP-glucose, 5 mM $MgCl_2$, 0.6 mM $NADP^+$, 3 mM sodium pyrophosphate, 0.02 mM Glc-1,6-P_2, 50 mM bicine (pH 8.5), 0.5 U of PGM, 5.6 U of Glc-6-P-dehydrogenase and aliquots of the supernatant liquid containing UGPase activity, in a final volume of 1 ml. The production of NADPH was monitored at 340 nm in a UV 2 UNICAM spectrophotometer. A unit of UGPase activity is defined as the amount of enzyme required to reduce 1 µmol of $NADP^+$ per min at 24°.

Glycogen synthase. GS activity was measured by following radioactive glucose incorporation in vivo from UDP[6-^{3}H] glucose into glycogen, in the absence of Glc-6-P, according to Báez et al. [20]. Briefly, 50 nl saline containing 3 mM UDP-6-^{3}Hglc (50,000–100,000 cpm) were microinjected into individual oocytes followed by incubation in 75 l saline during 10 min at 22°. After, 500 l of 30% KOH were added and the mixture was heated at 100° for 30 min. The solution was transferred to a glass tube and 100 l of 2% Na_2SO_4, 40 l of carrier glycogen (5 mg/ml) and 2 ml of 96% ethanol were added for glycogen precipitation overnight. The mix was centrifuged at 3,020×g for 2.5 min in a Sorvall RC-5 centrifuge and the pellet was resuspended in 2 ml of water and reprecipitated during 8 h with 6 ml of 96% ethanol. After centrifugation, the pellet was resuspended in 1.2 ml of water and the radioactivity present in 1 ml aliquots was measured in a 1600 TR Packard scintillation counter.

2.8. Purification of commercial UGPase

The freeze-dried commercial Sigma enzyme was not homogeneous by SDS PAGE, and, more important, it contained contaminating HK. Further purification by chromatography on a Sephacryl S-200 column (2.1 × 73 cm) was performed. A sample containing about 1 mg of commercial enzyme taken directly from the flask, 100 µl of glycerol and 200 µl of buffer (100 mM Tris–HCl pH 7.6, 100 mM KCl) was loaded to the column. HK and UGPase activities were measured in aliquots of the collected fractions as described. The fractions containing UGPase activity were pooled, concentrated and subjected to rechromatography on the same column.

2.9. Purity of the enzymes to be microinjected

Aliquots of HK, PGM and UGPase were loaded on a 10% polyacrylamide gel and submitted to SDS–PAGE to check for purity (Supplementary data). Also, the activity of the other enzymes involved in the pathway was checked in each enzyme sample.

3. Results

3.1. Endogenous activity of the enzymes involved in glycogen synthesis

Since measurement of the control coefficient of an enzyme implies changing enzyme activity in order to study the effect of this perturbation over the flux, previous knowledge of the oocyte intracellular enzymatic levels is essential. The activities of the four enzymes involved in the direct pathway for glycogen synthesis in oocytes are shown in Table 1. The data reveals that HK has the lowest activity (0.06 mU/oocyte), Comparison of the relative activity of the enzymes assigning the value of 1 to HK shows that PGM and UGPase have around 100 fold the HK activity, while GS is 1.5 times more active. The values obtained were used to estimate the amount of each enzyme to be microinjected into the cells.

3.2. Purity of the enzyme preparations to be microinjected

All three HK, PGM and UGPase appeared homogenous by SDS–PAGE (Supplementary Fig. S1). Also, contamination with any of the other enzymes of the pathway in each enzyme sample was discarded by direct measurement of the corresponding enzyme activities (results not shown).

3.3. Remaining intracellular activity of enzymes microinjected into the oocytes

Control experiments were performed in order to check that the microinjected enzymes remained active inside the cells. The same amounts of enzymes used for flux control coefficients estimation were microinjected into oocytes. After 1 h incubation under the same conditions used for flux control studies, groups of 10 oocytes were homogenized and centrifuged, and the corresponding enzyme activity was measured in the supernatant fraction. As shown in Table 2 for UGPase, the activity of the microinjected enzyme remained almost invariant after 1 h inside the cell, with values between 82% and 107% of the initial activity. This control was done for each enzyme every time a control coefficient was to be determined. In the case of GS, where the enzyme was activated by Glc-6-P microinjection, a slightly different control was performed. Groups of 10 oocytes treated under the same conditions as the ones used for flux control coefficient calculations (amounts of Glc-6-P microinjected and incubation times), were further microinjected with UDP-[6-^{3}H] Glc and GS activity was measured as described in Section 2.

3.4. Glc-6-P microinjection

Since no commercial GS was available, changes in the intracellular activity of the enzyme was achieved by microinjection if the allosteric activator Glc-6-P. At 2 mM injected Glc-6-P (6 nmoles per oocyte) a maximal activation of 2–3 times was obtained. The effect was transient (probably by the diversion of the compound

Table 1
Endogenous levels of enzymes involved in glycogen synthesis through the direct pathway in frog oocytes.

Enzyme	Activity (mU/oocyte)	Relative activity
Hexokinase	0.06 ± 0.004	1
Phosphoglucomutase	9.4 ± 0.93	157
UDPG-pyrophosphorilase	4.3 ± 0.65	72
Glycogen synthase	0.7 ± 0.03	12

Oocyte homogenates were prepared under the conditions described in Section 2 for each enzyme and centrifuged at 14,000 rpm for 20 min. Enzyme activities were measured in the obtained supernatant fraction as described in Section 2.

Table 2
UGPase activity remaining after 60 min microinjection of enzyme.

Microinjections	Microinjected	UGPase mU Total, 0 min	UGPase mU Total, 60 min	
None	0.0	3.5		
Barth saline	0.0	3.4		%
UGPase	1.8	6.1	5.0	82
	4.1	7.1	7.5	106
	6.2	9.5	9.6	101
	8.2	12.2	11.6	95

Groups of 10 oocytes were microinjected for each condition indicated in the table. At 0 and 60 min incubation, the oocytes were homogenized, centrifuged and UGPase activity was measured as indicated in Section 2.

to other pathways) and was maximal between 5 and 10 min (Supplementary Fig. S2).

3.5. Control coefficient determinations

The results of increasing enzyme activity over the glycogenic flux in oocytes are shown in Figs. 1A–4A for HK, PGM, UGPase and GS, respectively. The flux control coefficient with the highest value (0.61 ± 0.07, $n = 4$) was obtained for HK. Intermediate coefficients of 0.19 ± 0.03 ($n = 4$) and 0.13 ± 0.03 ($n = 3$) were calculated for PGM and UGPase, while the lowest coefficient obtained was for GS (−0.06 ± 0.08, $n = 4$). As a control of the specificity of the observed effects, the influence of changing these intracellular enzymatic activities was simultaneously analyzed for the pentose phosphate pathway. Previous results from our laboratory [25] have shown that CO_2 evolution is solely generated by glucose decarboxylation in this pathway. The control exerted by the glycogenic enzymes over the flux through the pentose phosphate pathway was almost null, as shown in Figs. 1B–4B. The flux control coefficients varied from −0.09 for PGM to 0.08 for GS. Another control included the estimation of the flux control coefficient for HK when glycogen synthesis proceeded mainly through the indirect pathway. This route is the prevailing one when glucose administered to the oocytes is as low as 0.5 nmoles microinjected per cell [17]. A flux control coefficient of 0.05 was estimated for HK in this condition, a result showing that although the enzyme significantly enhances the flux through glycogen synthesis by the direct pathway it has no effect over the flux through the indirect route.

4. Discussion

The results reported provide the first direct in vivo measurement of the flux control coefficients for the complete set of enzymes involved in a metabolic pathway. The values obtained also

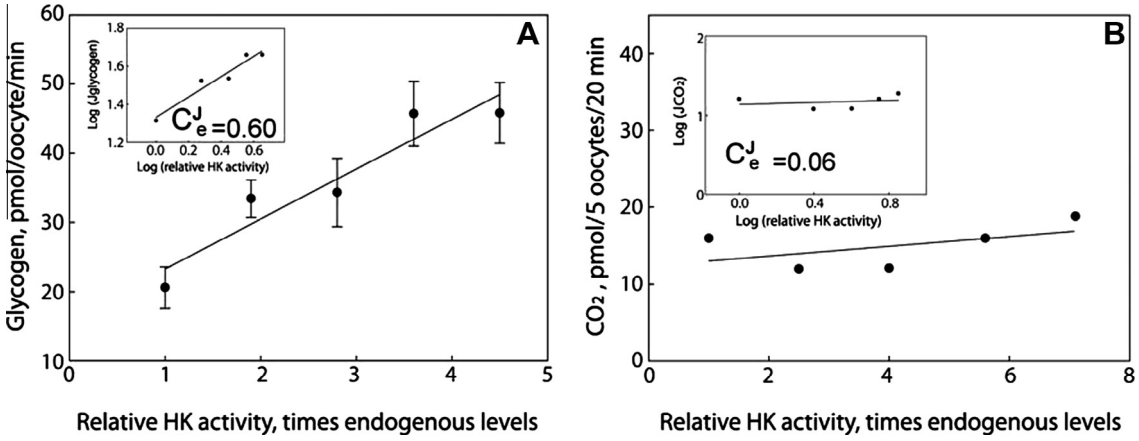

Fig. 1. Flux control coefficient for hexokinase. (A) Oocytes were microinjected with increasing amounts of HK and groups of 10 cells were preincubated for 1 h at 20°. Each cell was then reinjected with 6 nmol of [U-^{14}C]Glc and further incubated in groups of 5 for 20 min in the presence of oxygen. Glycogen was isolated from each oocyte and the radioactivity incorporated into the polysacharide was measured. The results correspond to the mean of 10 oocytes for each group of oocytes ± S.E.M. The inset shows the flux control coefficient calculation from the data shown in the figure. (B) In the same experiment CO_2 was collected from groups of 5 oocytes and the radioactivity incorporated was determined as a measure of the operation of the pentose-P pathway. The flux control coefficient for HK over this pathway was also calculated.

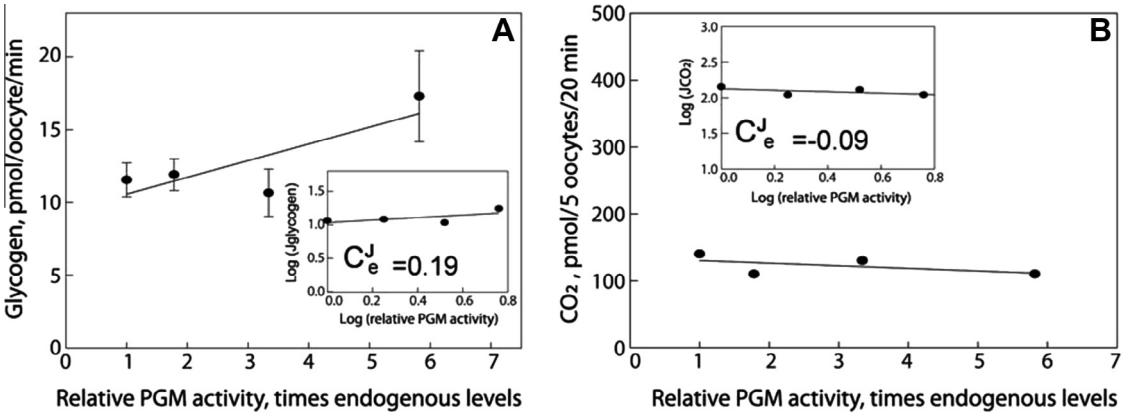

Fig. 2. Flux control coefficient for phosphoglucomutase. Oocytes were microinjected with increasing amounts of PGM and groups of 10 cells were preincubated for 1 h at 20°. The rest of the experiment and calculations were the same as described in Fig. 1.

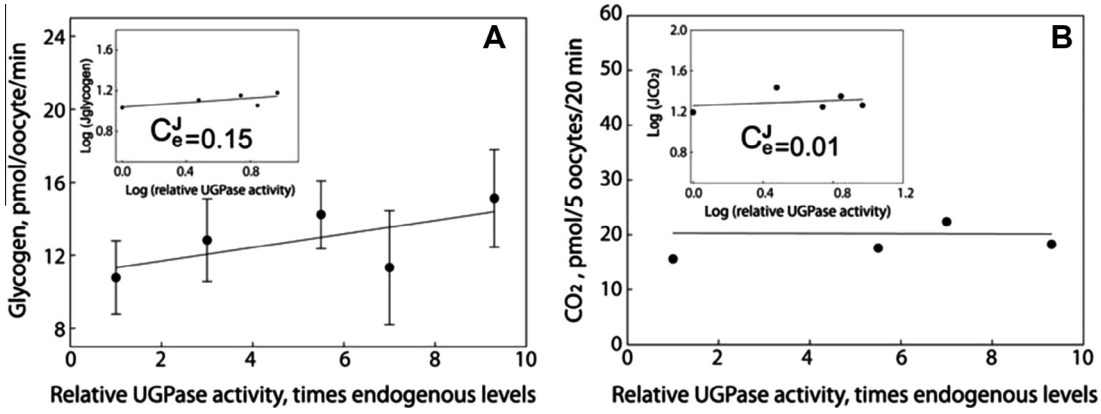

Fig. 3. Flux control coefficient for UDPG-pyrophosphorylase. Oocytes were microinjected with increasing amounts of UGPase and groups of 10 cells were preincubated for 1 h at 20°. The rest of the experiment and calculations were the same as described in Fig. 1.

fulfill the summation theorem, one of the fundamental principles of metabolic control analysis, since the sum of the control coefficients for all the enzymes of the pathway is 0.87 ± 0.05. We may thereby conclude that it is HK and not GS the enzyme primarily responsible for the control of the flux in the direct pathway for glycogen synthesis in amphibian oocytes. The finding that HK was the enzyme with the highest control coefficient and also with the lowest endogenous activity agrees with the data reported in the literature [13]. However, it should be born in mind that since glucose was microinjected into the cells, the influence of the glucose transport stage was not taken into account.

The feasibility of frog oocytes to be used as test tubes for in vivo metabolic studies has been strongly supported by this work. We were able to increase the intracellular activity of the first three individual enzymes (HK, PGM, UGPase) by directly microinjecting them into the oocytes and to activate GS by microinjection of Glc-6P, without affecting the remaining enzyme repertoire of the cells. Altering enzyme activity by microinjection proved to specifically affect a given pathway. Thus, although hexokinase significantly enhances the flux through glycogen synthesis by the direct pathway it has no effect over the flux through the indirect route. Also, enzyme microinjection does not affect the flux through the pentose-P pathway as shown by the respective experiments. Hence, repeated and reliable results were easily obtained by means of this experimental approach.

Microinjection of enzymes isolated from the same species would be the ideal situation for measuring control coefficients. In our experiments, the enzymes (excluding GS) were heterologous. Nonetheless, the yeast hexokinase has extensive sequence and three dimensional structure similarities with the vertebrate enzymes [26] and a similar nuclear-cytosolic localization [27]. Similar arguments apply to the other enzymes microinjected. Sequence comparison of the yeast *Saccharomyces cerevisiae* and *Xenopus laevis* UGPase by the program CLUSTAL W have shown a 50% amino acid sequence identity [28]. The sequences were obtained from Genbank with the following access numbers: AAH77213 (*Xenopus laevis*) and 2124302A (*Saccharomyces cerevisiae*).

GS activity was increased by microinjection of the allosteric activator Glc-6-P, which is also an inhibitor (competitive with ATP) of vertebrate hexokinases. Therefore the lack of control of GS on glycogen synthesis could perhaps be explained by the decrease of the flow through the pathway due to hexokinase inhibition. Several facts rule out this possibility. It is well known that the inhibitory effect of Glc-6-P is reversed by P_i [29]. When glycogen synthesis is operative, UGPase produces large amounts of P_i. High concentrations of P_i around 3 mM are found in unperturbed oocytes [30]. Moreover, ATP concentration in oocytes is high enough (~3.6 mM) to revert the inhibitory effect of Glc-6P [31]. Finally, microinjected glucose-6-P is also drained both by the indirect pathway for glycogen synthesis and the pentose-P pathway. Thus, hexokinase should not be inhibited by microinjected Glc-6-P.

Another possibility to be considered is that GS, being a highly regulated enzyme, could be inactive or not available for glycogen formation in our experiments. We have previously shown that

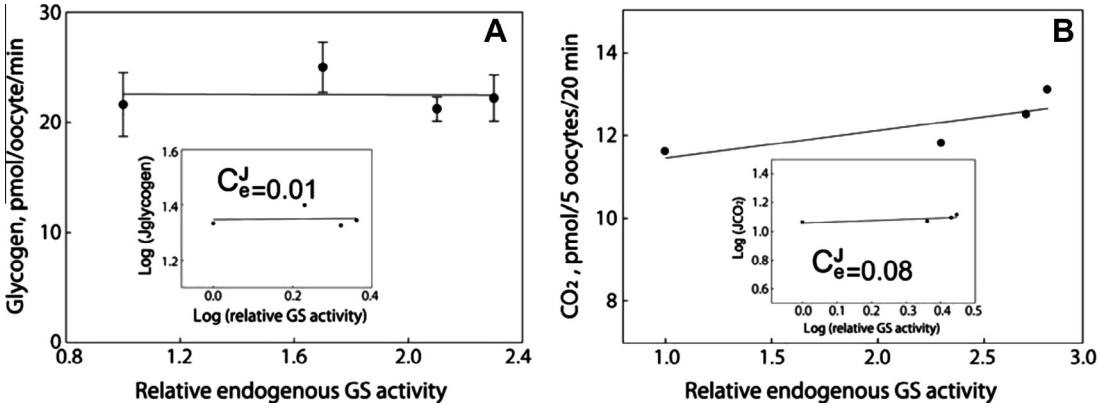

Fig. 4. Flux control coefficient for glycogen synthase. Oocytes were microinjected with increasing amounts of Glc-6P (0.13, 0.25, 0.5 and 0.75 mM, intracellular concentrations), and groups of 10 cells were preincubated for 8 min. Then, 6 nmol of [U-^{14}C]Glc were injected into each oocyte and the cells were incubated for 12 min. The rest of the experiment and calculations was as described in Fig. 1.

radioactive glucose microinjected into oocytes is quickly and efficiently incorporated into glycogen [15]. Besides, the results in this paper demonstrate that glycogen synthesis proceeds at least for 1 h in oocytes microinjected with HK and 20 min after glucose administration. Finally, the lack of control observed for GS could be influenced by phosphorylase since it has been reported that the enzyme has a high negative control coefficient (greater than 1.0) in hepatic glycogen synthesis [32]. However, unpublished results from our laboratory indicate that after 2 h of radioactive glucose microinjection, 90% of the label is recovered as glycogen, suggesting that the polysaccharide is very stable in frog oocytes, Also, phosphorylase should be dephosphorylated and hence inactive at high glucose concentration, which is the case in our experiments.

The lack of flux control exerted by glycogen synthase is fully consistent with the regulation of glycogen synthesis in mammals by the blood-glucose level, i.e. it is a pathway regulated by supply [33]. Although comparable information is lacking in other vertebrates it is realistic to think that similar principles should apply. Glycogen synthesis in oocytes will thus differ from the much more usual case of regulation by demand for end product, in which the principal effect of feedback inhibition is to provide information to the regulated enzymes early in the pathway about the demand for the end product [34]. This type of behavior is clearly seen in a recent study of feedback inhibition of aspartate metabolism in *Arabidopsis thaliana* [35,36].

A top-down approach to MCA which defines control over a few metabolic components of a pathway, each consisting of a number of enzymes, has been applied to glycogen synthesis in insulin stimulated rat skeletal muscle [37]. The authors studied the control of the flux for glucose disposal among glucose transport/phosphorylation [GLUT-4/hexokinase (HK)], glycogen synthesis, and glycolysis. A flux control coefficient of 0.55 ± 0.1 for the glut 4/HK component, and of 0.3 ± 0.06 for the glycogen synthesis component (PGM, UGPase and GS) was found, while the contribution of glycolysis was equal to 0.15 ± 0.02. These results are similar to the ones reported by us in this paper (0.61 for HK and 0.26 for the sum of PGM, UGPase and GS).

In agreement with these results is the proposal of Schafer and co-workers in muscle cells [38], stating that the control of the flux would be at the beginning of the pathway at the glucose transporter-HK level and that GS would allow, by means of its regulatory properties, the maintenance of constant metabolite concentrations. Thus, the glycogen synthesis pathway would accomplish the dual requirement of changing the flux while maintaining metabolite homeostasis.

The results presented in this work and others suggest that the role of hexokinase in controlling the flux in glycogen synthesis is not restricted to mammalian species (this work) nor to glycogen metabolism. It has been shown that changes in the activity of glucokinase in mouse are responsible for high metabolic control on glycolysis [39] and that hexokinase has an important control in the glycolytic flux in *Drosophila* [40].

As far as we know, flux control coefficients for PGM and UGPase are only available in plants. The control exerted by these enzymes on starch synthesis from sucrose in potato tubers was reviewed by Geigenberger et al. [41]. For cytosolic and plastidial PGM, flux control coefficients reported were 0.15 and 0.23, respectively. In the case of UGPase, the value reported was near 0.0. Although these findings cannot be directly extrapolated from plants to amphibia, the flux control coefficients for PGM are similar to the one determined in our study, reinforcing the idea that PGM has a low control in polysaccharide synthesis.

Finally, we would like to state that, beyond the results obtained in this paper, we are aware that glycogen synthesis constitutes a subset of the networks and networks interactions that occur in a whole cell or system (for a review see [42]. The contribution of different components of the network to regulation will vary, depending on the perturbation of the system. An in-depth understanding of the regulation of a metabolic process therefore requires an analysis of how it responds to all of the major perturbations to which it is typically exposed [41]. Nevertheless, network-based pathway analysis is beyond the scope of this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.037.

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