In vivo measurements of control coefficients for hexokinase and glucose-6-phosphate dehydrogenase in *Xenopus laevis* oocytes

Tito Ureta[a,*], Waldy Y. Fernández[b,1], Josep J. Centelles[b], Marta Cascante[b,2]

[a] *Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile*
[b] *Departament de Bioquímica y Biología Molecular, Facultat de Química, Universitat de Barcelona, Barcelona, Spain*

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Abstract Hexokinase and glucose-6-phosphate dehydrogenase activities were increased in *Xenopus laevis* oocytes by microinjection of commercial pure enzymes. The effect of increased fractional activities on glycogen synthesis or on the production of $^{14}CO_2$ (the oxidative portion of the pentose phosphate pathway) was investigated by microinjection of [1-^{14}C]glucose and measurements of the radioactivity in glycogen and CO_2. Control coefficients calculated from the data show that hexokinase plays an important role in the control of glycogen synthesis (control coefficient = 0.7) but its influence on the control of the pentose phosphate pathway is almost nil (control coefficient = −0.01). Glucose-6-phosphate dehydrogenase injections did not affect the production of $^{14}CO_2$ by the pentose phosphate pathway, indicating that other factors control the operation of this pathway. In addition, an almost null control of this enzyme on glycogen synthesis flux was observed.

Key words: Metabolic control analysis; Control coefficient; Hexokinase; Glucose-6-phosphate dehydrogenase; Glycogen synthesis; Pentose phosphate pathway; Frog oocyte

1. Introduction

In metabolic control analysis the control properties of a metabolic pathway are quantitatively expressed in terms of control coefficients, each defined as the fractional change in a systemic variable that results from an infinitesimal fractional change in the rate of an enzyme activity [1,2]. Control coefficients have been measured in different metabolic systems by indirect methods, e.g. from measurements of enzyme kinetic parameters and metabolite levels as first applied by Groen and coworkers [3,4] or by direct methods through the modification of the amount or activity of an enzyme [1].

Most measurements of control coefficients have been performed using titration with specific inhibitors [5–10] or titration with purified enzymes using tissue homogenates [11,12], as it is difficult to change the activity of an enzyme inside a cell. Manipulation at the genetic level, resulting in overexpression or deletion of an enzyme activity, is now possible and in principle is a powerful tool for changing an enzyme activity and computing the control coefficient, e.g. a high control coefficient has been reported for glucokinase in hepatocytes using adenovirus-mediated overexpression of the enzyme [13]. However, as summarized by Bailey [14], even in cases in which only one gene has been cloned into cells in an expression vector, a number of other proteins exhibit altered levels. Thus, several examples of two-dimensional electrophoresis and proteome analysis have been reported that clearly show that any change, including ones thought to be 'neutral', changes the relative levels of several, often many, individual proteins [14–18].

Amphibian oocytes may qualify as an ideal model system for in vivo measurements of control coefficients, as fractional changes of the rate of an enzyme can be accomplished by microinjection of the enzyme, ensuring that the necessary condition to measure a control coefficient is fulfilled: only one enzyme concentration is altered inside the cell. The fully grown oocyte of *Xenopus laevis* is a large cell, making manipulations easy and allowing injection of intermediates and larger biomolecules (enzymes, antibodies or mRNA). In frog oocytes, microinjected labelled glucose is incorporated into glycogen [19] and, to a minor extent, released as CO_2 through the pentose phosphate pathway only, since [6-^{14}C]glucose does not produce $^{14}CO_2$ [20–22].

In the research now described, several titers of hexokinase and glucose-6-phosphate dehydrogenase were microinjected into *Xenopus* oocytes, followed by labelled glucose microinjection and measurements of labelled glycogen and CO_2. From these experimental measurements we have computed the control coefficients and quantitatively assessed the potential of hexokinase and glucose-6-phosphate dehydrogenase to control glycogen synthesis and the oxidative arm of the pentose phosphate pathway fluxes in *Xenopus* oocytes and therefore to contribute to a greater understanding of the control of glucose metabolism in these cells. Also, we show that the amphibian oocyte is an ideal test tube for studies of control of metabolism and to test the feasibility of measuring control coefficients in vivo by efficiently perturbing the level of each enzyme independently.

2. Materials and methods

2.1. Materials

[1-^{14}C]Glucose was purchased from Amersham. Just before each experiment an aliquot containing the desired radioactivity was evaporated under N_2 to remove ethanol and resuspended on modified Barth's saline [23]. Hexokinase and glucose-6-phosphate dehydrogenase were from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

*Corresponding author. Fax: (56)-2-271 3891.
E-mail: tiureta@uchile.cl

[1] Pregraduate student of Universidad del Zulia, Maracaibo, Venezuela.
[2] Also corresponding author. Fax: (34)-93-4021219. E-mail: marta@sun.bq.ub.es

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2.2. Cells

Stage VI oocytes of *X. laevis* females were obtained by surgical excision under low temperature anesthesia and freed from membranes by blunt dissection with jeweller's forceps.

2.3. Preparation of oocyte extracts

One gram of oocytes (about 50 oocytes) was homogenized in 3 ml 50 mM Tris–HCl buffer pH 7.4 containing 0.3 mg/ml trypsin inhibitor. The homogenate was sonicated for 5 min and clarified by ultracentrifugation during 1 h at $100\,000 \times g$ and filtered through 0.2 μm filters. Hexokinase and glucose-6-phosphate dehydrogenase activities were measured in the extracts.

2.4. Measurement of enzyme activities

Glucose-6-phosphate dehydrogenase activity was measured as described by Tian et al. [24] using 2 mM glucose-6-phosphate and 0.5 mM $NADP^+$ in a 50 mM Tris–HCl buffer pH 7.6 at 37°C. The reduction of $NADP^+$, which is directly proportional to glucose-6-phosphate dehydrogenase activity, was followed by the increase in absorbance at 340 nm using a spectrophotometer (Shimadzu Cell Positioner CPS-260). Activity was expressed as units defined as μmol/min in the specified conditions.

Hexokinase activity was measured at 340 nm by a coupled assay with glucose-6-phosphate dehydrogenase as described by Ureta [25]. The assay mixture contained 10 mM glucose, 2 mM ATP-Mg, 1 mM $NADP^+$ and 1 mU of glucose-6-phosphate dehydrogenase in a 50 mM HEPES buffer pH 7.4.

2.5. Metabolic labelling

Oocytes were injected essentially as described by Ureta and Radojkovic [26] with 50 nl of Barth's saline solution alone or supplemented with a measured amount of either yeast hexokinase (type C-300, Sigma) or yeast glucose-6-phosphate dehydrogenase (type IX, Sigma). Enough enzyme was injected to increase the endogenous activity (measured in crude extracts as described above) by a factor of 1, 2, 3, 4, 5 or higher (up to 20 in some experiments). After about 60 min to allow for diffusion of the enzymes, the oocytes were reinjected with 50 nl of saline containing 6 nmol of [1-^{14}C]glucose (about 10 000 cpm/nmol) diluted in Barth's saline solution. Groups of four oocytes in the same experimental condition were then incubated at 22°C in 100 μl Barth's saline for 15 min. Incubation was performed in sealed tubes with a continuous flow of O_2. $^{14}CO_2$ was collected in vials containing 100 μl 0.3 M NaOH, 5% Triton X-100. Radioactivity in the vials was measured in a Wallac liquid scintillation spectrometer with 10 ml OptiPhase HiSafe (Wallac Scintillation Products).

Glucose incorporation into glycogen was measured by the procedure described by Chan and Exton [27]. Each oocyte was individually digested in 200 μl of 30% KOH at 100°C for 30 min. The whole incubation mixture was then applied to small squares (2×2 cm) of ET31 Whatman filter paper and washed once during 30 min in 66% (v/v) ethanol at −20°C, twice during 15 min in 66% (v/v) ethanol at room temperature, and briefly in acetone. Filters were dried in a microwave oven and the radioactivity measured in a scintillation counter using 0.5% (w/v) 2,5-diphenyloxazole in toluene as scintillant.

2.6. Estimation of flux control coefficients

Control coefficients are defined as:

$$C_e^J = \frac{(\partial J/J)}{(\partial e/e)} = \frac{\partial \log J}{\partial \log e} \quad (1)$$

where J is the flux of interest and e is the activity of the enzyme [28,29]. The flux control coefficients are defined for a reference steady-state flux that in general corresponds to the unperturbed system. They were estimated by two methods, either from the slope of log flux versus log enzyme activity, as in our experiments (see below) a linear dependence was obtained in the range of enzyme activities used, or by means of Small and Kacser's method [30] for large changes in enzyme activities. The flux control coefficient according to the latter method is computed as:

$$C_e^J = \frac{1-\left(\frac{J_i}{J_f}\right)}{\left(\frac{e_f}{e_i}-1\right)\Big/\left(\frac{e_f}{e_i}\right)} \quad (2)$$

where J_i and J_f are the initial and final metabolic flux respectively and e_i and e_f are the final and initial enzyme activities and C_e^J is the flux control coefficient at the initial steady state. J_i and e_i were measured according to the methods described above under basal conditions and J_f and e_f were measured after microinjecting the oocyte with an excess of the appropriate enzyme. As the control coefficient is a dimensionless measure, the enzyme activities may be expressed using arbitrary units, and in our case they are expressed as times the endogenous activities.

3. Results

3.1. Steady-state attainment of glycogen and CO_2 formation fluxes from glucose

Measurement of flux control coefficient with respect to any enzyme by any method requires that the basal flux of the system be at steady state. In order to determine the time interval in which this condition is fulfilled, time courses of glycogen synthesis and CO_2 formation were measured in oocytes microinjected with [1-^{14}C]glucose. Incorporation of label into glycogen and CO_2 was measured at different times from 0 to 30 min. The time course of glycogen and CO_2 formation was linear between 0 and 30 min (Fig. 1). Accordingly, all measurements were performed at 15 min after glucose injection.

3.2. Endogenous levels of hexokinase and glucose-6-phosphate dehydrogenase activities

The endogenous activities of oocyte hexokinase and glucose-6-phosphate dehydrogenase were 80 ± 7 μU/oocyte for hexokinase and 160 ± 14 μU/oocyte for glucose-6-phosphate dehydrogenase. The resulting values were used to calculate the amounts of hexokinase or glucose-6-phosphate dehydrogenase to be microinjected at the levels indicated below.

3.3. Control of hexokinase on glycogen synthesis and CO_2 production fluxes

To determine the relationship between glycogenic flux and hexokinase activity, hexokinase was microinjected in sufficient amounts to increase the enzyme activity from the endogenous value inside the oocytes by a factor in the range 2–5 (Fig. 2, left). A control coefficient value of 0.7 for hexokinase on glycogen flux was determined from the slope of log flux versus log hexokinase activity (inset in Fig. 2, left), which describes the sensitivity of glycogen synthesis to hexokinase. The control coefficient for the experiments in Fig. 2, left, was also estimated by the method of Small and Kacser [30] for large changes according to Eq. 2 for the different hexokinase titers; values between 0.7 and 0.8 were obtained for any of the titers considered as the final enzyme activity, showing the agreement between these two methods. To minimize possible errors in estimating the endogenous hexokinase activity, microinjection of 20 times the endogenous level was performed and the control coefficient was estimated by the method of large changes (Eq. 2). A value of 0.7 for the control coefficient was obtained showing the validity of the in vivo method also for a single large change in enzyme concentration. The value of around 0.7–0.8 obtained for the control coefficient is high, and indicates an important role of hexokinase in controlling glycogenic flux in oocytes. It is often assumed that the log flux versus log enzyme dependence is linear only for small changes in enzyme activity [1,28]. However, the data in the inset of Fig. 2, left, show a linear dependence on log flux versus log of

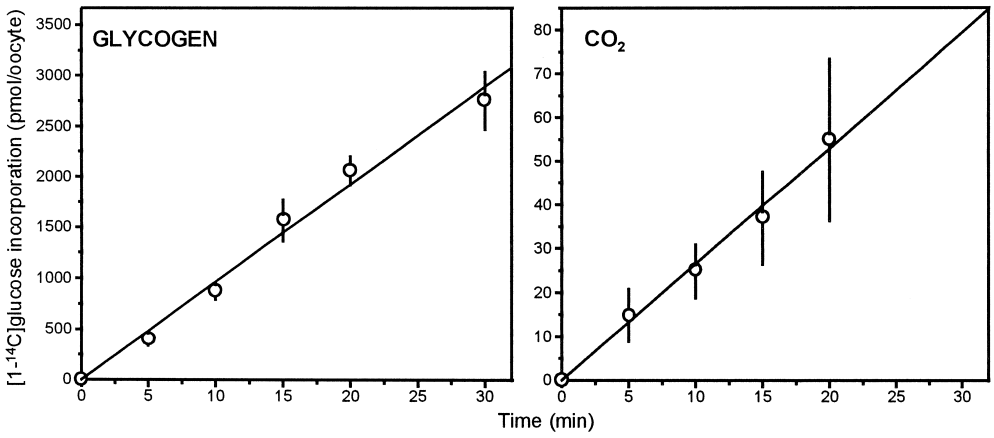

Fig. 1. Time course of glycogen and CO_2 labelling by oocytes microinjected with [1-^{14}C]glucose. Oocytes were microinjected with 50 nl of Barth's saline solution containing 6 nmol [1-^{14}C]glucose (~10 000 cpm/nmol). Groups of four oocytes in duplicated flasks were incubated during the indicated times. At the end of the incubation period, glycogen was isolated and its radioactivity measured as described in Section 2. $^{14}CO_2$ was measured at the end of the incubation period. Left: Incorporation of [1-^{14}C]glucose into glycogen. Right: Production of $^{14}CO_2$ from the same oocytes as in the left panel. Results are expressed as pmol of glucose equivalents in glycogen and CO_2 and presented as means ± S.E.M. of 6–8 observations.

hexokinase activity over a wide range of hexokinase levels. Thus, in this case the control coefficient of hexokinase on glycogenic flux measured in vivo is a good predictor of the effect of large changes in hexokinase activity. The linearity is only lost at concentrations of hexokinase activity higher than 10 times the endogenous level and the hyperbolic dependence of flux with regard to enzyme activity becomes apparent (data not shown).

The same experiments were used to determine the relation between the oxidative pentose phosphate pathway flux (measured as $^{14}CO_2$ formation from [1-^{14}C]glucose) and hexokinase activity (Fig. 2, right). CO_2 flux was not affected by increasing levels of hexokinase since a control coefficient value of −0.01 was obtained (see inset in Fig. 2, right). Thus, even though the conversion of glucose into glucose 6-phosphate is the first step of the oxidative pentose phosphate pathway it appears that glucose phosphorylation does not control the flux of the shunt.

3.4. Control of glucose-6-phosphate dehydrogenase on glycogen synthesis and CO_2 production fluxes

Similar experiments were performed to determine the sensitivity of glycogen synthesis and oxidative pentose phosphate pathway fluxes to increase of glucose-6-phosphate dehydrogenase activity in vivo.

To determine the relationship between glycogen flux and glucose-6-phosphate dehydrogenase activity we microinjected enough dehydrogenase to increase the endogenous values of enzyme activity inside the oocytes by factors of 2–6 (Fig. 3, left). The control coefficient value was practically zero (0.02) for glucose-6-phosphate dehydrogenase on glycogen flux (inset in Fig. 3, left) which describes the practically null sensitiv-

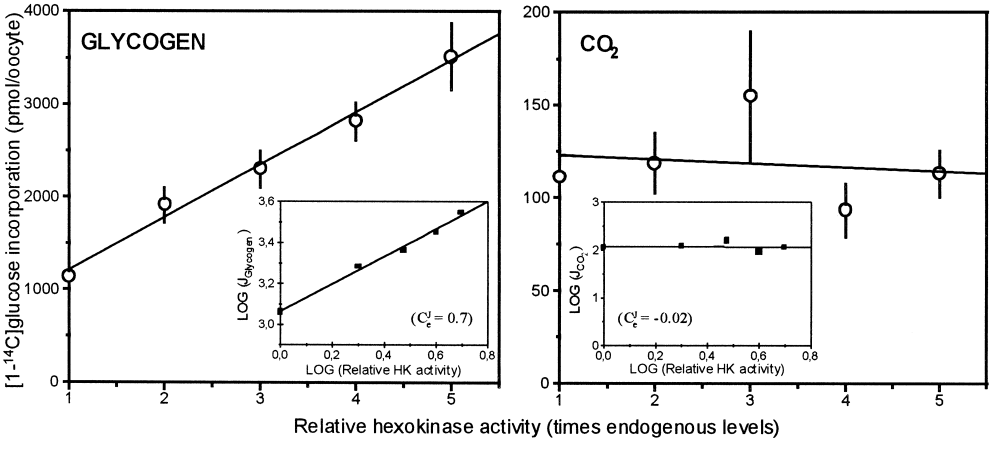

Fig. 2. Effect of hexokinase microinjection on [1-^{14}C]glucose metabolism by frog oocytes. Oocytes were microinjected with 50 nl of Barth's solution containing the desired amounts of enzyme such as to increase the hexokinase activity inside the cells by 2-, 3-, 4- or 5-fold. After incubation during 1 h, each oocyte was reinjected with 50 nl of saline containing 6 nmol [1-^{14}C]glucose (~10 000 cpm/nmol) and further incubated for 15 min. At the end of the second incubation period, radioactivity in glycogen and CO_2 was measured as described in Section 2. Left: Incorporation of [1-^{14}C]glucose into glycogen. Right: Production of $^{14}CO_2$ from the same oocytes as in the left panel. Results are expressed as pmol of glucose equivalents in glycogen and CO_2 and presented as means ± S.E.M. of 6–8 observations. Insets show the same data expressed as log–log graphs. Values in parentheses indicate the control coefficients determined as the slope of the linear fitting.

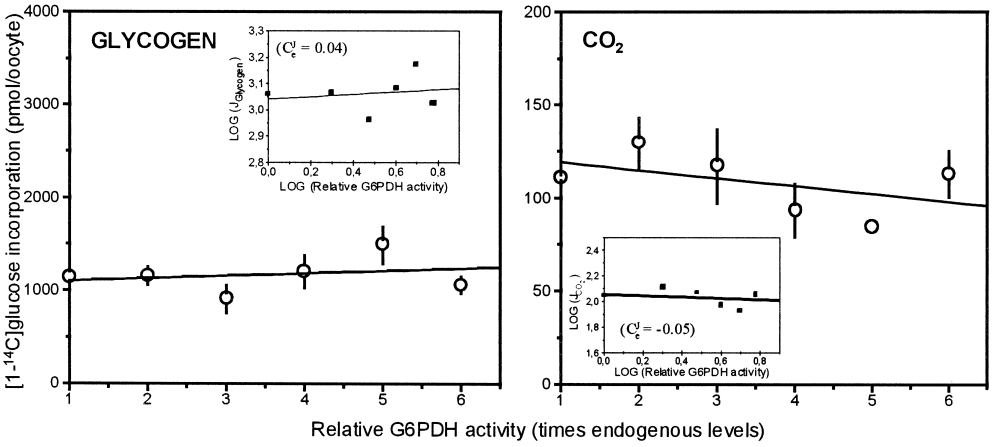

Fig. 3. Effect of glucose-6-phosphate dehydrogenase (G6PDH) microinjection on [1-^{14}C]glucose metabolism by frog oocytes. Oocytes were microinjected with 50 nl of Barth's saline solution containing the desired amounts of enzyme such as to increase the G6PDH activity inside the cells by 2-, 3-, 4- 5- or 6-fold. After incubation during 1 h the same treatment as in Fig. 2 was performed. Left: Incorporation of [1-^{14}C]glucose into glycogen. Right: Production of $^{14}CO_2$ from the same oocytes as in the left panel. Results are expressed as pmol of glucose equivalents in glycogen and CO_2 and presented as means ± S.E.M. of 6–8 observations. Insets show the same data expressed as log–log graphs. Values in parentheses indicate the control coefficients determined as the slope of the linear fitting.

ity of glycogen synthesis to the activity of the dehydrogenase.

The relationship between the oxidative pentose phosphate pathway flux and the glucose-6-phosphate dehydrogenase activity was determined by measuring $^{14}CO_2$ formation from [1-^{14}C]glucose (Fig. 3, right). A value very close to zero (−0.05) for the control coefficient of glucose-6-phosphate dehydrogenase on the oxidative pentose phosphate pathway flux (inset in Fig. 3, right) was obtained, which describes the null sensitivity of the shunt to glucose-6-phosphate dehydrogenase.

It should be noted that even though glucose-6-phosphate dehydrogenase is the first enzyme of the oxidative pentose phosphate pathway and is considered in practice to be an irreversible step, it does not control the flux of the pathway at all. In a linear chain such as the oxidative pentose phosphate pathway, the enzymes following an irreversible step must have a control coefficient value of zero if there is no feedback inhibition [1]. Thus, as neither hexokinase nor glucose-6-phosphate dehydrogenase exerts any control on the pathway flux, the following enzymes will have control coefficients equal to zero. Therefore, we conclude that in oocytes the oxidative pentose phosphate pathway is not controlled at the level of enzyme activities.

4. Discussion

The results described in this paper confirm that microinjection of enzymes in oocytes is a very powerful tool for in vivo titration of enzymes and show that it permits control coefficients to be measured in vivo with confidence that only one enzyme level has been changed. Overexpression of enzymes by genetic manipulation does not ensure that the expression of other proteins has not also internally changed to compensate for the perturbation [14].

Surprisingly, the control coefficient of hexokinase on glycogen synthesis flux is constant in a wide range of concentrations (from one to five times the endogenous levels) showing that control coefficients can in this case have a predictive value on the behavior of the system as a response to large changes in hexokinase levels. Thus, although theoreticians have often pointed out that the predictive value of control coefficients is limited only to infinitesimal changes around the operational steady state [1,2], it has been shown in this in vivo example that control coefficients can be constant over a large range of enzyme levels.

The lack of effect of microinjected glucose-6-phosphate dehydrogenase on the flux of the oxidative arm of the pentose phosphate pathway was not unexpected. The activity of the enzyme in oocytes of the frog *Caudiverbera* has a potential V_{max} of about 12 600 pmol/min/oocyte but in vivo measurements at saturating levels of labelled glucose or glucose 6-phosphate indicate a maximal production of $^{14}CO_2$ of at most 5 pmol/min/oocyte [22]. Therefore, the low in vivo activity of the pentose phosphate pathway in amphibian oocytes is not due to a low activity of the dehydrogenases but to other factors. Preller et al. [22] conclude that the unfavorable $NADP^+$/NADPH ratio effectively limits the operation of the pentose phosphate pathway as direct microinjection of $NADP^+$ raises 10-fold the production of $^{14}CO_2$ from labelled glucose.

The results presented in this paper on the lack of effect on oxidative pentose phosphate pathway of both hexokinase and glucose-6-phosphate dehydrogenase together with previous results of Preller et al. [22] in *Caudiverbera* are further evidence of the potential for physiological levels of NADPH demand to exert control over oxidative pentose phosphate pathway flux. An alternative explanation for the lack of effect of hexokinase on the oxidative pentose phosphate pathway flux is that the glucose 6-phosphate pool produced by the exogenous hexokinase does not mix with that available for the pentose phosphate pathway.

It should be stressed that hexokinase and glucose-6-phosphate dehydrogenase are enzymes with high homology throughout eukaryotes. Yeast hexokinase and glucose-6-phosphate dehydrogenase were used instead of *X. laevis* enzymes because it was easier to obtain the proper amounts to carry out microinjection studies, particularly in the case of hexokinase. The use of commercial enzymes from other sources is acceptable since our aim was to modify the hexokinase *activity* inside the oocyte which can be accomplished by injection of suitable available enzymes. The fact that a clear-cut effect

was indeed observed is consistent with the conclusion that the injected yeast hexokinase was able to use the metabolic set-up of the oocyte (i.e. endogenous ATP and the supplied glucose) and that the pool of glucose 6-phosphate thereby produced was directed to glycogen, the final product of the pathway. In fact, the main point to measure a control coefficient is that the activity of the enzyme inside the cell increases whereas other enzyme activities are not altered.

The application of control analysis to studies on metabolic regulation challenged the idea of the 'rate-limiting step' at the beginning of a metabolic pathway and has shown that it is not a general rule [31]. Our results show that the oxidative pentose phosphate pathway is another exception to this rule.

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