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GLYCOGEN SYNTHASE: A NEW ACTIVITY RATIO ASSAY EXPRESSING A HIGH SENSITIVITY TO THE PHOSPHORYLATION STATE

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1. Introduction

Since its introduction [1] the –glucose 6-phosphate/+ glucose 6-phosphate (–G6P/+G6P) activity ratio assay for glycogen synthase has been a very useful tool in the study of the activation state of this enzyme. The idea of glycogen synthase in two forms (one active in the absence of added G6P (I form), the other requiring it for activity (D form)) evolved from the first experiments in which the –G6P/+G6P assay was employed [1]. Shortly afterwards changes in the –G6P/+G6P activity ratio were correlated with phosphorylation and dephosphorylation of the enzyme [2].

At concentrations of metabolites close to their physiological levels it was shown [3] that the D form would be inactive whereas the I form would be active. Therefore conversion between D and I forms (i.e., changes in the –G6P/+G6P activity ratio) would correspond to changes in the 'in vivo' activity. Nevertheless, in physiological experiments rather small changes in the –G6P/+G6P activity ratio had been observed in response to the administration of hormones. These modest changes in the –G6P/+G6P activity ratio were rather hard to reconcile with an on–off control of glycogen synthase activity (reviewed [4]).

The enzyme has been shown to have a multiple phosphorylated subunit [5–11] and that phosphorylation produces pronounced effects on the apparent affinity of the enzyme for its substrate UDP-glucose and the activator glucose 6-phosphate [7,11–13]. In [13] we have demonstrated that when glycogen synthase is phosphorylated by glycogen synthase kinase-1, large changes in the kinetic properties are only scarcely reflected in the standard –G6P/+G6P activity ratio. It is worth noting that the magnitude of the changes in the –G6P/+G6P activity ratio resulting from the introduction of the third and fourth phosphates is similar to that of those observed in physiological experiments. Furthermore, these changes occur precisely in the same range of –G6P/+G6P activity ratio (from 0.20–0.10) as those provoked by epinephrine and glucagon in 'in vivo' experiments.

A remarkable consequence of studies [5–13] is the inadequacy of the I and D species model of synthase as defined by the –G6P/+G6P activity ratio, because this ratio cannot be interpreted as the measure of the mole fraction of the I (dephosphorylated) form. Moreover, the changes in the –G6P/+G6P activity ratio are not necessarily proportional to the changes in the capacity for glycogen synthesis in the cell. As pointed out [4] relatively small changes in the –G6P/+G6P activity ratio observed in response to a variety of stimuli could account for very large alterations in the rate of glycogen synthesis.

Here we propose a new simple type of activity ratio assay based on the measurement of glycogen synthase activity at two glucose 6-phosphate concentrations, one low and one high, using a low concentration of UDP-glucose. This low G6P/high G6P assay is very sensitive to changes in the enzymic properties caused by phosphorylation and thus would likely provide a more proper definition of the 'in vivo' activity state of the enzyme.

2. Methods

The procedures for obtaining glycogen synthase and glycogen synthase kinase-1 and the conditions for phosphorylation were as in [13].

2.1. *Glycogen synthase assay*

Glycogen synthase activity was measured at 30°C by the filter paper method [14] based on the incorporation of [U-^{14}C]glucose from UDP-[U-^{14}C]glucose into glycogen.

For the standard –G6P/+G6P activity ratio, assay samples of the enzyme (30 μl) were added to 60 μl solution containing 50 mM Tris–HCl (pH 7.8); 20 mM EDTA; 25 mM KF; 7 mg glycogen/ml; 6.7 mM UDP-[U-^{14}C]glucose (spec. radioact. 100 cpm/nmol) and in the +G6P assay 10 mM glucose 6-phosphate. In the low G6P/high G6P assay UDP-[U-^{14}C]glucose was present at 200 μM final conc. (spec. radioact. 7000 cpm/nmol). The reaction mixture also contained 50 mM Tris–HCl (pH 7.8); 12.5 mM EDTA; 7 mg/ml rabbit liver glycogen and in the 'high' G6P assay 10 mM glucose 6-phosphate. The concentration of G6P in the 'low' G6P assay varied as indicated in the figure legends. For standard conditions 0.25 mM may be recommended. In all cases care was taken to maintain the consumption of substrate at <10%. If the incubation time is equally set for both types of assay, samples 5–10-times more diluted must be used in the low G6P/high G6P assay than in the standard –G6P/+G6P assay.

2.2. *Hepatocyte isolation and incubation*

Male Sprague-Dawley rats (220–250 g) were deprived of food 24 h prior to hepatocyte isolation as in [15]. Cells were finally resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) pre-gassed with O_2/CO_2 19:1. Aliquots (3.5 ml, 9 × 10^6 cell/ml) were poured into stoppered 30 ml vials and incubated at 37°C with shaking (100 strokes/min). Hepatocytes were allowed to equilibrate with the medium for 30 min prior to glucagon addition. At the end of the incubation the contents of each vial were centrifuged (1000 × *g*, 20 s) and the cell pellet was immediately homogenized with 230 μl ice-cold 15 mM KF/15 mM EDTA (pH 7.0). The cell homogenates were centrifuged for 15 min at 6000 × *g* and the supernatents assayed for glycogen synthase activity.

3. Results and discussion

It has seemed appropriate to assume that the –G6P/+G6P activity ratio is a function of the sensitivity of the different forms of glycogen synthase to glucose 6-phosphate. However, this is not so. Changes in the –G6P/+G6P activity ratio are due to the increase in the $S_{0.5}$ value for UDP-glucose produced by phosphorylation of the glycogen synthase subunit. In effect, this becomes evident upon observing that in the presence of 6.6 mM G6P (the concentration present in the standard +G6P assay), values of $S_{0.5}$ for UDP-glucose are reduced to very low levels even for the more phosphorylated forms (table 1). Therefore, in the +G6P assay the concentration of UDP-glucose (4.4 mM if the concentrations indicated in [14] are used) becomes almost saturating and values close to V_{max} are observed. On the contrary, in the –G6P assay, as the $S_{0.5}$ for UDP-glucose increased with the phosphate content, the UDP-glucose concentration present in the assay becomes less and less saturating and the rate of the reaction falls well below the V_{max}. Therefore, the largest changes in the –G6P/+G6P activity ratio will be observed when the $S_{0.5}$ values are fluctuating around the concentration of UDP-glucose in the assay mixture. On the contrary, larger changes in $S_{0.5}$ at values far greater than the concentration of UDP-glucose in the assay mixture will result in only very small changes in the –G6P/+G6P activity ratio.

Table 1
Values of $S_{0.5}$ for glycogen synthase as a function of the degree of phosphorylation

^{32}P/85 000 (mol/subunit)	$S_{0.5}$ (mM) for UDP-glucose	
	Without G6P	6.6 mM G6P
0	1.5	0.07
0.6	2.2	0.16
1.6	6.5	0.25
2.2	10.0	0.50
4.3	200	0.54

$S_{0.5}$ was determined as in [13] in the absence and in the presence of 6.6 mM G6P

In a very simple manner the –G6P/+G6P activity ratio can be calculated from the $S_{0.5}$ values by applying the Michaelis-Menten equation:

$$-\text{G6P}/+\text{G6P activity ratio} = V/V_{max} = [S]/[S]+S_{0.5}$$
$$= 4.4/4.4+S_{0.5}$$

Although the Michaelis-Menten equation represents an oversimplification, as it does not take into account the negative cooperativity observed in the kinetics of glycogen synthase for UDP-glucose [7], the values observed are in keeping with the values predicted from this equation (fig.1). This helps to make clear the concept indicated above, i.e., that changes in –G6P/+G6P activity ratio are in fact a function of the $S_{0.5}$ for UDP-glucose. It would be theoretically possible to calculate $S_{0.5}$ from the –G6P/+G6P activity ratio but this would be equivalent to calculating the $S_{0.5}$ by Lineweaver-Burk plots obtained with only two measurements of the reaction rate, one of them at saturating concentrations of substrate.

In order to make the –G6P/+G6P assay more sensitive to changes in $S_{0.5}$, such as those produced by the introduction of the fourth phosphate by glycogen synthase kinase-1, a much higher concentration of UDP-glucose in the assay mixture (~100 mM)

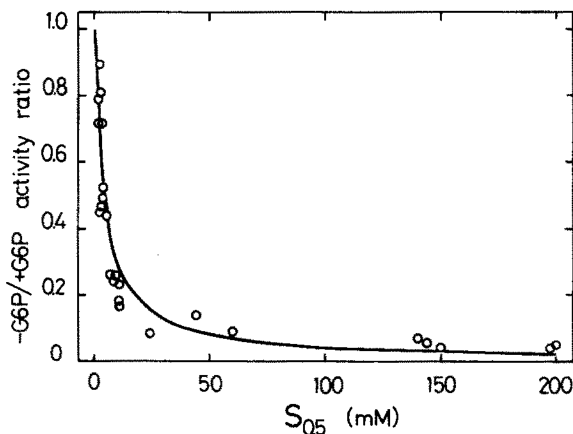

Fig.1. Relationship between the standard –G6P/+G6P activity ratio (at 4.4 mM UDP-glucose) and the $S_{0.5}$ for UDP-glucose. Each point corresponds to a different sample. The curve corresponds to the equation:

$$-\text{G6P}/+\text{G6P activity ratio} = 4.4/4.4+S_{0.5}$$

would be necessary. Of course, it is not possible to use such a concentration for obvious practical considerations. As a result, it is difficult to modify the –G6P/+G6P assay in order to make it more sensitive to extensive phosphorylation of the glycogen synthase subunit.

However, a sensitive assay can be easily devised on the basis of two facts:

(i) $M_{0.5}$ for G6P also changes with phosphorylation;

(ii) the concentration of G6P in the assay can be varied over a wide range of values without many of the inconveniences involved in modifying the level of the substrate.

Using these principles, we have developed the low G6P/high G6P activity ratio assay. It consists of the measurement of glycogen synthase activity at two G6P concentrations, one low and one high. The concentration of UDP-glucose has been fixed at a low level (0.2 mM) providing maximum sensitivity of the enzyme to the activator.

In the 'high' assay the concentration of G6P is high enough to be practically saturating for all samples, regardless of their degree of phosphorylation. Thus, the activity measured in the 'high' assay should be almost constant for one sample when phosphorylated to different degrees. This is a very convenient feature in an assay intended to measure interconversions between differently phosphorylated species. On the other hand, by fixing the concentration of the G6P in the 'low' assay at a level almost saturating for the non-phosphorylated forms, but very low when compared with the $M_{0.5}$ of the most phosphorylated species, maximum sensitivity would be obtained. The 'low' level may then be adjusted to obtain maximum sensitivity for any given system if the changes in $M_{0.5}$ for G6P with phosphorylation are known. However, G6P at ~100–250 μM may be satisfactory for most systems.

As shown in fig.2, the low G6P/high G6P assay is more sensitive to changes in enzymic properties caused by phosphorylation with glycogen synthase kinase-1 than the –G6P/+G6P assay. The introduction of the third and fourth phosphates by glycogen synthase kinase-1, which results in very small changes in the –G6P/+G6P activity ratio, provoked larger variations in the low G6P/high G6P assay.

It is worth noting that if the 'low' assay is performed at higher concentrations of G6P, the system

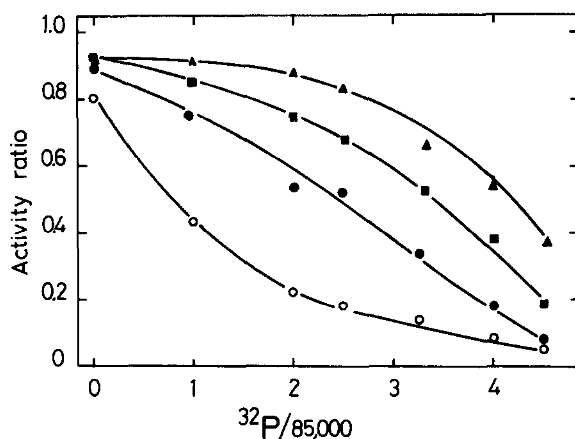

Fig.2. Changes in the glycogen synthase standard –G6P/+G6P (○) and low G6P/high G6P (●, ▲, ■) activity ratios with phosphate incorporated. The concentrations of G6P at the different low/high ratios were: (●) 0.1 mM/10 mM; (■) 0.25 mM/10 mM; (▲) 0.5 mM/10 mM.

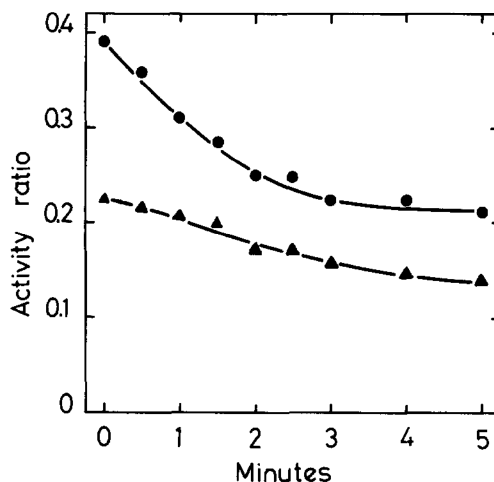

Fig.3. Changes in the standard –G6P/+G6P (▲) and low G6P/high G6P (●) activity ratios of glycogen synthase in rat hepatocytes incubated with 10^{-7} M glucagon. The concentrations of G6P at the low/high ratio were 0.25 mM and 10 mM, respectively.

becomes less sensitive to the introduction of the first phosphates (fig.2) since, in that case, the concentration of G6P in the 'low' assay is almost equally saturating for the non-phosphorylated as far as the species containing 1 or 2 mol phosphate/subunit.

The low G6P/high G6P assay is not only useful in the study of phosphorylation 'in vitro'. Figure 3 shows that the changes in rat hepatocyte glycogen synthase produced by incubation with glucagon are more outstanding in the low G6P/high G6P assay than in the –G6P/+G6P assay. In addition, the low G6P/high G6P activity ratio varies over a wider range of vaues (0.39–0.21) than the –G6P/+G6P activity ratio (0.22–0.14). The changes in the –G6P/+G6P activity ratio produced 'in vivo' by epinephrine and glucagon usually fell within 0.25–0.07. This is precisely the region of the curve that appeared less sensitive to increasing phosphorylation. However, in the low G6P/high G6P assay the changes introduced by physiological effectors are magnified as a steeper slope in the activity ratio versus phosphorylation curve, precisely in the range of phosphorylation where the greatest changes in enzymic parameters occur [13]. Furthermore, the 'low' assay is performed at concentrations of substrate (UDP-glucose) and activator (G6P) close to their physiological values. It is then tempting to speculate that the changes observed in the low G6P/high G6P assay reflect better than those calculated from the –G6P/+G6P assay the variations in the capacity for glycogen synthesis produced by phosphorylation of the enzyme 'in vivo', offering a closer approximation to the actual state of activation of glycogen synthase in the cell.

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References

[1] Villar-Palasí, C. and Larner, J. (1960) Biochim. Biophys. Acta. 39, 171–173.
[2] Friedman, D. L. and Larner, J. (1963) Biochemistry 2, 669–675.
[3] Piras, R., Rothman, L. B. and Cabib, E. (1968) Biochemistry 7, 56–66.
[4] Roach, P. J. and Larner, J. (1977) Mol. Cell. Biochem. 15, 179–200.
[5] Smith, C. H., Brown, N. E. and Larner, J. (1971) Biochim. Biophys. Acta 242, 81–88.

[6] Takeda, Y. and Larner, J. (1975) J. Biol. Chem. 250, 8951–8956.
[7] Roach, P. J., Takeda, Y. and Larner, J. (1976) J. Biol. Chem. 251, 1913–1919.
[8] Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) Eur. J. Biochem. 68, 31–44.
[9] Itarte, E., Robinson, J. C. and Huang, K.-P. (1977) J. Biol. Chem. 252, 1231–1234.
[10] Soderling, T. R., Jett, M. T., Hutson, N. J. and Khatra, B. S. (1977) J. Biol. Chem. 252, 7517–7524.
[11] Brown, J. H., Thompson, B. and Mayer, S. E. (1977) Biochemistry 16, 5501–5508.
[12] Roach, P. J. and Larner, J. (1976) J. Biol. Chem. 251, 1920–1925.
[13] Salavert, A., Itarte, E., Massagué, J. and Guinovart, J. J. (1979) FEBS Lett. 106, 279–283.
[14] Thomas, J. A., Schlender, K. K. and Larner, J. (1968) Anal. Biochem. 25, 486–499.
[15] Massagué, J. and Guinovart, J. J. (1978) Biochim. Biophys. Acta 543, 269–272.