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Enzymatic phosphorylation of muscle glycogen synthase: A mechanism for maintenance of metabolic homeostasis

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ABSTRACT **We recently analyzed experimental studies of mammalian muscle glycogen synthesis using metabolic control analysis and concluded that glycogen synthase (GSase) does not control the glycogenic flux but rather adapts to the flux which is controlled by the activity of the proximal glucose transport and hexokinase steps. This model did not provide a role for the well established relationship between GSase fractional activity, determined by covalent phosphorylation, and the rate of glycogen synthesis. Here we propose that the phosphorylation of GSase, which alters the sensitivity to allosteric activation by glucose 6-phosphate (G6P), is a mechanism for controlling the concentration of G6P instead of controlling the flux. When the muscle cell is exposed to conditions which favor glycogen synthesis such as high plasma insulin and glucose concentrations the fractional activity of GSase is increased in coordination with increases in the activity of glucose transport and hexokinase. This increase in GSase fractional activity helps to maintain G6P homeostasis by reducing the G6P concentration required to activate GSase allosterically to match the flux determined by the proximal reactions. This role for covalent phosphorylation also provides a novel solution to the Kacser and Acarenza paradigm which requires coordinated activity changes of the enzymes proximal and distal to a shared intermediate, to avoid unwanted flux changes.**

The control of metabolism is entering a flourishing period of understanding and quantitation. The work of Kacser and colleagues (1, 2) has made available a quantitative method for characterizing the control of flux. This method, called metabolic control analysis (MCA), gives a quantitative measure of flux control and defines experimental conditions needed for interpretable results. It defines a flux control coefficient for each enzyme in a pathway as the fractional change in flux over the fractional change in enzyme concentration. These changes must be small and are to be measured while all other activities of the pathway remain constant. A limitation of MCA as originally formulated is that it dealt only with perturbations about an established flux. In many situations the cell must make large alterations in flux in response to changes in the environment. Several mechanisms may alter the flux including allosteric regulation, covalent modification of enzyme activity, modification of enzyme activity by cofactor binding or translocation, and genetic induction. Based on MCA, the most effective way for the cell to alter flux is to modify the activity of the enzymes with the highest flux control coefficients. However, large changes in enzyme activity may result in redistribution of flux control coefficients so as to act against the intended change in flux.

Recently Kacser and Acarenza (3) addressed the problem of how a cell may make large, stable changes in flux. They reviewed experimental attempts to increase biosynthetic flux in cells through alteration in the activity of one or more enzymes in a pathway which, in most cases, either failed to increase the flux or proved deleterious to the survival of the cell. They pointed out that the failure to increase the flux could be explained by MCA, which indicates that the majority of enzymes make small contributions to flux control while increasing the activity of a particular enzyme with a high control coefficient might redistribute control. They further proposed that the deleterious nature of some of these activity alterations may be due to disturbance of the concentration of intermediates that were shared with other biosynthetic pathways. From theoretical considerations they derived a universal method of altering flux without affecting concentrations of shared intermediates. This would involve altering the intrinsic activities of all of the enzymes proximal and distal to the shared intermediate by the same fraction. The result of these alterations would be a smooth increase in flux in the targeted pathway without changing the concentration of intermediates shared with other biosynthetic pathways. In addition the method would avoid redistribution of control in the targeted pathway.

Our *in vivo* NMR studies of muscle glycogen synthesis have led us to study the control of this flux (4) by MCA. Glycogen synthase (GSase) is an extensively studied example of an enzyme in the glycogenic pathway that is regulated by covalent phosphorylation. Under conditions favoring muscle glycogen synthesis, such as the high plasma insulin and glucose concentrations following a meal, GSase is activated by eight or more signaling pathways containing kinases and phosphatases (5). The activity of GSase depends upon the number of phosphorylated sites on the enzyme and on allosteric regulation by glucose 6-phosphate (G6P). The two forms of GSase measured in the standard *in vitro* assay are the active G6P independent (I) form and the less active G6P dependent (D) form. The I form is active at physiological concentrations of G6P while the D form requires saturating concentrations of G6P to exhibit activity (6, 7). Removal of phosphates from the enzyme increases the percentage in the I form. The enzymatic activity is often described by the ratio I/I + D (%I), which is the relative velocity of the enzyme under conditions of low and saturating G6P. Both forms are inhibited by *in vivo* concentrations of ATP, an inhibition allosterically lifted by G6P (6).

Since the fraction of GSase in the active form is found to increase with increasing rates of glycogen synthesis it has been generally believed that GSase activity determines the flux through the glycogen synthesis pathway (8). However, we recently reanalyzed experimental studies of muscle glycogen synthesis regulation using MCA and concluded that GSase does not control the glycogenic flux but rather adapts to the flux that is controlled by the activity of the proximal glucose transport and hexokinase steps (GT/Hk) (4). A limitation of this model is that it did not provide a role for the dependence of GSase velocity upon covalent phosphorylation, because allosteric control by G6P alone was capable of matching the

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Abbreviations: MCS, metabolic control analysis; GSase, glycogen synthase; GT/Hk, glucose transport/helokinase; %I, enzyme activity ratio; G6P, glucose 6-phosphate.

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flux. We propose that the correspondence between GSase %I and glycogen synthesis rate may be reconciled with proximal flux control by viewing the regulation of GSase phosphorylation as a mechanism for controlling the concentration of G6P instead of controlling the flux. By being a shared intermediate in several pathways including glycolysis and the pentose phosphate shunt the concentration of G6P must be held near constant to allow independent control of the glycogen synthesis flux. In this paper, we show how covalent phosphorylation of GSase fulfills some of the requirements of Kascer and Acarenza (3) without requiring activity changes in all enzymes of the pathway.

A Model for Coordinate Regulation of the Muscle Glycogen Synthesis Pathway. Our previous model was based upon data showing that the muscle glycogen synthesis flux is determined by the activity of GT/Hk steps of the pathway (4). We suggested that the rate of GSase is forced to match the activity of the proximal steps by feed forward to G6P that allosterically regulates GSase activity. Previously Roach and Larner (9) discussed how allostery and phosphorylation reflect local and systemic controls of the flux. In this paper we consider the roles of both allostery and covalent phosphorylation in buffering changes in G6P concentration. In addition to the feed forward allosteric regulation by G6P on GSase activity there is feed back of GSase activity, modulated by phosphorylation, upon the concentration of G6P. In this way covalent phosphorylation of GSase enables the muscle glycogen synthesis pathway to approximate Kacser and Acarenza's criteria for a universal system for flux control as shown in Fig. 1. When the rate of muscle glycogen synthesis increases such as under conditions of high plasma insulin and glucose concentrations, the coordinated regulation of enzymatic activities in the pathway J_1, proximal to G6P, is achieved by changes in the activity of glucose transport and hexokinase. These enzymes in the proximal portion of the pathway exert the majority of flux control (4) and are activated by an insulin mediated signaling pathway (5, 10). If the activity of GSase remained constant the G6P concentration would rise until GSase velocity matched the transporter flux. However the concentration of the shared intermediate, G6P, is buffered by coordinated regulation of the activity of GSase distal to G6P (J_3). The removal of phosphates from GSase increases the sensitivity of the enzyme to allosteric regulation by G6P so that an increase in glycogen synthesis flux, determined by the proximal enzymes, is matched with a smaller increase in G6P concentration. Regulation of the phosphorylation of GSase may occur either by direct signaling from external effectors such as insulin, cAMP, Ca^{2+} etc operating through numerous kinases and phosphatases (5) or as discussed below by feed forward regulation from the G6P concentration (11). Other enzymes in the pathway are present at very high activity and do not require modification to maintain homeostasis and proximal flux control.

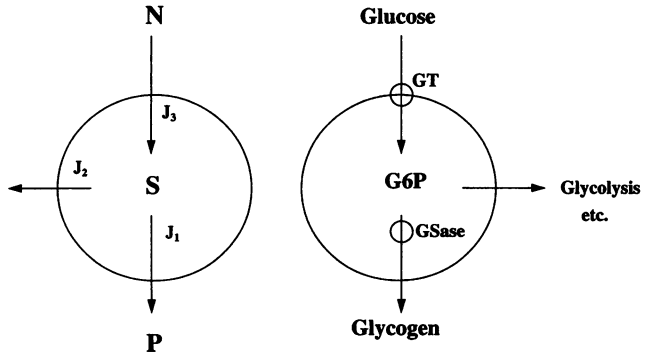

FIG. 1. Model of coordinated control proposed by Kacser and Acarenza (3) to accommodate changes in flux from N to the product (P) while maintaining the concentration of the shared intermediate (S) constant. In this way the branching flux J_2 will be independent of J_3. The analogy with the glycogenic pathway is shown where the shared intermediate is G6P.

Quantitative Predictions of the Model. To fulfill the criteria of Kacser and Acarenza when the flux of the glycogen synthesis pathway is changed both the concentration of G6P and the flux control coefficients of enzymes in the pathway must remain constant. As discussed by Fell and Thomas (12) and Srere (13) coordinated increase of enzyme activity by genetic induction can fulfill these criteria by increasing the total activity of all of the enzymes in the pathway by the same fraction. Whether covalent phosphorylation of GSase meets this criterion as proposed earlier (4, 12), has to be justified since the alteration in %I changes the *in vivo* kinetics of GSase rather than total GSase activity. To test the model it is necessary to show in terms of MCA that (*i*) the concentration of G6P is maintained within a tighter range during changes in flux than if GSase %I were maintained constant and (*ii*) the flux control coefficient for GT/Hk is close to 1 under all physiological conditions of glycogen synthesis. To allow experimental assessment of the predictions of the model we derive below these conditions in terms of MCA.

Theoretical Dependence of G6P on GSase Fractional Activity and Glycogen Synthesis Flux. The *in vivo* velocity of GSase as a function of G6P in the %I range where glycogen synthesis occurs physiologically may be represented by

$$V = V_0 F\,[\text{I, G6P, other effectors}]. \qquad [1]$$

V_0 is the velocity under reference conditions and F is some function of the variables. The %I is itself a function of the external effectors such as insulin. In addition to activation of GSase by signaling pathways a mechanism has been proposed by Villar-Palasi and demonstrated *in vitro* in which the rate of phosphatase attack upon GSase (4, 11) depends upon G6P concentration.

As shown by Piras for a fixed %I in this region the *in vivo* activity of the enzyme will be proportional to G6P concentration (6). Under conditions of insulin stimulated glycogen synthesis and late phase resynthesis of glycogen after exercise our NMR studies have shown that the major effectors of GSase other than G6P remain constant (4). Therefore the enzyme velocity V may be simplified to

$$V = V_0 \left(\frac{\text{G6P}}{\text{G6P}_0}\right) F(I), \qquad [2]$$

where V_0 is the velocity and $G6P_0$ is the concentration under reference conditions. The effect of a change of flux on G6P concentration is then given by:

$$\frac{[\text{G6P}]}{[\text{G6P}_0]} = \left(\frac{V}{V_0}\right)\left(\frac{1}{F(I)}\right). \qquad [3]$$

If F(I) were constant then the G6P concentration would be proportional to glycogen synthesis rate. However G6P is kept closer to constant by changes in %I brought about by changing phosphorylation state. This is shown in Fig. 2, which is a plot from Roach and Larner (15) of the velocity of extracted muscle glycogen synthase at different constant G6P concentrations as a function of %I under *in vivo* conditions of ATP, pH, and other effectors of GSase. The velocity of the enzyme is sigmoidal at a G6P concentration of 100–200 μM when %I is in the range of 40–50% as normally found during insulin stimulation. On the other hand the enzyme is almost completely inhibited at levels of %I $\approx$ 20% found in muscle in the absence of insulin. This supralinear dependence of GSase velocity on the phosphorylation state provides a powerful homeostatic mechanism since even small changes in phosphor-

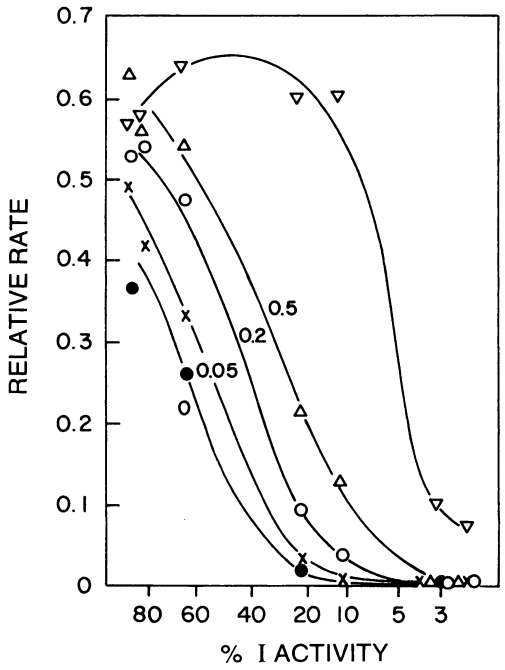

FIG. 2. Plot showing the strong dependence of the relative rate of GSase upon %I at different concentrations of G6P. The sensitivity of the rate under usual glycogenic conditions of ≈50%I activity is seen to be highest at G6P ≈ 0.2 mM showing the combined rates of phosphorylation and feed forward from glucose in establishing the tight coupling required for holding G6P at close to constant concentration [adapted from Roach and Larner (15)].

ylation state will accommodate large increases in flux while maintaining an almost constant G6P.

Effect of GSase Phosphorylation on Relative Flux Control Coefficients. The effect of phosphorylation upon flux control coefficients may be determined from the relative elasticities of GSase and the proximal GT/Hk enzymes to a shared intermediate (1). The elasticity coefficient of an enzyme i is defined by

$$\varepsilon_s^i = \frac{\partial V_i/V_i}{\partial S/S}, \qquad [4]$$

where V_i is the enzyme velocity under *in vivo* condition and S is the concentration of a substrate or allosteric effector. For the equivalent two enzyme top down (16) model of the muscle glycogen synthesis pathway the flux control coefficients of GSase and the proximal steps are related by the connectivity theorem of MCA that for the common intermediate G6P is given by

$$C_{\text{GSase}}\varepsilon^{\text{GT/Hk}} = -C_{\text{Gt/Hk}}\varepsilon_{\text{G6P}}^{\text{GSase}} \qquad [5]$$

This theorem indicates that the enzyme that is more sensitive to G6P (high elasticity coefficient) will exert less control on the flux.

If the internal effectors of the enzyme other than G6P are held constant (e.g. ATP, Pi, and pH) then the elasticity with respect to G6P is given by differentiating Eq. **4**

$$\varepsilon_s^{\text{i}} = \frac{\partial V/V}{\partial \text{G6P}/\text{G6P}} = \left(\frac{\partial F}{\partial I} \times \frac{\partial I}{\partial \text{G6P}}\right)(F(I))^{-1} + 1 \qquad [6]$$

Provided that any dependence of %I on G6P is positive the elasticity of GSase will be 1 or greater under *in vivo* conditions. If the elasticity of the GT/Hk step to G6P continues to be much lower than 1 the GT/Hk step will control the flux of the pathway throughout the physiological range of %I.

Experimental Evidence. We propose that the regulation of GSase activity by phosphorylation and allostery, which has been previously explored by Roach and Larner (9, 15), is coordinated with activity changes of the flux controlling GT/Hk step to maintain a near constant concentration of G6P at physiological rates of glycogen synthesis flux. The experimental test for this model would be to measure the flux control coefficients of the enzymes in the glycogen synthesis pathway as well as the fractional activity of GSase and the G6P concentration under the full range of physiological conditions of muscle glycogen synthesis. However since MCA has only recently been applied to the study of muscle glycogen synthesis (4) the study design of only a small set of experiments fulfill these criteria. We have previously reanalyzed the literature on the *in vivo* control of muscle glycogen synthesis and shown within experimental limitations it is largely consistent with the majority of flux control being at the proximal GT/Hk steps. Unfortunately in only a few studies was G6P measured accurately either by ^{31}P NMR or by fast freezing. However these studies are all consistent with the concentration of G6P being maintained in a small range about the concentration under basal nonglycogen synthesizing conditions (4). Using ^{31}P and ^{13}C NMR we have looked at G6P concentration and the rate of muscle glycogen synthesis under both basal and high plasma insulin conditions in human subjects. Even under the highest observed rates of glycogen synthesis under conditions of high plasma glucose and insulin concentrations the G6P concentration was only observed to double relative to basal conditions (17). We have also recently shown that the late phase of glycogen synthesis following glycogen depleting exercise in human subjects occurs at basal concentrations of G6P (18).

Using rapid biochemical assays, so as to minimize errors in G6P concentration, Rossetti and Giaccari (19) measured glycogen synthesis in euglycemic, conscious rats at plasma insulin concentration of ≈30, 70, 120, and 450 μunits/ml. The glycogen synthesis rates and G6P concentrations measured for each insulin level studied are expressed as a fraction of the value at 70 μunits/ml insulin in the bar graph in Fig. 3. In the physiological insulin range, 30–120 μunits/ml, the G6P concentration is close to constant despite a large change in glycogen synthesis flux. The relative G6P concentrations as a function of insulin calculated under the assumption of constant %I are plotted in Fig. 3 with respect to the fractional activity at 70 μunits/ml and are seen to differ dramatically from the small changes in G6P observed *in vivo*. Only at insulin concentrations far above the physiological range (450 μunits/ml) is there an appreciable increase in G6P concentration over basal conditions observed. The role of GSase phosphorylation in limiting changes in G6P concentration is supported by a separate study of Farrace and Rossetti (20) who measured %I under similar conditions and showed that there was a 2.6-fold increase in %I over the range of insulin levels in which the measurements of Rossetti and Giaccari (19) were performed. The nonlinearity between glycogen synthesis rate and %I may be explained by the nonlinearity in the Roach and Larner curve (Fig. 2) under basal concentrations of G6P. Further studies will be needed to assess whether %I and G6P can explain the observed flux changes quantitatively under these conditions.

Molecular Mechanisms to Maintain Homeostasis. The rise in G6P, with increased glycogen synthesis flux, while it does occur transiently during nonphysiological conditions such as recovery from intense glycogen depleting exercise (18), will be mediated by three molecular mechanisms that increase GSase activity under these conditions.

First as illustrated above (19) there is a coordinated increase in GSase activity stimulated by insulin that results in the enzyme being dephosphorylated and an increase in %I. This well-known change of GSase phosphorylation state is synchronous with flux changes and led to the concept of GSase as rate limiting. The second mechanism is that G6P is a positive allosteric effector of GSase decreasing K_m for uridine diphosphoglucose. This has been measured by Piras (6, 21) under *in*

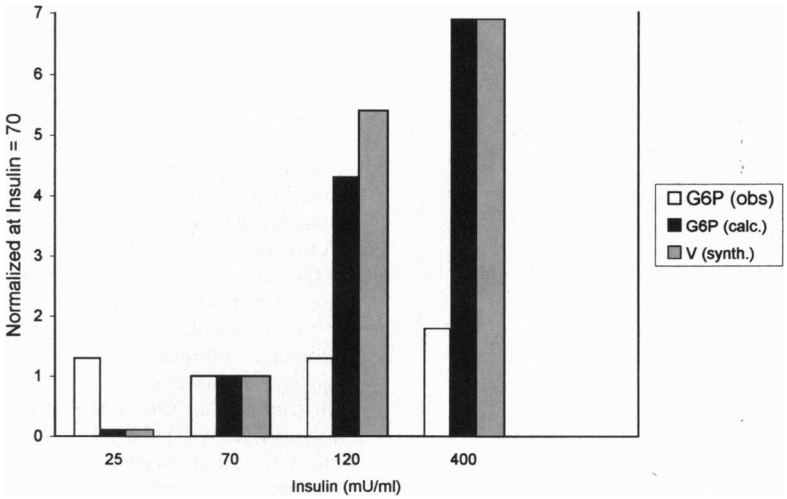

FIG. 3. Results replotted from Rossetti and Giaccari (19) of an experiment measuring rates of glycogen synthesis (V) in rat muscle vs. [G6P] at different plasma insulin concentrations. The observed values of [G6P] and V were set to unity at the basal insulin concentration of 70. The concentrations of G6P observed change very little over the wide range of insulin, only changing significantly at supraphysiological plasma insulin levels despite a large increase in flux. The values of G6P calculated on the assumption of constant GSase %I are seen to change significantly over this range being linear with flux. The increase in GSase activity with insulin levels eliminates the need for a large increase in G6P with flux.

vivo conditions where the normal ATP level, which acts as a negative effector in the absence of G6P almost completely inhibits GSase activity. While G6P restores activity of both the I and D forms the former overwhelmingly dominates the flux under *in vivo* G6P concentrations. Hence the increased percentage of I increases GSase activity and limits the rise in G6P concentration needed to match the flux. A third mechanism proposed by Villar-Palasi (11) on the basis of *in vitro* experiments is that increasing G6P concentrations will expose GSase to faster phosphatase attack. A fourth mechanism is the possible increase of uridine diphosphoglucose with G6P. All four of these mechanisms are sensitive to G6P concentrations in the physiological range of 0.1–0.2 mM.

The effectiveness of direct insulin stimulated increase of GSase %I in regulating G6P concentration is illustrated by the Giacarri and Rossetti data in which the glycogen synthesis flux increases with insulin with minimal increase in G6P. The completeness of these molecular mechanisms in explaining the data can be evaluated by recent experiments in which muscle glycogen synthesis and G6P were measured with insulin levels held constant while the plasma glucose levels were increased. These experiments selectively test the last three proposed mechanism because at constant insulin there should be no exogenous change in %I, while the change in plasma glucose concentration will increase the glycogen synthesis flux. In an NMR experiment of the human gastrocnemius muscle, Rothman *et al.* (22) measured glycogen synthesis and G6P at constant insulin concentration at two different glucose levels. A 2.7-fold increase in glycogen synthesis rate was accompanied by only a 1.4-fold in G6P. The increase in G6P is not as rapid as the increase in flux indicating an increase in GSase activity. Interestingly, no change in GSase %I was measured in a study (23) performed in humans under similar conditions to the Rothman *et al.* study. This apparent inconsistency with a model of covalent phosphorylation being the main buffering mechanism might be explained by the high dependence of glycogen synthesis velocity on %I under *in vivo* conditions as shown in Fig. 1. In the steepest part of the curve a 2.7 fold increase in flux as observed in these studies may be the result of less than a 15% change in %I. The small change in %I required buffer the G6P concentration may not be detected by the standard GSase activity assay in which conditions are chosen so that velocity is linear with %I. However in the absence of more quantitative studies we cannot be sure that changes in the GSase activity with increasing flux and at constant insulin are all mediated by G6P allostery plus %I. It may be that some additional feed forward mechanism is operative. Whether this is so will have to be experimentally determined. However it is clear that experimentally a substantial increase in GSase activity occurs during changes in flux, at constant insulin, and these changes tend to reduce changes in G6P concentrations.

Conclusions. We have proposed that rather than control glycogen synthesis flux the phosphorylation/dephosphorylation of GSase adapts its activity to changes in the flux so as to maintain G6P concentrations close to constant during flux changes. The control of flux exercised by GT/Hk feeds glucose forward to G6P during increases in flux and this allosterically increases GSase activity. To strive for homeostasis of this shared intermediate the dephosphorylation of GSase accommodates the increased flux by increasing GSase activity. This feeds back upon G6P limiting changes in its concentration. Accordingly phosphorylation provides some of the coordinated changes in activity required in Kacser and Acarenza's model for producing changes in a flux without impacting other biochemical pathways through concentrations of a shared intermediate. The degree to which the conditions of Kacser and Acarenza (3) are met under the full physiological range of muscle glycogen synthesis remains to be established as well as the quantitative contribution of covalent phosphorylation vs. noncovalent mechanisms for altering GSase activity to maintain G6P homeostasis. However from the limited data available it is clear that covalent phosphorylation contributes substantially to keeping the G6P concentration within a narrow range. This previously neglected function of enzymatic phosphorylation provides a novel explanation, beyond flux control, of the many activity changes through phosphorylation that are observed during flux changes in biochemical pathways.

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