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Studies on Frog Muscle Glycogen Synthetase. Factors Affecting its Activity *

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Frog muscle glycogen synthetase is a D form, that is, it depends on glucose 6-P for activity. In this paper data are presented on the effects of Mg^{2+} and ATP-Mg, as well as the influence of pH on the enzyme activity and stability.

Mg alone produces different effects on crude enzyme extracts or more purified enzyme preparations obtained after 90 minutes of ultracentrifugation at 100.000 × g. In crude extracts it produces an activation time and cation concentration dependent. At high cation concentrations some activity appears without added glucose-6-P, which is parallel to the increase experimented by the D activity. Such Mg concentrations are not physiologycal and this independent-like activity may be due to the small concentrations of endogenous glucose-6-P available to the enzyme.

In the purified particulate enzyme Mg^{2+} always activates strongly without appearance of any independent-like activity. This activation, apparent even without preincubation of the enzyme, can be of 4-5 fold. A Ka of activation of 10-12 mM can be determined in our assay conditions.

Addition of ATP-Mg produces an inactivation of the D-enzyme dependent both on time and concentration of ATP. This inactivation is sensitive to the addition of 3′,5′-cycloadenylate at the 10^{-5} M level. This seems evidence of the action of a kinase that inactivates the D-enzyme by extraphosphorylation.

The optimum pH for enzyme activity seems to be in the range 7.5-7.8 provided the enzyme is left at room temperature or a little higher. At this pH the enzyme has poor stability at temperatures near 0° C, showing maximum activity at pH 6.5 in these conditions. Warming of the enzyme to 30° C at this pH produces an inactivation which is almost total in 60 minutes.

The glycogen synthetase (UDPG: α-1,4-glucan α-4-glycosyltransferase; E.C. 2.4.1.11; synthetase of transferase) extracted from frog skeletal muscle *(Rana pipiens, R. esculenta)* is a D form of the enzyme, that is, it shows an absolute dependence on the presence of Glucose-6-P for activity (16, 19). When enzyme extracts are incubated in the presence of mercaptoethanol they show no increase nor does the independent activity appear, as happens with similar treatment on enzyme systems extracted from muscles of rat

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(20), rabbit (17), dog (18) or human subjects (14).

It has been shown that Mg ions stimulate the appearance of the I activity (independent of Glucose-6-P) of glycogen synthetase in enzyme preparations obtained from human lymphocytes (9), human diabetic polymorphonuclear leucocytes (4) or rat leucocytes (15).

Addition of ATP and Mg^{2+} to enzyme crude extracts from rat (5, 6) or dog (18) muscle induces a transformation of the I form of the enzyme (independent) into the D form (dependent) by a mechanism that incorporates phosphate into the D enzyme protein (6). The transformation of the D into the I form of the enzyme involves a release of inorganic phosphate and therefore is produced by a phosphoprotein phosphatase (6), the synthetase D-phophatase.

The present paper shows experimental data demonstrating that the D-form of glycogen synthetase from frog muscle can be converted to or inactivated into an inactive intermediate by a time dependent reaction initiated by addition of ATP-Mg, also dependent on ATP concentration and stimulated by 3',5'-cycloadenylate. This effect is not produced by ATP or Mg^{2+} alone.

Addition of Mg ions in sufficient amounts to crude enzyme extracts induces the appearance of an I-like activity by incubation in the presence of mercaptoethanol, but also stimulates the D activity, in a time dependent activation which also depends on Mg^{2+} concentration. Addition of Mg^{2+} to particulate enzyme fractions obtained by ultracentrifugation produces some distinct and different effects. The effect of pH on enzyme activity and activations is also shown.

Materials and Methods

Enzyme preparations were crude extracts or 100,000 × g particulate glycogen pellets as described previously (16, 19). Measurements of enzyme activity, assay mixtures and incubation times were also standard for these kinds of experiments and were also reported (16, 19). Other conditions are described in the text or the graphs.

Results

INFLUENCE OF THE PRESENCE OF Mg^{2+}. On crude enzyme extracts Mg ions stimulate the glucose-6-P dependent activity (D-form) of frog muscle glycogen synthetase in a time-concentration dependent reaction as shown in a typical experiment depicted in figure 1. It can be seen that a certain activity in the absence of glucose-6-P also appears with certain concentrations of Mg^{2+}. Kinetic analysis of Mg^{2+}

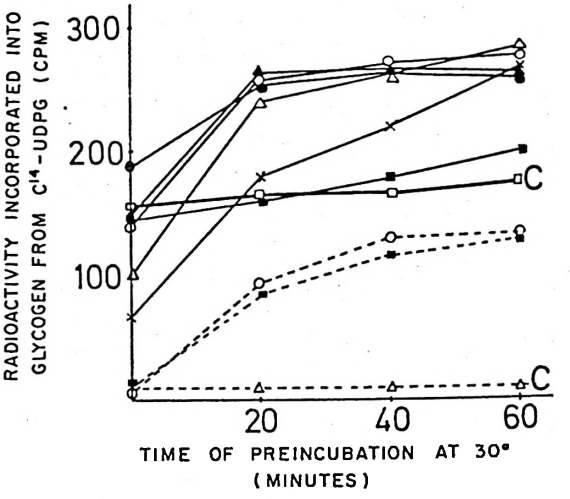

Fig. 1. *Action of Mg on glycogen synthetase from frog muscle.*
Different concentrations of Mg^{2+} were added to the enzyme crude extracts which were preincubated at 30° C for 20, 40 and 60 minutes and aliquots of the enzyme tested for activity at the end of each period. Full lines represent activity measured in the presence of 10 mM glucose-6-P. Broken lines mean activity measured without glucose-6 P. C and C are controls with no Mg^{2+}. Concentrations of Mg^{2+} are: 3 mM ■—■; 7 mM ×—×; 10 mM △—△; 12 mM ○—○; 15 mM ▲—▲, and ○····○; 20 mM ●—● and ■---■.

in the presence of glucose-6-P gives values of 10-12 mM for the Ka of Mg^{2+} in our conditions of assay (EDTA 5 mM).

When enzyme preparations are purified by ultracentrifugation at 100.000 × g, for 90 to 120 minutes, the precipitate enzyme preparations resuspended in the original buffer do not show any I-like activity nor any time dependent activation, although at any time of preincubation the presence of Mg^{2+} produces a 4-5 fold stimulation dependent on Mg^{2+} concentration. The particulate enzyme preparations incubated at 30° C in the presence of 50 mM mercaptoethanol and Mg ions either remain stable with time in some preparations, or show a decrease in activity through the time of incubation (Fig. 2).

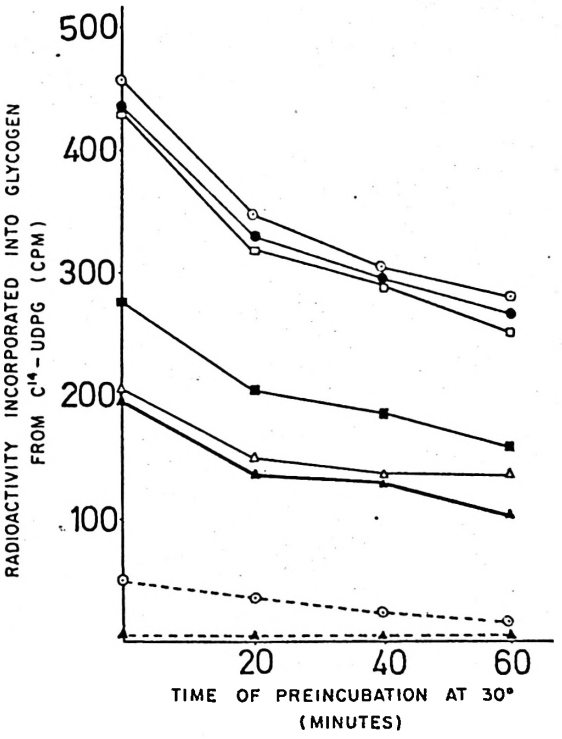

Fig. 2. *Effect of Mg^{2+} addition on the activity of frog muscle glycogen synthetase partially purified by ultracentrifugation at 100.000 × g.* Mg was added this time to the suspension of the particulated enzyme which was preincubated at the same time and temperature described in Fig. 1. Full and broken lines have the same meaning.
▲=====▲ are controls with no Mg. The concentrations of Mg^{2+} are: 6 mM △——△; 7 mM ■——■; 10 mM □——□; 12 mM ●——●; 15 mM ○——○.

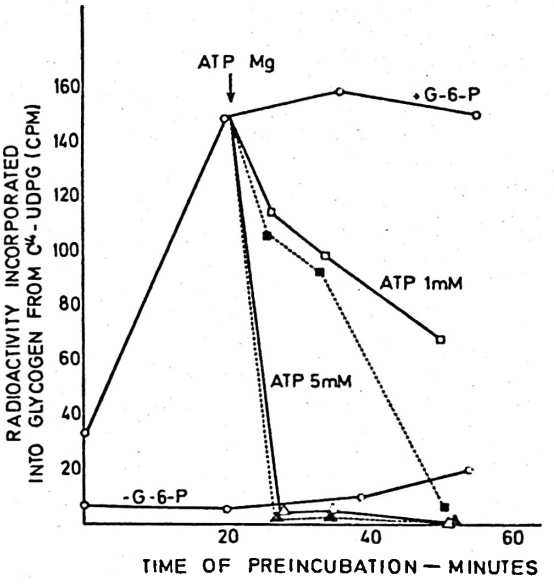

Fig. 3. *Effect of ATP-Mg addition on the D form of frog muscle glycogen synthetase.* Crude extracts of the enzyme were preincubated at 30° C in the presence of 50 mM mercaptoethanol and after 20 minutes the additions indicated were made. Aliquots of the enzyme were tested for activity at each point indicated. ——= Mg 10 mM; ----= 3',5'-AMP 3×10^{-3} mM.

EFFECT OF THE ADDITION OF ATP AND Mg^{2+} TO ENZYME PREPARATIONS. Addition of ATP and Mg to crude enzyme extracts, after 20-30 minutes of preincubation at 30° C, produces a decrease of the activity present (D-activity) dependent on time and amount of ATP (Fig. 3). The presence *in vitro* of 3',5'-AMP in the range of 10^{-5} M enhances this action, when added to amounts of ATP-Mg that do not cause a total inactivation. Similar results are obtained if additions are made to enzyme extracts previously treated with Mg^{2+}. This seems to be evidence that the glycogen synthetase D-form of frog mus-

cle is inactivated by a phosphorylating reaction induced by ATP-Mg and enhanced by the 3',5'-cycloadenylate.

The addition of ATP-Mg to 100.000×g particulate enzyme preparations has a quite different effect, depending on the concentrations of ATP and Mg^{2+}. This behaviour is depicted in figure 4. In these preparations activity without glucose-6-P is nil. The presence of Mg^{2+} on the suspended enzyme preparations increases the D activity strongly (2-4 fold). After 20 or 40 minutes of preincubation at 30° C different additions were made. ATP-Mg in concentrations 5-10 mM respectively produced a decrease of activity dependent on time in both D activities (with or without Mg^{2+} previously added), as in the crude extracts. If ATP concentration is reduced to 1 mM, no effect can be observed on the enzyme preparation with Mg^{2+} previously added and an increase in activity, instead of a decrease, can be seen in the preparation without Mg^{2+}. That this increase is due to the Mg^{2+} can be proved by adding Mg^{2+} alone at the same point (Fig. 4 *b*). The D activity rises practically to the levels of the one with Mg^{2+} previously added

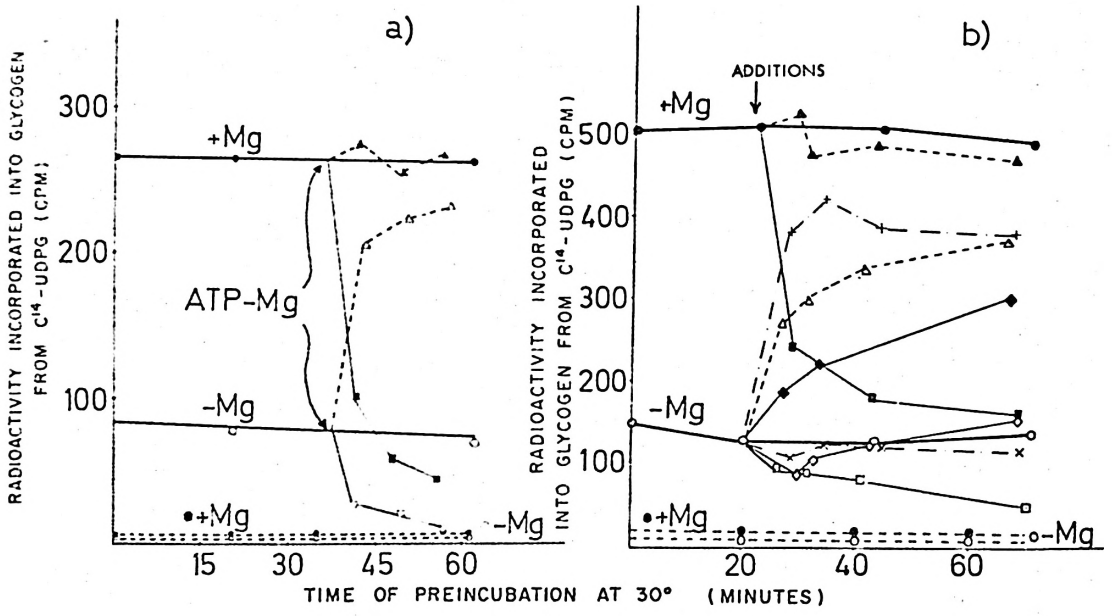

Fig. 4. *Effect of ATP-Mg additions on the particulate fraction of frog muscle glycoge synthetase.*

Enzyme preparations were suspended in Tris-EDTA buffer after 100.000 × g centrifugation for 90 minutes. 50 mM mercaptoethanol was added, the suspension divided in two equal parts and Mg^{2+} 10 mM added to one. Both were preincubated and at the times shown in the figures the different additions were made. At each point aliquots of the enzyme were tested for activity. The meaning of the lines is the following: Thick solid lines = activity in the presence of 10 mM glucose-6-P. Broken lines between circles = activity with no glucose-6-P. Concentrations of ATP-Mg: 5 mM ■—■; 10 mM □—□; 1 mM ▲···▲; 10 mM △···△; 1-10 mM plus 5.10^{-5} M 3',5'-AMP: ◆——◆; 1-5 mM plus 5.10^{-5} M 3',5'-AMP: ◇——◇;
Mg^{2+} alone: 10 mM: +—·—·—+; 5 mM: ×—·—·—×.

(10 mM). With 5 mM Mg^{2+} no rise can be observed, or a smaller one in other preparations, because of the complexing power of our Tris-EDTA buffer.

Addition of 3',5'-AMP (5×10^{-5} M) with low ATP concentrations (1 mM) modifies the action of Mg^{2+}. The rise in D activity without Mg ions previously added is diminished strongly. ATP alone, without Mg^{2+} has no effect dependent on time.

EFFECT OF pH ON THE ACTIVITY AND STABILITY OF THE ENZYME. The reported activation of frog glycogen synthetase (16, 19) by preincubation at 30° C in the presence of mercaptoethanol was now tested as a function of the pH of the enzyme. Enzyme extracts were made in buffer of different pH and tested for activity at different times of preincubation in the presence of 50 mM mercaptoethanol.

As shown in figure 5 the maximum activity is obtained at pH 6.5 with no preincubation, that is, when the enzyme preparations are kept in a cool ice-bath, but at this pH, preincubation of the enzyme produces an inactivation with time. If the pH is increased the activity and stability of the enzyme not preincubated (on ice-bath) decrease with increasing pHs.

On the other hand, when the enzyme has been preincubated for some minutes at 30° C, the maximum activity is reached at pH 7.5 no matter how long the time of preincubation. Maximum activity is reached after 30 minutes of preincubation at pH 7.5. Over pH 8 the enzyme has very poor activity and no increases in activity by preincubation are observed. Thus preincubation of the enzyme at 30° C at pH between 7 and 8 can result in a 2-3 fold increase in activity depending on pH.

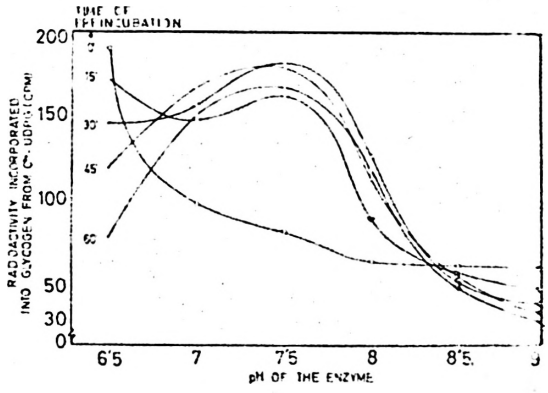

Fig. 5. *Effect of pH and preincubation on enzyme stability and activity.*
The different curves unite the points which express enzyme activity at each pH. The 0' (minutes) curve represents enzyme with no incubation at all, kept in an ice-cool bath before testing its activity. The other curves show enzyme activity at different pH when it was preincubated in the presence of 50 mM mercaptoethanol at 30° C during the times indicated.

Discussion

The great stimulation of the D activity of frog glycogen synthetase by Mg^{2+} first described by ROSELL-PÉREZ and LARNER (16), was later explained by ROSELL-PÉREZ and VILLAR (19) as an action modifying the affinity of the activator glucose-6-P for its allosteric site in the enzyme molecule.

The stimulation by Mg^{2+} of the D to I form transformation in human lymphocytes (9), diabetic human polymorphonuclear leucocytes (4) or rat leucocytes (15) could be explained either by a stimulation on an ATP-ase or on a glycogen synthetase D-phosphatase (4). Recently, HICKENBOTOM (10) has demonstrated that muscle glycogen synthetase D-phosphatase can became Mg^{2+} dependent by treatment with NaF. More recently we have found that the phosphatase system of human platelets is highly dependent on Mg ions (1).

HUIJING and LARNER (11) have reported that Mg^{2+} causes an allosteric activation of transferase I kinase and that adenosine 3',5'-cyclophosphate increases the degree of affinity of the allosteric site of the enzyme for Mg^{2+}. GOLD has described re-

cently (7) a differential stimulation of Mg^{2+} on two forms of liver glycogen synthetase. In the non activated form, Mg ions increase the affinity of the enzyme for glucose-6-P. This is similar to our fiindings in the D-form from frog muscle (19).

All these data show that Mg ions have strong influence at several levels on the glycogen synthetase systems, and probably, will influence other enzyme systems in different ways.

The action that Mg ion exerts on the enzyme D form of frog glycogen synthetase seems to be of more than one type. One is the increased affinity for the allosteric activator glucose-6-P already mentioned (19). A second could be a conformational modification of the enzyme molecule itself. Still a third could be a stimulation of a phosphoproteinphosphatase. In crude extracts this action by Mg^{2+} seems a slow one that depends both on time and concentration of cation. Probably this is due to the competition of other proteins and metabolites for the metalic ion. This action of Mg^{2+}, seen in crude extracts, seems to be double in that it depends on Mg concentration and on time of preincubation. In fact, low concentrations of Mg^{2+} seem to be a little inhibitory when the enzyme is kept cooled in an ice-bath (Fig. 1). The rise in the I-like activity at high concentrations of Mg^{2+} is parallel to the rise in D activity and seems to be a reflexion of the small quantities of endogenous glucose-6-P available in those extracts. In the 100.000 × g particulate fraction this rise is never found. On these more purified preparations, the Mg activation on the D form is very quick as it is already present at 0 minutes of preincubation. However the rise in this activity can be observed in experiments of incubation after addition of ATP-Mg. When ATP concentration is sufficiently high, the combination ATP-Mg for the kinase action is very effective and there is no Mg available to the glycogen synthetase molecule for its activation. Then we can see the inactivation time-dependent (due to a sinthetase D-kinase) that extraphosphorylizes the D form. If ATP, on the contrary, is low, without varying amounts of Mg, there is enough cation available to the enzyme and then a stimulation with time instead of an inactivation can be seen (Fig. 4). This activation can be produced by addition of Mg^{2+} alone in sufficient concentration (10 mM) to overcome the concentration of EDTA (5 mM) used in our experimental conditions. Now the activation is quicker as there is no competition of ATP for the metalic ion. If, in addition to ATP, there is some 3′,5′-adenosylcyclophosphate, the activation due to Mg^{2+} is still lower and slower than with low ATP alone, as Mg is also bound to 3′,5′-AMP and there is more competition for the cation.

No inhibition of ATP alone (with no Mg) of the type described by PIRAS *et al.* (12) could be observed. However our experiments were done in Tris-EDTA buffer at pH 7.5-7.8 and those reported by the Argentinian group were at pH 6.6. At this pH the activity of our enzyme would be very poor unless it would stay at a temperature near 0° C (Fig. 5) which certainly is not the frog's temperature when this animal is active. In this connection, ANDERSON (2) showed diminished glycogen content in livers of frogs kept at 4° C for some days. Besides, ATP inhibition by itself would be meaningless as Mg^{2+} always will be present in the cell and then regulation of enzyme activity can be undertaken by the kinase system. GOLD and SEGAL (8), in the rat liver enzyme, have seen that stability of the enzyme varies with pH depending on whether the enzyme was kept cool or at room temperature. Our enzyme can be maintained at low pH provided that it is cool. A rise in temperature at this pH produces an inactivation with time. At higher pHs, on the contrary, incubation at 30° C increases its activity.

The action of ATP-Mg on frog synthetase D-form shows that this form can be inactivated by a reaction time and ATP concentration dependent, enhanced in certain experimental conditions by 3',5'-cycloadenylate (Fig. 3). These are conditions that define a protein kinase, and, in this case, a glycogen synthetase D-kinase. The action of this enzyme will produce an inactive molecular species of the synthetase more phosphorylated than the D-form. The activation of the D form in crude homogenates when they are incubated in the presence of mercaptoethanol could mean the transformation of some of the molecular inactive species into the D form by a proteinphosphatase, as in other systems it can be prevented by NaF addition (13).

The transformation of active glucose-6-P dependent form into completely inactive more phosphorylated form and viceversa, as well as the presence of different levels of glucose-6-P and Mg, must play an important role in the regulation of glycogen biosynthesis in those cells in which no system of D to I form transformation is operational.

Recently, BLATT *et al.* (3), have reported that in liver of tadpoles, glycogen synthetase is absolutely dependent on glucose-6-P for activity and that this activity is stimulated by insulin. This could be an indication that the amount of this D form of the enzyme could be regulated hormonally.

Resumen

La glucógeno sintetasa de músculo de rana es una forma D, es decir, dependiente de glucosa-6-P para su actividad (16, 19). Se ha estudiado en este trabajo los efectos del ion Mg^{2+} y del ATP-Mg, así como el pH sobre la actividad y estabilidad del enzima.

El Mg solo produce efectos diferentes según se observe su acción en extractos enzimáticos crudos o purificados por ultracentrifugación a 100.000 × g. En extractos crudos produce una activación dependiente del tiempo y de la concentración del catión. A altas concentraciones aparece una cierta actividad en ausencia de glucosa-6-P, que es paralela al aumento que experimenta la actividad D. Las concentraciones de Mg, donde esto ocurre, no parecen fisiológicas y esta actividad pseudoindependiente puede ser debida a las pequeñas concentraciones de glucosa-6-P endógenas asequibles al enzima.

En el enzima purificado en forma particulada el Mg siempre activa fuertemente sin aparición de actividad pseudoindependiente. Esta activación, que aparece aun sin preincubar al enzima, puede ser de unas 4 ó 5 veces. Una Ka de alrededor 10-12 mM puede ser determinada en nuestras condiciones de ensayo.

La adición de ATP-Mg produce una inactivación del enzima D, dependiente del tiempo y la concentración de ATP. Esta inactivación es acentuada al añadir adenosín 3',5'-ciclofosfato a niveles 10^{-6} M. Esto parece una fuerte indicación de la actuación de una cinasa que produce la inactivación de la forma D por extrafosforilación.

El pH óptimo de actuación del enzima parece estar situado entre 7,5-7,8 siempre que el mismo esté a temperatura ambiente o quizá ligeramente superior. A temperaturas próximas a 0° C a ese pH el enzima tiene poca estabilidad, presentando máxima actividad en estas condiciones a pH 6,5. El calentamiento del enzima a 30° C a ese pH produce una inactivación que hace perder la actividad del enzima casi totalmente en 60 minutos.

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