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Selective Inactivation of the D or I Forms of Glycogen Synthetase from Rat Skeletal Muscle

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The inactivation of the D or I forms of glycogen synthetase (dependent or independent of glucose-6-phosphate) was demonstrated in this laboratory for the enzyme systems from frog skeletal muscle, heart and kidney from rat and also for human polimorphonuclear leucocytes and lymphocytes. A scheme of metabolic interconversions between different molecular forms of glycogen synthetase was suggested (10) in order to explain such phenomena. In the scheme coexisted enzyme forms completely active (I-forms) or less active (D-forms) with other completely inactive (X-forms).

In this paper experimental data are shown which demonstrate the possibility of selectively inactivating either the I or the D forms of glycogen synthetase from rat skeletal muscle, by reactions promoted by the addition of ATP and Mg^{2+} to the enzyme extracts. Neither of these inactivating reactions observed could be attributed to the Mg^{2+} or ATP independently, therefore they must be attributed to phosphorylating reactions brought about by protein kinases.

These inactive molecular species fit adequately in the scheme of interconversions proposed for other enzyme sources (8, 16) and can be logically explained with the recent knowledge obtained on these enzyme systems.

It is well known that the enzyme glycogen synthetase (UDPG: glycogen α-4-glucosyltransferase. EC. 2, 4, 1, 11) exists in two protein enzyme forms that can be interconverted by phosphorylation and dephosphorylation reactions (6, 11, 12). During the last four or five years, evidence has accumulated demonstrating that perhaps more than two forms of this enzyme could exist. Completely inactive precursors were postulated by ROSELL PÉREZ and LARNER as early as 1962 (13) for the frog muscle enzyme, and the formation *in vitro* of such inactive forms has been demonstrated by ROSELL PÉREZ and his collaborators in several sources such as frog muscle (2) cardiac muscle of rat (16) and rat kidney (8) as well as in several types of human white blood cells (1, 5, 7). Inactive forms

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have also been postulated recently for the liver enzyme (15, 20) and a physiologically inactive, glucose-6-dependent form (D' form), obtained by phosphorylation driven by ATP-Mg, has been demonstrated lately in the rat chloroma enzyme extracts (3).

In this paper data are presented which demonstrate the possibility of inactivating either the I or the D forms of the rat skeletal muscle glycogen synthetase systems, by reactions that involve the collaboration of ATP together with Mg ions, as neither alone is able to produce them. These data seem to confirm the findings discovered in the enzyme systems from other sources which support the idea of the existence of intermediate complete inactive forms produced by extraphosphorylization of the active I or D forms of glycogen synthetase.

Materials and Methods

Enzyme sources. Rat skeletal muscle crude extracts were made by homogeneizing fresh rat muscle (1:10 w/v) in 50 mM Tris-5 mM EDTA 50 mM mercaptoethanol buffer at *p*H 8. The white rats were of a local strain bred in the animal house of our school of Pharmacy. After homogeneization 1 to 3 minutes in a motor driven teflon pestle homogenizer, the muscle suspensions were centrifuged at 3,000-3,600 *g* for ten minutes in a MSE refrigerated clinical centrifuge. The sediment was discarded and the supernatants were used as source of enzyme for the experiments.

Glycogen synthetase assays. These were esentially done as described by VILLAR-PALASÍ *et al.* (21).

UDP-glucose, glucose-6-P, UTP, and rabbit glycogen were purchased from Sigma Chemical CO. C^{14} uniformely labeled glucose UDPG was supplied by New England Nuclear Corp. NaF, ortophosphate, $MgCl_2$ and other chemicals were analytical grade from Merck.

Results

The reaction that converts the I to the D form of muscle glycogen synthetase was discovered when a mixture of ATP-Mg was added to some enzyme crude preparations that already had a high percent of activity measured without glucose-6-phosphate, and without any other previous addition. In these conditions the I activity (measured without glucose-6-phosphate) diminishes with preincubation of enzyme crude extracts in a time course reaction while the D activity (measured in the presence of 10 mM glucose-6-phosphate) remains more or less stable during time of preincubation. The following experiments show conditions in which this type of transformation can be altered *in vitro*.

Inactivation of the activity in absence of glucose-6-phosphate (I form) without transformation to the D form.

Figure 1 depicts an experiment in which to the enzyme crude extract produced as described in methods, was added $MgCl_2$ up to 8 mM, tested at 0 time of preincubation, preincubated at 30° C and tested for activity at various intervals. After 30 minutes of such preincubation, a mixture of ATP-Mg (up to 5 and 6.5 mM respectively) was added to some aliquot parts of the preincubated enzyme and glycogen synthetase activity was assayed after different intervals. It can be seen that after the D to I form transformation produced during the first 30 minutes of preincubation, the addition of ATP-Mg did not elicit an I to D form transformation. Instead, an absolute inactivation of the glucose-6-phosphate independent activity present (I form), was produced as at the end of 25 minutes, after the ATP-Mg addition almost all the I activity had practically disappeared without the appearance of the activity in the presence of glucose-6-phosphate. The magnitude of counts per minute which diminished during these 25 minutes after the addition of ATP was

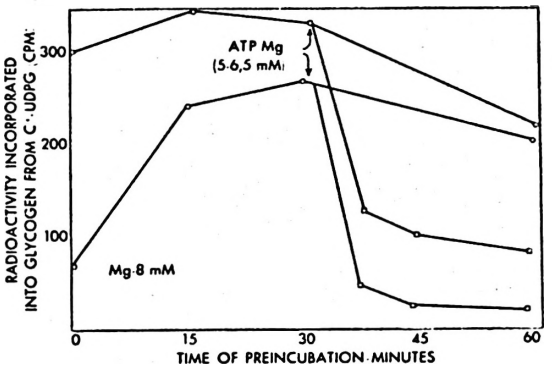

Fig. 1. *Selective inactivation of the I-form of glycogen synthetase from rat skeletal muscle.*
Muscle enzyme extracts (1:10, W/v) were made in Tris-EDTA buffer. Mercaptoethanol up to 50 mM and Mg^{2+} up to 8 mM were added and then preincubated and tested for enzyme activity at the intervals shown in the graph. ATP-Mg mixture at the concentration indicated was added after 30 minutes of preincubation to some aliquot parts and enzyme activity tested again at the intervals shown. Upper lines show the activity measured in the presence of 10 mM glucose-6-P and lower lines the activity measured without the activator (I-activity).

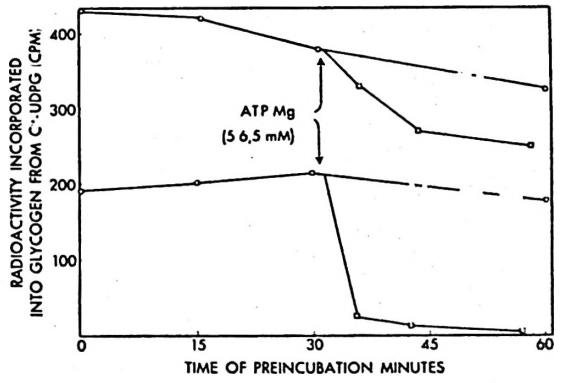

Fig. 2. *Inactivation of the I-form together with some I to D form transformation.*
Muscle enzyme extracts were an aliquot part of those described in Figure 1. The addition of Mg^{2+} was omitted and instead of it NaF up to 50 mM was added after 20 minutes of standing at room temperature. The addition of ATP-Mg at the same concentration as in the previous one induced an inactivation of the I activity bigger than the D.

exactly the same in the activity of glycogen synthetase measured either with or without glucose-6-phosphate.

Inactivation of the I activity with some transformation into the D activity.

Figure 2 shows now a different pattern of the transformation induced by the ATP-Mg addition.

The crude extract was an aliquot part of the same shown in the previous figure but the addition of $MgCl_2$ was omitted. Instead of that, after being left 20 minutes at room temperature, NaF up to 50 mM was added, tested for glycogen activity at 0 time, preincubated at 30° C and tested at intervals. After 30 minutes of preincubation ATP-Mg (5-6,5 mM respectively) was added to aliquot parts of the preincubated crude extract and tested for glycogen synthetase activity at intervals as described in the previous experiment. As the glycogen synthetase D-phosphatase was inhibited by the NaF, no D to I form transformation can be seen during the first 30 minutes of preincubation. The addition of ATP-Mg mixture inactivates the activity measured in the absence of glucose-6-phosphate (I form), but also some inactivation, time dependent, can be seen in the activity measured with added glucose-6-phosphate. In magnitude the I activity diminished about 190 cpm. while total activity (measured in the presence of glucose-6-phosphate) decreased some 100 cpm. Therefore it can be said that in these conditions, perhaps 50 % of the I form was transformed into the D form, but the other 50 % was inactivated, without being converted into the D form, or may be that part of the D form produced was also inactivated.

Inactivation of the glucose-6-phosphate dependent activity (D-form).

The glycogen synthetase D-phosphatase could be inhibited earlier if we prepared the enzyme crude extracts with buffer containing NaF (50 mM). Figure 3 depicts an ex-

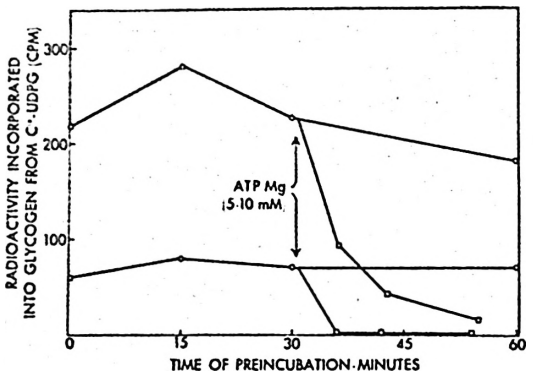

Fig. 3. *Preferential Inactivation of the D form of glycogen synthetase from rat skeletal muscle.*

Enzyme extracts (1:10, w/v) were made in Tris-EDTA buffet that had 100 mM NaF. $MgCl_2$ up to 8 mM and mercaptoethanol up to 50 mM were added and then preincubated at 30° C and tested for glycogen-synthetase activity at the intervals shown. As in the two previous experiments ATP-Mg mixture (5-10 mM respectively) was added after 30 minutes of preincubation. Upper lines depict the activity in the presence of glucose-6-P (10 mM) and lower lines show the activity without the sugar phosphate. Lines between squares represent the activities after the addition of ATP-Mg.

periment done in these conditions, in which prior to preincubation $MgCl_2$ (8 mM) was also added to this crude extract. As can be seen, there is very little I activity at 0 time of preincubation and the D to I form transformation does not appear during preincubation. The addition of ATP-Mg at concentrations 5-10 mM respectively to some aliquot part of the preincubated extract did inactivate the very low initial I activity, but also the glucose-6-P dependent activity (D form) was reduced in a time dependent reaction, to a much higher degree than the I form. Quantitatively the reduction of I activity was of about 50 cpm. in 20 minutes after the ATP-Mg addition, whereas the inactivation on the D activity was of about 200 cpm. in the same time, or 4-fold bigger than the I. At the end of this period practically all glycogen synthetase activity had disappeared. We have seen here mostly an inactivation of the D form of rat muscle glycogen synthetase.

A similar kind of preferential inactivation of the D-form can be seen in the experiment depicted in figure 4 done under different conditions. In this case the glycogen synthetase D-phosphatase was not inhibited by the addition of NaF and therefore the rise of I activity during the first 30 minutes of preincubation is easily observed. The previous addition of Mg ions to the extracts was also omitted, and now ATP-Mg mixture up to 5 and 8.6 mM respectively was added and enzyme activity of the aliquot added and that of controls tested at intervals for glycogen synthetase activity. The I activity decreased in the ATP-Mg treated samples during time of preincubation but so did the D activity on a bigger scale and with a quicker reaction. After 20 minutes of the addition of ATP and Mg^{2+}, practically all the acti-

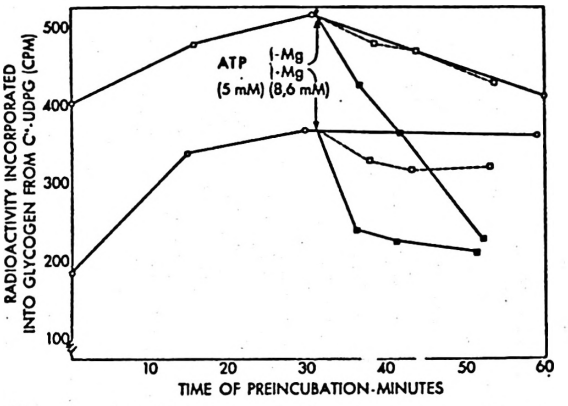

Fig. 4. *Inactivation of the D form without complete desappearance of the I form.*

Enzyme extracts were similar to those in the previous figures. No $MgCl_2$ was added previously. After 30 minutes of preincubation 5 mM ATP with 8.6 mM $MgCl_2$ (black squares) or without $MgCl_2$ (broken lines between white squares) was added to an aliquot part of the preincubated extract and glycogen synthetase activity with (upper lines) or without (lower lines) 10 mM glucose-6-P was measured at the intervals shown in the graph.

vity that remained was an I activity. This was not obtained by transforming the D into I by a protein-phosphatase type of reaction, but by selective inactivation of the D activity by a reaction promoted by ATP-Mg addition. That this type of reaction was driven by the presence of ATP together with Mg^{2+} can be proved, as the addition of ATP alone (broken lines) did not produce any effect, or perhaps, at most, a slight I to D type of reaction.

Intermediate steps during the transforming and inactivating reactions.

The inactivation of the I or D activities, shown up to here, could involve more than one step, as has been postulated for other enzyme sources.

The experiment shown in figure 5 seems to confirm this hypothesis. This was done on an aliquot part of the crude extract used for the experiment depicted in figure 3, but omitting the addition of NaF to the extracting buffer and that of $MgCl_2$ to the extract. The D to I transformation can be seen during the first 30 minutes of preincubation, although the I activity was already high (near 60 %) at 0 time of preincubation. The addition of ATP-Mg (up to 1-10 mM respectively) brought about a peculiar type of reaction. First the I activity was inactivated during the first 5 minutes after the addition as also was the D activity, but to a much lesser degree. The net result was a transformation of the I to the D form, with some inactivation of the D form also. During the next five minutes this change was more or less stable but afterwards the I activity began to reactivate while the D activity was inactivated, reaching levels near the I activity at the final point tested. It seems that, during this experiment, a multistep reaction was visualized that was initiated when the ATP-Mg was added and went through the following phases:

I activity ⟶ Inactive form ⟶
⟶ D activity ⟶ Inactive form

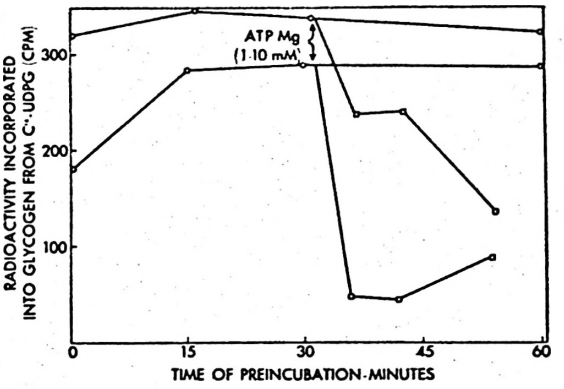

Fig. 5. *Intermediate steps during the transforming reactions of rat skeletal muscle glycogen synthetase systems.*
Enzyme extracts were an aliquot part of the extract shown in Figure 3 in which the $MgCl_2$ and NaF was omitted. In the ATP-Mg mixture the concentration of ATP was reduced to 1 mM but other conditions, intervals of preincubation and test of glycogen synthetase activity were the same.

The conditions for repeating this type of experiment were difficult as the balance between the concentrations of ATP and Mg^{2+} necessary to induce this trend of transformations, that could be analyzed during the time course of the preincubation period, varied from one crude extract to another. However, figure 6 reproduces a similar type of transformation obtained with another crude enzyme extract which reproduced the multistep reaction described in the above mentioned experiment. In this experiment the buffer was also devoid of NaF and $MgCl_2$ was not added either to the extract. The reaction was initiated by the addition of ATP-Mg (5 and 8 mM respectively) and the net final changes resemble very much those seen in the experiment of figure 4, but the sequential steps with the various kinds of transformations and inactivations could be visualized. Again the broken lines represent what happened with the addition of ATP alone to some aliquot part which demonstrates that the changes are induced by the combined action of ATP plus Mg^{2+}.

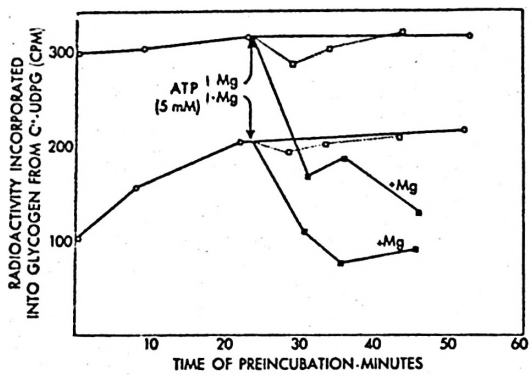

Fig. 6. *Sequential steps in the inactivating and transforming reactions of rat muscle glycogen synthetase.*

Enzyme extracts (1:10, w/v) were made in Tris-EDTA buffer. Mercaptoethanol up to 50 mM was added and the extracts were preincubated and tested for enzyme activity at the intervals shown. The addition of ATP (5 mM) with or without 8 mM $MgCl_2$ was made after the 30 minutes of preincubation. The meaning of the lines and symbols is the same as in Figure 4.

In vitro *inactivations induced by UTP-Mg.*

Some years ago ROSELL-PÉREZ and LARNER (12) showed that, in dog skeletal muscle, UTP-Mg could be more effective than ATP-Mg in decreasing the activity in the absence of glucose-6-P. However the UTP-Mg did not convert the I into the D form but produced a parallel decrease in the activity measured in the presence of glucose-6-P.

Figure 7 depicts what was observed after different amounts of UTP and Mg (8 mM) were added to a crude extract from rat skeletal muscle, compared with the effect produced by ATP-Mg (10-8 mM respectively) on the same enzyme crude extract. The extract had neither Mg^{2+} nor NaF added, and produced a good D to I form conversion during the first 30 minutes of preincubation. The additions were done on aliquot parts of the preincubated extracts. The addition of ATP-Mg mixture in these conditions, produced an inactivation time-dependent of the I activity with a parallel decrease in total activity, which seemed to be a selective inactivation of the I form. UTP-Mg produced what apparently were similar effects but more efficiently, that is to say, smaller concentrations had similar effects to that of ATP-Mg, and equimolar concentrations produced bigger effects than the ATP-Mg mixture, in such a way that the remaining activity after the addition of the highest concentration of UTP-Mg mixture (10-8 mM respectively) was exclusively glucose-6-phosphate dependent (D-form). It is necessary to emphasize here, that the UTP effect was not always time dependent, and that similar effects, although rather indiscriminate, could be observed by the addition of UTP alone to the extracts. The addition of ATP alone never succeeded in producing effects similar to those observed when added together with Mg^{2+} (Figs. 4 and 6), nor was Mg^{2+} alone responsible for such inactivations.

Influence of physiological metabolites on these transformations and inactivations.

As the extracts (1:10 w/v) represent a

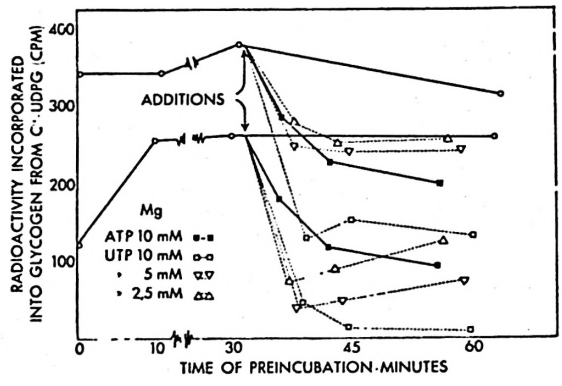

Fig. 7. *The action of UTP-Mg on the enzyme crude extracts of rat skeletal muscle glycogen synthetase.*

After the extracts were preincubated for 30 minutes different additions of ATP and UTP with 8 mM $MgCl_2$ were made and glycogen-synthetase activity with and without 10 mM glucose-6-P was measured at the intervals shown in the graph.

10 fold dilution of metabolites found in the cell sap, in addition to the disruption and desaggregation of cell microestructures and compartments, similar experiments were done supplementing the crude extracts with inorganic phosphate (up to 5 mM), glucose-6-phosphate (up to 5 mM), Mg^{2+} (up to 5 mM) separately or in any kind of combination. From all these data it could be deduced that only the concentrations of Mg^{+2} had influence in the rate and completness of the D to I form transformation reaction, and also in accentuating or balancing the selective inactivating reactions which also depended on the concentration of ATP used in each case.

Discussion

Recently it has been found in this laboratory (2) that the D-form (the only one present) of frog skeletal muscle could be inactivated by a reaction time and concentration of ATP-dependent, sensitive to the presence of 3', 5'-AMP and driven by the addition of ATP-Mg mixture to preincubating crude frog muscle extracts. During preincubation, prior to the addition of ATP-Mg it was also found that Mg ions stimulated both the appearance and the increase of this D activity (13, 14) and in addition increased 3-4 fold the activity of the D form of the enzyme without any preincubation (2). Mg ions also increased the activity of the 25-50 fold purified glycogen particulate enzyme preparation obtained after 100,000 × g centrifugation of crude extracts.

In the case of cardiac muscle extracts (16) and rat kidney enzyme preparations (8) ROSELL-PÉREZ and his collaborators found conditions to show *in vitro* inactivations of the D or I activities of glycogen synthetase from those sources. They were dependent on the addition of ATP-Mg to the preincubating extracts, dependent on time and sensitive to the cyclic nucleotide 3', 5'-AMP. These reactions could be reversed by arresting the protein kinase activity. The reverted reaction could no longer be seen if phosphoprotein-phosphatases were inhibited by the presence of NaF in the extracts.

All this data led us to postulate a multistep interconversion system between various forms of enzyme species of glycogen synthetase which differenciate from each other by different degrees of phosphorylation, some of them being active (the I or D forms) and some inactive, the transformations carried out by phosphoprotein phosphatases ($X' \rightarrow D \rightarrow X \rightarrow I$), or by protein kinases ($I \rightarrow X \rightarrow D \rightarrow X'$) (10, 16).

The data in the present paper seem to confirm the formation of some of these molecular species in the glycogen synthetase obtained from rat skeletal muscle. The inactivation of the I-form without being transformed into D or the inactivation of the D by what could be an extra-phosphorylation have been especially well demonstrated in the experimental data shown here.

The increases in D activity by preincubation of enzyme extracts in the presence of Mg^{2+} and mercaptoethanol have also been found by ASSAF and YUNIS (3) in the glycogen synthetase extracts from rat chloroma tumors. These authors also found a third form of glycogen synthetase that they call the D' form because it is active only in the presence of very high, unphysiological amounts of G-6-P (60 mM). This form was produced by addition of ATP-Mg to the preincubating cell extracts and would be similar to our X' form of rat cardiac muscle (16) kidney (8) or skeletal muscle of frog (2) and rat, as shown by the data presented here. Of course the D' form of ASSAF and YUNIS would be a completely inactive form in our conditions of assay.

Data in support of the presence of an inactive species of glycogen synthetase in rat liver extracts are found in the work of VARDANIS (20). During his NaF sensitive D to I conversion a substancial amount of

the D activity (that responding to the presence of 2 mM glucose-6-P) was also produced if the transformation took place at 0° C. He postulated an X (inactive form) even in the presence of glucose-6-P. On the other hand the existence of inactive precursor (S) of glycogen synthetase was postulated as early as 1962 by ROSELL-PÉREZ and LARNER (13).

Another type of data which appeared recently in the scientific press is of obvious interest relative to the possible existence of these inactive forms. The laboratories of Krebs and of Larner have been able to obtain data on the molecular weight and composition of the rabbit muscle glycogen synthetase.

KREBS' group have described (18) the enzyme I-form as a tetramer of sub-units of molecular weight 90,000-100,000. This would give a molecular weight for the I form of rabbit muscle of 360,000-400,000. They have also reported that during the D to I form transformation, brought about by a purified protein kinase, 1 mole of Pi was incorporated from ATP per 91,000 g of protein.

LARNER's group have reported (4, 17) a molecular weight for the D form of the rabbit muscle enzyme of 250-270,000, but the D or I purified forms preparations gave a single band of mobility by gel electrophoresis. The sub-unit had a molecular weight of 90,000 and they found that the D form has 6 mmoles of phosphate more than the I form per unit molecular weight or 90,000 mg. This would represent at least 18 mmoles of phosphate if the D form is a trimer or 24 if it is a tetramer, and differs widely from the data of KREBS' group. This is also a marked difference between the *a* and *b* forms of muscle phosphorylase in quantity of phosphate.

The data in LARNER's paper (4, 17) suggest the existence of a smaller sub-unit of molecular weight 13-15,000. As the sulfhydryl content of 6 mmoles/90,000 mg is similar to that of phosphate incorporated, and also because only one type of phosphorylated sequence was found by LARNER and SANGER (9), they feel that the phosphate groups occupy identical sites and that the small protein subunit of 15,000 is a component of that of 90,000. From these data it can be speculated that perhaps six small sub-units aggregate to form the 90,000 sub-unit.

The molecular weight for the rat muscle enzyme reported by STANELONI and PIRAS (19) is similar to that of rabbit muscle described by BROWN and LARNER (4). The former also showed data demonstrating aggregation-desaggregation phenomena produced by such factors as UDPG, UDP, glucose-6-P, ATP, AMP and KCl. With no effectors the enzyme preparations behave on sucrose gradient centrifugation as dimers of m.w. 186,000. UDPG and UDP favour trimerization or tetramerization, especially if glucose-6-P is present. ATP, AMP or KCl counteract the oligomerization produced by those effectors.

Aggregation-desaggregation phenomena have also been observed by SANADA and SEGAL (15) in the glycogen synthetase from rat liver. They concluded that either the *b* or the *a* forms of liver glycogen synthetase are capable of undergoing reversible conformational changes between active and inactive (or less active) states.

The inactivating reactions reported in this paper and those already described for other enzyme sources (5, 7, 8, 16) would be compatible with the structural profiles for the glycogen synthetase molecular species which are beginning to emerge from the efforts of different research teams. It seems that there are several phosphorylatable sites in the enzymic active molecular species. The incomplete phosphorylation of the I species, or at the wrong places, induced by the *p*H or ionic strength or cation influence, could lead to inactive (X) species that could be converted into complete D forms if the correct and adequate phosphorylation takes place. In the same way, extraphosphorylation of the D-conformed and phosphorylated species could

lead again to extraphosphorylated inactive forms (X') (10, 16).

In fact, the inhibitions described for ATP, metabolite that has been endowed with regulatory properties, could result from its binding at the different phosphorylating sites of the sub-units.

It must be borne in mind that glycogen synthetase must have, as phosphorylase has, several important sites in its molecule, which must be repeated in each allosteric protomer: *a*) Active center, where UDPG and the end branches of glycogen must fit, and where it is probable that UDP can produce some effect. *b*) The site for glucose-6-P, at least one per sub-unit 90,000. *c*) Several serine sites where phosphorylation takes place. It seems very likely that at least six (if not more) must exist per sub-unit of 90,000. *d*) One or two sites or zones of aggregation that can be identical or slightly different for the I or D molecular species, and that also can be different if dimer, trimer or tetramer species could exist.

Effectors such as glucose-6-P or inorganic phosphate do not seem to play a role during the inactivating reactions described here. The inactivation of the protein-phosphatase systems, or the conformational changes produced by the presence of cations such as Mg^{2+} may have much more influence in these *in vitro* inactivations produced or induced by ATP-Mg.

It is not yet known if these *in vitro* observed reactions can have a physiological meaning *in vivo*, but there is no doubt that, if produced, they represent a complete shut-off mechanism for the biosynthetic pathway of glycogen formation, that would avoid the futile recycling that would take place albeit slowly, even when the glycogen synthetase would be in the D form.

Resumen

La inactivación de la actividad glucógeno-sintetásica en sus formas D o I (dependiente o independiente de glucosa-6-fosfato) ha sido demostrada en este laboratorio para los enzimas procedentes de músculo esquelético de rana, corazón y riñón de rata y también para los de leucocitos y linfocitos humanos. Para explicar esos fenómenos se sugirió un esquema de interconversiones metabólicas de las diversas formas de la glucógeno sintetasa en el que coexistirían junto a formas completamente activas (forma I) o menos activas (formas D), otras totalmente inactivas (formas X) con diverso grado de fosforilización (10).

En este trabajo se muestran datos experimentales que demuestran la posibilidad de inactivar selectivamente, bien sea las formas I o las D, de la glucógeno sintetasa de músculo esquelético de rata por reacciones provocadas por la adición a los extractos enzimáticos de diversas mezclas de ATP-Mg. Ninguno de estos fenómenos de inactivación puede atribuirse al Mg^{2+} o al ATP independientemente, por lo que deben ser atribuidos a reacciones fosforilantes llevadas a cabo por protein-cinasas.

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