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On the Process of Activation and Inactivation of Dog Skeletal Muscle Glycogen Synthase *

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The presence of additional forms of glycogen synthase (UDPG: α-1,4-glucan α-4-glucosyltransferase) besides the I form (independent on glucose-6-P for activity) and the D form (dependent on glucose-6-P for activity) long ago described, is inferred from patterns of their interconversions obtained by processes of phosphorylation and dephosphorylation.

An intermediate form more phosphorylated than the I form and less than the D form, which is completely inactive in these assay conditions, and a superphosphorylated form, more phosphorylated than the D form and also inactive even in presence of 0.01 M glucose-6-P are described.

Glycogen synthase (UDPG: α-1,4-glucan α-4-glucosyltransferase; E.C. 2.4.1.11) is an interconvertible enzyme whose forms were described from the beginning as the I form (independent of glucose-6-P for activity) (1) and the D form (dependent of glucose-6-P for activity) (20). It is now well established that both forms are interconverted in a phosphorylation-dephosphorylation reaction, sequence catalyzed by a set of primary regulatory enzymes (6, 19, 21, 22).

ROSELL-PÉREZ and associates have found, while studying several aspects of glycogen synthesis, a considerable amount of data that suggests the presence of more than two forms of glycogen synthase. More especifically a form that seems to be more phosphorylated than the I form and less than the D form and for that reason called intermediate form, and anoth-

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er form which seems to be more phosphorylated than the D form and thus called superphosphorylated form. Both forms would be inactive in the regular assay conditions of the enzyme and their presence has been inferred from interconversion experiments with frog muscle (2, 20), dog skeletal muscle (4, 21), rat heart muscle (23), rat kidney (9), rat skeletal muscle (12), human lymphocytes (7), and leukocytes (5).

On the basis of all these data ROSELL-PÉREZ has proposed and enlargement of the initial scheme of the several forms of glycogen synthase and their interconversions (18). The scheme shown in figure 1, will use the same enzymatic mechanisms which, through a sequence of several steps of phospho and dephosphorylation would explain the existence of the inactive forms mentioned.

The existence of additional forms of glycogen synthase has also been reported by other laboratories (1, 3, 10) and lately work from ROACH and LARNER (15) has shown that each subunit of glycogen synthase may undergo multiple phosphorylation, thus modifiying the enzymatic activity and the phsiological role of the enzyme.

In this report we present experiments of interconversion performed with dog skeletal muscle extracts that can be interpreted as showing further evidence of the presence of the already described intermediate and superphosphorylated forms of glycogen synthase.

Materials and Methods

Male dogs with free access to food and water were killed by intravenous administration of 1 g sodium seconal and a necropsy from the hind legs was immediately taken, cut in small pieces and stored in plastic bags at —25° C.

Crude extracts were prepared by mechanical disruption (Potter-Elvehem) of the muscle in 0.05 M Tris-0.005 M EDTA buffer pH 7.8 containing mercaptoethanol (0.05 M), at a 1/7 w/v proportion and subsequent centrifugation at 3,000 × *g* during 10 minutes in order to remove the cell debris. Samples obtained by this procedure averaged 5 to 7 mg/ml of protein as measured by the method of LOWRY *et al.* (11).

Glycogen synthase activity was measured by the method of THOMAS *et al.* (25) using 10 minutes incubation time at 30° C for the regular assay and addition of glucose-6-P to 0.01 M concentration when the total activity (I + D) had to be recorded. Linearity of the assay over a broad range of protein concentration and up to 20 minutes of incubation time was checked as well as the influence of the incubation time on the interconversion reactions carried over on the preincubation time, which appeared to be negligible.

The interconversions were analyzed measuring the I form and total (I + D) activities of aliquot samples withdrawn at the indicated times from an extract that was being preincubated at 30° C. For phosphorylation conditions ATP and Mg^{++} were added to the extracts at the concentrations and times indicated.

Tris-hydroximethyl-aminomethane, UDP-glucose, ATP, glucose-6-P, glycogen and 3',5'-AMP were obtained from Sigma

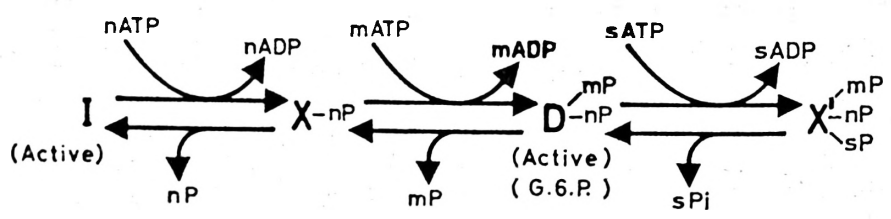

Fig. 1. *Proposed scheme for the interconversion reactions of glycogen synthase.* See text for explanations.

Chemical Co. Mercaptoethanol was obtained from Eastman Kodak and ^{14}C uniformly labeled glucose UDP-glucose from Amersham Radiochemicals. Other Chemicals were analytical grade from Merck.

Results and Discussion

Dephosphorylation of an inactive intermediate form of glycogen synthase. The behaviour depicted in figure 2 has been observed often when an extract is preincubated in the presence of 0.05 M mercaptoethanol in order to have a high level of I form activity. Both activities increase in the first minutes of reaction, until the total activity reaches a plateau while the I form activity keeps increasing at a somewhat slower rate.

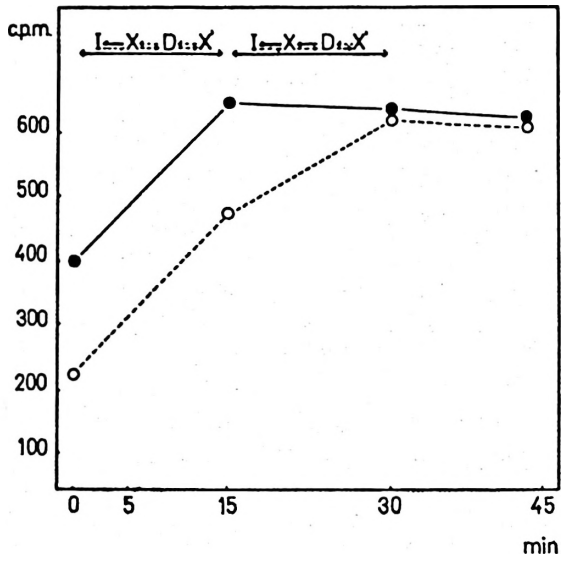

Fig. 2. *Dephosphorylation of an inactive intermediate form of glycogen synthase.* The time course of conversion is shown for an enzyme preparation containing 0.05 M mercaptoethanol which is beeing preincubated al 30° C. Solid lines and closed circles represent the activity measured in presence of glucose-6-P (0.01 M) and broken lines and closed circles the activity measured in absence of glucose-6-P. The scheme on the top corresponds to the one presented in figure 1 and shows the main conversion taking place at the time indicated by the horizontal arrows.

The parallel increase in both activities during the first minutes of incubation may be interpreted as a activation of an inactive form to the I form in a dephosphorylation reaction process that would account for the parallel increase in total activity. When the inactive molecular species of the enzyme being converted is exhausted, the increase in total activity stops. At the same time the I form activity keeps increasing as the D form molecules are dephosphorylated to I form molecules, presumably through the inactive intermediate state, until complete conversion is accomplished.

In experiments with dog muscle presented here, the conversion of I form to an inactive intermediate, less phosphorylated than the D form, seems to occur as in many other tissues, by means of a phosphorylation reaction that will be shown later. But here the glycogen synthase phosphatase of dog muscle being particularly active, it has been possible to observe the reverse conversion that would mean, the dephosphorylation of the intermediate to a I form molecular species. This reversibility of the phenomenon would endow the system with more mobility and would further evidence its functionality and versatility.

It is also possible to observe the conversion of the intermediate to the D form and the D form to an inactive superphosphorylated form in a phosphorylation reaction, but in neither of these two last instances the reverse reaction has been observed experimentally.

Phosphorylation of I form activity through the inactive intermediate. The addition of ATP and Mg^{++} to extracts with a high ratio I to total activity promoted an immediate I to D form conversion with a rate proportional to the amount of ATP added and significantly stimulated by cyclic AMP at a 10^{-5} M concentration

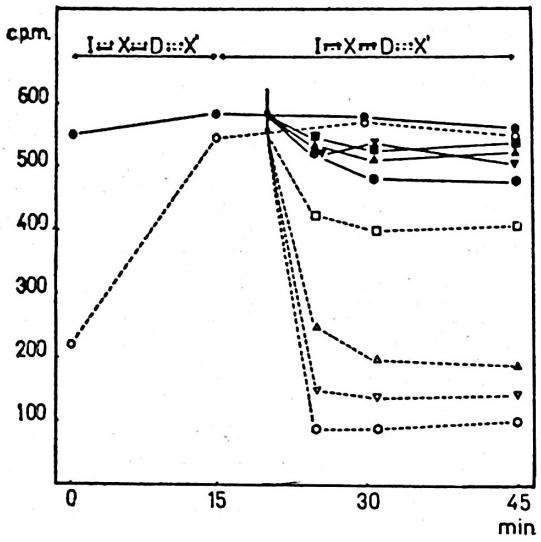

Fig. 3. *I form to D form activity conversion.* The time course of the conversion before and after addition of several concentrations of ATP-Mg^{++} at the time indicated by the arrow is shown for a preparation containing 0.05 M mercaptoethanol. Solid lines and closed symbols represent the activity measured in presence of glucose-6-P (0.01 M); broken lines and open symbols the activity measured without glucose-6-P. (●○) control, (■□) ATP 0.002 M-Mg^{++}, (▲△) ATP 0.005 M-Mg^{++} 0.005 M (▼▽) ATP 0.010 M-Mg^{++} 0.002 M, (⬟⬠) ATP 0.005 M-Mg^{++} 0.005 M-3',5'-AMP 2.0×10^{-4} M. For the scheme on the top see figure 2.

as described by ROSELL-PÉREZ and LARNE, (21).

Figure 3 shows such an experiment performed in conditions that, according to the late model of glycogen synthase interconversions (18), would be interpreted as a phosphorylation of the I form molecules to the D form molecules through the intermediate state of phosphorylation. This could be carried out by the common protein kinase (24) or perhaps by other kinases (13). Nevertheless in some unpredictable circumstances a different pattern is obtained in which both I and total activities decrease simultaneously showing in some instances a complete inactivation of both activities that results in an absolute desapearance of any glycogen synthase activity from the assay (figure 4).

Such inactivation may be interpreted as a conversion by phosphorylation of I form molecules to an inactive form which would not be recorded in the assay even in the presence of 1.0×10^{-2} M glucose-6-P, thus being different from the D form.

It is not known the exact situations and conditions in which each interconversion takes place. One can speculate that the rates of each step in the sequential process of interconversion will depend on the relative proportions of each form present and also on the disponibility of many metabolites affecting the phosphorylation and dephosphorylation reactions. One has to keep in mind that the occurrence of one

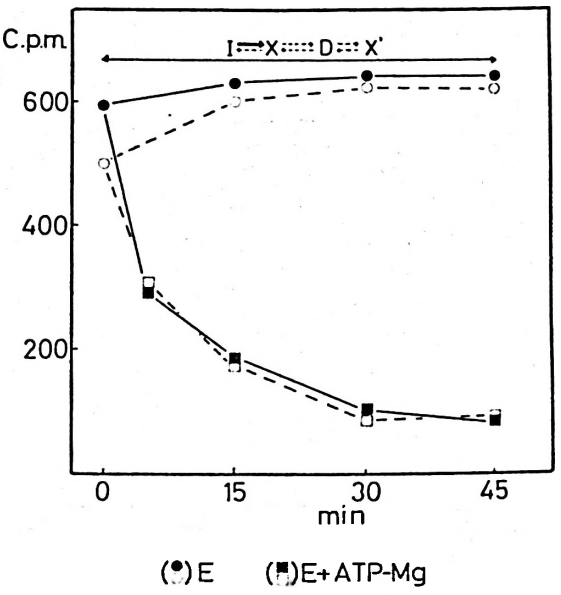

Fig. 4. *Phosphorylation of the I form to an Inactive Intermediate.* The time course of this conversion is shown for a preparation with high percentage of I form activity. Solid lines and closed symbols represent the activity measured in presence of glucose-6-P (0.01 M); broken lines and open symbols the activity measured without glucose-6-P. Circles represent the control where no ATP-Mg^{++} was added and squares represent activities after addition of ATP 0.005 M-Mg^{++} 0.005 M. For the scheme on the top see fig. 2.

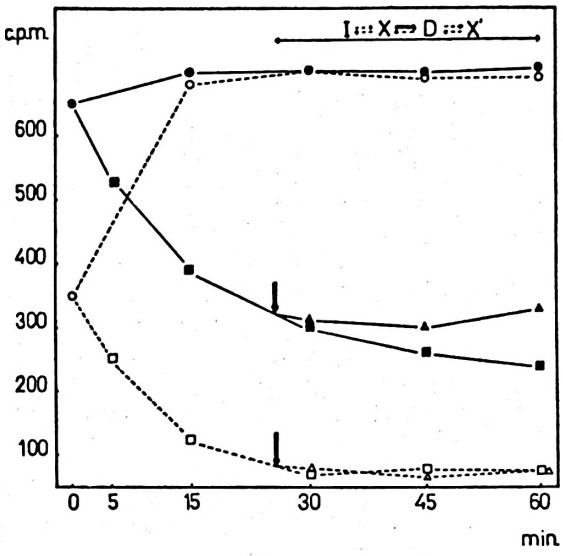

Fig. 5. *Phosphorylation of the intermediate form to D form activity.*
The time course of this interconversion is shown for a preparation to which a first pulse of ATP 0.005 M-Mg^{++} 0.005 M was added a time cero. A second pulse was added at the time indicated by the arrow. Solid lines and closed symbols represent the activity measured in presence of glucose-6-P (0.010 M); broken lines and open symbols the activity measured without glucose-6-P. Circles represent control activity, squares activity after first addition and triangles activity after second addition. For the scheme on the top see figure 2.

type of conversion does not necessarily mean that other types of conversions are completely turned off. Consequently what is seen in our experimental conditions is the result of the summation of all reactions taking place in a specific physiological situation which will be the overall conversion leading to a new situation in response to the physiological requirements.

The possibility of both primary regulatory enzymes acting simultaneously though coordinately regulated cannot be discarded. In this sense reports from other authors (8) seem to admit the existence of «futile cycles» such as the one that could possibly work between the phospho and dephosphorylated forms of glycogen synthase and its benefit for the regulation of the cell metabolism.

Further phosphorylation of the inactive intermediate X (figure 1) would yield a molecular species that would correspond to the D form activity. This seems indeed to be the explanation for experiments such as that shown in figure 5, where the addition of a second pulse of ATP to an extract had already phosphorylated all molecules of I form results in an increase of the total activity rather than in a further decrease while the I form activity remains unchanged.

Phosphorylation of D form activity to a superphosphorylated form of glycogen synthase. Another pattern of interconversion was observed when a second pulse of ATP was given to a preparation with a low I to total activity ratio obtained by the effect of a first pulse of ATP.

Figure 6 shows a first step phosphorylation interpreted as a clasical conversion of total to I form activity followed by a second step, triggered by the second addition of ATP, in which the completely inactivated I form remains virtually inactive and the D form is inactivated in turn.

This second step phosphorylation may be interpreted as a conversion of the D form to a more phosphorylated molecular species, inactive even in presence of 1×10^{-2} M glucose-6-P responsible for the complete disappearance of the glucogen synthase activity in the assay.

The sequential phosphorylation of a multiple phosphorus site protein and the concomitant changes in enzyme activity offer a model with wider possibilities in the regulation of the biochemical functions performed by the system and since it uses the same enzymatic equipment and therefore the same genetic information, may present some evolutionary advantages.

One may speculate on the possibility of the inactive forms of the enzyme being a reservoir of the enzymatic protein that would be activated and inactivated in

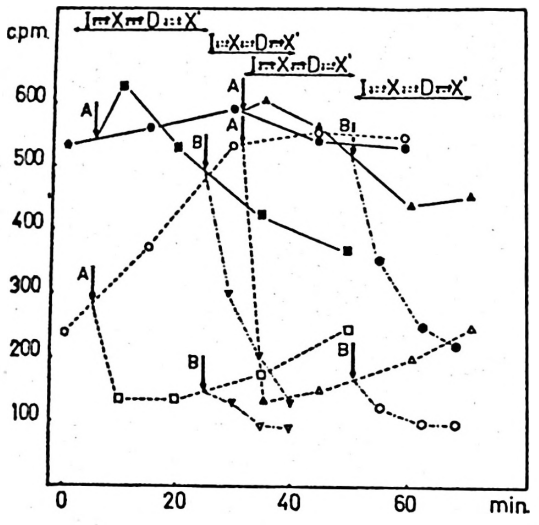

Fig. 6. *Superphosphorylation.*
The time course of the conversion of the D form to an inactive superphosphorylated form is shown for a preparation containing 0.05 M mercaptoethanol. The preparation was first deprived of I form activity by addition of ATP 0.005 M-Mg^{++} 0.005 M (arrows A) and after 20 minutes, when the I form was completely converted to D form, a second pulse of ATP-Mg^{++} at the same concentrations was added. The experiment was performed in the same preparation having low I form activity and high I form activity as can be seen in the graph. The solid lines and closed symbols represent the activity measured in presence of glucose-6-P (0.01 M); the broken lines and open symbols the activity without glucose-6-P. Circles represent the control where no additions were made and the rest of the symbols the activities after additions as indicated. The schemes on the top correspond to the one presented in figure 1 and show the main conversion taking place at the time indicated by the corresponding arrow.

more rapid way than if it had to be synthesized *de novo* or degraded in response to hormonal and non hormonal stimulus and giving in this way a more rapid physiological response too.

It is now possible to manipulate the purified glycogen synthase and its primary regulatory enzymes, that would permit a molecular approach in the study of the system, such as the one presented by LARNER and associates (14, 16, 17), with which experiments the scheme of several step phosphorylation of glycogen synthase presented here seems to be in good agreement.

Resumen

Se describe la existencia de estados de fosforilación de la Glucógeno sintasa (UDPG: α-1,4-glucan α-4-glucosiltransferasa) distintos de los correspondientes a las dos formas conocidas: forma I (independiente de glucosa-6-P para su actividad) y forma D (dependiente de glucosa-6-P para su actividad).

Se presentan experiencias que indican la existencia de una forma intermediaria entre la forma I y la forma D en cuanto a su grado de fosforilación y que resulta inactiva en las condiciones de ensayo. Ello se infiere a partir de experimentos de interconversión entre forma I y forma D tanto por actuación de la fosfatasa en la reacción de defosforilación como por actuación de la kinasa en la reacción de fosforilación. En ciertas condiciones ambos tipos de transformaciones (forma D a forma I o forma I a forma D) presentan comportamientos sólo explicables por la existencia de un intermediario como el que se describe.

Igualmente se presenta cierta evidencia sobre una forma de glucógeno sintasa más fosforilada que la forma D y por ello llamada superfosforilado, que resultaría también inactiva. Dicha forma parecería explicar experiencias en las que por actuación de la kinasa sobre preparaciones con la totalidad del enzima en forma D, ésta desaparece del ensayo no modificándose la forma I.

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