Regulation of Rat Liver Glycogen Synthetase

RELATIONSHIP OF THE HORMONAL ACTIVATION AND THE TIME-DEPENDENT IN VITRO ACTIVATION*

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SUMMARY

The activation of glycogen synthetase by insulin proceeds via the transformation of the less active D form to the active I form, with essentially no change in total activity. Although the enhancement of glycogen deposition has also been attributed to glucocorticoid activation of glycogen synthetase, insulin-induced activation is counteracted by hydrocortisone in diabetic and starved rats. The time-dependent activation of glycogen synthetase in crude liver extracts, which has been used as a model for activation of the enzyme by various agents, has now been demonstrated with more purified preparations. With the purified system the characteristics of the in vitro activation have been compared to the in vivo hormonally induced activation. In contrast to the in vivo case, the total activity of glycogen synthetase increased during activation in vitro in addition to the increase in the I activity. The effect of various cellular metabolites on the in vitro activation kinetics is profoundly altered by EDTA. Certain properties of the synthetase system, such as the ratio of D to I form, the $K_m$ for UDP-glucose, and the in vitro activation, are temperature-dependent.

Uridine diphosphate glucose:α-1,4-glucosyltransferase (EC 2.4.1.11) (glycogen synthetase), the rate-limiting enzyme for liver glycogen synthesis occurs in two forms: a less active form dependent on glucose-6-P (D form) and an active form independent of glucose-6-P (I form) (1-6). Activation of the enzyme by the conversion of the D to the I form is one of the early and biochemically important effects of insulin (7-12). In different biological systems, the ratio of the two activities varies markedly (13). Enzymatic modification of glycogen synthetase via phosphorylation and dephosphorylation, under endocrine control, is thought to be an important mechanism regulating hepatic glycogen synthesis (9, 10). Another mechanism, referred to as "metabolite control" has been studied in tadpole liver in vitro (4), rat liver (14), muscle (15), adrenal glands (16), and yeast (17). This control mechanism is based on the effect of fluctuations in the concentrations of cellular activators, inhibitors and substrates on the normally occurring enzyme forms. Thus, regulation of glycogen synthetase encompasses two important control mechanisms: (a) endocrine regulation of an enzyme occurring in interconvertible forms, and (b) metabolite regulation of an allosteric enzyme. However, since the cellular concentrations of UDP-glucose and glucose-6-P are generally too low to allow activity of the D form, the insulin-induced activation of the enzyme by conversion of the D to the I form is probably necessary for the enhancement of glycogen deposition (18).

The exact mechanism of hormonal regulation of the enzyme transformation in liver is still not clear. In addition to insulin (4, 7-12, 19), it has also been suggested that hydrocortisone or glucose or both are the agents responsible for enzyme activation (20-22). However, many factors can mimic the effects of insulin in the intact animal by sequences of reactions which lead to the release of insulin from the pancreas (4, 23, 24). Certainly, the activation of glycogen synthetase by insulin and hydrocortisone, hormones which normally exert opposite effects on carbohydrate metabolism, merits further clarification. It has been shown that hydrocortisone activation of glycogen synthetase is mediated by insulin in adrenalectomized, diabetic rats (6). In a minced tadpole liver system (4) hydrocortisone actually blocked insulin-induced activation. In this communication, it will be shown that hydrocortisone also blocks activation of glycogen synthetase in rat liver under certain conditions.

The study of the activation of glycogen synthetase by various agents has also been approached through the use of the time-dependent activation system introduced by Gold and Segal (25). It has been suggested that activation of the enzyme in this system is analogous to the in vivo hormonal activation (14). However, the in vitro activation, studied in essentially crude liver extracts, has not been well characterized, nor has the relationship between the in vitro and in vivo activation been rigorously examined. In this communication, we report the characterization of the in vitro activation in a purified preparation and compare the hormonally induced activation to the in vitro activation.

MATERIALS AND METHODS

Glucose-6-P, UDP-glucose, and other nucleotides were purchased from Nutritional Biochemicals. UDP-[U-14C]glucose...
used (Fig. 1C). Following insulin administration, the level of insulin and hydrocortisone, with respect to glycogen stimulation of I activity did not occur. This antagonism between the steroid hormone and insulin treatment glycogen synthetase activity, in the absence of glucose-6-P, was undetectable and remained below the control level throughout the experiment. It has been suggested that hydrocortisone stimulation of glycogen synthetase in diabetic, adrenalectomized animals requires a longer time period than in nondiabetics (21). However, no activation was observed as long as 10 hours after administration of hydrocortisone to diabetic rats. When hydrocortisone was administered with insulin, enzyme activation was blocked. Although 1 hour after hormone treatment the enzyme activity in animals given both hormones was about the same as in those receiving only insulin, by 3 hours the level of activity in those rats treated with both hormones remained at about the level of the untreated animals.

These experiments indicate that insulin is the primary hormone regulating glycogen synthetase. The effect of insulin can be demonstrated in fed or starved normal animals as well as in diabetics. Hydrocortisone, on the other hand, did not activate glycogen synthetase under these conditions. Although the stimulation of hepatic glycogen deposition in the rat has been ascribed to a steroid-induced transformation of D to I form (20, 21), it appears more likely that the apparent steroid effect is indirect; that is, the gluconeogenic hormone induces a hyperglycemic condition which leads to the release of insulin. Hepatic glycogenesis is then stimulated by the insulin-induced D to I transformation of glycogen synthetase. The failure of hydrocortisone to activate glycogen synthetase, reported here, confirms the results of Kreutner and Goldberg's experiments with adrenalectomized, diabetic rats (6).

Although hydrocortisone alone had no significant effect on glycogen synthetase in normal fed rats, administration of the steroid hormone with insulin enhanced the D to I transformation. As shown in Fig. 2, 3 hours after hormone treatment the I activity in groups of animals receiving both hormones was greater than in those receiving only insulin. The total glycogen synthetase activity did not change significantly with any of the hormone treatments.

**Effect of Insulin on in Vitro Activation**—It has recently been suggested that hydrocortisone is necessary for the maintenance of the enzyme system which activates glycogen synthetase (20). This conclusion was reached with the use of an in vitro system in which a time-dependent activation of glycogen synthetase in crude rat liver homogenates is observed. This system has been exploited as a model for the activation of glycogen synthetase by various agents (14, 18, 20, 21). It has been suggested that the in vitro activation is analogous to the hormonally induced D to I conversion in vivo (14). Thus, it is important to further characterize this in vitro system and its relationship to the hormonally induced activation.

As shown in Fig. 3A, no difference in the activation pattern was found when homogenates from control and insulin-treated rats were incubated and assayed at 25°C. In agreement with previous reports (18, 25, 27, 28), there is an apparent lag phase before glycogen synthetase activation is observed. Activation in either homogenate is essentially complete after 60 min of incubation at 25°C. During in vitro activation both the I and the total activity increased about 4-fold, but the extent of activa-
Fig. 1. Effect of insulin and hydrocortisone on glycogen synthetase. Rats were injected intraperitoneally with insulin (•), hydrocortisone (□), or both hormones (▲), killed at the times indicated, and the livers were removed. Glycogen synthetase was assayed at 37°C in the crude homogenate with (A', B', C') or without (A, B, C) glucose-6-P. The following dosages (per 100 g of body weight) were used: A, normal fed rats, insulin, 1.2 i.u., hydrocortisone, 1 mg; B, starved rats, insulin, 1.2 i.u., hydrocortisone, 2 mg; C, diabetic rats, insulin, 4.8 i.u., hydrocortisone, 2 mg.

Fig. 2. Effect of insulin and hydrocortisone on normal, fed rats. Hormones were administered to normal, fed rats 1 or 3 hours prior to removal of the livers. Glycogen synthetase was assayed at 37°C in the crude homogenate without (A) or with (B) glucose-6-P. The following dosages (per 100 g of body weight) were used: a, insulin, 1.2 i.u.; b, hydrocortisone, 2 mg; c, insulin, 1.2 i.u., plus hydrocortisone, 1 mg; d, insulin, 1.2 i.u., plus hydrocortisone, 0.5 mg; e, insulin, 1.2 i.u., plus hydrocortisone, 2 mg.

Fig. 3. Effect of incubation at 25°C on glycogen synthetase from insulin-treated and control animals. Normal, fed rats were injected with insulin (1.2 i.u./100 g, body weight) 3 hours prior to removal of the liver. Controls received only the carrier solution. The enzyme was incubated in a 25°C water bath and samples were taken every 20 min. Glycogen synthetase was assayed at 37°C for 10 min. ——, insulin treated, with (●) glucose-6-P; — —, control, with (□) or without (●) glucose-6-P. Experiment A, crude enzyme; Experiment B, purified enzyme.

The apparent deviation from the postulated D to I conversion varies in different preparations. This contrasts with insulin-induced activation in vivo in which total activity remains unchanged while the activity of the I form increases about 2- to 3-fold; a pattern consistent with the proposed D to I conversion. However, the data obtained with the in vitro system differ from the hormonally induced D to I conversion.

The apparent deviation from the postulated D to I conversion has been explained on the assumption that the increase in total activity is due to the differential effect, on the I form, of metabolites, such as inorganic phosphate, formed during the incubation period (27). Therefore, to characterize the in vitro activation it was necessary to develop a system in which the
Fig. 4. Effect of incubation at 25° on glycogen synthetase from diabetic rats. Diabetic rats were injected with either carrier solution (A) or insulin, 4.8 i.u. per 100 g of body weight (B) 3 hours prior to removal of the livers. Partially purified enzyme was incubated in a 25° water bath and samples were taken every 20 min. Glycogen synthetase was assayed with (O—O) or without (●—●) glucose-6-P, at 25°, for 10 min. The activity in crude homogenate assayed without glucose-6-P, before (□) and after (●) 60 min of incubation at 25° is indicated.

Fig. 5. Effect of assay temperature on in vitro activation pattern of glycogen synthetase. Partially purified glycogen synthetase in glycylglycine buffer with 10 mM EDTA was incubated at 25°. Aliquots were removed at the time intervals indicated and assayed at either 37° for 2 min (A) or 25° for 5 min (B). I activity, O—O; D activity, △—△; D + I activity, ○—○.

effect of such metabolites and other effectors could be examined individually.

Effect of Insulin on Activation of Glycogen Synthetase in Purified Preparations—Since many factors such as metal ions, salts, nucleotides, glucose, etc. (14, 20, 27–29) reportedly affect the

glycogen synthetase system, the in vitro activation and the effect of insulin were re-examined in a more purified system. The activation observed with the partially purified enzyme was similar to that of the crude extract. However, the lag phase usually observed with crude preparations is eliminated in the purified preparations (Fig. 3B). The activation kinetics of purified enzyme from untreated and insulin-treated rats were essentially the same. In both cases, the total activity increased in addition to the increase of I activity.

In Vitro Activation of Glycogen Synthetase from Diabetic Rats—The in vitro activation was also examined in extracts from diabetic rats since a deficiency of the activating system has been reported in the case of diabetic (14) and starved, adrenal-ectomized animals (20). In the diabetic rat, although almost no activation of the I form was noted in the crude extract, significant activation was obtained with the purified preparation (Fig. 4A). If diabetic rats were treated with insulin 3 hours prior to the experiment (Fig. 4B), activation of the enzyme was obtained with the crude homogenate as well as with the partially
pursed preparation. The initial activation kinetics of purified preparations from untreated and insulin-treated diabetic rats are similar, but the extent of activation is greater in the preparation from the insulin-treated rats. Again, during activation in vivo the total activity and the I activity increased.

One explanation of these observations is that insulin may affect the amount of synthetase that can be activated rather than some component responsible for the activation. This interpretation may have some relevance to the observation made by Steiner and King that insulin induces the synthesis of glycogen synthetase (30). It is also possible that, in addition to changes in the proteins involved, metabolites or inhibitors present in the diabetic liver may affect the activation.

**Table I**

**Effect of assay temperature on glycogen synthetase D and I activities**

Glycogen synthetase activity was assayed either with or without glucose-6-P in the crude homogenate and in the partially purified preparation as described under "Materials and Methods." The enzyme was incubated for 5 min at the temperatures indicated below.

<table>
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<tr>
<th>Sample</th>
<th>Temperature</th>
<th>Glycogen synthetase activity</th>
<th>I</th>
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As shown in Table I, the expression of the D and I activities is dependent on temperature and stage of purification. At lower

**Fig. 7. Double reciprocal plot for UDP-glucose concentration and activity of glycogen synthetase.** Partially purified enzyme in glycylglycine buffer with 10 mM EDTA was used. Enzyme activity was measured at 37° for 2 min (○) or at 25° for 5 min (△) at the UDP-glucose concentrations indicated. The double reciprocal plots for: A, the I form as measured in the absence of glucose-6-P; B, the D form, calculated from the difference between the activity with and without glucose-6-P; C, total glycogen synthetase activity measured in the presence of glucose-6-P. (ε = micromoles of [14C]glucose incorporated into glycogen from UDP-[14C]glucose in 5 min at 25° or 2 min at 37°.)

**Effect of Temperature on Glycogen Synthetase**—We have found that the D form of glycogen synthetase is extremely temperature-dependent for the expression of its activity. Although there have not been any systematic studies of the effect of temperature on glycogen synthetase, temperatures of 0–37° have been used for the assay of glycogen synthetase by different investigators (15, 25, 27). As shown in Fig. 5, the activation pattern is similar if glycogen synthetase is assayed at 25° or 37°. In all preparations, increases in the I and total activity were noted in either case. The D activity either remained the same or increased slightly. When assayed at 37°, the D form is the preponderant species (about 90%); however, at 25°, the D form is generally 50%, or less, of the total activity.

The activation in liver extracts was also temperature-dependent. Surprisingly, the activation was not directly correlated with temperature, that is, increasing the temperature did not necessarily result in an increased rate of activation. As shown in Fig. 6A, if the incubation temperature was 37° and the aliquots were assayed at 25°, a rapid loss of activity was noted. When the crude homogenate was incubated at 30° and assayed at 25°, no activation was observed (Fig. 6B). When both incubation and assay were conducted at 25°, the usual activation kinetics were observed (Fig. 6C). The effect of incubation temperature on the activation of the partially purified enzyme was somewhat different. Incubation at 37° resulted in the rapid loss of the total activity, while the I activity was slightly activated (Fig. 6D). The total and the I activity were both activated when incubation was carried out at 30° and aliquots were assayed at 25° (Fig. 6E). The usual activation kinetics were obtained when the incubation temperature was 25° and the assay was performed at 25° (Fig. 6F).

As shown in Table I, the expression of the D and I activities is dependent on temperature and stage of purification. At lower
Effect of Temperature on $K_m$ of Glycogen Synthetase for UDP-Glucose—The $K_m$ for UDP-glucose was determined at 25° and 37°, as shown in Fig. 7. In the presence of glucose-6-P, normal double reciprocal plots were obtained for the D activity and the total activity at both temperatures. Normal Michaelis-Menten kinetics were obtained for the I form, in the absence of glucose-6-P, at 25°. However, the $K_m$ for UDP-glucose could not be determined at 37° in the absence of glucose-6-P since saturation with respect to UDP-glucose is not reached at reasonable substrate concentrations. At 37° the line intersects the y axis to the right. That is, there is a greater increase in velocity at higher UDP glucose concentrations than would be predicted by the simple kinetic equation. This deviation from normal kinetics may indicate a substrate-induced activation of the I form which is only observed at 37°.

It has been emphasized (18) that meaningful values for the relative D and I activities can only be obtained with rather high concentrations of substrate and activator. The failure to use saturating concentrations of glucose-6-P and UDP-glucose in the assay of glycogen synthetase might lead to activation curves with a spurious increase in total activity. Therefore, although the concentration of UDP-glucose in our assay is already saturating, experiments were conducted with UDP-glucose concentrations as high as 12 mM in the assay system. The activation kinetics observed were the same whether the enzyme activity was assayed with 2 or 12 mM UDP-glucose. Increases in both the I and the total activities still obtained. Furthermore, the percentage of the activity in the I form was not significantly altered by the 6-fold increase in the substrate concentration used in the assay. The concentration of glucose-6-P, 20 mM, is well above the $K_m$ for either the nonactivated form (2 mM) or the in vitro activated form (0.06 mM) reported by Mersmann and Segal (18). Therefore, the increase in total activity during in vitro activation does not appear to be dependent on the substrate and activator concentrations used in the assay system.

Effect of in Vitro Activation on $K_m$ for UDP-Glucose—The apparent increase in total activity during in vitro activation has been explained as a reflection of the differential effect of metabolites, formed during incubation of the crude homogenate, on the D and I activities (27). This interpretation of the data is not applicable to the increase in total activity observed with the purified system. An alternative hypothesis is that the apparent increase is due to the conversion of the I form into a more active form during incubation. This possibility was examined by determining the $K_m$ for UDP-glucose before and after in vitro activation. A fresh partially purified preparation and the same preparation after 50 min of incubation at either 0° or 25° were used. As shown in Fig. 8, the $K_m$ and $V_{max}$ were the same for the fresh preparation and the sample kept at 0° for 50 min, in the
Fig. 10. Effect of EDTA on in vitro activation of glycogen synthetase. Glycogen pellets from rat liver homogenates prepared in glycylglycine buffer were resuspended in either the same buffer (A) or in buffer with 10 mM EDTA (D). Similarly, glycogen pellets from homogenates prepared in buffer with EDTA were resuspended in either the same buffer (C) or buffer without EDTA (B). The partially purified enzyme preparations were incubated at 25°C. Aliquots were removed at the time intervals indicated and assayed with or without glucose-6-P. I activity, O--O; D activity, A--A; D + I, O--O.

These observations suggest two possibilities: (a) a time- and temperature-dependent conformational change leading to a more active form of the enzyme, or (b) a third form of glycogen synthetase which can be activated in the in vitro system. In the case of UDP-glucose pyrophosphorylase, a time-dependent increase in activity was also accompanied by an increase in Vₘₐₓ with no change in the Kₘ. Fitzgerald, Chen, and Ebner (31) suggested the increased enzyme activity was due to a slow, time-dependent change in enzyme structure which led to the production of a more active form of the enzyme.

Effect of EDTA and Cellular Metabolites on in Vitro Activation—EDTA has a profound effect on the in vitro activation in the purified system and on the effect exerted by various metabolites on the activation process. As shown in Fig. 9A, no activation occurs in the purified system when glycylglycine buffer, with no further additions, is used. However, incubation of the enzyme with AMP or Mg²⁺, resulted in enzyme activation. Glucose, which reportedly enhances activation of Sephadex-treated homogenates (28), did not promote activation in the purified system. This suggests that some metabolite of glucose, formed during incubation in the crude system, may have affected the activation rather than glucose per se. If buffer containing 10 mM EDTA is used, activation occurs during incubation without further additions to the partially purified enzyme preparation. In the presence of EDTA, the addition of AMP or glucose has no significant effect on activation. However, incubation with Mg²⁺ in the presence of EDTA, resulted in the loss of glycogen synthetase activity. This loss of activity was not caused by an irreversible change in the enzyme since when the enzyme was recovered as a glycogen pellet and resuspended in fresh buffer containing EDTA, enzyme activity was restored and activation could proceed normally.

The nature of the synthetase complex was not irreversibly changed by preparation of the partially purified enzyme in EDTA. This was demonstrated by preparing pellets from homogenates containing EDTA and then resuspending the pel-
Fig. 12. The effect of NaF and NaCl on the in vitro activation of glycogen synthetase. The effect of NaF and NaCl was studied in crude homogenates (A) and in partially purified preparations in glycylglycine buffer either with (B) or without (C) 10 mM EDTA. The enzyme samples were incubated at 25°C and assayed in the absence of glucose-6-P at 25°C for 5 min. The NaF concentrations in the enzyme samples were as follows: a, none; b, 25 mM; c, 50 mM; d, 100 mM; e, 200 mM. The NaCl concentrations were: f, 50 mM; g, 200 mM.

Discussion

The data presented in this paper support the hypothesis that the in vivo activation of glycogen synthetase, by the transformation of the D to the I form, or less active to more active form, is mediated by insulin. Treatment of fed, starved, and diabetic rats with insulin results in a 2- to 3-fold increase in the I activity with no significant change in the total activity. Although enhanced glycogen deposition following glucocorticoid treatment of adrenalectomized rats has been attributed to a steroid-induced activation of glycogen synthetase, hydrocortisone did not increase the I activity in either fed or starved normal rats or in alloxan-diabetic rats. In starved or diabetic rats, hydrocortisone blocked the insulin-induced activation of glycogen synthetase in accordance with the normally antagonistic physiological roles of these hormones. The glycogenic response to glucocorticoids apparently derives from their stimulatory effect on gluconeogenesis (32, 33) and is dependent on the action of insulin, as also suggested by Kreutner and Goldberg with their experiments on diabetic adrenalectomized rats (6). The kinetics of insulin release from the pancreas support this indirect mode of action, with respect to the activation of glycogen synthetase, for factors which increase blood glucose levels. Since the initial phase of insulin release was noted 45 sec after glucose infusion into the perfused pancreas (34), insulin release would not be a rate-limiting step in the response to alterations of blood glucose levels.

Clearly, further work is needed to determine the steps by which insulin causes the transformation of D to I form of glycogen.
synthetase is activated by insulin, but not by other factors which mimic the effect of insulin in vivo. Recently, insulin bound to Sepharose was found to be equivalent to free insulin as a mediator of glycogen synthetase activation in the in vitro liver system (35). This suggests that, even under circumstances in which entry into the cells is precluded, insulin can activate glycogen synthetase. Thus, studies of the effect of Sepharose-bound insulin on liver tissue in vivo have brought into focus an early step in the mechanism of insulin action, i.e. interaction of the hormone with the cell membrane. Another case in which insulin and hydrocortisone both appear to increase the level of the same enzyme is that of tyrosine transaminase. In this case, de novo protein synthesis and degradation regulate the level of enzyme activity. However, insulin and hydrocortisone act on this enzyme by different mechanisms (36). Whereas glucocorticoids increase enzyme activity by induction of enzyme synthesis, the insulin effect is the result of a decrease in the rate of enzyme degradation, possibly as part of a general antiproteolytic effect on liver (37).

To gain further insight into the mechanisms regulating glycogen synthetase the time- and temperature-dependent in vitro activation of the enzyme was investigated. Due to the development of a more purified system, the in vitro activation could be further characterized. Although the in vitro system has been used as a model for the activation of glycogen synthetase by various factors (14, 15, 20, 21, 26), our studies revealed significant differences between the hormonally induced activation in vivo and the in vitro activation. The in vitro system, as presently used, cannot adequately reflect the in vivo activation by insulin. Although the activation kinetics of glycogen synthetases from control and insulin-treated rats were the same, chronic hormone deprivation may indeed affect the activating enzymes of the glycogen synthetase system. However, more drastic treatment of the animals is required to demonstrate these effects. For example, glucocorticoid involvement in the maintenance of the synthetase-activating system could be demonstrated only in the starved, adrenalectomized rat (20). It has also been suggested that insulin plays some role in the maintenance of the activating system (14).

A major difference between in vivo and in vitro activation is the increase in the total activity observed during the latter process. Several possible explanations of this phenomenon have been considered, but none of them proved sufficient to nullify the conclusion that a change other than a straightforward D to I conversion is occurring in vitro. (a) The differential effects on the D and I forms of metabolites formed during in vitro incubation is not applicable to the purified system. (b) Increasing the concentration of substrate in the assay system did not affect the activation kinetics. Furthermore, if an insufficient concentration of substrate is responsible for the apparent increase in total activity the same problem should have developed with respect to the in vivo hormonal activation. However, no increase in total activity was found following insulin treatment although the I activity increased about 3-fold. (c) The inhibition of activation by NaF, which has been taken as evidence of a phosphatase-catalyzed D to I conversion in vitro, is also obtained with equivalent concentrations of NaCl. In the purified preparation, both NaF and NaCl, when incubated with the enzyme, caused activation. As demonstrated with the purified preparations, many compounds commonly added to either the homogenization medium or the assay system, such as Mg++, EDTA, AMP, NaF, etc. exert a profound effect on the in vitro activation.

With the partially purified in vitro activation system, effects of the various cellular activators and inhibitors on the activation of glycogen synthetase can be more rigorously examined.

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