Regulation of Hepatic Glycogen Synthetase of Rana catesbeiana

STUDIES OF ADENOSINE TRIPHOSPHATE INHIBITION WITH REFERENCE TO INSULIN ACTIVATION OF GLYCOGEN SYNTHETASE*

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SUMMARY

Treatment of Rana catesbeiana tadpoles with insulin results in activation of glycogen synthetase. Glycogen synthetase from insulin-treated animals has a lower $K_m$ for uridine diphosphoglucose and the nature of ATP inhibition is changed from cooperative to strictly competitive with substrate and with the activator, glucose 6-phosphate. A change in the mechanism of ATP inhibition from cooperative to strictly competitive with substrate and activator is also obtained by photo-oxidation of the untreated enzyme. Other properties of the insulin-activated and photo-oxidized enzymes are also similar: (a) cooperativity for glucose-6-P and UDP-glucose in the presence of ATP is no longer observed; (b) inhibition by ATP is more completely reversed by glucose-6-P in these enzymes compared with untreated glycogen synthetase.

It is proposed that in the untreated enzyme ATP interacts with an allosteric regulatory site in addition to the UDP-glucose and glucose-6-P sites. Treatment with insulin in vivo or photo-oxidation in vitro abolishes the functional allosteric regulatory site for ATP without affecting the other ATP sites.

It is not yet clear whether the insulin-mediated enzyme modification that results in a reduced $K_m$ for UDP-glucose is separate from that which inactivates the allosteric regulatory site for ATP.

In higher animals, glycogen synthetase (UDP-glucose:glycogen α-1,4-glucosyl transferase, EC 2.4.1.11) exists in two forms; D form and I form. The D form has a high $K_m$ for the substrate UDP-glucose, and requires glucose-6-P as an activator, whereas the I form has a lower $K_m$ for UDP-glucose and is independent of glucose-6-P for activity. It is generally believed that only the I form is significantly active under normal physiological conditions (1). Thus, the hormone-controlled interconversion of the D and I forms exerts a major role in the regulation of glycogen synthesis in higher animals (2-4). The tadpole, on the other hand, is unique in that all glycogen synthetase activity is dependent on glucose-6-P. Insulin treatment of tadpoles mediates the transformation of a less active form (high $K_m$ for UDP-glucose) to a more active form (lower $K_m$ for UDP-glucose) without affecting the requirement for glucose-6-P (3).

One of the most important changes in the properties of the enzyme accompanying this transformation in tadpoles or other animals is the nature of ATP inhibition (3, 5). For example, the enzyme from control tadpoles is strongly inhibited by ATP and shows cooperative kinetics with ATP at low concentrations of glucose-6-P or UDP-glucose. However, the activated enzyme from insulin-treated animals shows weak inhibition by ATP and no cooperativity for ATP (3). This type of differential effect of ATP together with other cellular metabolites such as glucose-6-P probably functions to control glycogen synthesis in liver, muscle (5), and yeast (6, 7).

Although similar ATP inhibition has been reported for glycogen synthetase from many sources and appears to be retained throughout the evolutionary tree (3, 5, 6-8), the mode by which ATP exerts its effect is not yet clear. Kinetic studies indicate that ATP inhibition is competitive with the substrate, UDP-glucose, and the activator, glucose-6-P. Thus, the question arises whether the cooperativity for ATP inhibition is due to interaction of ATP at the sites for both glucose-6-P and UDP-glucose or whether it is due to interaction with independent allosteric sites in addition to those of the activator and the substrate.

In an attempt to characterize the number and type of sites required for inhibition by ATP and the role of insulin in modifying the kinetics for inhibition by ATP, we have examined the kinetics for ATP inhibition of glycogen synthetase of Rana catesbeiana tadpoles. Our results indicate that the enzyme from untreated tadpoles has an ATP binding site or sites independent of glucose-6-P or UDP-glucose and this binding site is rendered nonfunctional by insulin treatment.

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1 For further information on the nomenclature of glycogen synthetase, see Reference 1.
EXPERIMENTAL PROCEDURE

Materials

Animals—R. catesbeiana tadpoles, weighing 7 to 10 g, were purchased from Lemberger Company, Oshkosh, Wisconsin. The stock animals were kept in a tank of dechlorinated water at 15°C.

Chemicals—Nucleotides and sugar derivatives were purchased from Sigma Chemical Company. Radioactive [14C]UDP-glucose was purchased from Schwarz BioResearch, Inc., and had a specific activity of 200 μCi per pmole. Glass fiber filters (934 A/II) were the product of Reeve Angel, Clifton, New Jersey.

Methods

Protein Determination—Protein was determined by the method of Lowry et al. (9) with crystalline bovine serum albumin as the standard.

Preparation of Enzyme and Assay—Procedures for the purification and assay of glycogen synthetase were the same as previously described (10). The standard reaction mixtures contained the following components: 0.67 μmole of [14C]UDP-glucose (6000 cpm), 1.00 mg of shellfish glycogen, 10 μmoles of glucose-6-P, 3.5 μmoles of sucrose, 2 μmoles of EDTA (pH 7.4), and an appropriate amount of enzyme in a final volume of 0.5 ml. The reaction mixture was incubated at 37°C for 10 min and then inactivated by the addition of 0.5 ml of 20% trichloroacetic acid containing 2 mg per ml of LiBr. Glycogen, precipitated by the addition of 2 volumes of 95% ethanol, was washed twice with 5-ml portions of 67% ethanol on a glass filter. The dried filter was then counted in a scintillation counter using 10 ml of toluene scintillation fluid. The reaction rate was a linear function of protein concentration and time up to 20 min. This linear portion represented approximately 75% of the total reaction observed. The first 10 min of this linear portion was used for the assay of enzyme activity. The enzyme concentration was varied to obtain, when possible, glucose incorporation into glycogen of the order of 500 to 1000 cpm. Shellfish glycogen and tadpole liver glycogen showed no difference as the primer of the reaction.

After appropriate corrections for background counts, the amount of glucose incorporated into glycogen was calculated. Glycogen synthetase activity was expressed as moles of glucose transferred to glycogen per 10 min per g of liver tissue (or mg of protein). A unit of enzyme is defined as the amount of enzyme necessary for the incorporation of 1 μmole of glucose moiety from UDP-glucose into glycogen in 10 min under the standard assay conditions.

Photosensitization of Glycogen Synthetase—A 10 ml beaker with 3 ml of partially purified enzyme (0.5 mg per ml) containing 2.7 × 10⁻⁷ M methylene blue, 0.4 M sucrose and 0.01 M EDTA, and 30 μM glucose-6-P or 6.7 μM UDP-glucose was placed in an ice bath. The enzyme solution was then illuminated with a 150-watt light bulb at a distance of approximately 30 cm with constant stirring for 10 min. In the absence of glucose-6-P or UDP-glucose, illumination resulted in total inactivation. Illuminated enzyme was then reisolated by centrifugation at 144,000 × g for 75 min in a Spinco centrifuge or by gel filtration of the enzyme solution through a small column of Sephadex G-25. The recovery of activity was about 80%.

RESULTS

Characteristics of ATP Inhibition—Tadpole glycogen synthetase is inhibited by ATP. This inhibition was found to be competitive with the substrate, UDP-glucose as shown by the double reciprocal plot in Fig. 1. Inhibition by ATP was also competitive with the activator glucose-6-P (Fig. 2). The concave upward curvature at 4 mM and 8 mM ATP is consistent with cooperativity for inhibition by glucose-6-P as well as for UDP-glucose under these conditions. These double reciprocal plots indicate that ATP increases the Kₘ for both UDP-glucose and glucose-6-P without changing the Vₘₕ. To further examine the nature of these competitive kinetics, the dependence of the initial velocities on inhibitor concentration at fixed levels of glucose-6-P and UDP-glucose was checked by means of Dixon plots. In Fig. 3a, the dependence of the initial velocities on ATP concentration at various levels of glucose-6-P and a saturating amount of UDP-glucose is shown. The apparent straight lines obtained at glucose-6-P concentrations above 20 mM indicate that ATP inhibition is truly competitive with glucose-6-P or that the cooperative inhibition by ATP is negligible under these conditions. Below 20 mM glucose-6-P, however, ATP inhibition is cooperative.

A similar examination with respect to ATP and UDP-glucose at a saturating amount of glucose-6-P indicates that ATP inhibits competitively with UDP-glucose only when the ratio of ATP to UDP-glucose is less than 1 (Fig. 3b). The cooperativity of ATP inhibition under these conditions is negligible, if any. As the ratio of ATP to UDP-glucose becomes bigger, the lines become steeply curved upward. Upward curvature indicates cooperativity for inhibition by ATP even though there is a saturating amount of glucose-6-P. The Kᵢ values obtained from Fig. 3, A and B, are identical at 1.5 mM. These studies indicate cooperative interaction between ATP molecules on the enzyme. These cooperative interactions may be due to interaction of ATP...
FIG. 2. Double reciprocal plot for glycogen synthetase with respect to glucose-6-P (G-6-P) in the presence of the following levels of ATP: 0 (●), 1 (○), 2 (■), 4 (▲), and 6 mM (△). The substrate UDP-glucose concentration was 4 mM.

at glucose-6-P and UDP-glucose sites even though excess glucose-6-P or UDP-glucose was used to saturate their sites. Alternatively, ATP may have a binding site independent of the glucose-6-P and UDP-glucose sites; then cooperative inhibition would be the result of ATP acting at both the glucose-6-P or UDP-glucose site and its own regulatory site.

To further examine the possibility that ATP binds at a site independent of glucose-6-P and UDP-glucose, we have studied the multiple inhibitor kinetics by the method of Yonetani and Theorell (11). Yonetani and Theorell have devised a simple graphic method to analyze the kinetics of interaction between two competitive inhibitors. This kinetic approach has been used in the investigation of substrate or coenzyme sites for several enzymes (12, 13). Since glycogen synthetase requires a substrate and an activator, and ATP inhibits competitively with both the substrate, UDP-glucose, and the activator, glucose-6-P, the analysis of multiple kinetics is extremely complex. Therefore, the analysis of multiple inhibitor kinetics at the activator site was made under conditions in which the substrate concentration is not rate limiting and the activator concentration is kept sufficiently low so that the initial rate of the reaction is controlled only by the activator concentration. When the substrate site was examined, the conditions for the activator and substrate concentrations were reversed. Under these conditions, if two inhibitors are interacting at the same site, 1/ is plotted against the concentration of one inhibitor at several levels of the other should give rise to a series of essentially parallel lines. On the other hand, if they interact at different sites, then the slopes obtained from the above functions should be different.

In order to perform the inhibition analysis as described it is necessary to use true competitive inhibitors for the glucose-UDP and glucose-6-P sites. UDP inhibits the enzyme activity competitively with the substrate, UDP-glucose, in the presence of a saturating amount of glucose-6-P (Fig. 4A). Fructose-1,6-P inhibits competitively with the activator, glucose-6-P (Fig. 4B).

Using the following sets of multiple inhibitors, UDP and ATP for the UDP-glucose site in the presence of a saturating amount of glucose-6-P, and fructose-1,6-P and ATP in the presence of a saturating amount of UDP-glucose for the glucose-6-P site, the multiple inhibitor kinetics were studied. In Fig. 5, 1/ is plotted against the concentration of UDP at several levels of ATP and a family of straight lines is obtained. At low concentrations of ATP, the lines are essentially parallel, indicating that UDP interacts competitively with ATP at the UDP-glucose. The increased slopes at higher ATP concentrations (above 1 mM) indi-
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Fig. 5. Kinetic plot for the multiple inhibition of glycogen synthetase using UDP as variable inhibitor at 0 (●), 0.25 (○), 0.5 (■), 1.0 (□), and 2.0 mM (△) of ATP. Glucose-6-P and UDP-glucose concentration was 30 mM and 0.57 mM, respectively.

Fig. 6. Kinetic plot for the multiple inhibition of glycogen synthetase using fructose-1,6-P₂ (F-1,6-diP) as variable inhibitor at 0 (●), 0.5 (○), 1.0 (■), and 2.0 mM (△) of ATP. Glucose-6-P and UDP-glucose concentration were 10 mM and 3.35 mM, respectively.

Fig. 7. A, double reciprocal plot for the photo-oxidized glycogen synthetase with respect to UDP-glucose (UDPG) at variable concentrations of ATP: 0 (●), 1 (○), 2 (■), and 4 mM (△). Glucose-6-P concentration was 20 mM. B, double reciprocal plot for the photo-oxidized glycogen synthetase with respect to glucose-6-P (G-6-P) at variable concentrations of ATP: 0 (●), 0.5 (○), 1 (■), 2 (□), and 4 mM (△). UDP-glucose concentration was 3.35 mM.

Fig. 8. A and B, Dixon plots for the photo-oxidized enzyme as described below. The family of straight lines indicates that ATP inhibition is truly competitive with glucose-6-P and UDP-glucose. The Kᵢ values obtained from these Dixon plots were identical, 0.5 mM.

Furthermore, when the photo-oxidized enzyme was subjected to the multiple inhibitor kinetics analysis described below, a family of essentially parallel straight lines was obtained as shown in Fig. 9, A and B. This experiment indicates that ATP interacts only at the glucose-6-P and UDP-glucose sites of the photo-oxidized enzyme.
These results clearly establish that tadpole glycogen synthetase has at least one independent ATP binding site and that cooperative inhibition is the result of the presence of this allosteric ATP binding site or sites. Competitive inhibition by ATP with glucose-6-P and UDP-glucose alone does not show cooperative inhibition by ATP.

**Fig. 8.** A, Dixon plot for inhibition of photo-oxidized glycogen synthetase by ATP at 3.38 mM UDP-glucose and the following concentrations of glucose-6-P: 5 (●), 10 (○), 20 (■), 40 (□), and 50 mM (△). B, Dixon plot for inhibition of photo-oxidized enzyme by ATP at 20 mM glucose-6-P and the following concentrations of UDP-glucose: 0.67 (●), 1.34 (○), 2.01 (■), 2.98 (□), and 3.35 mM (△).

**Fig. 9.** A, multiple inhibitor kinetic for the photo-oxidized enzyme. UDP was used as variable inhibitor at 0 (●), 0.25 (○), 0.5 (■), 1.0 (□), and 2.0 mM (△) of ATP. Glucose-6-P was 20 mM and the concentration of UDP-glucose was 1.35 mM. B, fructose-1,6-P₂ (F-1,6-dsP) was used as variable inhibitor at the same series of ATP concentration as A. Glucose-6-P and UDP-glucose concentration were 10 mM and 3.35 mM, respectively.

**Fig. 10.** Effect of glucose-6-P (G6P) ATP inhibition. Glucose-6-P reversal of ATP inhibition (0.5 mM) with the control (●), and the desensitized enzyme (○) was studied at different concentrations of glucose-6-P. Percentage of activity is calculated from that obtained in the absence of ATP compared with that obtained with ATP at the glucose-6-P concentration indicated.

**TABLE I**

Properties of glycogen synthetases

The $K_m$ for UDP-glucose was determined from the double reciprocal plots at different concentrations of UDP-glucose with 20 mM glucose-6-P. The $K_m$ for glucose-6-P was determined similarly except that the UDP-glucose concentration was 3.35 mM. The kinetic data of ATP inhibition obtained from a Dixon plot were examined. Effectiveness of glucose-6-P in reversing ATP inhibition (1 mM) is expressed as a percentage of the activity obtained in the absence of ATP compared with that obtained with ATP.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ for UDP-glucose</th>
<th>$K_m$ for glucose-6-P</th>
<th>Kinetic ATP inhibition</th>
</tr>
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<tbody>
<tr>
<td>Control enzyme</td>
<td>1.3 (mM)</td>
<td>2.5 (mM)</td>
<td>Normal</td>
</tr>
<tr>
<td>Insulin-activated enzyme</td>
<td>0.1 (mM)</td>
<td>2.5 (mM)</td>
<td>Normal</td>
</tr>
<tr>
<td>Photo-oxidized enzyme</td>
<td>1.3 (mM)</td>
<td>2.5 (mM)</td>
<td>Normal</td>
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Glucose-6-P Reversal of ATP Inhibition of Photo-oxidized Enzyme—The above studies indicate distinct properties for the allosteric ATP regulatory site or sites. (a) ATP bound to the regulatory site or sites promotes cooperativity for UDP-glucose and glucose-6-P (Figs. 1 and 2). (b) ATP bound to the regulatory site causes cooperative inhibition of enzyme activity (Fig. 3). Further evidence that the independent ATP regulatory site or sites is distinct from the site for glucose-6-P is shown by the data in Fig. 10. These data show that glucose-6-P completely reverses ATP inhibition of the photo-oxidized enzyme but not of the untreated enzyme. With the photo-oxidized enzyme, complete reversal of ATP inhibition by glucose-6-P implies that both reagents compete for the same site. With the untreated enzyme, residual inhibition by ATP in the presence of glucose-6-P would result from interaction of ATP with the distinct regulatory site or sites.
Properties of Photo-oxidized Enzyme and "Insulin-activated" Enzyme—As previously discussed, one of the changes in the properties of the enzyme isolated from insulin-treated tadpoles is the loss of cooperativity for ATP inhibition. In addition, glycogen synthetase from insulin-treated tadpoles has a lower $K_i$ for UDP-glucose (3). To explore the possible relationship between activation of the enzyme by insulin treatment and modification of the ATP regulatory site, we have compared the properties of the "insulin-activated" enzyme and the photo-oxidized enzyme. The data in Table I show that photo-oxidation of the ATP site is not accompanied by a decrease in the $K_i$ for UDP-glucose. However, other properties of the photo-oxidized enzyme are extremely close to those of "insulin-activated" enzyme. Both the photo-oxidized and "insulin-activated" enzymes have the same $K_i$ for ATP, 0.5 mM compared with 1.5 mM for the control enzyme. Both enzymes are no longer cooperatively inhibited by ATP. ATP inhibition of the photo-oxidized enzyme is completely reversed at 20 mM glucose-6-P, whereas only a partial effect is observed with hormone-activated enzyme, even though the degree of reversal was high in comparison to that obtained with the control enzyme. This may be due to incomplete activation of all enzyme molecules by insulin treatment, since the degree of glucose-6-P effect on ATP inhibition of the insulin-activated enzyme varies depending on the preparation.

These studies indicate that glycogen synthetase from insulin-treated tadpoles differs from the control enzyme in two important respects. First, the ATP regulatory site of the insulin-activated enzyme is modified similar to in vitro modification by photo-oxidation. Second, the insulin-activated enzyme is altered so that it has a lower $K_i$ for UDP-glucose compared with the control enzyme. It is not yet apparent whether these two changes in the insulin-activated enzyme are the result of a single or multiple chemical modifications.

**DISCUSSION**

ATP inhibits glycogen synthetase from all the biological systems studied thus far (3,5,6-8). The degree of ATP inhibition and the effect of glucose-6-P on reversal of ATP inhibition differs depending on the state of the enzyme. Thus, active enzyme, I form, is less strongly inhibited by ATP than the less active enzyme, D form, and ATP inhibition of the I form is readily reversed by glucose-6-P. These differential effects of ATP and glucose-6-P are the basis for the thesis that the ratio of ATP to glucose-6-P in the cell exerts a role in the control of glycogen synthesis with (5) or without hormone-mediated enzyme transformation (15).

ATP is also clearly involved in the transformation of I to D form; ATP-dependent phosphorylation of I form yields D form (14). Dephosphorylation of D form to I form is mediated by insulin or glucocorticoid treatment (16) of the animals.

In view of the significant role of ATP in the regulation of glycogen synthetase, the examination of the nature of ATP inhibition is imperative in understanding the regulation of glycogen synthesis. In this report, we have examined the nature of ATP inhibition in three ways. (a) We have demonstrated homotropic interaction between ATP molecules on the enzyme by Dixon plots of the initial velocities. (b) We have established the existence of an independent ATP binding site or sites. ATP also inhibits enzyme activity by interaction with the UDP-glucose and glucose-6-P sites. (c) We have shown that interaction of ATP with the allosteric regulatory site promotes cooperativity for UDP-glucose and glucose-6-P. Apparently, the independent allosteric site or sites for ATP does not interact with glucose-6-P since an excess amount of glucose-6-P fails to reverse ATP inhibition completely in the presence of a saturating amount of UDP-glucose. ATP inhibition of the photo-oxidized enzyme is completely reversed by glucose-6-P. An increased capacity for glucose-6-P reversal of ATP inhibition was also observed with insulin-activated enzyme. Piras, Lochman, and Cabib (5) also reported that ATP inhibition of the I form of rat and rabbit muscle enzyme was almost completely reversed by increasing amounts of glucose-6-P. Our studies then clearly explain the action of glucose-6-P in reversing ATP inhibition. Glucose-6-P completely reverses ATP inhibition when the ATP regulatory site is nonfunctional. When the ATP regulatory site is functional, glucose-6-P only reverses inhibition by ATP related to the glucose-6-P and UDP-glucose sites. Thus it is clear that one of the important effects insulin has on glycogen synthetase is at the independent ATP regulatory site.

Working with muscle glycogen synthetase from rat and rabbit, Piras et al. (2) suggested that the binding sites for ATP and glucose-6-P were closely interrelated even though it was difficult to decide if both compounds actually bind to the same site. However, as they noted, the fact that ATP inhibition of the D form could not be completely reversed by glucose-6-P favored the view that rat or rabbit muscle glycogen synthetase also have an independent ATP site similar to the tadpole enzyme.

Two mechanisms for the regulation of glycogen synthetase have been noted in liver or muscle; (a) transformation of the enzyme mediated by hormones and (b) metabolic regulation of enzyme activity. From our studies it is clear that both of these regulatory mechanisms are related to the presence of an allosteric ATP regulatory site on the enzyme. Insulin-mediated activation of glycogen synthetase results in loss of the functional ATP regulatory site and in enzyme having a lower $K_i$ for UDP-glucose. It is not presently clear if these two effects result from a single or from multiple alterations. Loss of the ATP regulatory site in the hormone-activated enzyme accounts for its catalytic properties.

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