Regulation of Rat Liver Glycogen Synthetase

CARBOXYLIC ACID ACTIVATION OF LIVER GLYCOGEN SYNTHETASE*

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SUMMARY

Rat liver glycogen synthetase is strongly stimulated by cellular carboxylic acids which are intermediates of glycolysis and the tricarboxylic acid cycle. Activation of glycogen synthetase by the carboxylic acids may coordinate hepatic glycogen deposition with the functional status of the tricarboxylic acid cycle. At physiological concentrations of the carboxylic acids, the I form is preferentially stimulated. Whereas citrate, isocitrate, succinate, etc., stimulate total glycogen synthetase activity about 25 to 50%, these compounds stimulate the I activity 6- to 8-fold. Citrate was used as a model compound in studies of the mechanism of glycogen synthetase activation. Stimulation of the enzyme is the result of modification of the glucose-6-P binding site which accompanies a conformational change mediated by citrate.

The subtlety and complexity of cellular regulatory mechanisms are well illustrated in the case of hepatic glycogen synthetase (uridine diphosphate glucose: α, 1, 4-glucosyltransferase, EC 2.4.1.11), the rate-limiting enzyme for glycogen deposition. In higher animals, the enzyme is found in two forms: the D form, which requires glucose-6-P for activity, and the I form, which is active in the absence of glucose-6-P (1-5). One level of regulation is the conversion of the D to I form without increasing the total enzyme activity, which is mediated by hormones (2, 3, 5-9). Transformations between the two forms of glycogen synthetase involve phosphorylation and dephosphorylation reactions catalyzed by a protein kinase and phosphatase (10, 11). Increased affinity for both UDP-glucose and glucose-6-P accompanies activation of the enzyme (4, 5, 12, 13). Glycogen synthetase also exhibits a diurnal rhythm in its peak activity which, unlike insulin activation, requires new protein synthesis and results in a change in total enzyme activity (14).

Although both forms of the enzyme are subject to allosteric regulation by various cellular metabolites (15-20), because of the low cellular concentration of UDP-glucose and glucose-6-P, and the relative stability of the level of glucose-6-P under different physiological conditions, the conversion of the D to the I form is thought to be physiologically important (12). Since the enzyme occurs in two forms and glucose-6-P strongly affects the activity of the I form, in addition to being required by the D form, most studies of the enzyme have been directed toward understanding the effect exerted by glucose-6-P on the enzyme.

In this communication, we present evidence that regulation of glycogen synthetase is further integrated into the complex regulatory network of cellular metabolism through carboxylic acid intermediates of glycolysis and the tricarboxylic acid cycle which stimulate the enzyme, particularly the I forms, several-fold. These data indicate a relationship which may serve to coordinate glycogen deposition in the liver with the functional status of the tricarboxylic acid cycle. A preliminary report on this subject has appeared previously (21).

MATERIALS AND METHODS

Chemicals—Glucose-6-P, UDP-glucose, shellfish glycogen, and bovine insulin (24.1 i.u per mg) were purchased from Sigma Chemical Co. Radioactive UDP-[U-14C]glucose, obtained from Schwarz BioResearch, Inc., had a specific activity of 200 μCi per μmole. Glass fiber filters (923 AH) were the product of Grove Electric. Bovine insulin (24.1 i.u per mg) was purchased from Sigma Chemical Co. Radioactive UDP-[U-14C]glucose, obtained from Schwarz BioResearch, Inc., had a specific activity of 200 μCi per μmole. Glass fiber filters (923 AH) were the product of Grove Electric.

Animals—Experimental animals were 200-g female Wistar albino rats, from the Biochemistry Department rat colony, fed a standard laboratory diet.

Preparation and Assay of Glycogen Synthetase Partially purified glycogen synthetase (glycogen pellet stage) was prepared as described previously (5). Glycogen synthetase phosphatase was removed from the glycogen pellet by sonication as described by Hizukuri and Larner (11). Glycogen synthetase, free of phosphatase, was stored in 0.02 M glycylglycine buffer, pH 7, containing 30% glycerol at -20°. This procedure consistently yielded a stable preparation of glycogen synthetase with an activity of about 2 units per mg of protein. The enzyme was assayed as described previously (5). The standard reaction mixture contained the following components: 0.67 μmole of UDP-[U-14C]glucose (6000 cpm), 1.2 mg of shellfish glycogen, 10 μmoles of glucose 6-phosphate when the total activity was measured, 3.5 μmoles of sucrose, 1 μmole of EDTA, and an appropriate amount of the enzyme in a final volume of 0.5 ml. The reaction mixture was incubated at 37° for 10 min and inactivated by the addition of 1 ml of 10% trichloroacetic acid containing 2 mg of LiBr per ml. Glycogen, precipitated by the addition of 2 volumes of 95% ethyl alcohol, was washed twice with 5-ml portions of 67% ethyl alcohol on a glass filter. The
and fumarate. In our studies of the mode of action of these carboxylic acids, citrate was used as a model compound.

Activation of glycogen synthetase by carboxylic acids is reversible. If the enzyme is reisolated as a glycogen pellet from medium containing any of the carboxylic acids, the effect of the test compound is no longer observed. This indicates that the enzyme is not converted to a stable new form in the presence of the carboxylic acids. Further evidence against protein modification involving an enzymatic reaction is the fact that activation by the test compounds is not time-dependent. Comparable concentrations of the analogous amino acids had no effect on activation.

$K_m$ of Glycogen Synthetase for Various Carboxylic Acids—To determine whether various carboxylic acids could be effective activators of glycogen synthetase when present at about their physiological concentration, the concentration required for half-maximal activation as reflected by the apparent $K_m$ values, was determined (Table II). When total enzyme activity was assayed, the apparent $K_m$ for citrate was about $10 \text{ mM}$. However, the apparent $K_m$ of the I form for citrate was much lower, that is $2 \text{ mM}$. The apparent $K_m$ of the I form for other carboxylic acids, such as isocitrate and malate, was about the same as the apparent $K_m$ for citrate.

The total physiological concentration of the carboxylic acids in the liver is about $2 \text{ mmol per g of liver}$ (22). Although the subcellular distribution of the various carboxylic acids is difficult to assess, it is apparent that their intracellular level is in the range at which the I form could be significantly activated. The high $K_m$ for the total activity indicates that the D form has a lower affinity for the carboxylic acids or the added carboxylic acids is less effective as an activator in the presence of glucose-6-P. In the case of tadpole liver glycogen synthetase, the insulin-activated enzyme has a $K_m$ for citrate which is about one-seventh that of the control enzyme (19).

Effect of Citrate on Binding of Glucose-6-P—To examine the mode by which citrate activates glycogen synthetase, we studied the effect of citrate on the apparent $K_m$ values of glucose 6-P and UDP-glucose. Citrate greatly decreased the $K_m$ for glucose-6-P. As shown in Fig. 1, even a low concentration of citrate, which has little or no effect on $V_{max}$, markedly lowers the $K_m$ for glucose-6-P. For example, in the presence of $5 \text{ mM}$ citrate, the $K_m$ for glucose-6-P is lowered from 2.2 to $0.7 \text{ mM}$. This suggests that citrate increases the affinity of the enzyme for glucose-6-P. The kinetic pattern for glucose-6-P itself is quite complex, as shown in Fig. 2. Although it is known that glucose-6-P affects both the I and D forms of glycogen synthetase and that these two forms have different affinities for glucose-

### Table I

**Effect of Carboxylic Acids on Glycogen Synthetase**—The stimulatory effect of various carboxylic acids on glycogen synthetase activity, measured in the presence and absence of glucose-6-P, is shown in Table I. Citrate, isocitrate, succinate, fumarate, oxalacetate, and lactate stimulate total activity 25 to 50% in the presence of a saturating concentration of glucose-6-P (20 mM).

Greater stimulation of total activity could be obtained by increasing the concentration of the carboxylic acids. For example, $100 \text{ mM}$ citrate stimulated the total activity 2-fold. The effect of the carboxylic acids on the I form of glycogen synthetase is more striking. In the absence of glucose-6-P, most of the compounds tested activated the enzyme several-fold. Stimulation ranged from about 3-fold for pyruvate to 8-fold for malate and fumarate.

### Table II

$K_m$ for Carboxylic Acids

The apparent $K_m$ values of the I form for carboxylic acids were determined from the double reciprocal plots obtained from the initial velocity at different concentrations of carboxylic acids. The $K_m$ for the total activity (D + I) was determined in the presence of $20 \text{ mM}$ glucose-6-P.

<table>
<thead>
<tr>
<th>Acids</th>
<th>$K_m$ (I form)</th>
<th>$K_m$ (D + I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
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<tr>
<td>Acetate</td>
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FIG. 1. The effect of citrate on glycogen synthetase at different concentrations of glucose-6-P (G-6-P). The partially purified enzyme was used either with 5 mM citrate (○) or in its absence (●) at different concentrations of glucose-6-P. A, saturation curves for glucose-6-P; B, double reciprocal plot of the data in Part A.

FIG. 2. The effect of citrate on glycogen synthetase. Activity of glycogen synthetase was assayed in the presence of 5 mM (○) or in the absence (●) of citrate at a wide range of glucose-6-P (G-6-P) concentrations. Other reaction conditions were the same as in the standard reaction mixture (see Table I).

6-P, most kinetic studies of the enzyme have used a relatively narrow range of glucose-6-P concentrations. When a wide range of glucose-6-P concentrations is examined, the complexity of the effect of the activator becomes quite apparent. Although the saturation curves for glucose-6-P appear to be normal in either the presence or absence of citrate, and citrate activation is obvious only at low concentrations of glucose-6-P (Fig. 2A), examination of the double reciprocal plot reveals apparent negative cooperativity of glucose-6-P binding when a very broad concentration range of the activator is considered. As the concentration of glucose-6-P is increased, the affinity of the enzyme toward glucose-6-P decreases, as indicated by the increase in the apparent $K_m$ values. In the presence of citrate, the apparent negative cooperativity for glucose-6-P is eliminated and, except at very high concentrations of glucose 6-P, a straight line is obtained for the double reciprocal plot (Fig. 2B). Although determining the exact mechanism for this apparent negative cooperativity requires detailed studies as indicated by the work of Levitzki and Koshland (23), one factor involved could be the different glucose-6-P requirements of the two forms of glycogen synthetase. As noted previously, in the presence of citrate the $K_m$ for glucose-6-P decreases, suggesting that citrate exerts some effect on the activator binding site.

Effect of Citrate on Binding of UDP-glucose—To further explore the relationship between citrate and glucose-6-P activation of the enzyme, the effect of these compounds on UDP-glucose binding was studied. When the activity of the I form, in the presence of different concentrations of UDP-glucose, is plotted, the resultant curve is sigmoidal (Fig. 3). Very low activity is obtained at physiological concentrations of UDP-glucose (about 0.2 mM) in the absence of glucose-6-P. These data are in agreement with the report by Mersman and Segal (12) that even the I form is not fully functional in the presence of low concentrations of UDP-glucose and that for full activity glucose-6-P is required. However, a low concentration of citrate activates the enzyme and normalizes the kinetics in the absence of glucose-6-P (Fig. 3A). The cooperativity of UDP-glucose binding is clearly shown in the double reciprocal plot in Fig. 3B. In contrast, when citrate is present this cooperativity is eliminated. Thus, with respect to enhancing UDP-glucose binding, the effect of citrate is similar to that reported for glucose-6-P (12).

Effect of Citrate on ATP Inhibition—To further characterize the citrate activation of glycogen synthetase, we studied the effect of citrate on ATP inhibition. Although both forms of glycogen synthetase are inhibited by ATP, the I form is less strongly inhibited by ATP than the D form and ATP inhibition of the I form is readily reversed by glucose-6-P (15–18). This is partly due to the lack of independent ATP binding sites on the I form (18). As shown in Fig. 4A, 8 mM ATP inhibits the I form about 50%. The degree of inhibition is shown more clearly in Fig. 4B, where enzyme activity at different concentrations of ATP is plotted as a percent of the activity in the absence of ATP. It should be noted that the activities obtained in the absence of ATP with both the control and citrate-activated enzyme are taken as 100%. Surprisingly, the citrate-stimulated enzyme activity is strongly inhibited by ATP. In the presence of low concentrations of ATP (2 mM), the citrate-activated enzyme level is reduced to 40% of that obtained with 5 mM citrate in the absence of ATP. When the ATP concentration is increased to 8 mM, the activity of the enzyme is reduced to the level found in the absence of citrate (Fig. 4A). As shown in
Fig. 4. The effect of ATP on the I form of glycogen synthetase. The effect of ATP on the activity was examined in the presence (●) and absence (○) of 5 mM citrate. A, the effect of different concentrations of ATP on the velocity is plotted. B, percentage of activity based on the velocity obtained in the absence of ATP.

Fig. 4B, the relative inhibition of glycogen synthetase I form is more marked in the case of the citrate-activated enzyme than in the absence of citrate.

These data show that the effect of citrate on the enzyme is not entirely analogous to that of glucose-6-P. Whereas glucose-6-P relieves ATP inhibition (15, 18), the citrate-activated enzyme activity, in contrast, is extremely sensitive to inhibition by ATP.

As shown in Fig. 5, 5'-AMP activates glycogen synthetase. In the presence of 7 mM AMP, the activity of the I form is about 3 times the control level. However, the citrate-activated enzyme is inhibited by the addition of AMP, which is analogous to the case of ATP inhibition. The relative stimulation of glycogen synthetase activity caused by AMP is counteracted by citrate, as shown in Fig. 5B.

In all the experiments discussed above, the enzyme was added last to a reaction mixture containing both citrate and ATP or AMP. However, it was found in separate experiments that the order of addition of citrate and ATP or AMP to a reaction mixture containing the enzyme did not change the inhibitory kinetics.

Effect of Temperature on Glycogen Synthetase—As we have previously reported (24), the activities of the D and I forms of glycogen synthetase are very sensitive to the presence of various cellular metabolites and to changes in the temperature of assay. Because of the differential effect of temperature on the two enzyme forms, the apparent percentage of enzyme in the I form is highly dependent on the assay temperature. The effect of assay temperature on the percentage of I form observed is shown in Fig. 6. As reported previously, the apparent percentage of

Fig. 5. The effect of 5'-AMP on the I form of glycogen synthetases. Enzyme activity at different concentrations of 5'-AMP was examined in the presence (●) and absence (○) of 5 mM citrate. A, the effect of 5'-AMP in the standard reaction mixture in the absence of glucose-6-P. B, percentage of activity based on the activity obtained in the absence of 5'-AMP was plotted at different concentrations of 5'-AMP.

Fig. 6. The effect of temperature on the ratio of the activities of the D and I forms. The glycogen-pellet stage of glycogen synthetase was assayed at different temperatures in the presence (●) and absence (○) of citrate (10 mM). The percentage of I activity was calculated on the basis of activities obtained in the presence (the total) and absence (I activity) of 20 mM glucose-6-P.

I form is higher at lower temperatures, e.g. at 5° the activity is about 90% I form, whereas at 37° the percentage of I form is about 10%. The difference in the relative activity of the D and I forms, with respect to temperature, is still observed in the
presence of citrate. As shown in Fig. 6, in the presence of citrate the percentage of I form is greater than in its absence at all temperatures. However, the relationship between temperature and percentage of I form is similar, i.e. the percentage of I form is greater at lower temperatures. For example, at 5° the apparent percentage of I form is about 80%, while at 37° about 25% of the activity appears to be in the independent form.

Conformational Changes in Glycogen Synthetase Induced by Citrate—Citrate activation of hepatic glycogen synthetase from Rana catesbeiana is accompanied by aggregation of the enzyme (19). Although sucrose density gradient studies of the rat liver enzyme do not show comparable changes in the aggregation pattern (data not shown), a conformational change in the rat enzyme caused by citrate is suggested by other experiments. In Fig. 7, the effect of citrate on the inactivation kinetics of glycogen synthetase during trypsin digestion is shown. The enzyme was significantly protected from trypsin digestion by glucose-6-P and citrate (Curve 3). In the absence of glucose-6-P, the enzyme is inactivated to about 70% within 4 min (Curve 1). In contrast, less than 20% inactivation was observed in the presence of citrate and glucose-6-P together. This experiment suggests that the enzyme undergoes a subtle structural change in the presence of citrate and glucose-6-P which renders it less susceptible to trypsin digestion.

A similar study of the effect of citrate on enzyme conformation was carried out by examining the active sulfhydryl group of glycogen synthetase. Since the enzyme was not purified to homogeneity, the number of active and other sulfhydryl groups on glycogen synthetase cannot be accurately measured at this time. We have recently observed that rat liver glycogen synthetase undergoes a sulfhydryl-disulfide exchange which accompanies inactivation (25). The SH group of the enzyme forms a mixed disulfide derivative with oxidized glutathione and the enzyme becomes inactive as shown in Fig. 8. However, in the presence of citrate and glucose-6-P the formation of the mixed disulfide derivative is retarded as shown in Curve 3 of Fig. 8. Glucose-6-P alone could protect the enzyme from both tryptic digestion and disulfide inactivation, although to a lesser degree than with citrate present.

These experiments suggest that citrate activation of glycogen synthetase is accompanied by a conformational change which may in turn significantly affect the binding site for glucose-6-P.

DISCUSSION

The data presented in this paper show that the activity of glycogen synthetase is subject to a regulatory mechanism involving carboxylic acids which are intermediates of the tricarboxylic acid cycle and glycolysis. This linkage between glycogen synthetase and the tricarboxylic acid cycle provides further evidence for the integration of important biosynthetic pathways by cellular metabolites. Studies of the effect of citrate on individual enzymes have led to an understanding of the role of this key metabolite in the control of glycolysis via its influence on phosphofructokinase and on fatty acid synthesis by the activation of acetyl-CoA carboxylase (26). Recently, in a discussion of the strategic position which citrate occupies in metabolism, Atkinson noted seven enzyme-modifier interactions involving citrate and predicted that other regulatory interactions would be discovered (27). Activation of glycogen synthetase by citrate is another example of this kind of regulatory mechanism and also illustrates how the study of effectors of key enzymes can lead to concepts of the unifying mechanisms which regulate interdependent metabolic pathways. Activation by citrate of glycogen synthetase and acetyl-CoA carboxylase, both known to be sensitive rate-limiting enzymes, links the regulation of the pathways leading to the deposition of the cellular storage compounds, glycogen and fat.

These findings also extend our knowledge of metabolite control for the special case in which an enzyme exists in two forms which are interconvertible by modification of the protein. It has been assumed that the physiological significance of the insulin-mediated transformation of glycogen synthetase D to I form is to allow the enzyme to be more functional at the low concentrations of glucose-6-P found in the cell. However, even in the absence of glucose-6-P, the I activity can be increased many-fold by carboxylic acids. Evidence presented here and for the tadpole enzyme previously (19) suggests that the im-
importance of the enzyme transformation may be in the amplification of allosteric control exerted by cellular metabolites.

Activation of glycogen synthetase by citrate is similar to the effect of glucose-6-P in certain respects. Citrate relieves the cooperativity of UDP-glucose binding and, at low concentrations of UDP-glucose, citrate activates the enzyme. Thus citrate, like glucose-6-P, normalizes the kinetics of UDP-glucose binding from sigmoidal to hyperbolic. The carboxylic acids increase the affinity of the enzyme for glucose-6-P. Although the kinetics of binding is quite complex, citrate eliminated the negative cooperativity of glucose-6-P binding and lowered the $K_m$ for glucose-6-P. Thus, the carboxylic acids apparently exert same effect on the glucose-6-P binding site. However, the effect of citrate differs from that of glucose-6-P with respect to ATP inhibition. Unlike glucose-6-P, citrate cannot protect the enzyme from inhibition by ATP. Even very high levels of citrate were unable to relieve ATP inhibition. Activation by the carboxylic acids extends the list of cellular anions known to affect the activity of glycogen synthetase (12). However, activation by citrate appears to be unique since the further effect of citrate was observed even in the presence of $P_i$ (data are not shown). This indicates that the simultaneous interaction of many cellular metabolites with the two forms of glycogen synthetase can profoundly affect the enzyme activity. These factors must be taken into account in studies of glycogen synthetase, particularly when crude preparations are used. Changes in the concentration of cellular metabolites, due to metabolism of glucose to various carboxylic acids during in vitro incubation of the enzyme may be partially responsible for the apparent time-dependent activation of glycogen synthetase observed in crude preparations (8, 28).

The exact mode of citrate activation of glycogen synthetase is not clear. However, the results of the present studies of the rat enzyme and previous studies of the amphibian enzyme suggest that the activation accompanies conformational changes in the enzyme. The gross conformational changes in the case of the tadpole enzyme which could be detected by ultracentrifugation studies were not shown by the rat enzyme. The importance of changes in the state of conformation and aggregation have become increasingly clear in recent studies of the activation of glycogen synthetase. It has been reported recently that the activator, glucose-6-P, promotes aggregation of glycogen synthetase. In this connection, Smith and Larner (29) reported that dephosphorylated enzyme from rabbit muscle exists in an aggregated form. Also, we have demonstrated a similar aggregation pattern for the active form (I form) of the rat liver enzyme (30).

Glucose-6-P alone can protect the enzyme from inactivation by trypsin and oxidized glutathione, however, with citrate also present more significant protection was observed.

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