Control of Glycogen Synthase Phosphatase from Rat Heart

THE ROLE OF SUBSTRATE*

JAMES A. THOMAS AND CHIHARU NAKAI

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010

SUMMARY

Glycogen synthase phosphatase from rat heart has been partially purified by ultrafiltration techniques, and the properties of this enzyme have been examined by using an assay based on the conversion of glycogen synthase D into I. As observed in other tissues, the phosphatase from heart was activated by both Mg$^{2+}$ and glucose-6-P. The specificity of this activation was examined and it was shown to be virtually identical with the specificity for activation of glycogen synthase D. In addition, Mg$^{2+}$ decreased the requirement for glucose-6-P approximately 10-fold in both the phosphatase reaction and in the synthase D reaction, and both inorganic sulfate and phosphate inhibited the glucose-6-P activation of both reactions. These results were interpreted as evidence for the regulation of the phosphatase reaction by the conformational state of the substrate, glycogen synthase D. Glycogen was shown to be an inhibitor of the phosphatase and this inhibition was independent of the activation of this reaction by Mg$^{2+}$ and glucose-6-P. These results indicate a complex pattern of interaction between various cellular metabolites in the regulation of glycogen synthase D dephosphorylation in heart tissue.

The interconversion of glycogen synthase (UDP-glucose: glycogen α-4, glucosyltransferase EC 2.4.1.11) between a phosphorylated (D) and nonphosphorylated (I) form is an important method of regulation of glycogen synthesis in mammalian tissues and in some microorganisms. The phosphorylation reaction, catalyzed by a kinase, is directly activated by cyclic 3',5'-adenylic acid (2, 3). The dephosphorylation reaction, catalyzed by glycogen synthase phosphatase, is affected by hormones (4, 5), by the content of tissue glycogen (6, 7), glucose-6-P (7, 8), Mg$^{2+}$ (4, 8, 9), and phosphorylase a (10). Thus, the regulation of each of these interconverting enzymes (i.e. protein kinase and glycogen synthase phosphatase) is an important part of the control of glycogen synthesis.

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The synthase phosphatase reaction has been studied in several mammalian tissues, but little is known of the nature of this enzyme or of the mechanism of regulation of its activity. Since the substrate, glycogen synthase D, is known to interact strongly with most of the molecules known to affect the phosphatase reaction in various tissues, (i.e., Mg$^{2+}$ (11, 12), glucose-6-P (13), and glycogen (14)) the substrate may well be involved in the regulation of the phosphatase reaction. In the work reported here, the phosphatase reaction in heart tissue was studied, the specificity for activators was examined, and several properties of the phosphatase and glycogen synthase D reactions were compared in an attempt to understand the role of the substrate in the control of glycogen synthase D dephosphorylation.

EXPERIMENTAL PROCEDURES

Assays—Glycogen was determined by the phenolsulfuric method (15), and protein by the Folin-Lowry method (16). Glycogen synthase was assayed by the filter paper method described previously (17) except that synthase I was assayed in the presence of 28 mM Na$_2$SO$_4$ to measure the activated form of this enzyme, and total synthase was assayed with 20 mM glucose-6-P. The concentration of activators in these assays was approximately three times that normally used in order to eliminate effects of any endogenous activators present in impure preparations of the phosphatase. Other components of the reaction mixture were 5 mM UDP-glucose, 10 mg per ml of rabbit liver glycogen, and 50 mM Tris-5 mM EDTA (pH of Tris-EDTA adjusted to 7.8 with HCl). The quantity referred to in this report as "% synthase I" was determined by dividing the activity with 28 mM Na$_2$SO$_4$ by the activity with 20 mM glucose-6-P and multiplying by 100. To test the ability of this assay method to give reliable data on the molecular ratio of synthase I to total synthase, purified glycogen synthase I and glycogen synthase D were mixed together to give the theoretical % synthase I desired and the mixtures of enzymes were assayed by the above procedure. The observed % synthase I corresponded well with that expected.

Glycogen synthase phosphatase was assayed by the change in activity of glycogen synthase D into I. Frozen, purified glycogen synthase D (prepared as described below) was thawed, and incubated at 30° at a concentration of approximately 2.5 units per ml (units defined in Table I) in 50 mM Tris-5 mM EDTA (pH 7.8), and 20 mM dithiothreitol for 30 min to fully activate the glycogen synthase. Forty to fifty millimicrons of this enzyme were incubated at 30° in a final volume of 50 μl containing 50 mM
Tris-5 mM EDTA (pH 7.8), 20 mM dithiothreitol, 10 mM MgCl₂, 2 mM glucose-6-P, and the phosphatase preparation. (These conditions of assay are subsequently referred to as “standard conditions.”) The reaction was started by the addition of the phosphatase, and a 10-µl aliquot was immediately removed for the determination of the % synthase I at zero time. At suitable intervals, additional 10-µl aliquots were removed for determination of the % synthase I. All aliquots were immediately diluted 8- to 10-fold by pipetting directly into ice-cold buffer (50 mM Tris-5 mM EDTA (pH 7.8), 20 mM dithiothreitol, 10 mg per ml of rabbit liver glycogen, and 50 mM KE). After all aliquots were collected on ice, glycogen synthase was assayed by the procedure just described.

Enzyme Preparations—Glycogen synthase was purified from bovine heart tissue by methods similar to those used with rabbit skeletal muscle (17a). The % synthase I in these preparations was variable, and, to decrease the content of synthase I, the enzyme was incubated with protein kinase (prepared from bovine heart by the method of Reimann et al. (18)), 5 mM ATP, 7.5 mM MgCl₂, and 10 mM cyclic AMP at 4° by methods similar to those used by Brown and Larner (14). The mixture was incubated for periods up to 10 days in the refrigerator to obtain less than 5% synthase I. There was no loss in activity during this incubation. The final enzyme was precipitated with ethanol (20% v/v), collected by centrifugation (15,000 g, 10 min), and redissolved in 50 mM Tris-5 mM EDTA (pH 7.8) buffer. The enzyme contained approximately 0.3 mg of glycogen per unit of enzyme and could be stored frozen at −80° for several months.

Synthase phosphatase was partially purified from male Wistar strain rats weighing approximately 500 g each. The rats were anesthetized by subcutaneous injection of 0.5 ml of sodium secobarbital (50 mg per ml), and the hearts were removed as quickly as possible. All subsequent steps were carried out at 4° and all dialysis, concentration, and purification steps were done in an Amicon TCF-10 or UF-12 ultrafiltration cell (Amicon Corp., Lexington, Mass.). The tissue was finely diced and homogenized with a Teflon-glass homogenizer in 4 volumes of 50 mM phosphate buffer (pH 7.4) containing 50 mM mercaptoethanol. Debris was removed by centrifugation (15,000 × g, 20 min) and the supernatant was placed in the Amicon TCF-10 ultrafiltration chamber. The sample was diluted to approximately 20 ml with the same buffer used in homogenization and dialyzed with 400 to 500 ml of the buffer using an XM-300 membrane at 20 p.s.i.

In this step and in succeeding steps the red color of the extract was used for a visual check of the progress of the dialysis. We had previously determined that this colored material (presumably a heme protein) had a molecular size that was close to that of the phosphatase and could be used as a marker for that enzyme. The effluent from the above dialysis step was then placed in the same ultrafiltration chamber using a XM-100A membrane, concentrated to approximately 20 ml, and dialyzed with approximately 100 to 200 ml of the same buffer at 20 p.s.i. The final step was to collect the effluent, concentrate it to a final volume of approximately 1 to 2 ml using a PM-30 membrane, and then dialyze with 50 mM Tris-5 mM EDTA (pH 7.8), 50 mM mercaptoethanol. The enzyme could be stored in a refrigerator for 5 to 6 days.

RESULTS

Purification of Glycogen Synthase Phosphatase from Heart—Table I shows data from a typical preparation of this enzyme. All assays were done on fractions that were retained by the various ultrafiltration membranes since enzyme concentrations in the effluent fractions were too low to measure. All of the retained fractions were concentrated to 3 to 5 ml with a PM-30 membrane and dialyzed with 50 mM Tris-5 mM EDTA (pH 7.8), 50 mM mercaptoethanol, to remove phosphate and any other contaminating small molecules before assay.

The final preparation gave a good recovery of activity, but more important, it was free of glycogen synthase and glycogen. The synthase phosphatase was purified 4-fold. Retention of the synthase phosphatase by the PM-30 membrane but not by the XM-100A membrane indicates that this enzyme is similar in size to a globular protein with a molecular weight between 30,000 and 100,000. In additional experiments the phosphatase was not able to penetrate an XM-50 membrane indicating a size greater than 50,000. A substantial portion of the phosphatase in the crude extract was retained by the XM-300 membrane. The amount of enzyme retained by this membrane was somewhat variable in several enzyme preparations. The retained enzyme may result from either a tight complex between the phosphatase and some large components of the crude extract, or a second form of phosphatase with a high molecular weight.

Conditions for Assay of Glycogen Synthase Phosphatase—The data shown in Table II compare the activity of synthase phosphatase under standard assay conditions (with both Mg²⁺ and glucose-6-P), without Mg²⁺, and without glucose-6-P. The enzyme had approximately one-fourth the maximum activity in the absence of both activators and the effect of the activators together was considerably more than the sum of either activator acting alone.

Progress curves of the phosphatase reaction were linear for at
least 20 min, during which time the glycogen synthase could be half converted into the D form. The reaction was inhibited completely by 50 mM KF added either at the start of the experiment or after 10 min. The pH optimum for the phosphatase reaction under standard conditions was 7.3, and in the absence of glucose-6-P it was 7.5.

**Specificity of Activation of Synthase Phosphatase and Glycogen Synthase D**—Tables III and IV show the specificity for activation of both synthase phosphatase and glycogen synthase D by cations and anions. Table III shows that a divalent cation activated both reactions. Mn²⁺ usually gave slightly more activity than Mg²⁺, but we chose to use Mg²⁺ routinely in our assays because of the greater physiological significance of this ion at the concentrations required for activation. Calcium was not as effective as either Mg²⁺ or Mn²⁺ for the phosphatase reaction, and K⁺ and Na⁺ had no effect on either reaction.

The specificity for an anion activator is shown in Table IV. Glucose-6-P was the most active compound tested with both reactions, but glucosamine-6-P was also quite effective. Glucose-1-P stimulated the phosphatase reaction nearly as well as glucose-6-P but had less effect on the glycogen synthase reaction. It was subsequently found that glucose-1-P could be converted to glucose-6-P by contaminating enzymes in the phosphatase preparation at a rate sufficient to account for the inordinate stimulation of the phosphatase reaction by glucose-1-P. No other compound tested was very effective in activation of either reaction studied.

**Comparison of Activation of Glycogen Synthase D and Synthase Phosphatase**—The similarity in the activation specificity (for both an anion and a cation) of both glycogen synthase D and synthase phosphatase, led to the idea that the activators of the phosphatase reaction may be acting directly on the substrate, glycogen synthase D. Therefore, the activation constants for both the synthase phosphatase and glycogen synthase D were compared.

Fig. 1 shows the effect of glucose-6-P on both reactions in the presence of MgCl₂, and Fig. 2 shows the effect of glucose-6-P in the absence of MgCl₂. In all instances glucose-6-P gave linear reciprocal plots over the range of concentrations studied and the activation constants obtained from these plots are summarized in Table V. The A₁/₂ values for both reactions in the absence of MgCl₂ were similar and quite high, while the values in the presence of 5 mM MgCl₂ were decreased 15- to 18-fold. Although there was very little effect of MgCl₂ on the maximum velocity of the synthase reaction, there was a 4-fold increase of the maximum velocity of the phosphatase reaction.

Since it was known that both inorganic phosphate and sulfate inhibit the glucose-6-P activation of the D form of glycogen synthase (13, 19), the effect of these ions on the phosphatase reaction was examined. Fig. 3 shows that both inorganic phosphate and sulfate were potent inhibitors of the glucose-6-P activation of heart glycogen synthase D. Glucose-6-P reversed this inhibition to some extent and in the presence of 1 mM glucose-6-P, the phosphate and sulfate inhibition was far from complete. Fig. 4 shows the results of the same experiment with synthase phosphatase. For this reaction the activation of glucose-6-P was completely inhibited by both inorganic phosphate and sulfate at all concentrations of glucose-6-P tested.

### Table III

**Cation specificity for phosphatase and glycogen synthase D**

The activity was measured in the presence of 2.0 mM glucose-6-P and without EDTA as described under "Experimental Procedure." All activities are expressed relative to that in the presence of Mg²⁺. Synthase phosphatase gave a 28% increase in % synthase I in 20 min with Mg²⁺ and glycogen synthase D activity was 20.9 nmoles of glucose incorporated into glycogen in 10 min.

| Addition (5 mM) | Synthase phosphatase activity % | Glycogen synthase D activity % |
|----------------|---------------------------------|--------------------------------|
| Mn²⁺           | 118                             | 137                            |
| Mg²⁺           | 100                             | 100                            |
| Ca²⁺           | 65.8                            | 99.1                           |
| K⁺             | 26.5                            | 36.9                           |
| Na⁺            | 25.2                            | 37.7                           |
| None           | 26.9                            | 34.2                           |

### Table IV

**Anion specificity for phosphatase and glycogen synthase D**

The activity was measured in the presence of 5 mM MgCl₂ and without EDTA as described under "Experimental Procedure." All activities are expressed relative to that with glucose-6-P. Synthase phosphatase gave a 25% increase in synthase I in 20 min with glucose-6-P and glycogen synthase D activity was 19.1 nmoles of glucose incorporated into glycogen in 10 min.

| Addition (2 mM) | Synthase phosphatase activity % | Glycogen synthase D activity % |
|----------------|---------------------------------|--------------------------------|
| Glucose-6-P    | 100                             | 100                            |
| Glucose-1-P    | 77.5                            | 12.5                           |
| Glucosamine-6-P| 65.5                            | 40.6                           |
| 6-P glucose    | 25.2                            | 1.3                            |
| Fructose-1,6-P | 20.0                            | 1.3                            |
| α-Glycerol-P   | 19.0                            | 0.9                            |
| Citrate-P      | 18.4                            | 0.6                            |
| Pi             | 29.4                            | 0.6                            |
| SO₄            | 29.8                            | 3.8                            |
| None           | 20.4                            | 0.5                            |

**Fig. 1.** Kinetic plots of glucose-6-P activation of glycogen synthase D and synthase phosphatase in the presence of Mg²⁺. The velocity of glycogen synthase D was measured as described under "Experimental Procedure" and is expressed as the increase in % synthase I in a 20-min incubation. All assays were done in the presence of 10 mM MgCl₂.
Fig. 2. Kinetic plots of glucose-6-P activation of glycogen synthase D and synthase phosphatase in the absence of Mg$^{2+}$. Data were obtained as described in Fig. 1. The ordinate values for the synthase phosphatase have been multiplied by 0.2 in order to put the data for both enzymes into one figure.

TABLE V

| Enzyme        | $A_{1/2}$ for glucose-6-P | $V_{max}$ |
|---------------|---------------------------|-----------|
| Synthase D    | 1.6 0.11                  | 11.1 12.8 |
| Synthase phosphatase | 1.1 0.06               | 5.0 21.0 |

Since Mg$^{2+}$ had large effects on the glucose-6-P activation of both enzyme reactions, the requirement for this cation was examined. Fig. 5 shows reciprocal plots for both enzyme reactions in the presence of 2 mM glucose-6-P. The $A_{1/2}$ for Mg$^{2+}$ of the synthase D-catalyzed reaction was 1.6 mM, and that for the phosphatase-catalyzed reaction was 1.4 mM.

Inhibition of Synthase Phosphatase by Glycogen—The relationship of the activators of synthase phosphatase to the inhibition of the enzyme by glycogen is shown in Fig. 6. As shown in Fig. 6a, the reaction was inhibited by glycogen under all assay conditions even though there was a considerable difference in the activity of the phosphatase. These data were replotted (Fig. 6b) to determine the degree of inhibition of glycogen under the different assay conditions. The inhibition by glycogen was the same with or without Mg$^{2+}$ or glucose-6-P. Additional experiments confirmed the observation of Villar-Palasi (6) with the phosphatase from skeletal muscle, that the inhibition of glycogen was competitive with respect to the substrate, glycogen synthase D.

DISCUSSION

Control of the reaction catalyzed by heart glycogen synthase phosphatase may occur by interaction of any effector molecule primarily with the substrate of the reaction (glycogen synthase D), primarily with the synthase phosphatase, or with both of these proteins. To determine the role of both substrate and catalyst in the control of this reaction, we studied the reactions catalyzed by each protein, (i.e. the synthase and phosphatase) to see if there was some similarity in the two reactions with respect to (a) specificity of activation by effector molecules; (b) activation constants for the activators of these reactions; or (c) interaction between the various effector molecules in the regulation of the reaction rate.

It seems that the activation of the heart synthase phosphatase
by both glucose-6-P and by Mg\(^{2+}\) is largely due to the effects of these molecules on the substrate, glycogen synthase D. First, the specificity of activation of both the glycogen synthase D and synthase phosphatase is quite similar. Table III shows that Mn\(^{2+}\), Mg\(^{2+}\), K\(^{+}\), and Na\(^{+}\) had very similar effects on both the synthase phosphatase and glycogen synthase D reactions, while Ca\(^{2+}\) activated the glycogen synthase reaction slightly more than the phosphatase reaction. The differential effect of Ca\(^{2+}\) on these reactions may indicate that there is an effect of these ions on both proteins involved in the phosphatase reaction, but further work will be needed to clarify this point. Table IV shows that the specificity for an anionic activator is nearly identical for the two reactions. The large effect of glucose-1-P on the phosphatase reaction may be explained by the presence of contaminating phosphoglucomutase that produced glucose-6-P during the enzyme assay. Second, the activation constants for both glucose 6-P and Mg\(^{2+}\) (Table V and Fig. 5) for both glycogen synthase D and synthase phosphatase are similar. Third, the effect of Mg\(^{2+}\) (Table V), and of inorganic sulfate and phosphate (Figs. 3 and 4) on the activation of either glycogen synthase D or synthase phosphatase by glucose-6-P was similar. Mg\(^{2+}\) increased the affinity of both reactions for glucose-6-P approximately 10-fold, indicating that binding of glucose-6-P to glycogen synthase D was enhanced by the cation, producing a better substrate for the phosphatase. However, Mg\(^{2+}\) also increased the \(V_{\text{max}}\) of the phosphatase reaction approximately 4-fold, while there was no effect on the \(V_{\text{max}}\) of the glycogen synthase D reaction. Therefore, Mg\(^{2+}\) may have a second role in the phosphatase reaction that is not related to the binding of glucose-6-P to the substrate. It is not possible at present to determine if this Mg\(^{2+}\) effect is the result of binding of the cation to the substrate, glycogen synthase, or to the enzyme, synthase phosphatase. The effect of inorganic sulfate and phosphate on the activation by glucose 6-P was similar for both reactions, but not identical. It is quite obvious that the interaction between glycogen synthase D and synthase phosphatase is a complex one, and small differences in the conformation of the substrate or effects on both substrate and catalyst may be important in explaining the differences in the effect of inorganic sulfate and phosphate on these two reactions.

Inhibition of the synthase phosphatase reaction by glycogen occurred both in the absence and presence of glucose-6-P or Mg\(^{2+}\) (Fig. 6b). This indicates that the glycogen inhibition site and the activation sites for both glucose-6-P and Mg\(^{2+}\) do not interact with each other. One might expect to obtain this data if glycogen bound to the phosphatase and the other effectors bound to the glycogen synthase. Glycogen synthase interacts strongly with glycogen (20–22), however, and it seems reasonable to suspect an effect of glycogen on the substrate in this reaction. The data presented here do not rule out the possibility that glycogen has a direct effect on glycogen synthase D.

This report indicates that the dephosphorylation of glycogen synthase D in heart tissue is under a complex pattern of metabolite regulation involving at least glucose-6-P, Mg\(^{2+}\), phosphate, and glycogen concentrations. There is some question whether or not any of these metabolite effects are due to direct binding of effectors to the phosphatase, but it is quite certain that the substrate plays an important role in the activation of the phosphatase by both glucose-6-P and Mg\(^{2+}\). A similar conclusion concerning the role of glucose-6-P and ATP as effectors of the phosphatase reaction has been reached independently by other research groups (23, 24). Further understanding of the control of the phosphatase reaction will be aided by studies of the conformation states of glycogen synthase and the role of effector molecules in the changes in glycogen synthase conformation.

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