THE ROLE OF ATP AND GLUCOSE 6-PHOSPHATE IN THE REGULATION OF GLYCOGEN SYNTHETASE D PHOSPHATASE

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SUMMARY. Glycogen synthetase D phosphatase catalyzes the conversion of synthetase D to synthetase I. The phosphatase reaction has been found to be inhibited by ATP, ADP and UDP; however, only ATP inhibited at a physiological concentration. ATP inhibition was enhanced by glycogen. Glucose 6-phosphate (G 6-P) stimulated the phosphatase reaction and at least partially relieved the ATP inhibition. A possible physiological role for ATP, G 6-P and glycogen in the regulation of the synthetase D phosphatase reaction has been proposed.

Glycogen synthetase exists in two interconvertible forms in skeletal muscle, synthetase I and synthetase D. Conversion of the I form to the D form is catalyzed by synthetase I kinase. The reverse reaction, conversion of synthetase D to synthetase I, is mediated by synthetase D phosphatase. The I form is considered to be the active form *in vivo* due to metabolite inhibition of the D form (1). Thus, regulation of synthetase phosphatase activity is important in the control of glycogen synthesis. Danforth (2) has shown an inverse relationship between glycogen concentration and the proportion of glycogen synthetase in the I form in mouse skeletal muscle and rat diaphragm. Villar-Palasi (3) reported inhibition of synthetase phosphatase by glycogen in crude extracts; however, an inhibitory effect of glycogen with partially purified synthetase

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phosphatase was not observed. He suggested that the loss of the glycogen effect could be ascribed to an alteration of the synthetase D phosphatase molecule during purification.

In the present study we report the potent inhibition of the synthetase phosphatase reaction by ATP which is independent of the glycogen inhibition previously described.

METHODS. Tissue extracts - Fresh thigh muscle from anesthetized (Seconal, 40 mg/kg) Holtzman strain rats (180-250 g), was immediately cooled on ice, trimmed and minced with scissors. Tissue was homogenized (1:3, w/v) using the buffers indicated in the figure legends. Homogenates were centrifuged at 10,000 x g for 10 minutes and the supernatants used for assay. In some cases supernatants were treated for 10 min at 0° (x2) with an anion exchange resin (AG1-X4, 100 mg/ml). In other experiments 150 µl of homogenates were treated with 25 µl of Apyrase solution (2 mg/25 µl) under the conditions indicated.

Phosphatase assay - Phosphatase activity was assayed by incubating the extracts at 30° and determining the conversion of synthetase D activity to synthetase I activity (Δ synthetase I). Endogenous synthetase D was used as substrate. The reaction was stopped by diluting a 25 µl aliquot in 100 µl of ice cold 200 mM KF or in a solution containing 200 mM KF, 10 mM Pi and 7.5 mM EDTA. Synthetase I activity and total synthetase activity (I+D) were determined using the method of Thomas et al (4). Results are given as units/gram wet weight of tissue. One unit is equivalent to 1 µmole of glucose incorporated into glycogen per minute.

MATERIALS. ATP, ADP, UDP, G 6-P, UDPG, rabbit liver glycogen, and imidazole were purchased from Sigma Chemical Company. Purified Apyrase (Grade 1) was also obtained from Sigma Chemical Company. Glycogen was further purified using the method of Villar-Palasi (5).

All reagents were the highest quality available and were neutralized before use. α-(N Morpholino) ethane-sulfonic acid (MES) was purchased from Calbiochem. UDPG-^{14}C (uniformly labeled) was purchased from New England Nuclear Corporation. AG1-X4 anion exchange resin was purchased from BioRad Laboratories.

RESULTS. In skeletal muscle extracts (1:3) prepared in 50 mM glycylglycine, 10 mM glutathione and 10 mM $MgCl_2$, pH 7.8, synthetase phosphatase was not measurable. Synthetase I activity remained constant while total synthetase activity decreased suggesting that under these conditions synthetase D, substrate for the phosphatase, was inactivated. A loss of substrate could account for the failure to detect phosphatase. Among the agents found to stabilize total synthetase activity was glycogen (0.33% in the phosphatase incubation) and EDTA (3.3 mM). In the presence of glycogen, total synthetase activity remained constant and synthetase I activity rose rapidly after a 10-15 minute lag (Fig 1). The lag was similar to that observed with liver extracts (6) but of shorter duration. Glycogen at this concentration produced little synthetase phosphatase inhibition.

Treatment of skeletal muscle extracts with an anion exchange resin resulted in the complete elimination of the lag period (Fig 1). Similar results were observed when the glycogen concentration in the phosphatase incubation mixture was raised to 1.3%. Incubation of the extract for up to 2 hours at 0° likewise eliminated the lag (results not shown).

It was concluded that the agent(s) responsible for the lag must be anionic and relatively unstable. A number of anionic agents known to play a role in glycogen metabolism were tested for their ability to inhibit phosphatase activity. Of the anions tested only ATP, ADP and UDP were inhibitory (Table I). Only ATP was inhibitory at a normal physiological concentration (Fig 2).

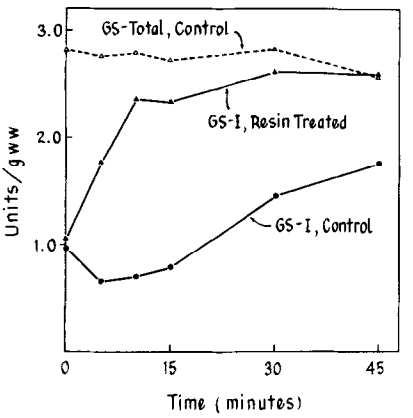

Fig. 1.

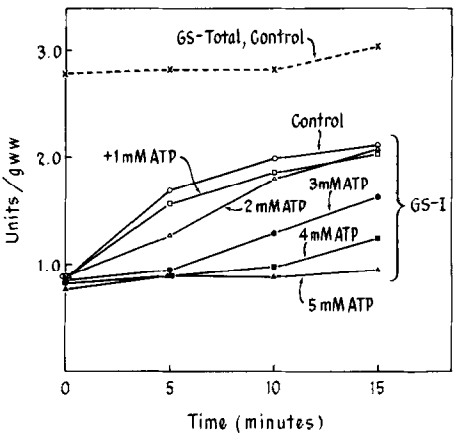

Fig. 2.

Figure 1 - The effect of glycogen and anion exchange resin treatment on synthetase phosphatase activity in crude skeletal muscle extracts. Extracts (1:3, weight/volume) were prepared in 50 mM glycylglycine, pH 7.8 and 0.5% glycogen (final concentration, 0.33%). One portion was treated with BioRad AG1-X4 resin (100 mg/ml) for 10 min (x2) at 0°. Untreated and treated extracts were assayed for phosphatase activity as described.

Figure 2 - The effect of ATP concentration on synthetase phosphatase activity. Muscle extracts were prepared in 50 mM glycylglycine buffer, pH 7.8 containing 0.5% glycogen and resin treated. Extract samples were diluted with a neutralized ATP solution to varying final ATP concentrations. Extracts were assayed for phosphatase activity.

The importance of ATP as the causative agent for the lag observed with added glycogen in tissue extracts was demonstrated in an experiment in which Apyrase (a mixed ATPase and ADPase) was used to treat muscle extracts. Purified Apyrase was added to freshly prepared, non-resin treated extracts and then immediately assayed for phosphatase activity. The lag in synthetase phosphatase activity was completely eliminated (Fig 3). Thus it appears that the lag in phosphatase activity seen in untreated extracts represents the time necessary to reduce ATP concentration to a non-inhibitory level.

An ATP-Mg complex is the substrate for skeletal muscle synthetase I kinase (8) although at a molar ratio of ATP to Mg^{++}

TABLE I

Compound	Phosphatase Activity Concentration		Physiological Concentration of Compound*
	10 mM	1 mM	
ATP	-	+	7 mM
ADP	-	+	0.7 mM
AMP	+	+	0.09 mM
UDP	-	+	<0.07 mM
UDPG	+	+	0.03 mM
G 1-P	+	+	0.015 mM

The effect of various anionic compounds on synthetase phosphatase activity. Muscle extracts were prepared with 50 mM glycylglycine pH 7.8 and 0.5% glycogen and resin treated. To portions of the extract were added neutralized solutions of each compound to yield the final concentration indicated. Extracts were then assayed for synthetase phosphatase activity. (-) indicates no measurable phosphatase activity; (+) indicates measured phosphatase activity equivalent to control activity without additive.

*Piras and Staneloni (7).

greater than 1:1 the reaction rate is reduced and at a ratio of 4:1 the reaction is completely inhibited. Dilution of a muscle extract with an EDTA-containing buffer routinely used to inhibit the kinase reaction (9,10,11) would result in an inactive synthetase kinase in the presence of ATP. To eliminate the possibility that ATP stimulated synthetase kinase in the phosphatase assay, dilute extracts (1:24) containing 5 mM EDTA were assayed for phosphatase activity in the presence and absence of 5 mM ATP. Assuming a Mg^{++} concentration of 11 mM (12) in skeletal muscle, the free Mg^{++} concentration in this system would be less than 0.5 mM with a molar ratio greater than 10:1. Under these conditions ATP was still completely inhibitory (Fig 4). Similarly, ATP at 10 mM was inhibitory. Neither in the absence nor presence of ATP was a net

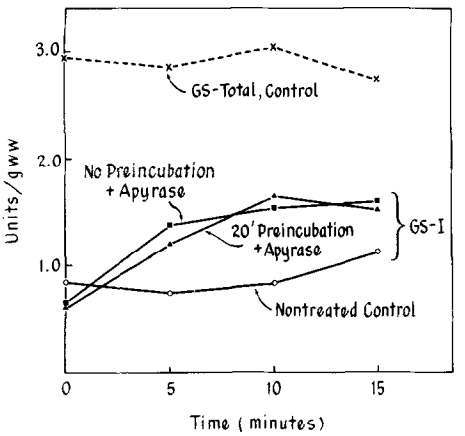

Fig. 3.

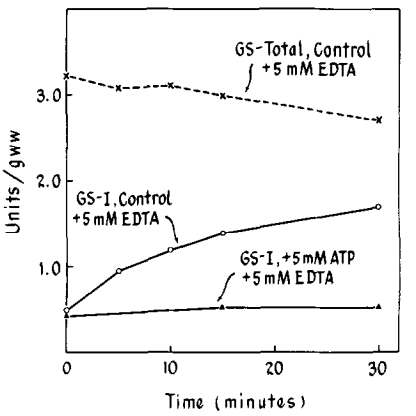

Fig. 4.

Figure 3 - Treatment of muscle extracts with Apyrase and the effect on synthetase phosphatase activity. An extract (1:3) was prepared with 50 mM glycylglycine buffer, pH 7.8 containing 0.5% glycogen. The extract was not resin treated. Twenty-five µl of Apyrase solution (2 mg/25 µl) were used to treat 150 µl of extract. One treated sample was assayed immediately for phosphatase activity and another was allowed to stand on ice for 20 min before the assay. Water was added to a separate sample prior to assay as a control.

Figure 4 - Synthetase phosphatase activity in the presence of EDTA and ATP. Muscle extracts (1:24) were prepared in 50 mM glycylglycine, 0.5% glycogen and 5 mM EDTA and resin treated. ATP (final concentration, 5 mM) was also added to part of the extract. Both samples were then assayed for phosphatase activity.

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increase in synthetase D ever observed to indicate that the synthetase kinase system was active.

The presence of glycogen increased the ATP inhibition (Fig 5). Extracts were prepared in 50 mM Imidazole and 7.5 mM EDTA, pH 7.0 with and without glycogen. Glycogen (0.1%) reduced the phosphatase activity in the presence of 5 mM ATP to one-half that observed in the absence of glycogen.

Glucose 6-Phosphate (0.5 mM) was found to stimulate synthetase phosphatase activity approximately two fold (Fig 6, Panel A) as noted by others (13,14). It also appears that glucose 6-phosphate

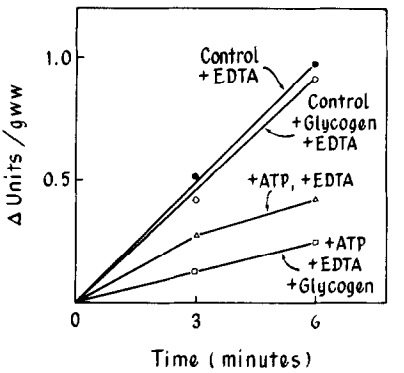

Figure 5 - The influence of glycogen on the ATP inhibition of synthetase phosphatase. Resin treated muscle extracts (1:10) were prepared with 50 mM imidazole buffer, pH 7.0, containing 7.5 mM EDTA with and without glycogen (0.1%, final concentration). ATP (final concentration, 5 mM) was added to part of each extract prior to phosphatase assay.

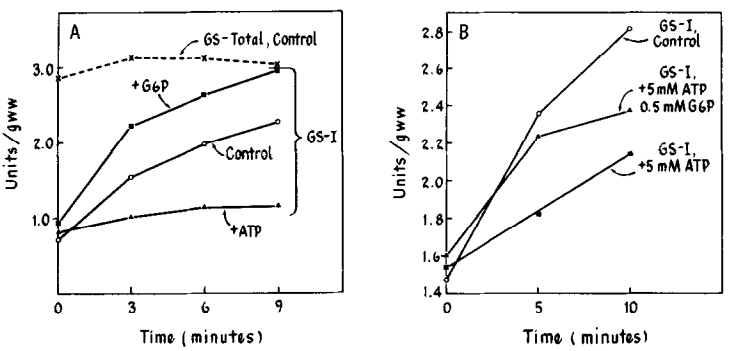

Figure 6 - The influence of glucose 6-phosphate on synthetase phosphatase activity in the presence and absence of ATP. A. A skeletal muscle extract (1:10) was prepared with 50 mM Imidazole buffer, pH 7.0, containing 7.5 mM EDTA and resin treated. Either water, ATP (final concentration, 5 mM) or G 6-P (final concentration, 0.5 mM) was added and the extracts were assayed for phosphatase activity. B. A muscle extract (1:10) was prepared with 50 mM Tris, pH 7.5, containing 7.5 mM EDTA and resin treated. Either water, ATP (final concentration, 5 mM) or glucose 6-phosphate (final concentration, 0.5 mM) and ATP (final concentration, 5 mM) were added. The extracts were then assayed for synthetase phosphatase.

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will reverse ATP inhibition of synthetase phosphatase but only in the absence of added glycogen (Fig 6, Panel B). However, the reversal of ATP inhibition by G 6-P has not been extensively studied as yet.

DISCUSSION. It is apparent that ATP at physiological concentrations inhibited the synthetase phosphatase reaction and the inhibition was enhanced by the presence of glycogen. In the absence of added glycogen, G 6-P stimulated phosphatase activity despite the presence of a physiological concentration of ATP. These observations suggest the presence of a sensitive regulatory mechanism for glycogen synthesis in skeletal muscle mediated through metabolite control of the synthetase phosphatase reaction and independent of hormonal stimulation.

This could possibly explain the increase in % synthetase I in muscle noted by Piras and Staneloni (7) following tetanic contraction. At this time glycogen concentration had decreased from 72 mM to 42 mM, glucose 6-phosphate had increased from 0.3 to 3.0 mM and ATP concentrations were unchanged.

We have previously shown an increase in % synthetase I in anoxic rat heart incubated in vitro (11). Under these circumstances a decrease in glycogen and increase in G 6-P concentration could possibly also play a role in the conversion of synthetase D to synthetase I through a stimulation of synthetase D phosphatase activity. However, preliminary data indicate no inhibition of heart synthetase phosphatase by ATP.

A number of questions remain, particularly with regard to the biochemical mechanisms by which glycogen, ATP and G 6-P interact with the glycogen synthetases and synthetase phosphatase. It is conceivable that the effect of ATP is either directed at synthetase phosphatase or at synthetase D thereby changing its character so that it no longer serves as a substrate for synthetase phosphatase. These questions are currently under investigation.

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