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PURIFICATION AND PROPERTIES OF HEPATIC GLYCOGEN SYNTHETASE OF *RANA CATESBEIANA**

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SUMMARY

Glycogen synthetase (UDPG:glucan glucosyl transferase, EC 2.4.1.11) has been purified about 700-fold from the liver of *Rana catesbeiana* tadpoles. The enzyme has an absolute requirement for glucose 6-phosphate (Glc-6-*P*). Glc-6-*P* lowers the K_m for the substrate, UDPG, without affecting the v_{max}. The reaction is inhibited by nucleotides. UDP and ATP are both competitive inhibitors. While UDP inhibition is completely reversed by high concentrations of Glc-6-*P*, ATP inhibition is only partially reversed. At different concentrations of Glc-6-*P*, the number of interacting sites for ATP has been determined from Hill plots. The *N* number for ATP is 1 at high Glc-6-*P* concentrations and approaches 4 as the Glc-6-*P* concentration is lowered.

INTRODUCTION

Glycogen synthetase (UDPG:glucan glucosyl transferase, EC 2.4.1.11) from various biological systems generally occurs in two forms: one glucose-6-phosphate (Glc-6-*P*) dependent (D- or b-form) and the other independent of Glc-6-*P* (I- or a-form)[1,2]. It has been suggested that the insulin-mediated transformation of the D-form to the I-form is one of the earliest biochemical effects of the hormone[3]. This transformation appears to involve a phosphorylation–dephosphorylation sequence reaction[4].

The hepatic glycogen synthetase of *Rana catesbeiana* tadpoles appears to be unique in that the enzyme is totally Glc-6-*P* dependent. We have previously reported the effect of insulin treatment on tadpole glycogen synthetase[5]. Insulin activates the enzyme by transformation to a form with higher substrate affinity. However, the insulin-transformed enzyme still has an absolute requirement for Glc-6-*P*.

In order to study the mechanism of the insulin-mediated enzyme transformation, we have partially purified tadpole liver glycogen synthetase. The purpose of this communication is to describe the purification procedure and some of the properties of the enzyme.

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MATERIALS AND METHODS

Chemicals

UDPG, other nucleotides and Glc-6-*P* were purchased from Nutritional Biochemical Corp. Shellfish glycogen was the product of Sigma Chemical Co. [^{14}C]UDPG purchased from Schwartz BioResearch had a specific activity of 200 μC/μmole. Glass fiber filters (934 AH) were the product of Wilkins–Anderson Co., Clifton, N.J.

Animals

R. catesbeiana tadpoles weighing 7–10 g were purchased from Lemberger Co., Oshkosh, Wisc. The stock animals were kept in a tank of dechlorinated water (15°) and fed boiled spinach once a week. Treatment of the animals with hormone was carried out, however, at 24°.

Protein determination

Protein was determined by the method of LOWRY *et al.*[6] with crystalline bovine serum albumin as the standard.

Glycogen synthetase and phosphorylase assay

Glycogen synthetase activity was assayed by the method described by VILLAR-PALASI *et al.*[1] with a slight modification. The standard reaction mixtures contained the following components: 0.67 μmole [^{14}C]UDPG (6000 counts/min), 1.00 mg shellfish glycogen, 10 μmoles Glc-6-*P*, 3.5 μmoles sucrose, 2 μmoles EDTA (pH 7.4) 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 0.5 ml of 20% trichloroacetic acid containing 2 mg LiBr per ml. Glycogen, precipitated by the addition of 2 vol. 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 was a linear function of protein concentration and time up to 20 min. This linear portion represented approx. 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 500–1000 counts/min. Shellfish glycogen and tadpole liver glycogen showed no difference as the primer of the reaction.

After appropriate corrections of background, the amount of glucose incorporated into glycogen was calculated. Glycogen synthetase activity was expressed as μmoles of glucose transferred to glycogen per 10 min per g liver tissue (or mg protein). A unit of enzyme is defined as the amount of enzyme necessary for the incorporation of 1 μmole of glucose moiety from UDPG into glycogen in 1 min under the standard assay conditions. Phosphorylase was assayed by the method of SUTHERLAND[7] except that [^{14}C]Glc-1-*P* (200 μC/μmole: New England Nuclear Corp.) was used. Incorporation of radioactivity into glycogen was measured as described for the glycogen synthetase assay.

Purification of glycogen synthetase

Pooled livers of several animals were washed once in 4 vol. of frog Ringer's solution. The washed livers were then homogenized in 4 vol. of 0.4 M sucrose (pH 7.4)

containing 0.01 M EDTA and glycogen (1.5 mg/ml). The tadpole enzyme was found be very unstable when frozen in Tris–HCl buffer; maximum stability was obtained, however, in 0.4 M sucrose solution containing EDTA. The crude homogenate was centrifuged at 30 000 × *g* for 10 min and the precipitates were rehomogenized with 2 vol. of sucrose solution. After centrifugation, the supernatants were combined and used for further purification. The combined supernatant was centrifuged at 144 000 × *g* for 2 h in a Spinco L-2 ultracentrifuge. After centrifugation, the brownish microsomal fraction which sedimented on top of the transparent glycogen pellet was carefully removed by washing with sucrose solution. Approx. 100–200-fold purification of the enzyme was obtained, depending on the preparation. The glycogen pellet, suspended in a small volume of 0.4 M sucrose containing 0.01 M EDTA (pH 7.4) and 0.5% shellfish glycogen, was applied to a DEAE-cellulose column (1.5 cm × 25 cm^2) which was previously equilibrated with 0.4 M sucrose (pH 7.4) containing 0.01 M EDTA. The protein was eluted by stepwise increases in the Tris concentration of the sucrose solution: 0.16, 0.32 and 1.0 M. The elution buffer also contained 0.5% glycogen. Glycogen synthetase activity was eluted by 1.0 M Tris–HCl buffer in 0.4 M sucrose with 0.01 M EDTA and 0.5% shellfish glycogen. Phosphorylase was eluted in the 0.32-M fraction. A summary of the enzyme purification is shown in Table I. Various attempts to isolate the tadpole enzyme free from glycogen have been unsuccessful so far.

TABLE I

PURIFICATION OF GLYCOGEN SYNTHETASE

Fractions	*Specific activity (μmoles/min per mg protein)*		*Total activity (μmoles/min)*	*Purification*	*Recovery (%)*
	+Glc-6-P	*−Glc-6-P*			
Crude extract	0.042	0	5.2	1	
Glycogen pellet (144 000 × *g* for 2 h)	3.92	0	2.94	92	58
DEAE-cellulose fraction	33.13	0	1.59	780	30

RESULTS

Effect of buffer and pH

The optimum pH for glycogen synthetase activity was markedly affected by the nature of the buffer used (Fig. 1). The optimum pH with Tris–HCl buffer was 7.0. Although the reaction rate was slightly greater with glycylglycine buffer, the pH profile was similar. On the other hand, the enzyme was only slightly active in histidine buffer in the range where the other two buffers were effective. An absolute requirement for Glc-6-*P* was observed throughout the pH range examined. A similar dependence of optimum pH on the nature of the buffer has been observed with glycogen synthetase from other biological sources[8–10].

Highly purified enzyme was extremely labile in Tris–HCl buffer upon storage; complete inactivation was observed at 24 h at −20°. However, the inclusion of 0.4 M

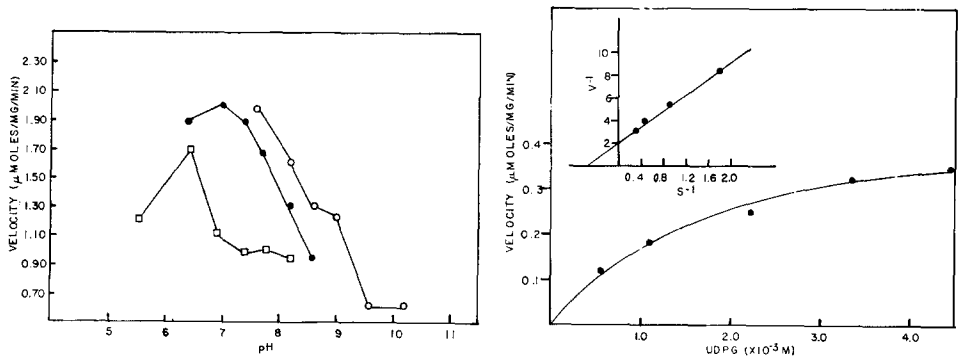


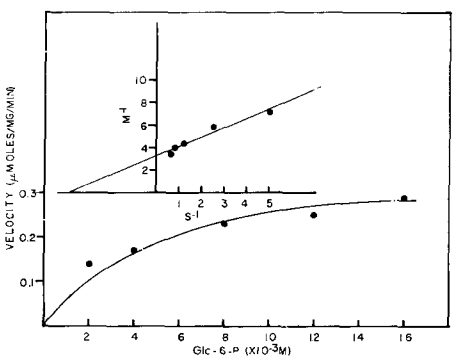
Fig. 1. The effect of pH and buffers on the activity of glycogen synthetase. Enzyme (1.2 μg protein) eluted from the DEAE-cellulose column as described in MATERIALS AND METHODS was used. Assay conditions were as described in MATERIALS AND METHODS. ●—●, 0.02 M Tris–HCl buffer; ○—○, 0.02 M glycylglycine buffer; □—□, 0.02 M histidine buffer.

Fig. 2. The determination of K_m for UDPG. Assay conditions as described in MATERIALS AND METHODS except that saturating amounts of Glc-6-*P* (20 mM) and 700-fold purified enzyme were used.

sucrose and 0.01 M EDTA protected the enzyme from inactivation. Thus, the purified enzyme could be stored at −20° without any loss of activity for up to 1 week.

Apparent K_m's for UDPG and Glc-6-P

The rate of reaction at different UDPG concentrations is shown in Fig. 2. Several determinations of the apparent K_m for UDPG in the presence of a saturating concentration of Glc-6-*P* (20 mM) gave (2.5 ± 0.7) mM. The apparent K_m for Glc-6-*P* was found to be 2.5 mM (Fig. 3). Glc-6-*P* affected the K_m for UDPG as shown in Fig. 4. Similar observations were made with rabbit muscle enzyme[11]. There were no significant changes in v_{max}. The apparent K_m for UDPG of yeast enzyme is not affected by Glc-6-*P*[11]. When the data for UDPG and Glc-6-*P* concentration effect on the rate



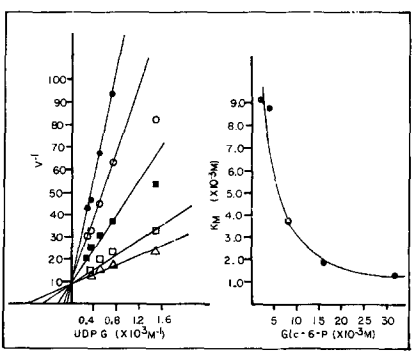


Fig. 3. The determination of the K_m for Glc-6-*P*. Assay conditions as described in MATERIALS AND METHODS, except that UDPG concentration was 3.35 mM.

Fig. 4. The effect of Glc-6-*P* concentration on the K_m for UDPG. The K_m for UDPG was determined at different concentrations of Glc-6-*P*, as described previously. Glc-6-*P* concn. (mM): △—△, 32; □—□, 16; ■—■, 8; ○—○, 4; ●—●, 2.

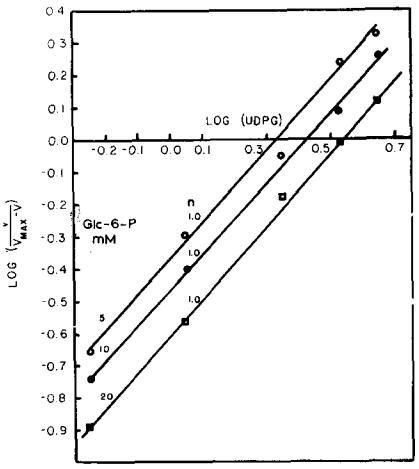


Fig. 5. Hill plot for UDPG at different levels of Glc-6-*P*. The data were plotted by the method of ATKINSON[13]. Glc-6-*P* concn. (mM): ○—○, 5; ●—●, 10; □—□, 20.

were drawn as a Hill plot[12], the slope (n) for UDPG was 1 within the concentration of Glc-6-*P* tested (Fig. 5). This observation, together with the fact that Glc-6-*P* affects the K_m for UDPG of glycogen synthetase, indicates that Glc-6-*P* exerts its effect on the UDPG binding site(s) independently, if there are more than one. Conversely, UDPG concentration did not affect the slope for Glc-6-*P* in the Hill plot (slope $n = 1$ for Glc-6-*P*).

Among the various phosphate esters tested as possible activators, Glc-6-*P*, fructose 6-phosphate (Fru-6-*P*) and fructose 1,6-diphosphate (Fru-1,6-P_2) were effective (Table II). Triose phosphate, such as 3-*P*-glycerate, activate the yeast enzyme[14]. The failure of triose phosphates and Glc-1-*P* to activate the tadpole enzyme suggests that in this respect, the tadpole enzyme is similar to mammalian glycogen synthetases. The structural aspects of the activator have been reported[15].

TABLE II

EFFECT OF ACTIVATOR ANALOGUES

Assays were performed as described in MATERIALS AND METHODS, with 700-fold purified enzyme. Activities with analogues are expressed as percent of that obtained with 10 mM Glc-6-*P*.

Activator	*Concn. (mM)*	*Activity (%)*
Glc-6-*P*	10	100
Glc-1-*P*	10	4
Fru-6-*P*	10	74
Fru-1,6-P_2	10	18
Creatine-*P*	10	4
α-Glycerol-*P*	10	2
3-*P*-Glycerate	10	0

Effect of nucleotides

In Table III, the inhibitory effect of various nucleotides on tadpole glycogen synthetase is shown. The most potent inhibitor among the nucleotides is UDP, the

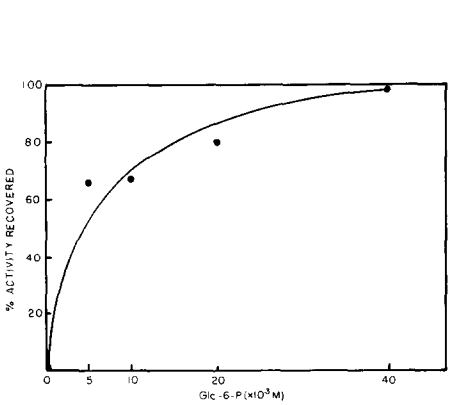
TABLE III

EFFECTS OF NUCLEOTIDES

Glycogen synthetase was assayed in the presence of 2 mM nucleotide as described in MATERIALS AND METHODS. Percentage inhibition was calculated on the basis of incorporation in the absence of these nucleotides.

Nucleotides	*Activity (μmoles/mg protein per 10 min)*	*Inhibition (%)*
None	4.47	0
ADP	0.96	79.8
UDP	0.31	93.5
UMP	1.39	71.0
AMP	2.81	41.1
CMP	4.38	8.2
GMP	2.97	37.7
CTP	3.71	22.2
UTP	0.68	85.7
GTP	2.82	40.8

reaction product. Similar observations have been reported with the enzyme from other species[8]. Simple kinetic studies indicated that UDP inhibition was of the true competitive type. However, UDP inhibition was almost completely reversed by Glc-6-*P* as shown in Fig. 6. Since it is unlikely that UDP and Glc-6-*P* have a common binding site, reversal of UDP inhibition by Glc-6-*P* should not be truly competitive. Indeed, Glc-6-*P* reversed UDP inhibition noncompetitively at lower concentrations, while it acted competitively at higher concentrations (Fig. 7). UDP inhibition of yeast[11] and rat muscle[16] glycogen synthetases was not reversed by Glc-6-*P*. In contrast, reversal of UDP inhibition is achieved for rat-liver enzyme by Glc-6-*P*. Thus, in this respect the tadpole enzyme behaves similarly to rat-liver glycogen synthetase.



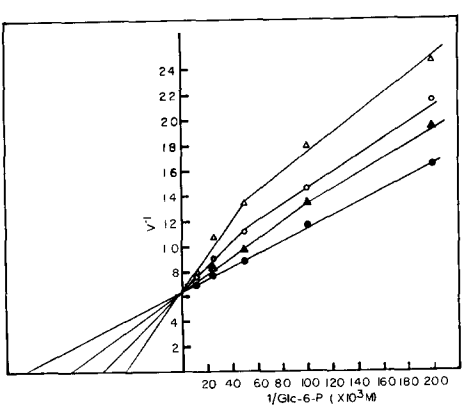
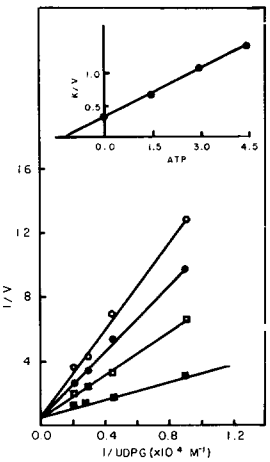


Fig. 6. Reversal of UDP inhibition by Glc-6-*P*. UDP was present at 0.1 mM. Activity recovered was calculated as the percent of that found without UDP.

Fig. 7. Kinetics of interaction between UDP and Glc-6-*P*. ●—●, control, (no UDP); ▲—▲, 0.1 mM UDP; ○—○, 0.2 mM UDP; △—△, 0.4 mM UDP. Inhibition was noncompetitive at low concentrations; competitive at high concentrations.



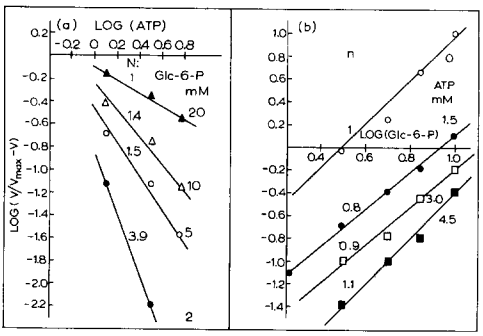


Fig. 8. Competitive interaction between high ATP concentrations and UDPG. ■—■, control with no ATP; □—□, 1.5 mM ATP; ●—●, 3.0 mM ATP; ○—○, 4.5 mM ATP.

Fig. 9. Hill plots[13]. a. Effect of variation of ATP concentration in the presence of several fixed concentrations of Glc-6-*P*. b. Effect of variation of Glc-6-*P* concentration in the absence and in the presence of several concentrations of ATP.

High concentrations of ATP also inhibit competitively with respect to UDPG (Fig. 8). However, ATP inhibition was reversed by Glc-6-*P* only partially (Table IV). The K_i for ATP was calculated to be 1.5 mM. While the general inhibitory effect of ATP is clear from Fig. 8, the cooperative effect of ATP becomes apparent at higher concentrations of ATP and lower concentrations of Glc-6-*P*. As shown in Fig. 9a, when the effect of ATP in the presence of different concentration of Glc-6-*P* is drawn as a Hill plot, cooperativity is very apparent. The extrapolated slope (n) decreased from 3.9 to 1 as the Glc-6-*P* concentration increased (Fig. 9a). However, cooperative effects were not observed with ATP (Fig. 9b). This indicated that in the absence of Glc-6-*P* there are multiple interacting sites for ATP which are modified by Glc-6-*P*. Similar decreases in the slope for ATP were also observed with the enzyme prepared from the insulin-treated animals[5].

TABLE IV

EFFECT OF Glc-6-*P* ON ATP INHIBITION

The reversal of ATP (1.5 mM) inhibition by different concentrations of Glc-6-*P* is shown. Percent activity is calculated from that obtained in the absence of ATP compared to that obtained with ATP, at the Glc-6-*P* concentration indicated.

Glc-6-P (mM)	*+ATP**	*−ATP**	*Activity (%)*
3	0.56	2.20	25.6
5	1.41	4.10	34.4
10	2.26	4.96	45.7
20	3.28	6.56	50.0
40	4.00	6.86	58.0

* μmoles/mg protein per 10 min.

TABLE V

EFFECTS OF VARIOUS ANIONS ON GLYCOGEN SYNTHETASE

Glycogen synthetase was assayed as described in MATERIALS AND METHODS except that 10 μmoles of the sodium salt of the anions listed was present in the assay system. Activity is reported as a percentage of the activity of the control.

Anion	*Activity (%)*
None	100
Acetate	100
Chloride	89
Citrate	100
Arsenate	72
Fluoride	92
Phosphate	52
Pyruvate	100
Sulfite	40

The effect of other anions

Since it has been reported that some metabolic anions have inhibitory effects on yeast glycogen synthetase[14], the effect of various anions on the tadpole enzyme was examined. As shown in Table V, certain anions exerted some degree of inhibition. Since these determinations were made in the presence of a rather high concentration of Glc-6-*P* (20 mM), it is possible that these anions could exert a greater inhibitory effect under other conditions. It is interesting to note that, at lower concentrations, inorganic phosphate (P_i) stimulated the enzyme, as shown in Fig. 10. Although P_i could not completely replace the requirement for Glc-6-*P*, it did lower the K_m for Glc-6-*P* from 2.5 to 1.1 mM. Further experiments are necessary to clarify the physiological significance of this effect.

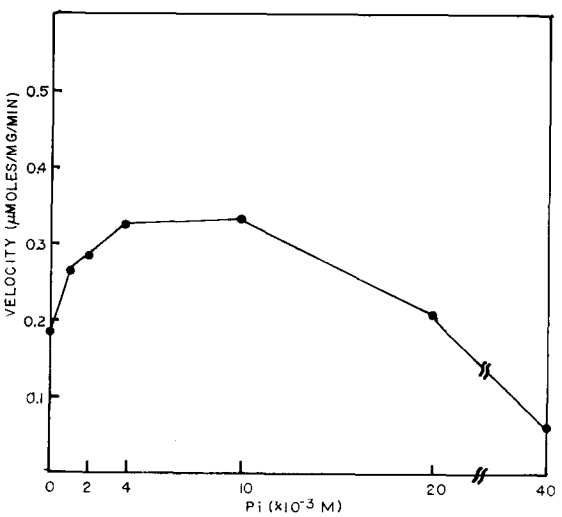


Fig. 10. Effect of P_i on glycogen synthetase. Assay conditions were the same as described in MATERIALS AND METHODS except that 10 mM P_i and a different concentration of Glc-6-*P* were present.

TABLE VI

EFFECT OF SULFHYDRYL REAGENTS

Glycogen synthetase was assayed as usual with the sulfhydryl reagents at the concentrations indicated and 20 mM Glc-6-*P*. Activities expressed as percentage of that obtained without the sulfhydryl reagents.

Reagents	*Concn. (mM)*	*Activity (%)*
None	—	100
Cleland's reagent	1	125
	0.5	114
Mercaptoethanol	50	123
	25	123
Reduced glutathione	1	124
	0.1	118
Cysteine	1	106
	0.1	114
Mg^{2+}	10	149
	1	144
Mercaptoethanol; Mg^{2+}	50; 10	126
	5; 1	120
Mercuribenzoate	0.33	0
	0.165	0
	0.082	0

Sulfhydryl reagents

Various sulfhydryl compounds stimulated the enzyme activity to different degrees (Table VI). These observations, together with the inhibitory effect of mercuribenzoate, suggest that a sulfhydryl group is involved in the functioning of the enzyme. Mg^{2+} stimulated activity by about 50%. The mechanism of this cation stimulation is not clear. However, the effect of this cation might be the result of Mg^{2+} chelation of UDP which is a reaction product and competitive inhibitor.

DISCUSSION

While tadpole hepatic glycogen synthetase exhibits kinetic properties generally similar to those of the enzyme from mammalian sources, it is unique in having an absolute requirement for Glc-6-*P*. Thus, tadpole liver glycogen synthetase can be controlled by multiple mechanisms. (1) Glc-6-*P* concentration: Glc-6-*P* can increase the substrate affinity; that is, the K_m for UDPG is decreased; (2) hormonal effect: insulin treatment of tadpoles transforms glycogen synthetase to a form with a higher substrate affinity; (3) metabolites: tadpole glycogen synthetase is also controlled by various metabolic anions.

Since Glc-6-*P* affects the apparent K_m for UDPG, it is still possible that an undetectably low rate of reaction occurs in the absence of Glc-6-*P*. However, it was not possible to detect any incorporation of the glucose moiety into primer glycogen when the specific radioactivity of UDPG was increased 10-fold. Thus, the tadpole enzyme can be defined as a glycogen synthetase with an absolute dependence on Glc-6-*P*. This definition is further supported by the fact that, although the K_m for UDPG of the enzyme from insulin-treated animals was 0.2—0.05 that of the control enzyme, it had the same K_m for Glc-6-*P* (2.5 mM) and also had an absolute requirement for Glc-6-*P*.

Our kinetic studies of the tadpole enzyme are in accordance with the general observation that this enzyme system is universally inhibited by ATP. This property has apparently been maintained throughout the evolutionary process. The inhibitory effect of ATP, other nucleotides, and anions make it tempting to adapt the hypothesis advanced for the yeast system; that is, in the absence of Glc-6-*P*, various metabolites keep the enzyme in an inhibited state. The level of Glc-6-*P* would then control the rate of glycogen synthesis by competing with the inhibitors.

In spite of the seemingly competitive nature of the ATP inhibition, it is difficult to determine whether ATP actually binds to the same site as the substrate, UDPG, in view of the fact that Glc-6-*P* almost completely reverses the probably true UDP competition. A similar situation is found with rat-liver enzyme. In this case, Glc-6-*P* reversal of UDP inhibition was explained on the basis of a decreased binding affinity for UDP in the presence of Glc-6-*P* by virtue of an increased negative charge in the vicinity.

A decrease in the multiple ATP interacting sites on the tadpole enzyme was observed in the presence of increased Glc-6-*P*. It is interesting to note that the enzyme prepared from insulin-treated animals showed a similar change in sensitivity to this modifier. Insulin treatment resulted in a decrease in interacting sites to 1.0 from a value of 3.9 for the control. However, the failure of high concentrations of Glc-6-*P* to completely reverse ATP inhibition, suggests that the binding sites of Glc-6-*P* and ATP are different.

ACKNOWLEDGMENTS

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