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INTERCONVERSION AND CHARACTERIZATION OF D AND I FORMS OF GLYCOGEN SYNTHASE FROM FROG MUSCLE

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Summary

Glycogen synthase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) from frog skeletal muscle appeared mostly in D form, but could be converted in vitro into I form by preincubation at 30° C of either crude extracts or glycogen particulate fractions. The optimum pH for D to I conversion was around 7.8. A great increase in the rate of conversion was observed in the presence of glucose-6-*P* with an $A_{1/2}$ for this process of about 1 mM.

Galactose-6-*P*, glucosamine-6-*P*, 2-deoxyglucose-6-*P* and ribose-5-*P* also stimulated the conversion, as well as Mg^{2+}, Ca^{2+} and Mn^{2+}. ATP, inorganic phosphate and glycogen were inhibitors of the conversion. Competitive inhibition patterns were observed between glucose-6-*P* and inorganic phosphate, but no competition was observed with glycogen.

Glucose-6-*P* stimulated the activity of both the I and the D forms although the $A_{1/2}$ value for the former was over 40 times lower than that for the latter. Glucose-6-*P* considerably decreased the K_m for UDPglucose on the I form but affected only the *V* on the D. ATP and high concentrations of inorganic phosphate inhibited differentially the two enzyme forms, the inhibition being competitive with respect to glucose-6-*P* in both cases. Stimulation of the I form and inhibition of the D form were observed at low concentrations of inorganic phosphate.

The conversion from the D to the I form of glycogen synthase from frog skeletal muscle is similar to that observed in other tissues where glycogen synthase-D phosphatase has been shown to be responsible for this transformation, although in frog muscle a high dependence on glucose-6-*P* is observed for this process.

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Introduction

In most tissues glycogen synthase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) exists in two different forms, D (or dependent on glucose-6-*P* for its activity) and I (or independent) [1,2], which are interconvertible by reactions of phosphorylation and dephosphorylation [3]. Although the characterization of these forms was based primarily on their dependence (D form) on glucose-6-*P* [4], some other characteristic differences have also been pointed out lately, such as their different kinetic behaviour [1], their different sensitivities toward ATP [5,6] and the differential effect that phosphate ions have on the two forms of the enzyme [7,8]. However, in frog skeletal muscle [9,10], as well as in toadfish [9], the enzyme was reported to be present only in the D form and no D to I conversion could be demonstrated. Instead, in these tissues, the presence of an inactive form of the enzyme that could be converted into the D form was described [9—11]. Since it is accepted that only I forms are active in vivo [6] and that the regulation of glycogen biosynthesis is based mainly on the interconversion between the D and I forms, the mechanism of control of glycogen biosynthesis in frog skeletal muscle has remained obscure.

In the present work we report that the D to I transformation can be observed in frog skeletal muscle preparations and that a change in the kinetic characteristics of the enzyme takes place during conversion. Similar D to I conversion has recently been observed in the liver of adult frogs, both in vivo [12] and in vitro [13].

Materials and Methods

UDPglucose, ATP, all the sugar phosphates and rabbit liver glycogen were obtained from Sigma Chem. Co.; $NADP^+$ and glucose-6-*P* dehydrogenase from Boehringer Mannheim, G.F.R. and UDP-[U-^{14}C]glucose from the Radiochemical Centre (Amersham, U.K.).

Enzyme preparation. Female *Rana ridibunda* (30—50 g) were pithed and the muscle from the hind legs was quickly removed and homogenized with 5 vols. of cold 0.05 M Tris · HCl buffer (pH 7.8)/0.005 M EDTA. The homogenate was centrifuged at 2500 $\times g$ for 20 min at 4°C. The resultant crude extract was centrifuged at 25 000 $\times g$ for 1 h at 4°C. The sediment was resuspended in the same buffer, except where indicated, and contained most of the glycogen synthase, which was purified about 20-fold upon completion of this step [14].

For the kinetic studies 25 000 $\times g$ pellets from non-treated frogs showing practically no glycogen synthase activity when assayed in the absence of glucose-6-*P* were used as the source of D activity. In order to obtain sources of I activity, the 25 000 $\times g$ sediments were preincubated after resuspension in Tris · HCl buffer at 30°C in the presence of 5 mM Mg^{2+} and 1 mM glucose-6-*P* for 2 h. After centrifugation of the incubated mixture at 100 000 $\times g$ for 1 h sediments were obtained containing glycogen synthase whose level of independent activity was at least 95% of the total. In both cases, the corresponding sediments were resuspended in 0.05 M Tris · HCl buffer (pH 7.8) to give a final activity of 0.1 unit/ml. Activity and percentage of the independent activity of the enzyme were stable in these preparations when stored at —20°C.

Analytical procedures. Glycogen synthase activity was assayed at 30°C by the method of Thomas et al. [15]. One unit of glycogen synthase is the amount of enzyme that incorporates 1 μmol of glucose from UDPglucose to glycogen per min.

The glycogen synthase D to I conversion was followed by determining the change in the glycogen synthase I activity upon incubation at 30°C under various experimental conditions. The concentration of glycogen synthase D was always set at 0.2 unit/ml and a time course of the reaction was run for each concentration of modifier. Total glycogen synthase activity remained constant except where indicated.

Protein was determined by the Folin-Lowry method [16] and glucose-6-*P* by the glucose-6-*P* dehydrogenase method [17].

Results

Glycogen synthase conversion

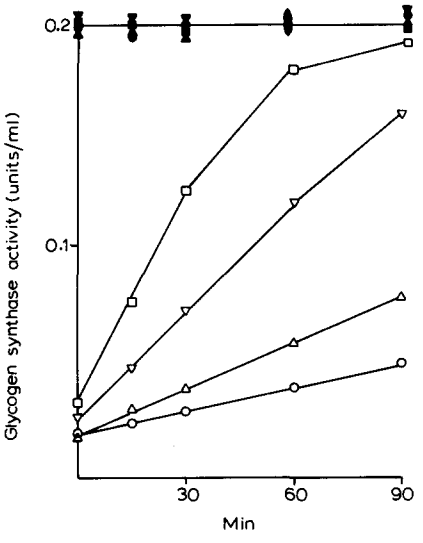
In crude extracts of frog muscle glycogen synthase I activity was only about 10—20% of total activity. When incubated at 30°C without additions, an increase of both the I and total activities was observed, with only a small change in the percentage of the I form, but, by incubation in the presence of 5 mM Mg^{2+} and 10 mM dithiothreitol, a time-dependent rise of the percentage of I form (3—5-fold) was observed (data not shown).

An increase in I activity was also observed by preincubation of 25 000 $\times g$ sediments in the presence of 5 mM Mg^{2+} and 10 mM dithiothreitol (Fig. 1). In both the crude extracts and the 25 000 $\times g$ particulate fractions, the rate of the conversion of glycogen synthase D to I was influenced by the pH of the media, showing a sharp peak at a pH of 7.8 (Fig. 2).

Effect of glucose-6-P. Glucose-6-*P* (at a concentration of 1 mM) greatly stimulated the conversion of glycogen synthase D to I in the 25 000 $\times g$ sediments (Fig. 1). The additional presence of 5 mM Mg^{2+} caused a further increase in conversion rate. From double reciprocal plots the $A_{1/2}$ value of glucose-6-*P* for the glycogen synthase D to I conversion was observed to be 1 mM.

The effect of glucose-6-*P* was not due to an apparent increase in I activity as a consequence of the endogenous glucose-6-*P* stimulation on the D activity in the synthase assay, since the 10 mM Na_2SO_4 present in the assay mixture would have completely counteracted such an effect *. Furthermore, the increase in I activity was clearly time dependent and, in addition, when samples were taken and centrifuged at 100 000 $\times g$ for 1 h at different points during the period of incubation, both I and D activities being assayed in the sediments, the values found were in agreement with those observed prior to centrifugation. Moreover, when incubation with 1 mM glucose-6-*P* was carried out at 0°C for 90 min with subsequent centrifugation at 100 000 $\times g$ for 1 h, no increase in the I activity in the sediment could be observed, which indicated that the increase in I activity, where observed, was not due to the binding of glucose-6-*P* to the D form enzyme. As is described later, a complete change in the kinetic properties of the enzyme took place during incubation.

* Unpublished data. In fact, though, this is evident also from Fig. 5 since phosphate usually behaves very similarly to sulphate with respect to synthase kinetic properties.



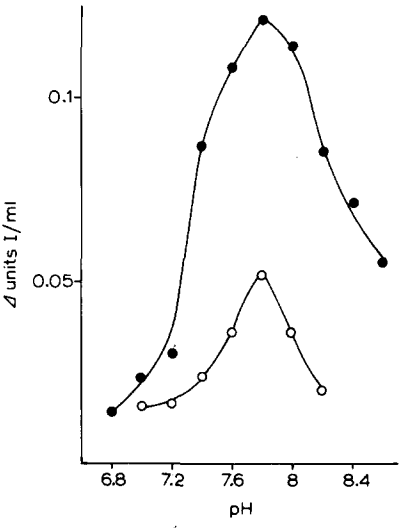


Fig. 1. Effect of activators on the glycogen synthase D to I conversion. The resuspended 25 000 × *g* glycogen particulate fraction was incubated at 30°C in the presence of 10 mM dithiothreitol alone (○, ●) and with the following additions: 5 mM Mg^{2+} (△, ▲), 1 mM glucose-6-*P* (▽, ▽), or 5 mM Mg^{2+} and 1 mM glucose-6-*P* (□, ■). Glycogen synthase I (empty symbols) and total activities (filled symbols) were assayed at the times indicated.

Fig. 2. Effect of pH on glycogen synthase D to I conversion. Both the crude extract (○) and the glycogen particulate fraction (●) were incubated at 30°C in 50 mM Tris · HCl buffer/5 mM EDTA at indicated pH values. Preincubation was carried out in the presence of 5 mM Mg^{2+} and 10 mM dithiothreitol in the case of crude extract, while in the case of the glycogen particulate fraction 1 mM glucose-6-*P* was also added. The incubation time was 1 h for the crude extract and 30 min for glycogen particulate fraction.

Effect of divalent cations. The effect of several divalent cations was studied both in the presence and absence of 1 mM glucose-6-*P* (Table I). Mg^{2+} was the most effective of those tested. Mn^{2+} slightly stimulated the D to I conversion in

TABLE I

EFFECT OF DIVALENT CATIONS ON GLYCOGEN SYNTHASE D TO I CONVERSION IN 25 000 × *g* GLYCOGEN PARTICULATE FRACTIONS

The buffer used for the incubation was 50 mM Tris · HCl, pH 7.8. The concentration of cation was always 5 mM.

	Activity (nmol/min per ml)				Δ%I
	0 min		30 min		
	+Na_2SO_4	+Glc-6-*P*	+Na_2SO_4	+Glc-6-*P*	
No additions	11.2	184	19.9	169	5.7
+Mg^{2+}	10.8	177	29.1	155	12.8
+Mn^{2+}	20.4	234	32.6	209	6.6
+Ca^{2+}	10.4	137	15.4	95	8.5
1 mM Glc-6-*P*	17.2	184	61.5	184	24.0
+Mg^{2+}	30.8	178	98.4	178	38.0
+Mn^{2+}	61.5	246	101.5	193	27.6
+Ca^{2+}	24.6	148	70.0	101.5	42.4

TABLE II

STIMULATION OF THE GLYCOGEN SYNTHASE D TO I CONVERSION BY SUGAR PHOSPHATES

The glycogen synthase D to I conversion was carried out for 30 min under standard conditions in the presence of the indicated metabolites at 1 mM. The results of the synthase conversion are refered to the conversion obtained in the control without additions. Concentrations of glucose-6-*P* in the incubation mixture measured at the end of the incubation are also presented.

	Without Mg^{2+}		5 mM Mg^{2+}	
	D to I conversion	Glc-6-*P* (mM)	D to I conversion	Glc-6-*P* (mM)
No additions	1.0	0	1.0	0
Glucose	1.3	—	0.8	—
Glucose-6-*P*	7.1	0.98	6.9	0.90
Galactose-6-*P*	5.6	0	4.6	0
Glucosamine-6-*P*	2.7	0	3.5	0
Mannose-6-*P*	0.9	0.03	1.7	0.04
2-Deoxyglucose-6-*P*	4.7	0	2.2	0
Ribose-5-*P*	4.1	0	1.7	0
Glucose-1-*P*	1.0	0.02	6.6	0.78
Glucose-1,6-P_2	—	—	0.5	—
Fructose-6-*P*	7.4	0.85	5.5	0.85

the absence of glucose-6-*P*. In addition, Mn^{2+} had a clear stimulatory effect on both the I and the total glycogen synthase activities. Ca^{2+} showed a stimulatory effect on the conversion, giving rise to percentages of I activity similar to or even greater than Mg^{2+}, but in this case interpretation will be complicated if Ca^{2+}-stimulated proteases are present in frog muscle as in rabbit muscle [18]. From double reciprocal plots it was evidenced that Mg^{2+} did not affect the $A_{1/2}$ value for glucose-6-*P* of the glycogen synthase D to I conversion, although its presence, at 5 mM, increased 4-fold the *V* of the reaction.

Effect of other sugar phosphates. Besides glucose-6-*P*, some other sugar phosphates were stimulators of the D to I conversion as shown in Table II. Measurements of glucose-6-*P* concentration in the incubation media were made to ensure that the effect of these sugar phosphates was not due to their conversion into glucose-6-*P* or the possible presence of contaminating glucose-6-*P* in the commercial products. Glucose-6-*P* was the best stimulator. Galactose-6-*P* and glucosamine-6-*P* were also stimulators, whereas mannose-6-*P* was not a stimulator in the absence of Mg^{2+} and had only a slight effect in its presence. Glucose-1-*P* had no effect on the glycogen synthase D to I conversion since stimulation was only observed in the presence of Mg^{2+} where conversion into glucose-6-*P* occurred. 2-Deoxyglucose-6-*P* and ribose-5-*P* also showed stimulatory effects, especially in the absence of Mg^{2+}. Neither glucose-1,6-P_2 nor glucose activated the conversion. The effect of fructose-6-*P* could not be evaluated since a clear conversion into glucose-6-*P* was always observed.

Effect of KF, inorganic phosphate, ATP and glycogen. In frog skeletal muscle KF was observed to be a strong inhibitor of the glycogen synthase D to I conversion. Mg^{2+} at 5 mM counteracted this effect to a certain extent, increasing from 12 to 25 mM the concentration of F^- necessary to cause a 50% inhibition. In all cases inhibition was complete at 100 mM KF.

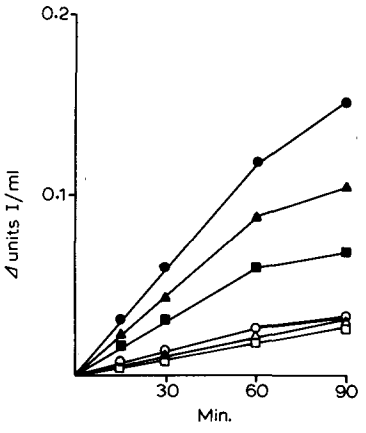


Fig. 3. Effect of ATP on the glycogen synthase D to I conversion. The resuspended 25 000 × *g* glycogen particulate fraction was incubated at 30°C in the presence of 10 mM dithiothreitol, either alone (○) or plus 0.2 mM ATP (△), 1 mM ATP (□), 1 mM glucose-6-*P* (●), 0.2 mM ATP and 1 mM glucose-6-*P* (▲) and 1 mM ATP and 1 mM glucose-6-*P* (■). At timed intervals, samples were taken for the measurement of glycogen synthase activity.

Phosphate ions were also inhibitors of the D to I conversion. From double reciprocal plots, their effect was seen to be competitive with respect to glucose-6-*P*, the $A_{1/2}$ value for this metabolite increasing from 1 mM in the absence of phosphate to 13 mM in the presence of 10 mM phosphate. ATP also showed an inhibitory effect, counteracting the activation by glucose-6-*P* (Fig. 3).

Glycogen was found to be an inhibitor of the D to I conversion, although even with 10 mg/ml glycogen this inhibition was less than 50%. Upon representation by double reciprocal plots it was seen that the glycogen effect was on the V of the conversion and not on the $A_{1/2}$ for glucose-6-*P*. The concentration of polysaccharide necessary to decrease the V to one-half should be about 11 mg/ml, an amount that, in fact, must be even higher due to the presence of endogenous glycogen (about 1.8 mg/ml) in the incubation media.

Kinetic behaviour of the incubated and non-incubated enzyme

Effect of glucose-6-P. At a concentration of 6.6 mM UDPglucose, glucose-6-*P* had no significant effect on the activity of the I form preparation, whereas its presence was necessary for the non-incubated one to be active. However at a low UDPglucose concentration (0.4 mM), both enzyme preparations were stimulated by the metabolite (Fig. 4). Nonetheless, the $A_{1/2}$ value corresponding to the I form preparation (0.01 mM) was 40 times lower than that for the D form (0.4 mM).

With the I form, glucose-6-*P* decreased the K_m value for UDPglucose from 0.6 to 0.08 mM, without modifying the V and this effect was already maximal at a concentration of glucose-6-*P* of 0.1 mM. On the other hand with the D form preparation, glucose-6-*P* greatly increased the V without modifying the K_m value for UDPglucose which was 0.3 mM. Furthermore, the increase of the V in the D form preparation was observed at all glucose-6-*P* concentrations tested (up to 5 mM).

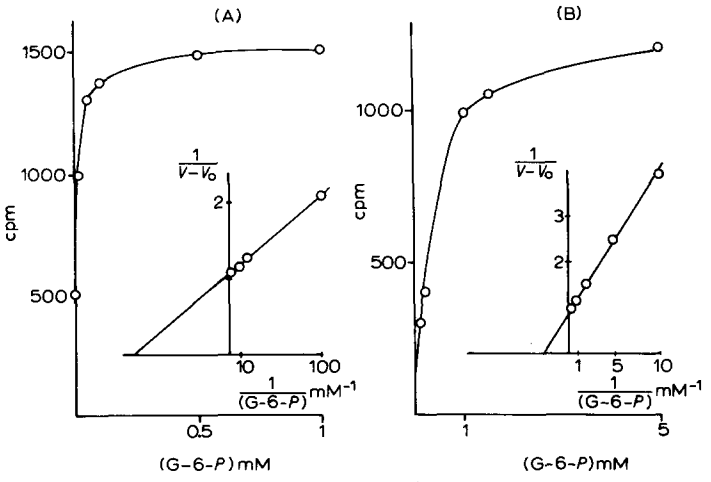


Fig. 4. Effect of glucose-6-*P* (G-6-*P*) on the activity of incubated (I form, A) and non-incubated (D form, B) enzyme preparations. UDPglucose concentration in the assay was 0.4 mM. In the inserts, the reciprocals of the reaction rate are plotted against the reciprocals of the concentrations of glucose-6-*P*.

Effect of Mg^{2+}. As can be observed in Table III, Mg^{2+} considerably enhanced the *V* of the D form preparation but no stimulation of the *V* of the I form preparation was observed. Besides this effect, Mg^{2+} also slightly increased the affinity of both enzyme preparations for glucose-6-*P*, decreasing by about one half their $A_{1/2}$ value when the cation concentration was 5 mM.

Effect of inorganic phosphate. The effect of inorganic phosphate on the activity of the I form preparation was "biphasic" (Fig. 5A). At concentrations below 2 mM, the anion stimulated the enzyme activity, but at higher concentrations it acted as an inhibitor. The extent of inhibition was clearly counteracted by the presence of increasing concentrations of glucose-6-*P*, which, at a concentration 1 mM, nullified the inhibition exerted by a 10 mM concentration of inorganic phosphate. With the D form enzyme preparation, inorganic phosphate was an inhibitor at any concentration tested (Fig. 5B). Also, in this case, glucose-6-*P* reversed the inhibition although much less effectively than with the I form preparation. From double reciprocal plots it was seen that

TABLE III

EFFECT OF Mg^{2+} ON THE *V* AND THE $A_{1/2}$ FOR GLUCOSE-6-*P* OF INCUBATED AND NON-INCUBATED ENZYME PREPARATIONS

Mg^{2+} (mM)	$A_{1/2}$ (mM)		$\frac{1}{V-V_0}$ ($\frac{1}{cpm} \times 10^3$)	
	Incubated enzyme	Non-incubated enzyme	Incubated enzyme	Non-incubated enzyme
0	0.010	0.40	1.1	1.0
0.2	0.008	0.39	1.1	1.0
0.5	0.006	0.26	1.3	0.94
1	0.006	0.25	1.3	0.74
5	0.005	0.22	1.2	0.48

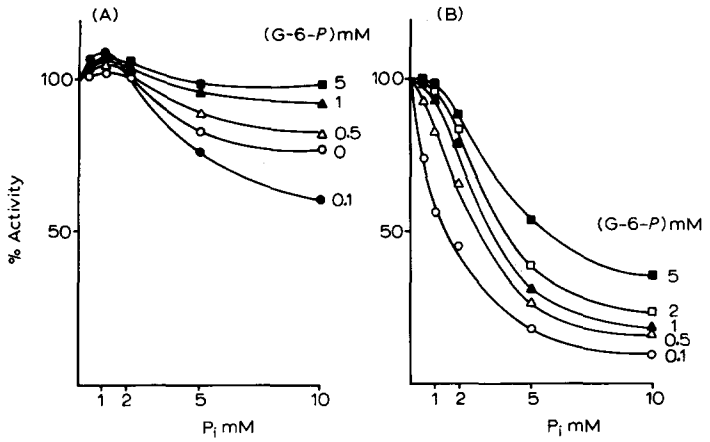


Fig. 5. Effect of inorganic phosphate on the activities of incubated (I form, A) and non-incubated (D form, B) enzyme preparations at the indicated concentrations of glucose-6-*P* (G-6-*P*).

inorganic phosphate increased the $A_{1/2}$ of both enzymes for glucose-6-*P*, without affecting the maximal rate.

Effect of ATP. ATP was also observed to be an inhibitor of both enzyme preparations from frog muscle, this effect being reversed by the simultaneous presence of glucose-6-*P*. However, at any given concentration of glucose-6-*P*, the inhibition exerted on the D form preparation was higher than that on the I form (Fig. 6). As can further be seen, the inhibition by ATP was more efficiently reversed for both enzyme preparations when, in addition to glucose-6-*P*, Mg^{2+} was present in the assay mixture. Similar to the observations with inorganic phosphate, ATP appeared to act by decreasing the affinity of both enzyme preparations for glucose-6-*P* without modifying the maximal rate.

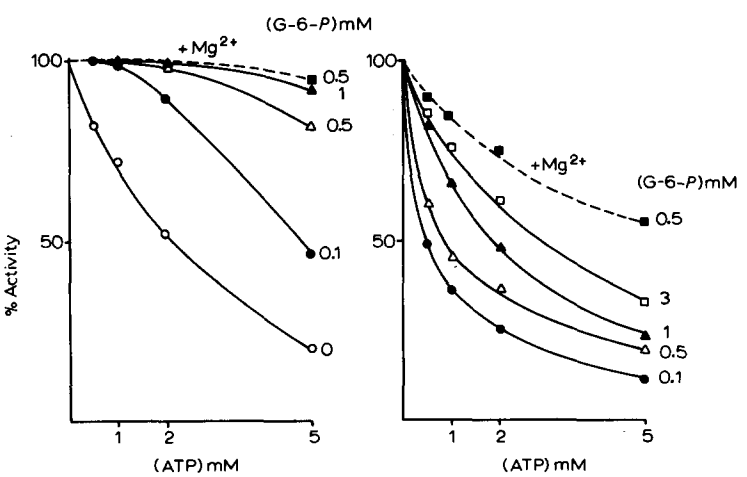


Fig. 6. Influence of ATP on the incubated (I form, A) and non-incubated (D form, B) enzyme preparations, in the presence of different concentrations of glucose-6-*P* (G-6-*P*). The broken lines represent the activity measured in presence of 5 mM Mg^{2+}. The results are expressed as percentage of the activity observed in absence of ATP at each concentration of glucose-6-*P*.

Discussion

The results reported in this paper prove that, contrary to what has previously been reported [9,10], frog muscle glycogen synthase can be converted from the D form normally found, into an I form, as for the enzyme from most tissues already studied [1,2].

The enzyme catalyzing the glycogen synthase D to I conversion was probably bound to the high molecular weight glycogen fraction since it sediments almost completely at 25 000 $\times g$. As we previously observed [14], most of the D form glycogen synthase activity is also bound to this fraction. The general characteristics of the glycogen synthase D to I conversion in frog skeletal muscle closely correspond to those described in other tissues where the presence of glycogen synthase-D phosphatase has been described [19—23], strongly suggesting that a similar enzyme is also present in frog skeletal muscle. However, in crude extracts of this tissue the conversion is never complete and may be masked by the concomitant increase in total activity and, furthermore, the enzyme shows a high dependence on glucose-6-*P*, what may explain why the D to I conversion had not been previously observed in frog skeletal muscle.

With regard to the biochemical mechanisms by which glucose-6-*P*, Mg^{2+}, ATP and inorganic phosphate affect the D to I conversion, it is interesting to point out that the $A_{1/2}$ for glucose-6-*P* on the glycogen synthase D to I conversion is in the same range as that for the D form of glycogen synthase from this same tissue and in both cases Mg^{2+} increases their $\hat{V}$. Moreover, the inhibition exerted by inorganic phosphate and ATP is, in both cases, reversed by glucose-6-*P* and, furthermore, the order in which the different sugar phosphates stimulate the conversion, particularly in the presence of Mg^{2+}, is the same as that previously described for the stimulation of the D form of glycogen synthase in mammalian muscle [5]. Thus, it is conceivable that, as previously postulated for rat heart [23], the effect of these metabolites in frog muscle could be due to their interaction with the D form of glycogen synthase and not only with glycogen synthase-D phosphatase. The inhibition by glycogen is analogous to that observed in muscle synthase phosphatase by Villar Palasi [20] and the effects of the polysaccharide are probably exerted on the converting enzyme.

The kinetic properties of the frog muscle enzyme before and after incubation coincide with those described for the D and I forms, respectively, in other muscle systems [1,2]. The incubated enzyme has an $A_{1/2}$ for glucose-6-*P* many times lower than the non-incubated enzyme; the effect of the activator in the former case was to decrease the K_m for UDPglucose (K effect) while, in the latter case, the effect of glucose-6-*P* was to increase the V without affecting the K_m (V effect). In most systems studied, the I forms behave as K systems while the D forms behave as V systems. The non-incubated enzyme is more sensitive to inhibition by ATP than the incubated enzyme. It is a general characteristic of I forms that they are less inhibited by ATP than D forms [6,24]. In the same way, the incubated enzyme is stimulated by low concentrations of inorganic phosphate as is generally the case with the I forms, whereas these ions inhibit the non-incubated enzyme in the presence of glucose-6-*P* as occurs with D forms [12,15,25].

Roach and Larner [24] have recently proposed a model depicting the activity of glycogen synthase as the result of two regulatory inputs, one through changes in the concentration of metabolites in the cell, and the other through phosphorylation-dephosphorylation of the enzyme. From the point of view of such a model, and weighing the effects of the physiological concentrations of metabolites in muscle tissue on both forms of frog muscle glycogen synthase, our results indicate that, in vivo, in conditions in which glycogen synthase is present only in the D form, the enzyme would be practically inactive. Therefore, the modulation of enzyme activity by the concentration of metabolites in the cell would be significant only when the D to I form conversion could take place.

Besides the effect of metabolites in modulating I form activity, it is worthwhile to note that, in frog muscle, these metabolites also modify the rate of D to I conversion. Therefore, in an integrated model of the regulatory mechanisms affecting glycogen synthase activity in vivo, we may postulate that the effect of metabolites will be greater than that indicated by the Roach and Larner model [24] since they affect both conversion between D and I forms, and the activities of these forms.

Work in progress shows that several hormones act on the interconversion between the D and I forms of frog muscle glycogen synthase, modifying the percentage of independent activity.

Acknowledgements

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