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GLYCOGEN SYNTHETASE OF RAT SKELETAL MUSCLE AND HEPATOMAS AND ITS COMPARISON WITH THE ENZYME OF RAT LIVER

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SUMMARY

1. Properties of glycogen synthetase of rat skeletal muscle and two ascites hepatomas (AH-66F and AH-130) were studied in comparison with rat liver enzyme. The standard assay employed the Tris–maleate buffer of pH 7.4 and 5 mM UDP-glucose.

2. When fresh extracts of muscle and hepatomas were incubated at 30 °C, glycogen synthetase underwent an activation due to the D to I conversion, but unlike the activation observed in the liver enzyme, only the activity measured without glucose 6-phosphate was increased with time.

3. The D and I forms of glycogen synthetase were prepared from muscle, AH-66F and AH-130. The pH–activity curves of the D forms from these tissues differed from that of liver enzyme in that in the presence of glucose 6-phosphate, the activity measured at pH 7.4 was as high as that at pH 8.6.

4. The apparent Michaelis constants for UDP-glucose of the D forms of muscle, AH-66F and AH-130 determined in the presence of glucose 6-phosphate were 0.50, 0.22 and 0.15 mM, respectively. These values are much lower than that reported for the D form of liver (8 mM).

5. Glycogen synthetases of muscle and liver are thus mutually different proteins. There was a close resemblance between the muscle and hepatoma enzymes, suggesting that hepatocarcinogenesis may have resulted in the replacement of liver-specific synthetase by some other type, possibly of muscle.

INTRODUCTION

In the preceding paper[1], some properties of rat liver glycogen synthetase as studied in the Tris-maleate buffer of pH 7.4 were described. The most striking finding was that when maleate was present, the D form of glycogen synthetase exhibited an extremely low affinity towards UDP-glucose at pH 7.4 even in the presence of glucose 6-phosphate (Glc-6-*P*). Inorganic phosphate (P_i) at physiological levels appears to exert the same effect[2]. Because of this peculiar property, the D to I conversion of liver synthetase is accompanied by a marked increase in activity measured

with Glc-6-*P* (refs 1–5), a phenomenon that has not been observed with the muscle enzyme[6–9].

Furthermore, the kinetic properties of glycogen synthetase of a few hepatomas reported in the previous papers[10,11] appear to differ from those of liver enzyme. This is not surprising since a number of key enzymes in carbohydrate metabolism are known to undergo isozymic changes upon hepatocarcinogenesis[12–16].

In the present work, the properties of glycogen synthetase of skeletal muscle and hepatomas of the rat were compared with the rat liver enzyme under assay conditions which employed the Tris–maleate buffer of pH 7.4. From the results obtained, it was concluded that the glycogen synthetase of muscle differs from that of liver while no marked difference was noted between the muscle and hepatoma enzymes.

MATERIALS AND METHODS

Animals, chemicals used and the method employed for the preparation of tissue 5000 × *g* supernatants were described in the preceding paper[1].

Hepatomas

Yoshida ascites hepatomas (AH) 66F and 130 were used. They were implanted into the peritoneal cavity of rats and harvested 4–6 days after. The tumor cells were collected by centrifugation and washed twice with cold physiological saline. The glycogen content and glycogenic and other biochemical capacities of these hepatomas were described previously[10,11].

Assay of glycogen synthetase

Glycogen synthetase was assayed by the method described in the preceding paper[1]. The standard assay mixture contained 50 mM Tris–maleate buffer (pH 7.4), 5 mM UDP-[$^{14}C_6$]glucose, 10 mM Glc-6-*P*, 40 mM NaF, 0.6 mg glycogen and 0.05 ml of enzyme in a final volume of 0.2 ml.

Preparation of glycogen synthetase from muscle

Glycogen synthetase D and I were prepared from hind leg muscle of the rat according to the method of Villar-Palasi *et al.*[17] In short, the preparation of the D form consisted of the following steps: (1) extraction of muscle with 0.04 M NaF; (2) precipitation of the enzyme by centrifugation at 105 000 × *g*; (3) incubation of the pellet (in suspension) at 30 °C; (4) centrifugation of the suspension at 30 000 × *g*; (5) finally, the supernatant was fractionated by DEAE-cellulose chromatography using stepwise elution technique. Purification from crude extract was 150-fold and the final product had a specific activity of 2.1 μmoles of [^{14}C]glucose incorporated into glycogen/min per mg protein when assayed under the standard conditions in the presence of Glc-6-*P*.

To obtain the I form, the crude extract prepared in the absence of NaF was incubated at 30 °C and after centrifugation at 105 000 × *g*, the supernatant was fractionated by DEAE-cellulose chromatography. The final product had a specific activity of 0.31 μmole/min per mg protein.

RESULTS

Effect of preincubation on glycogen synthetase activity of 5000 × g supernatant

In the experiments shown in Fig. 1, the 5000 × *g* supernatants of rat tissue homogenates were incubated at 30 °C prior to the assay of glycogen synthetase. The enzyme underwent an activation, the pattern of which, however, was quite different between the muscle and liver enzymes. With the liver enzyme, the activities measured with and without Glc-6-*P* were increased almost in parallel[1], whereas with the

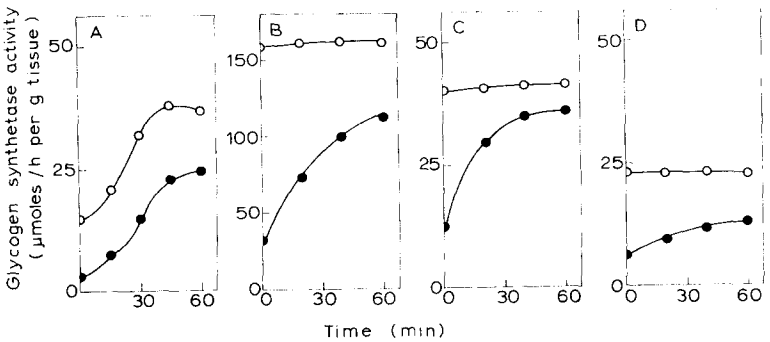

Fig. 1. Activation of glycogen synthetase of 5000 × *g* supernatant by preincubation. Supernatant from liver (A), skeletal muscle (B), AH-66F (C) or AH-130 (D) was incubated at 30 °C. At times indicated, glycogen synthetase was assayed under the standard conditions in the presence (○) or absence (●) of Glc-6-*P*.

muscle enzyme, only the activity measured without Glc-6-*P* was increased with time. Fig. 1 also shows that under the same conditions, the glycogen synthetase of AH-66F and AH-130 behaved just like the muscle enzyme, that is ,only the activity measured without Glc-6-*P* was increased.

Fig. 2 shows that the activation of glycogen synthetase in the 5000 × *g* supernatant of AH-66F is stimulated by Mg^{2+} and reversed by ATP *plus* Mg^{2+}. The results

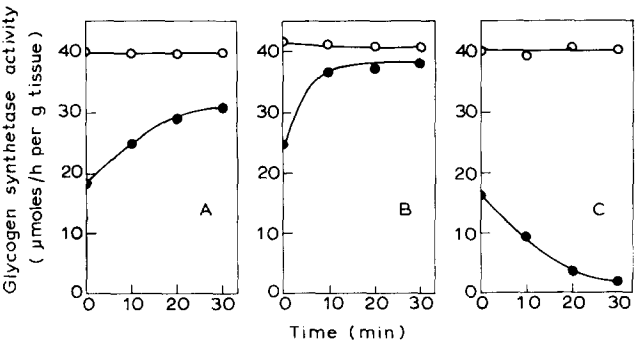

Fig. 2. D–I interconversion of AH-66F glycogen synthetase. 5000 × *g* supernatant was incubated at 30 °C in the absence of $MgCl_2$ (A), and in the presence of 10 mM $MgCl_2$ (B) or 8 mM ATP *plus* 10 mM $MgCl_2$ (C). At times indicated, the synthetase was assayed under the standard conditions in the presence (○) or absence (●) of Glc-6-*P*.

are compatible with the supposition that the activation is due to the D to I conversion catalyzed by glycogen synthetase D phosphatase (see ref. 1).

Preparation and partial purification of glycogen synthetase from AH-66F

In contrast to numbers of hepatomas including AH-130 which are devoid of glycogen, AH-66F stores a large amount of glycogen while growing in peritoneal cavity[11]. The previous studies[11] further revealed that in the latter hepatoma, glycogen synthetase was always found to be firmly bound to particulate glycogen. This provided a means for preparing and purifying the enzyme. All the operations were conducted at 0–4 °C.

For the preparation of the D form, it was necessary to carry out the entire purification in the presence of NaF. Saline-washed hepatoma cells were washed once with 0.4 M sucrose–50 mM Tris–HCl (pH 7.4)–5 mM EDTA–40 mM NaF and centrifuged at 900 × *g* for 5 min. The cells were then suspended in 5 vol. of the above medium and subjected to ultrasonic disintegration for 2.5 min at 10 kcycles. The mixture, approx. 10 ml in volume, was centrifuged at 5000 × *g* for 10 min and the resulting supernatant was further centrifuged at 105 000 × *g* for 60 min. The particulate glycogen was compacted into a firmly packed pellet, which was resuspended in 3 ml of 0.4 M sucrose–50 mM Tris–HCl (pH 7.4)–5 mM EDTA–40 mM NaF. The suspension was then layered over 7 ml of 2.1 M sucrose and centrifuged at 105 000 × *g* for 2 h as described by Luck[18]. The entire supernatant was poured off and the particulate glycogen remaining at the bottom of the tube was washed once with 0.4 M sucrose–50 mM Tris–HCl (pH 7.4)–5 mM EDTA.

The final product had a specific glycogen synthetase activity of 0.68 μmole/min per mg protein when assayed under the standard conditions in the presence of Glc-6-*P*. The extremely low—Glc-6-*P*/+ Glc-6-*P* activity ratio (0.03) indicated that the enzyme was entirely in the D form. Data for the purification are given in Table I.

The I form of glycogen synthetase was prepared by the same procedure, but NaF was omitted and the 5000 × *g* supernatant was preincubated for 60 min at 30 °C in order to complete the D to I conversion. The final glycogen pellet had a

TABLE I

PURIFICATION OF GLYCOGEN SYNTHETASE D FROM AH-66F CELLS

Fractionation: Fraction	*– Glc-6-P*	*+ Glc-6-P*			*(1)/(2)*
	Specific activity (1)*	*Specific activity* (2)*	*Purity (-fold)*	*Recovery (%)*	
5 000 × g supernatant	0.32	7.53	(1)	(100)	0.04
105 000 × g centrifugation					
Supernatant	0.19	1.10			0.17
Pellet	0.59	19.8	26	70	0.03
Sucrose density gradient centrifugation					
Microsome	0.55	1.16			0.47
Glycogen pellet	18.7	675	90	40	0.03

* nmoles/min per mg protein. Assays were made under the standard conditions in the presence or absence of 10 mM Glc-6-*P*.

specific activity of 0.53 μmole/min per mg protein in the presence of Glc-6-*P*. The −Glc-6-*P*/+ Glc-6-*P* activity ratio was 0.97, which may be contrasted to 0.03 found for the D form.

Preparation and partial purification of glycogen synthetase from AH-130

AH-130 cells, freshly harvested, lack glycogen and contain glycogen synthetase mostly in a soluble form[10]. 5000 × *g* Supernatant prepared in the absence of NaF was incubated for 1 h at 30 °C and centrifuged at 105 000 × *g* for 1 h. The I form of the enzyme was then precipitated from the supernatant between 30 and 50% saturation of $(NH_4)_2SO_4$. Further purification was effected by dissolving the precipitate in the sucrose medium and repeating the $(NH_4)_2SO_4$ fractionation. The final product had a specific activity of 0.013 μmole/min per mg protein in the presence of Glc-6-*P* and gave a −Glc-6-*P*/+ Glc-6-*P* activity ratio of 0.80.

In order to obtain the D form, freshly harvested hepatoma cells were first incubated with glucose under the conditions described by Saheki and Tsuiki[10]. During this incubation, glycogen was formed and the glycogen synthetase was converted into the D form and redistributed from soluble to particulate fraction[10]. The 5000 × *g* supernatant prepared from these cells was centrifuged at 105 000 × *g* for 1 h and the pellet was further purified as described for the AH-66F enzyme. The final product

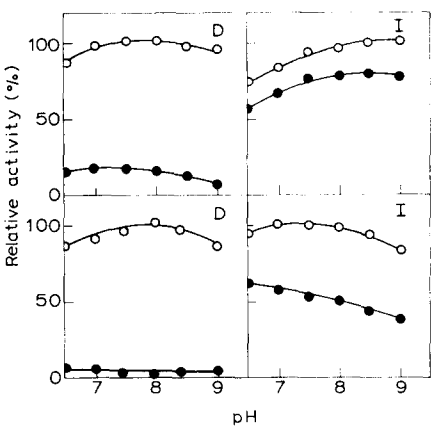

Fig. 3. pH-Activity curves of glycogen synthetase D and I purified from muscle (top) or AH-66F (bottom). Assays were made in the presence (○) or absence (●) of Glc-6-*P* using Tris–maleate buffer of indicated pH.

had a specific activity of 0.02 μmole/min per mg protein. Its −Glc-6-*P*/+ Glc-6-*P* activity ratio was found to be 0.10.

Properties of purified enzyme. Effect of pH

Fig. 3 shows the pH–activity relationship of the synthetase D and I purified from muscle and AH-66F. The data for the liver enzyme have been reported in the preceding paper[1]. All the D forms exhibited little activity when Glc-6-*P* was absent over a wide range of pH, whereas the I forms were active both with and without Glc-6-*P*. The pH–activity curves of the I forms were all broad although they showed a maximum activity at a pH value somewhat different from each other.

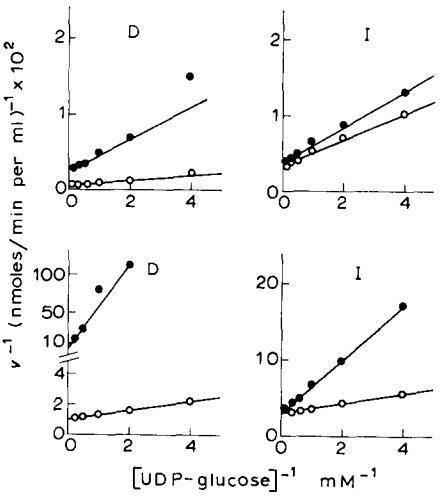

Fig. 4. Double reciprocal plots of velocity *vs* UDP-glucose concentration of the D and I forms of glycogen synthetase purified from muscle (top) or AH-66F (bottom). Assays were made under the standard conditions except that the concentration of UDP-glucose was varied. ○, Glc-6-*P* present; ●, absent.

A remarkable difference was demonstrated between the liver and muscle enzymes when the activity of the D form was measured with Glc-6-*P*. While the activity of the liver enzyme was very low in the physiological pH range and rose sharply above pH 8, the activity of the muscle enzyme was almost independent of pH between 7 and 9. The pH–activity curve of the synthetase D of AH-66F closely resembled that of muscle enzyme. Similar results were obtained with the AH-130 enzyme (not shown).

Affinity towards UDP-glucose and Glc-6-P

The affinity towards UDP-glucose of the D and I forms of glycogen synthetase purified from muscle and hepatomas was studied in the presence or absence of Glc-6-*P* with the Tris–maleate buffer of pH 7.4 as assay medium. Fig. 4 shows the double reciprocal plots of velocity *vs* UDP-glucose concentration of the muscle and AH-66F enzymes. The apparent K_m values derived from these and similar (AH-130) plots are given in Table II together with those of the liver enzyme determined under the same conditions[1].

It can be seen from Fig. 4 and Table II that the muscle and AH-66F enzymes remarkably resemble each other, not only in the general pattern of Glc-6-*P* stimulation of enzyme activity but also in that the apparent K_m values of the D and I forms measured in the presence of Glc-6-*P* are identical. This relationship also holds for the AH-130 enzyme, but is in sharp contrast to that seen for liver enzyme, whose apparent K_m is 20 times greater for the D form than the I form. It is to be noted that this discrepancy between the liver and other enzymes arises primarily from the unusually high K_m of the D form of liver; the apparent K_m values of the I forms were not very much different for these four enzymes.

The apparent K_m values for the Glc-6-*P* stimulation of glycogen synthetase

TABLE II

K_m FOR UDP-GLUCOSE AND GLUCOSE 6-PHOSPHATE OF PURIFIED GLYCOGEN SYNTHETASE ENZYMES IN TRIS–MALEATE BUFFER OF pH 7.4.

	*Liver****	*Muscle*	*Hepatomas*	
			AH-66F	*AH-130*
K_m for UDP-glucose*				
D	8.0	0.50	0.22	0.15
I	0.4	0.50	0.23	0.19
K_m for Glc-6-*P***				
D	0.60	0.23	0.13	0.29
I	0.08		0.009	0.017

* Determined at 10 mM Glc-6-*P* and given in mM.
** Determined at 1 mM UDP-glucose and given in mM.
*** Taken from ref. 1.

were determined for the muscle and hepatoma enzymes with 1 mM UDP-glucose as substrate. The results are given in Table II together with those of the liver enzyme. It is clear that the hepatoma enzymes differ from the liver enzyme, resembling more closely the muscle enzyme.

DISCUSSION

In the present work, the properties of glycogen synthetase enzymes from rat muscle and two rat hepatomas were compared with those of the rat liver enzyme described in the preceding paper[1]. The wide divergence in type and glycogen content of the tissues studied made it difficult to adopt a common method of purification for all of them. This, however, would not be a serious matter for comparison of enzyme properties since the preceding paper[1] demonstrated that the affinity of liver synthetase D towards UDP-glucose was extremely low irrespective of whether the enzyme was bound to particulate glycogen or present as a soluble protein. Under our experimental conditions, the K_m for UDP-glucose of liver synthetase D was found to be 8 mM whereas the corresponding value for the muscle D enzyme was only 0.5 mM. Since the K_m of the I form was 0.4 and 0.5 mM for the liver and muscle enzymes, respectively, the liver but not the muscle enzyme increases the affinity towards substrate as a result of the D to I conversion.

In our system, such an extremely low affinity of the D form of liver synthetase at neutral pH regions was brought about by maleate[1]; similar effect can be exerted by sulfate or P_i and the effect of the latter appears to be vitally important from the physiological point of view[2,5]. It therefore appears that the muscle enzyme is insensitive to these effectors or at least less sensitive than the liver enzyme. Since purified enzyme preparations were used in the present as well as preceding studies, participation of another factor that might potentiate or suppress the inhibition could not be expected. The glycogen synthetase enzymes of liver and muscle therefore are mutually different proteins or are a set of isozymes as are the phosphorylase enzymes of these tissues[19].

Another evidence that is compatible with this conclusion is the finding that the

glycogen synthetase of two ascites hepatomas differs from the liver enzyme, resembling more closely the muscle enzyme. In rapidly growing hepatomas such as those studied in the present work, a number of key enzymes in carbohydrate metabolism have undergone isozyme changes probably as a result of "dedifferentiation". Thus in AH-130, glucokinase, aldolase B and the liver-type pyruvate kinase, all unique to liver, have been replaced by hexokinase[13], aldolase A (ref. 14) and the muscle-type pyruvate kinase[15], respectively. It is therefore likely that upon hepatocarcinogenesis, glycogen synthetase also undergoes an isozymic alteration in which the original liver-specific enzyme is replaced by an isozyme which is insensitive to maleate inhibition. This isozyme might be or might not be identical to the muscle enzyme.

A possible significance of isozyme studies of neoplastic tissues is that they may provide molecular basis for the characteristic features of tumor metabolism. Thus the powerful glycolytic capacity of rapidly growing hepatomas is associated with the neoplastic change occurring in the glucokinase-hexokinase system described above.

Numbers of hepatomas are characterized by the lack of glycogen deposition, although these tumors are not devoid of a glycogen synthesizing system[11]. Hers *et al.*[20] mentioned that glycogen synthesis in liver is much less susceptible to inhibition by glycogen as compared with muscle[21]. This alone, however, could not be sufficient to account for the lack of glycogen in hepatomas, since AH-66F and AH-130 differ markedly in glycogen content yet they possess seemingly the same muscle-type glycogen synthetase. We have previously reported that the rate of glycogen synthesis in AH-130 declines as glycogen accumulates[10] whereas the glycogen synthesis of AH-66F occurs almost independent of cellular glycogen level[11]. Glycogen synthetase D phosphatase appears to be a site of inhibition of glycogen synthesis by glycogen. To account for glycogen deficiency in hepatomas, therefore, the multiple forms of the phosphatase and its alteration might be expected to occur during hepatocarcinogenesis.

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