BBA 66611

GLYCOGEN SYNTHETASE D AND I OF RAT LIVER AND THEIR INTERCONVERSION *IN VITRO*

KIYOMI SATO, NORIO ABE AND SHIGERU TSUIKI
Biochemistry Division, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai (Japan)

(Received November 12th, 1971)

SUMMARY

1. Properties and interconversion of the D and I forms of rat liver glycogen synthetase were studied with Tris–maleate buffer (pH 7.4) as assay medium.

2. The D to I conversion of glycogen synthetase observed in crude extracts was accompanied by a marked rise in the activity measured with glucose 6-phosphate.

3. The D form was partially purified. It was then converted to the I form by using glycogen synthetase D phosphatase.

4. The purified D form was extremely low in activity even at a high level of glucose 6-phosphate. Its apparent K_m for UDP-glucose was 8 mM under these conditions, whereas that of the I form was 0.4 mM. The omission of glucose 6-phosphate reduced the V of the D but not of the I form.

INTRODUCTION

The activation of hepatic glycogen synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) has been studied extensively both *in vivo* and *in vitro*[1–18]. Although the principal mechanism was postulated to be a conversion of a phospho to a dephospho form[19], analogous to that occurring in skeletal muscle, observation was often made that the activation brought about a marked rise even in the activity measured with glucose 6-phosphate (Glc-6-*P*)[1,10,19–21], a phenomenon that has not been observed for the muscle enzyme[22–24]. Bishop and Larner[2] studied this rise and found that the V for UDP-glucose was, in fact, not altered. Segal and his coworkers[1,5] reported that activation of hepatic glycogen synthetase observed in crude extracts was not to eliminate a Glc-6-*P* requirement for the enzyme but to increase the affinities for UDP-glucose and Glc-6-*P*. They concluded that the D–I interconversion of hepatic glycogen synthetase has the characteristics of an on-off process[5].

De Wulf and Hers[7,8] pointed out that inorganic phosphate (P_i) might play an important role in this liver system: in the presence of a physiological concentration (5 mM) of P_i, the synthetase D was inactive whatever the concentration of Glc-6-*P*, whereas the I form was nearly fully active[7]. Using 10 mM sulfate in place

of P_i, they were able to show that the D to I conversion of hepatic glycogen synthetase was a process by which an inactive enzyme became fully activated[8].

The present communication reports the properties and interconversion of the partially purified D and I forms of rat liver glycogen synthetase as studied with Tris–maleate buffer (pH 7.4) as assay medium. The use of this buffer was adopted for the purpose of studying the liver enzyme from physiological standpoint since maleate was found to replace P_i in depressing the glycogen synthetase D activity. In addition, the use of this buffer has enabled a differentiation between the liver and muscle enzymes to be made, as will be shown in the following paper[25].

MATERIALS AND METHODS

Preparation of 5000 × g supernatants

Male Donryu rats weighing 120–180 g and fed a commercial rat diet *ad libitum* were used. The animals were killed between 9 and 11 a.m. so as to minimize daily changes in liver glycogen. The livers were quickly removed and placed in cold 0.4 M sucrose containing 50 mM Tris–HCl (pH 7.4) and 5 mM EDTA. This solution will be referred to as the sucrose medium. All the following experiments were conducted at 0–4 °C unless otherwise specified.

The livers were drained, blotted and homogenized in a glass-Teflon homogenizer using 4 vol. of 0.5 M sucrose containing 62.5 mM Tris–HCl (pH 7.4) and 6.25 mM EDTA. The homogenate was centrifuged at 5000 × *g* for 10 min and the supernatant was used for the further experimental procedure.

Partial purification of glycogen synthetase D

Rats were fasted for 24 h and then re-fed for 24 h. 5000 × *g* supernatant was prepared as described above except that the homogenizing medium contained 40 mM NaF. The synthetase D was then purified according to the method of Hizukuri and Larner[19]. In short, the above supernatant was centrifuged at 50 000 × *g* for 60 min. Particulate glycogen sedimented was washed with sucrose medium containing 40 mM NaF, resuspended in the same medium and after centrifugation at 5000 × *g* for 10 min, the supernatant was subjected to ultrasonic disintegration for 30 s at 10 kcycles. The sonicate was then centrifuged at 50 000 × *g* for 60 min. The glycogen pellet thus obtained could be stored at −20 °C for a month without losing glycogen synthetase D activity.

Partially purified glycogen synthetase I

The 5000 × *g* supernatant prepared without using NaF was centrifuged at 105 000 × *g* for 1 h; the resulting supernatant was then used as glycogen synthetase D phosphatase. The glycogen pellet purified as described above was suspended in the sucrose medium to half the original volume and incubated with the same volume of phosphatase solution at 30 °C in the presence of 10 mM $MgCl_2$. After 1 h, the mixture was chilled and centrifuged at 50 000 × *g* for 40 min. The glycogen pellet recovered was washed with the sucrose medium and used as partially purified glycogen synthetase I.

Assay of glycogen synthetase

The standard assay mixture contained 50 mM Tris–maleate buffer (pH 7.4)*, 5 mM UDP-[$^{14}C_6$]glucose (50 000 cpm/μmole), 10 mM Glc-6-*P* (when indicated), 40 mM NaF, 0.6 mg glycogen and 0.05 ml of enzyme in a final volume of 0.2 ml. After incubation for 5 min at 30 °C, the reaction was stopped by addition of 2.5 ml of 30% KOH. Glycogen was isolated and the amount (μmoles or nmoles) of [$^{14}C_6$]-glucose incorporated into glycogen from UDP-[$^{14}C_6$]glucose was determined by the method of Steiner *et al.*[21]. Protein was determined by the phenol reagent[26].

Chemicals

UDP-[$^{14}C_6$]Glucose was obtained from New England Nuclear Corp., Boston. UDP-Glucose, Glc-6-*P* and glycogen (rabbit liver) were the products of Boehringer. Tris was purchased from Sigma.

RESULTS

Effect of maleate on glycogen synthetase activity

Fig. 1 shows that maleate exerts about the same effect as P_i on the glycogen synthetase activity of fresh liver extracts although the latter was 10 times more potential on a molar basis than maleate. They specifically reduced the activity measured with Glc-6-*P* in physiological pH range. Similar results were obtained for purified synthetase D.

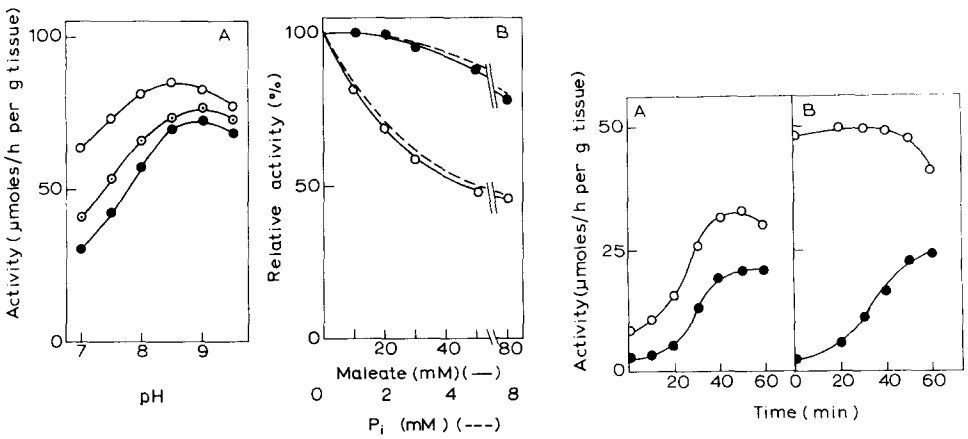

Fig. 1. Effects of maleate and P_i on glycogen synthetase activity of fresh 5000 × *g* supernatant. Assays were made under the standard conditions (with 10 mM Glc-6-*P*) except that 80 mM glycylglycine buffer was used. A. ○, none; ◉, 5 mM P_i; ●, 50 mM maleate. B. Assays were made at pH 7.4 (○) or 8.8 (●) in the presence of maleate. For comparison, the effect of P_i is shown by broken lines (— — —).

Fig. 2. Activation of glycogen synthetase in extract. 1 ml of fresh 5000 × *g* supernatant prepared as described in text was incubated at 30 °C. At the times indicated, 0.05-ml portions were withdrawn and assayed for glycogen synthetase in Tris–maleate buffer of pH 7.4 (A) or 8.6 (B) with 1.5 mM UDP-glucose as substrate. 10 mM Glc-6-*P* was present (○) or absent (●).

* The buffer contained 50 mM Tris and 50 mM maleate, the pH being adjusted to 7.4 by NaOH.

Activation of glycogen synthetase in crude extracts

In the experiments shown in Fig. 2, fresh 5000 × *g* supernatant was preincubated for various duration times at 30 °C and glycogen synthetase was assayed with Tris–maleate buffer of pH 7.4 or 8.6 as medium. At pH 7.4, activities measured with and without Glc-6-*P* were increased concurrently, whereas at pH 8.6, activity measured with Glc-6-*P* remained high throughout the period of preincubation. The activation was totally suppressed by 20 mM NaF showing that the D to I conversion of glycogen synthetase catalyzed by glycogen synthetase D phosphatase was involved.

Partial purification of glycogen synthetase D

In the liver of well-fed rats, glycogen synthetase is predominantly in the D form and is associated with particulate glycogen[19], thereby providing a means for purifying the D form. All the steps were carried out in the presence of 40 mM NaF so as to accelerate the I to D conversion and minimize the D to I conversion. The purification from crude extracts was 275-fold with a recovery of 51% and the final glycogen pellet had a specific activity of 266 nmoles/min per mg protein in the presence of Glc-6-*P*. The ratio of activities measured without and with Glc-6-*P* was 0.08. The pellet still exhibited considerable phosphorylase activity, but this does not affect the glycogen synthetase reaction since the assay mixture contained no P_i.

Partially purified glycogen synthetase I

When the purified glycogen pellet was incubated with $MgCl_2$ at 30 °C, no activation took place, confirming the report of Hizukuri and Larner[19]. The conversion of purified synthetase D to the I form, however, could be achieved if 105 000 × *g* supernatant of liver extract was added. As shown in Fig. 3, the conversion required Mg^{2+} (ref. 19), was blocked by NaF and reversed by ATP and Mg^{2+}. The pellet

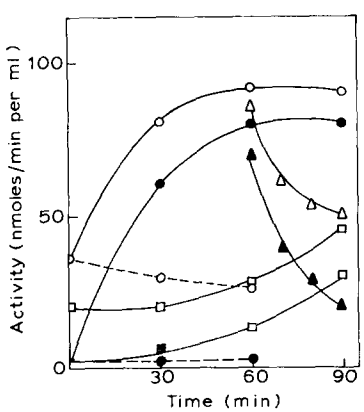

Fig. 3. Conversion of purified glycogen synthetase D to the I form by glycogen synthetase D phosphatase. ○, ●; the purified glycogen pellet was incubated with glycogen synthetase D phosphatase and $MgCl_2$ as described in the text in the absence (———) or presence of 40 mM NaF (— — —). △, ▲; at 60 min, a portion of the incubation mixture was added with 5 mM ATP. □, ■; incubation was conducted in the absence of $MgCl_2$. At the times indicated, 0.05-ml portions were withdrawn and assayed for glycogen synthetase under the standard conditions in the presence (○, △, □) or absence of 10 mM Glc-6-*P* (●, ▲, ■).

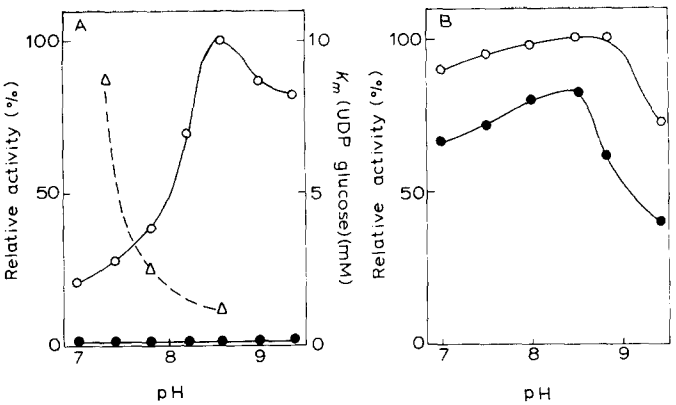

Fig. 4. pH activity curves of partially purified glycogen synthetase D (A) and I (B). Assays were made in 80 mM Tris–maleate buffer with (○) or without 10 mM Glc-6-*P* (●). For synthetase D, the apparent K_m for UDP-glucose was determined at different pH values in the presence of Glc-6-*P* and plotted (△—△).

recovered after incubation had a specific activity of 150 nmoles/min per mg protein when Glc-6-*P* was present. The —Glc-6-*P*/ + Glc-6-*P* activity ratio was 0.87.

Properties of partially purified glycogen synthetase D and I. Effect of pH

The pH–activity relationship of purified synthetase D and I is shown in Fig. 4. When Glc-6-*P* was present at a high level, the most characteristic feature of synthetase D was its extremely low activity in physiological pH range. The activity, however, rose sharply above pH 8 and when compared at pH 8.6–9.0, no significant difference could be noted between the activities of D and I forms. For synthetase D, apparent K_m for UDP-glucose was determined at several pH values and plotted in

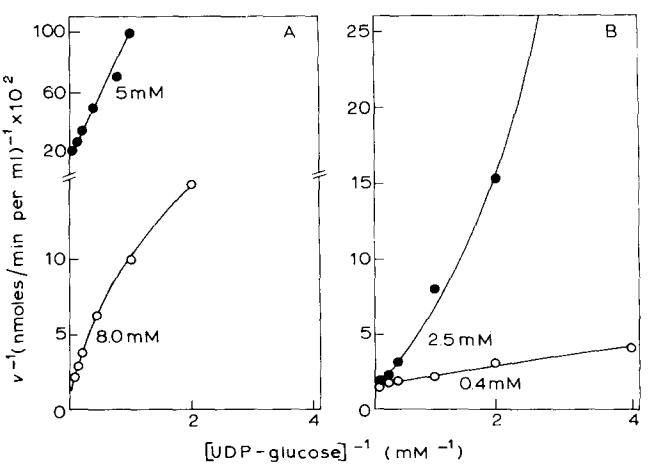

Fig. 5. Double reciprocal plots of velocity *vs* UDP-glucose concentration of partially purified glycogen synthetase D (A) and I (B). The analyses were made at pH 7.4 in the presence (○) or absence of 10 mM Glc-6-*P* (●). The apparent K_m values obtained are listed in the figure.

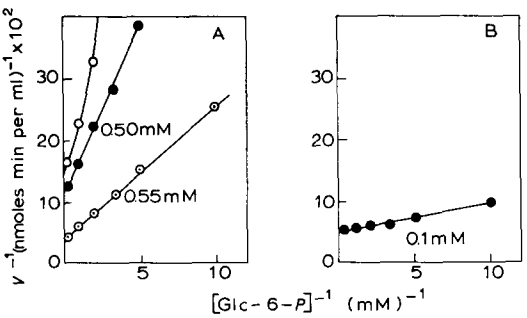

Fig. 6. Double reciprocal plots of velocity increment *vs* Glc-6-*P* concentration of partially purified glycogen synthetase D (A) and I (B). The analyses were made at pH 7.4 with 0.25 (○), 1.0 (●) or 5.0 mM UDP-glucose (◉) as substrate. The apparent K_m values obtained are listed in the figure.

Fig. 4. The K_m was thus an important factor accounting for the low activity of D form in physiological pH range.

Affinity towards UDP-glucose

The kinetic analysis of purified enzymes made at pH 7.4 (Fig. 5) revealed that V of the D form was increased markedly by Glc-6-*P*; for the I form, Glc-6-*P* did not affect V but did reduce the K_m for UDP-glucose. These results were grossly similar to those obtained for muscle enzyme[22-24]. The liver enzyme, however, was unique in that in the presence of Glc-6-*P*, the K_m for UDP-glucose of D form (8 mM) was 20 times as great as that of I form (0.4 mM).

Affinity towards Glc-6-P

The effect of Glc-6-*P* concentration on purified glycogen synthetase D activity was determined at three different levels of UDP-glucose. The kinetic data shown in Fig. 6 revealed that rise in UDP-glucose concentration did not affect the affinity of enzyme towards Glc-6-*P*. At 1 mM UDP-glucose, the K_m of synthetase I was only 1/5 that of synthetase D.

DISCUSSION

Extensive studies were made recently of *in vitro* activation of hepatic glycogen synthetase[1,5,7,8]. The most characteristic feature of liver enzyme appears to be that in concentrated extracts or in the presence of physiological concentrations of P_i, glycogen synthetase D is almost inactive even at high levels of Glc-6-*P* (refs 7, 8). Since maleate exerts almost the same effect as does P_i, use of Tris–maleate buffer, pH 7.4, should provide valuable information as to the kinetic and regulatory behavior of liver glycogen synthetase under physiological conditions.

The data on the partially purified D and I forms suggested that a major effect of maleate on the *D* form was to cancel the stimulatory effect of Glc-6-*P* in neutral pH range. Thus the K_m for UDP-glucose of synthetase D but not of I remained extremely high even at high levels of Glc-6-*P*. This alone would be sufficient to account for the marked rise in activity measured with Glc-6-*P* during the D to I con-

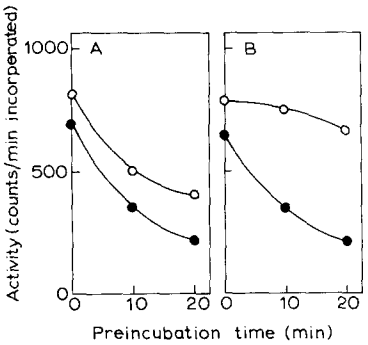

Fig. 7. Conversion of synthetase I not bound to particulate glycogen to D. The synthetase was incubated with glycogen synthetase I kinase, 8 mM ATP and 10 mM $MgCl_2$ at 30 °C. At the times indicated, 0.05-ml portions were withdrawn and assayed for glycogen synthetase in the Tris–maleate buffer of pH 7.4 (A) or 8.6 (B) in the presence (○) or absence of 10 mM Glc-6-*P* (●). The concentration of UDP-glucose was 1 mM. The assay mixture also contained 10 mM EDTA and 40 mM NaF. The soluble synthetase I was prepared as follows. The crude glycogen pellet was incubated in the sucrose medium for 1 h at 30 °C and then for 10 min at 37 °C (ref. 21) and centrifuged at 105 000 × *g* for 1 h. The synthetase I appearing in the supernatant was precipitated at 50% saturation of $(NH_4)_2SO_4$, dissolved in the sucrose medium and incubated for 50 min at 30 °C with 4 mM dithiothreitol. The final product had a specific activity of 81 nmoles/min per mg protein in the presence of 10 mM Glc-6-*P*. The recovery was approx. 10%. Synthetase I kinase was prepared from 105 000 × *g* rat liver supernatant by the method of Bishop and Larner[27].

version of hepatic glycogen synthetase. Under similar conditions, little rise of activity measured with Glc-6-*P* was observed for the skeletal muscle enzyme[25]. In Fig. 7, I form not bound to particulate glycogen was incubated with glycogen synthetase I kinase, ATP and $MgCl_2$. The activity measured with Glc-6-*P* was reduced progressively when assayed at pH 7.4 but not at 8.6, suggesting that the unusually low affinity of hepatic D form towards UDP-glucose at neutral pH values in the presence of Glc-6-*P* was independent of association with particulate glycogen.

In contrast to previous observation[5], *V* of hepatic synthetase D was found to be markedly increased by Glc-6-*P*, as was *V* of muscle synthetase D (refs 22–24). The discrepancy could not be ascribed to the use of maleate since in the previous work also, synthetase D was apparently in an inhibited state probably due to the occurrence of P_i. Segal's[1,5] and Hers's groups[7,8] emphasized that liver synthetase D was inactive whereas the I form was fully active under physiological conditions and recommended the use of *b* and *a* terminology instead of the D and I. The less active phospho form of hepatic glycogen synthetase studied in the present work can be referred to either as D, because of the dependence of *V* on Glc-6-*P*, or as *b*, because of its inactive nature under physiological conditions.

REFERENCES

1 A. H. Gold and H. L. Segal, *Arch. Biochem. Biophys.*, 120 (1967) 359.
2 J. S. Bishop and J. Larner, *J. Biol. Chem.*, 242 (1967) 1354.
3 H. De Wulf and H. G. Hers, *Eur. J. Biochem.*, 2 (1967) 50.
4 H. De Wulf and H. G. Hers, *Eur. J. Biochem.*, 2 (1967) 57.
5 H. J. Mersman and H. L. Segal, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1688.
6 A. H. Gold, *Biochem. Biophys. Res. Commun.*, 31 (1968) 361.
7 H. De Wulf, W. Stalmans and H. G. Hers, *Eur. J. Biochem.*, 6 (1968) 545.

8 H. De Wulf and H. G. Hers, *Eur. J. Biochem.*, 6 (1968) 552.
9 H. De Wulf and H. G. Hers, *Eur. J. Biochem.*, 6 (1968) 558.
10 A. Vardanis, *Arch. Biochem. Biophys.*, 130 (1969) 413.
11 H. J. Mersman and H. L. Segal, *J. Biol. Chem.*, 244 (1969) 1701.
12 H. B. Buschiazzo, J. H. Exton and C. R. Park, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 383.
13 A. H. Gold, *J. Biol. Chem.*, 245 (1970) 903.
14 J. S. Bishop, *Biochim. Biophys. Acta*, 208 (1970) 208.
15 H. De Wulf, W. Stalmans and H. G. Hers, *Eur. J. Biochem.*, 15 (1970) 1.
16 W. Glinsman, G. Pauk and E. Hern, *Biochem. Biophys. Res. Commun.*, 39 (1970) 774.
17 K. Gruhner and H. L. Segal, *Biochim. Biophys. Acta*, 222 (1970) 508.
18 W. Stalmans, H. De Wulf and H. G. Hers, *Eur. J. Biochem.*, 18 (1971) 582.
19 S. Hizukuri and J. Larner, *Biochemistry*, 3 (1964) 1783.
20 D. F. Steiner, *Biochim. Biophys. Acta*, 54 (1961) 206.
21 D. F. Steiner, L. Younger and J. King, *Biochemistry*, 4 (1965) 740.
22 D. L. Friedman and J. Larner, *Biochim. Biophys. Acta*, 64 (1962) 185.
23 M. Rosell-Perez and J. Larner, *Biochemistry*, 3 (1964) 81.
24 W. H. Danforth, *J. Biol. Chem.*, 240 (1965) 588.
25 K. Sato, N. Abe and S. Tsuiki, *Biochim. Biophys. Acta*, 268 (1972) 646.
26 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
27 J. S. Bishop and J. Larner, *Biochim. Biophys. Acta*, 171 (1969) 374.