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STUDIES ON GLYCOGEN SYNTHASE D PHOSPHATASE OF RAT LIVER— MULTIPLE NATURE

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SUMMARY

More than 70% of the glycogen synthase D phosphatase activity of rat liver homogenate was recovered in the postmicrosomal supernatant. Using the phosphatase preparations partially purified from the supernatant, conditions have been developed whereby the conversion of rat liver glycogen synthase D into synthase I is proportional both to time and to phosphatase concentration.

The partially purified D phosphatase was resolved into six peaks by the use of DEAE-cellulose chromatography. Sephadex G-200 chromatography resolved the same phosphatase into three fractions with molecular weights of approx. $1.15 \cdot 10^5$, $3.4 \cdot 10^5$ and above $5 \cdot 10^5$, respectively.

The six phosphatase forms separated by DEAE-cellulose chromatography were not identical to each other with respect to molecular weight and tissue distribution. These findings suggest that multi-isozymic forms of synthase D phosphatase exist in rat liver.

INTRODUCTION

Glycogen synthase (UDP-glucose:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) exists in two different forms, synthase I and synthase D. Synthase I, the active form, is converted into synthase D, the inactive form, by a phosphorylation reaction catalyzed by cyclic AMP-dependent protein kinase. It has recently been shown that in skeletal muscle [1, 2] and heart [3], the conversion of synthase D into synthase I is catalyzed by phosphoprotein phosphatase capable of dephosphorylating numbers of proteins that have been phosphorylated by the protein kinase. Little is known, however, of the nature of the enzyme that catalyzes the synthase D to synthase I conversion of glycogen synthase in liver. Hizukuri and Larner [4] have reported that the factor responsible for the conversion was released from the crude glycogen pellet by ultrasonic disintegration.

In the previous papers from this laboratory [5, 7], glycogen synthase D phosphatase activity was shown to be present in the postmicrosomal supernatant of rat liver. Attempts to purify the soluble phosphatase have led to the postulation that

there are multi-isozymic forms of the enzyme. The present communication reports the results of these studies.

MATERIALS AND METHODS

The animals and preparation and purification of rat liver glycogen synthase D free of D phosphatase were described previously [5, 6]. A unit of synthase was defined as before [6].

Assay of synthase D phosphatase

Synthase D phosphatase activity was measured by the formation of synthase I from synthase D at 30 °C. The standard assay mixture contained the following components in a final volume of 0.5 ml: 50 mM glycylglycine buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM Na_2SO_4, 0.5 mM caffeine, 0.2 ml of synthase D (approx. 1.2 unit) and 0.2 ml of synthase D phosphatase appropriately diluted in cold 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM mercaptoethanol. The reaction was started by the addition of synthase D and after 10 and 30 min, 0.05-ml aliquots were removed and transferred to 0.15 ml of a synthase test mixture that contained 50 mM Tris—maleate buffer (pH 7.4), 1 mM UDP-[U-^{14}C]glucose, 40 mM NaF and 0.6 mg of rabbit liver glycogen. NaF effectively stopped the phosphatase reaction. The resulting mixture was then incubated for 5 min at 30 °C and the extent to which ^{14}C was incorporated into glycogen was determined as described previously [5]. Since the synthase test mixture contained no glucose 6-phosphate, synthase D was inactive; so that the difference in ^{14}C incorporation between the 10-min and 30-min samples was due to the formation of synthase I. As will be shown below (see Fig. 1), there was a lag period of about 10 min in the onset of the D phosphatase reaction. This was the reason why the measurements were made between 10 and 30 min. A unit of D phosphatase was defined as the amount which gave rise to 1 unit of synthase I in 1 min under the above conditions.

Chemicals and commercial enzymes

Commercial enzymes were purchased from Boehringer. DEAE-cellulose was obtained from Brown and Sephadex gels were from Pharmacia. The sources of other chemicals were described in the previous paper [6].

RESULTS

Subcellular distribution of synthase D phosphatase in rat liver

The subcellular distribution of rat liver synthase D phosphatase was studied by employing a differential centrifugation technique. As shown in Table I, more than 70% of the original activity was recovered in the postmicrosomal supernatant (the 105 000 $\times$ *g* supernatant). The crude glycogen pellet (the 50 000 $\times$ *g* pellet) contained only a very small fraction of activity, most of which was released from the pellet by ultrasonic disintegration.

Partial purification of D phosphatase

The postmicrosomal supernatant of rat liver was prepared as described in Table I except that the centrifugation at 50 000 $\times$ *g* was omitted. D phosphatase was then

TABLE I

SUBCELLULAR DISTRIBUTION OF GLYCOGEN SYNTHASE D PHOSPHATASE ACTIVITY IN RAT LIVER

The liver from a single rat was homogenized in a glass–Teflon homogenizer for 2 min using 4 vol. of 0.5 M sucrose–62.5 mM Tris–HCl buffer (pH 7.4) —6.25 mM EDTA and centrifuged at 5000 × *g* for 10 min. The supernatant was then centrifuged successively at 50 000 × *g* and 105 000 × *g* each for 60 min and the pellet fractions were resuspended in the above buffer. All these steps were performed at 0–4 °C. Protein concentrations were measured using a phenol reagent [8].

Fractions	Protein (mg)	Activity (units)	(%)	Spec. act. (units/mg)
5 000 × *g* supernatant	590.4	161.7	(100)	0.274
50 000 × *g* pellet	17.3	0.7	0.4	0.038
105 000 × *g* pellet	110.4	27.8	17.2	0.252
105 000 × *g* supernatant	446.4	121.0	74.8	0.271

partially purified from the supernatant by an acid treatment followed by $(NH_4)_2SO_4$ fractionation. The detailed procedure was given previously [6]. In a representative experiment shown in Table II, the purification was 2.3-fold over the postmicrosomal supernatant with a recovery of 53%. The final product had a specific activity of 0.413 unit/mg protein and was free of glycogen synthase, phosphorylase and phosphorylase *b* kinase.

Assay of D phosphatase

When the partially purified D phosphatase was incubated with rat liver synthase D, a time lag of about 10 min in the onset of synthase I formation was observed (Fig. 1). The synthase D was subsequently found to be contaminated by phosphorylase *a*, which was totally converted to the *b*-form upon preincubating the synthase D for 10 min at 30 °C in the presence of 5 mM $MgCl_2$ and 0.5 mM caffeine. Since no lag was observed when the preincubated synthase D was used, it was concluded that the phosphorylase *a* was responsible for the lag. The enzyme has been shown by Stalmans et al. [9] to be a potent inhibitor of D phosphatase reaction in liver.

After the lag was completed, the formation of synthase I was proportional to the time during the following 20 min (Fig. 1). The proportionality, however, could not be maintained if Na_2SO_4 was omitted from the assay mixture, indicating that the salt

TABLE II

PARTIAL PURIFICATION OF GLYCOGEN SYNTHASE D PHOSPHATASE FROM RAT LIVER

Steps and fractions	Total protein (mg)	Spec. act. (units/mg)	Purification (-fold)	Recovery (%)
1. 105 000 × *g* supernatant	430	0.178	(1)	(100)
2. Acid treatment	392	0.153	0.86	78
3. $(NH_4)_2SO_4$ precipitate	98	0.413	2.32	53

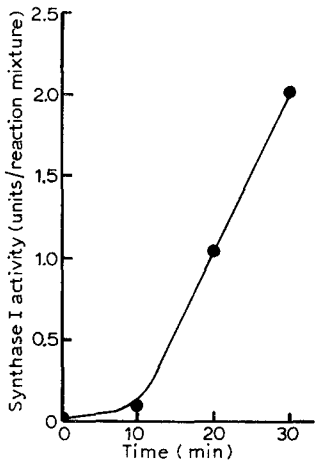

Fig. 1. Time course of rat liver synthase D phosphatase reaction. The partially purified D phosphatase was incubated under the standard conditions and aliquots were removed at times indicated for the mesaurement of glycogen synthase I activity.

acts to stabilize synthase I. Unlike the muscle enzyme, liver D phosphatase was inhibited by mercaptoethanol at concentrations greater than 10 mM.

The phosphatase required Mg^{2+} for activity. Mn^{2+} was not usable because of the reaction rate which decreased rapidly after 10 min. The velocity of the phosphatase reaction also varied markedly with the type of buffer employed; the highest velocity was obtained by using glycylglycine and other buffers such as Tris—HCl, Tris—maleate and phosphate were all inhibitory. In glycylglycine, the maximum activity was obtained at pH 7.4 (Fig. 2).

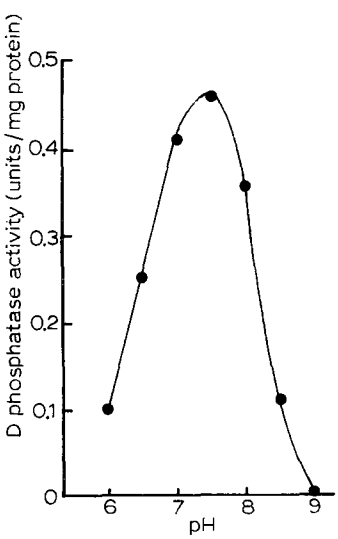

Fig. 2. D phosphatase activity as a function of pH. The activity of partially purified D phosphatase was assayed in 100 mM glycylglycine buffer of indicated pH values.

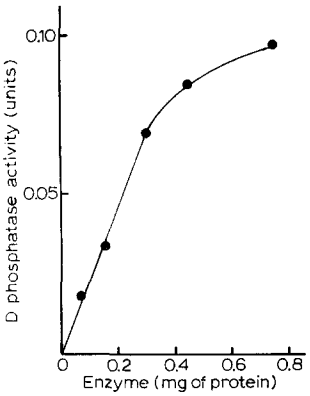

Fig. 3. Relationship of D phosphatase activity and enzyme concentration. Assays were made under the standard conditions except that the concentration of partially purified D phosphatase was varied as indicated.

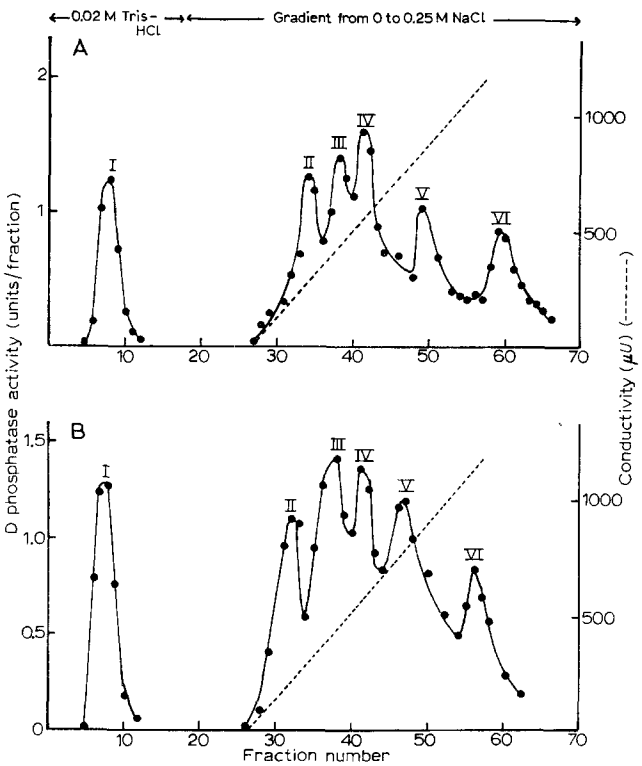

Fig. 4. DEAE-cellulose chromatography of rat liver D phosphatase. (A) The partially purified D phosphatase (6.5 ml, 49 mg protein) was applied to a column (1.5 cm × 15 cm) of DEAE-cellulose previously equilibrated with 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM mercaptoethanol. The column was first washed with 80 ml of the same buffer; a portion of the D phosphatase (I) was eluted under these conditions. Elution was then conducted with a 200-ml linear gradient of NaCl (0–0.25 M) added to the above buffer. As NaCl was inhibitory to D phosphatase at higher concentrations, the eluate was first passed through a continuous-flow counter current dialyzing apparatus (Biomed Instruments, Model D-1) for dialysis against the starting buffer. Fractions of 5 ml were then collected at a flow rate of 20 ml/h. D phosphatase was assayed in each of these fractions under the standard conditions. (B) Freshly prepared postmicrosomal supernatant of rat liver (6 ml, 103 mg protein) was chromatographed on DEAE-cellulose under the conditions similar to those described above.

Under the standard assay conditions thus developed, D phosphatase activity could be proportional to the amount of enzyme (Fig. 3). The reason why the proportionality was lost beyond a certain enzyme concentration (Fig. 3) has not been elucidated.

DEAE-cellulose chromatography

In attempts to further purify rat liver D phosphatase, the partially purified enzyme prepared as described above was chromatographed on DEAE-cellulose. The elution pattern of D phosphatase activity is shown in Fig. 4A. There were six distinct peaks of activity, arbitrarily designated I, II, III, IV, V and VI in order of elution. Overall recovery and specific activity of D phosphatase activity from this step were more than 90% and 0.650 units/mg protein, respectively. It should be noted that none of these peak fractions released P_i from β-glycerophosphate.

To test the possibility that these multiple peaks might be caused by artifact, the chromatography was repeated with freshly prepared postmicrosomal supernatant. As shown in Fig. 4B, the same six peaks were observed, thereby showing that they did not arise from enzyme degradation or modification during purification. The experiment also suggested that the partial purification described above resulted in no selective loss of certain enzyme forms.

Sephadex G-200 chromatography

Multiple forms of rat liver D phosphatase have also been demonstrated by a gel filtration study. In the experiment shown in Fig. 5, the partially purified D phosphatase was chromatographed on Sephadex G-200. The activity was resolved into three

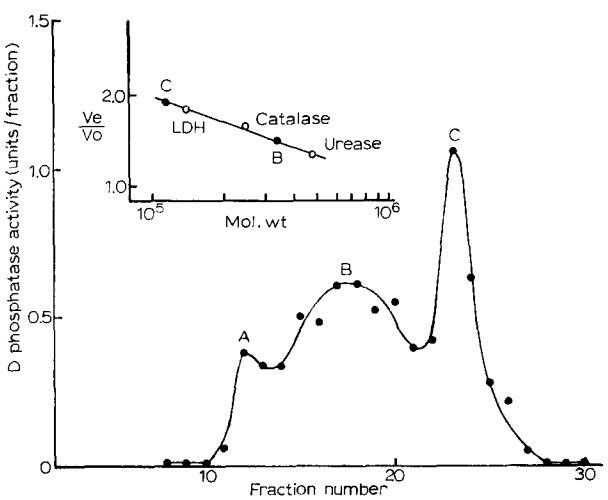

Fig. 5. Sephadex G-200 chromatography of rat liver synthase D phosphatase. The partially purified D phosphatase (1 ml, 17 mg protein) was applied to a column (1.5 cm × 45 cm) of Sephadex G-200 previously equilibrated with 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM mercaptoethanol and eluted with the same buffer. Fractions of 2 ml were collected and D phosphatase was assayed in each of these fractions. The insert shows the molecular weight estimation, where rabbit muscle lactate dehydrogenase (LDH, mol. wt $1.3 \cdot 10^5$–$1.4 \cdot 10^5$), beef liver catalase ($2.3 \cdot 10^5$) and soybean urease ($4.8 \cdot 10^5$) were used as markers. Peak A was eluted at the void volume.

peaks, of which the first one eluted in the void volume. They were designated A, B and C in order of elution. When each of the three peaks was rechromatographed on Sephadex G-200, an almost single peak of activity that eluted in the original volume was observed. It, therefore, appears that these multiple forms did not arise from a reversible aggregation–disaggregation of the enzyme.

The elution volumes of phosphatase activities from the column were compared with those of protein markers of known molecular weight. As shown in Fig. 5 (the insert), the apparent molecular weights were $3.4 \cdot 10^5$ for B and $1.15 \cdot 10^5$ for C. Peak A probably has a molecular weight of more than 500 000.

The various D phosphatase peaks as separated from each other by DEAE-cellulose (I–VI, see Fig. 4A) were then chromatographed separately on Sephadex G-200. The elution patterns obtained are shown in Fig. 6. If allowance is made for prob-

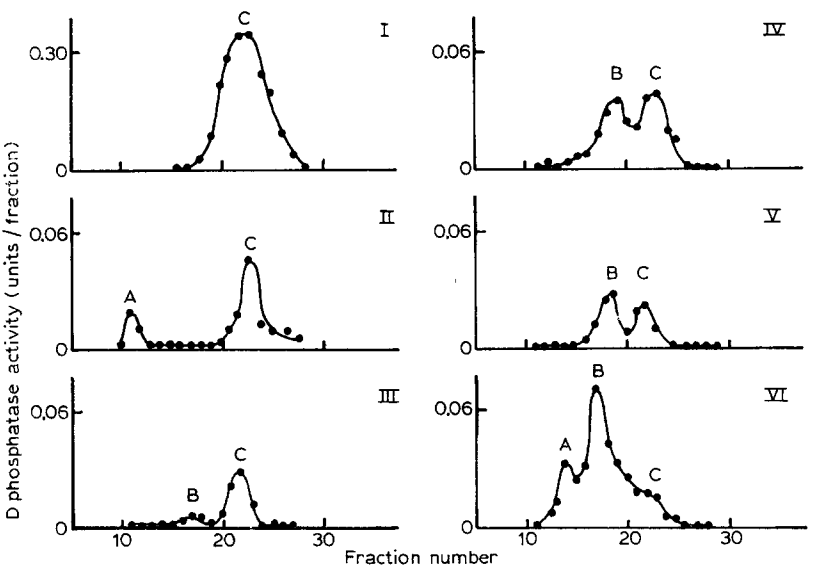

Fig. 6. Sephadex G-200 chromatography of the peak activities as separated from each other by DEAE-cellulose. Fractions representing each peak activity in Fig. 4A were pooled, precipitated at 60% satn of $(NH_4)_2SO_4$, dissolved in 1 ml of 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM mercaptoethanol and applied to a column (1.5 cm × 45 cm) of Sephadex G-200. Other conditions were the same as those described in the legend to Fig. 5.

able overlapping, it is possible to state that Peaks I, II and III have the chromatographic properties of Peak C of Fig. 5 and IV, V and VI those of Peak B. A minor fraction having the chromatographic properties of Peak A is present in Peaks II and VI.

DEAE-cellulose chromatography of skeletal muscle D phosphatase

D phosphatase was partially purified from rat skeletal muscle and chromatographed on DEAE-cellulose. The results are shown in Fig. 7. There were four distinct peaks of D phosphatase activity, which eluted at ionic strengths equal to those for Peaks I, III, IV and VI of liver phosphatase, respectively (Fig. 4A). No activity was

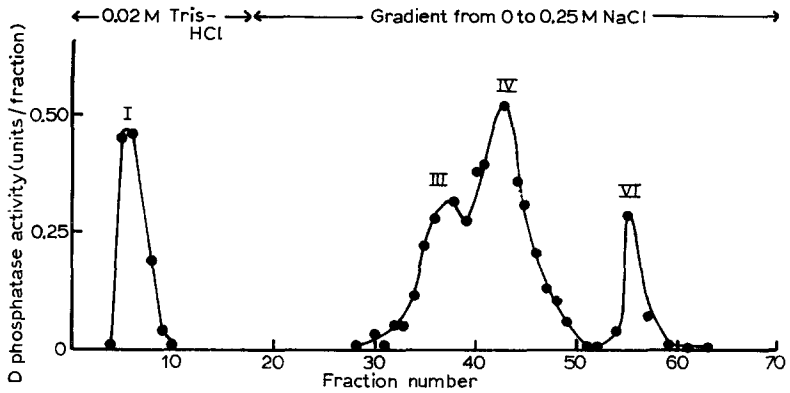

Fig. 7. DEAE-cellulose chromatography of rat skeletal muscle D phosphatase. D phosphatase was partially purified from the postmicrosomal supernatant of rat skeletal muscle by the procedure similar to that used for liver D phosphatase. The enzyme (7 ml, 34 mg protein) was then chromatographed on DEAE-cellulose and assayed for activity as described in the legend to Fig. 4.

detected in the fractions corresponding to Peaks II and V, while Peak IV appears to exist in a much larger proportion than in liver.

DISCUSSION

In addition to the soluble enzymes investigated in the present work, rat liver contains synthase D phosphatase bound to particulate (Table I). Its activity, however, is low, being less than 20% of the total activity. In confirmation of the results of the previous workers [4], the activity associated with the crude glycogen pellet was released by ultrasonic disintegration. Due to rather small activities, chromatographic examinations have not been performed on the particulate-bound enzymes. Therefore, it is not clear if these enzymes are still another form or they are the supernatant enzymes which have been adsorbed on the particulates.

The substrate employed in the present work was the glycogen synthase D that had been purified from rat liver to a level essentially free of D phosphatase activity [5, 6]. But the enzyme was still bound firmly to glycogen particle. The association with glycogen particle, however, might be favorable to synthase D for maintaining the conformational state suitable for the binding with D phosphatase. In this regard, it is of interest that glucose 6-phosphate [10] or ATP [11], known to modulate D phosphatase activity, has been shown to interact with synthase D rather than with D phosphatase.

Previous workers [1, 12] studied the D phosphatase of rabbit skeletal muscle by DEAE-cellulose or Sephadex G-200 chromatography. Although their data are indicative of the multiple nature of the enzyme, the multiplicity was not further investigated; the identical distribution of synthase D phosphatase and phosphohistone phosphatase activities was taken by Kato and Bishop [1] as evidence that the two activities reside in the same protein. Whether or not the same is true for the liver enzyme must await further investigation. Meisler and Langan [13] repotred that the phosphohistone phosphatase of rat liver was resolved into two fractions by DEAE-

cellulose chromatography. Their molecular weights were identical ($1.9 \cdot 10^5$). Synthase D was not tested as substrate.

In the present work, multiple forms of rat liver D phosphatase have been demonstrated by DEAE-cellulose and Sephadex G-200 chromatography. Partially purified D phosphatase of rat liver was resolved into six forms by DEAE-cellulose (Fig. 4A) and a similar experiment made on fresh liver supernatant (Fig. 4B) excluded the possibility that they have arisen from enzyme degradation or modification during purification. The possibility that the multiplicity as revealed by Sephadex G-200 is due to different states of aggregation is also unlikely, because no spontaneous transformation has been observed upon rechromatography.

Preliminary studies on the various forms of rat liver D phosphatase resolved by DEAE-cellulose have suggested that they are not identical to each other in properties and may have different physiological functions. For example, Forms IV, V and VI were shown to have much higher molecular weights than I, II and III (Fig. 6). Secondly, Forms II and V failed to exhibit themselves in a muscle fraction (Fig. 7). These forms may, therefore, be absent from muscle.

These data suggest that multi-isozymic forms of glycogen synthase D phosphatase exist in rat liver. More detailed studies on their properties and physiological significance are now in progress.

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