GLYCOGEN SYNTHASE PHOSPHATASE OF RAT LIVER. ITS SEPARATION FROM PHOSPHORYLASE PHOSPHATASE ON DE-52 COLUMNS

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SUMMARY: Glycogen synthase D was prepared from rat liver by chromatographing the glycogen pellet on DE-52 columns. It was free of glycogen and phosphorylase and converted readily into synthase I upon incubation with glycogen synthase phosphatase. With this synthase D as substrate, the identity of rat liver glycogen synthase phosphatase was studied by means of DE-52 column chromatography. Under the conditions developed, synthase phosphatase emerged from the columns as a sharp, single peak, and phosphorylase phosphatase came off later. The two phosphatases were also different from each other in stability, synthase phosphatase being less stable than phosphorylase phosphatase.

INTRODUCTION

The conversion of glycogen synthase from the D into the I form is catalyzed by glycogen synthase phosphatase (1). Although this phosphatase appears to be a major determinant of the rate of glycogen synthesis (2-5), little is yet known of its nature and properties. Recently, several laboratories presented evidence that in certain bovine and rabbit tissues, synthase phosphatase and phosphorylase phosphatase reside in a single protein (6-9). An earlier paper from this laboratory (10), however, showed that the enzyme from rat liver could be resolved into several peaks on DEAE-cellulose columns. Furthermore, all the fractions did not exhibit phosphorylase phosphatase activity (unpublished result).

A major difficulty encountered with such studies on rat liver synthase phosphatase concerns substrate. Rat liver synthase D is firmly associated with particulate glycogen (11,12) and cleavage of this association usually leads to a modification of the enzyme such that it is no longer a substrate for synthase phosphatase. Accordingly, the previous studies (10) employed as substrate

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glycogen pellets of rat liver homogenates, which were contaminated heavily by phosphorylase as well as by glycogen.

The present report describes a chromatographic study on rat liver synthase phosphatase with special reference to its relationship to phosphorylase phosphatase. The substrate employed was rat liver synthase D freed of glycogen and phosphorylase and the procedure for its isolation is also reported.

MATERIALS AND METHODS

Isolation of glycogen synthase D. Glycogen pellet was prepared from rat liver by homogenizing tissue with 4 volumes of 0.5 M sucrose—62.5 mM Tris-HCl (pH 7.4)—6.25 mM EDTA—50 mM NaF and centrifuging the homogenate at 50,000 ×g for 1 hr. Details of the procedure were described elsewhere (13). Over 70% of the homogenate glycogen synthase was recovered in the pellet, exclusively in the D form. The pellet from approximately 25 g of fresh liver was suspended in 15 ml of 10 mM glycylglycine (pH 7.4)—5 mM mercaptoethanol—2% (V/V) glycerol using a glass-Teflon homogenizer and applied to a column of DE-52 (1.5 × 12 cm) previously equilibrated with the same buffer. The column was washed with 70 ml of the same buffer and a linear gradient made with 250 ml-portions of the buffer with and without 0.5 M NaCl was applied. 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 10 ml were then collected at a flow rate of approximately 30 ml/hr and assayed for glycogen synthase in the presence of glucose 6-phosphate. Active fractions were united, concentrated approximately 5-fold using a concentration column of DE-52 (1.5 × 1.0 cm) and stored at -20° until use. All these and the following preparative procedures were carried out at 2-4°.

Phosphorylase a. The above fractions were also assayed for phosphorylase and active fractions were united and kept frozen until use.

Assay of glycogen synthase. Glycogen synthase was assayed by measuring the incorporation of glucose from UDP-[U-^{14}C]glucose into glycogen. The standard assay mixture contained 50 mM glycylglycine (pH 8.5), 2.5 mM UDP-[U-^{14}C]glucose, 10 mM glucose 6-phosphate (when indicated), 10 mM EDTA, 80 mM NaF, 0.6 mg of rabbit liver glycogen and enzyme in a final volume of 0.2 ml. After incubation at 30° for 15 min, glycogen was isolated as described previously (14), suspended in 1 ml of water, and its radioactivity was assayed in a Beckman liquid scintillation counter after addition of 10 ml of scintillation mixture containing Triton X-100—toluene (1:2). One unit of enzyme was defined as the amount which catalyzed the incorporation of 1 μmol of glucose per hr.

Assay of phosphorylase. Phosphorylase was measured by the incorporation of glucose from [U-^{14}C]glucose 1-phosphate into glycogen. The standard assay mixture contained 50 mM Tris-maleate (pH 6.1), 25 mM [U-^{14}C]glucose 1-phosphate, 10 mM EDTA, 80 mM NaF, 0.6 mg of rabbit liver glycogen and enzyme in a final volume of 0.2 ml. After 15 min-incubation at 30°, glycogen was isolated and counted as described for glycogen synthase. The conditions were such that only phosphorylase a was detectable. One unit of the enzyme was the amount which catalyzed the incorporation of 1 μmol of glucose per hr.

Partial purification of glycogen synthase phosphatase. The enzyme was partially purified from rat liver by a procedure similar to that of Abe and Tsuiki (15). In short, rat liver was homogenized with 4 volumes of 0.5 M

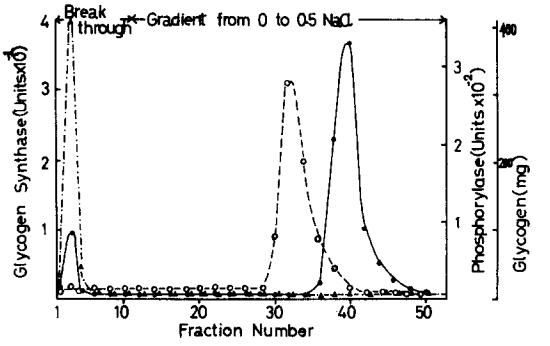

Figure 1. Separation of glycogen synthase D from glycogen and phosphorylase a on a DE-52 column. Conditions are as described in the text. Each fraction was assayed for glycogen synthase (in the presence of glucose 6-phosphate) (—●—), phosphorylase (- -○- -) and glycogen (-·-Δ·-). Glycogen was determined by the anthrone reaction.

sucrose—62.5 mM glycylglycine (pH 7.4)—6.25 mM EDTA and the homogenate was centrifuged at 105,000 ×g for 1 hr. The pH of the supernatant was adjusted to 5.2; the solution was centrifuged: and the resulting supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ after neutralization. The precipitate was then collected, dissolved in a minimum volume of 10 mM glycylglycine (pH 7.5)—5 mM mercaptoethanol—2% (V/V) glycerol and passed through a column of Sephadex G-25.

Column chromatography of synthase phosphatase. Partially purified synthase phosphatase, amounting to 50 mg in protein, was applied to a column of DE-52 (1.5 × 12 cm) previously equilibrated with 10 mM glycylglycine (pH 7.4)—5 mM mercaptoethanol—2% (V/V) glycerol. The column was first washed with 50 ml of the equilibrating buffer and a linear gradient made with 100 ml-portions of the buffer with and without 0.5 M NaCl was applied. The eluate was dialyzed and collected just as described for glycogen synthase; and the fractions were assayed for synthase- and phosphorylase-phosphatase.

Assay of synthase phosphatase. This was conducted by measuring the formation of synthase I from synthase D. The standard assay mixture contained 20 mM glycylglycine (pH 7.4), 5 mM $MgCl_2$, 2 mM Na_2SO_4, 1 mM caffeine, 0.2 mM glucose 6-phosphate (when indicated), 25% (V/V) glycerol, 0.5 unit of substrate (synthase D) and enzyme in a final volume of 0.2 ml. After 10 min-incubation at 30°, 0.05 ml was removed and transferred to 0.15 ml of a synthase test mixture containing 16 μmol of NaF. Glycogen synthase activity was then determined as described above. Since the test mixture contained no glucose 6-phosphate, synthase D was inactive; so that the activity found in the 10 min-sample was due to the formation of synthase I. One unit of synthase phosphatase was defined as the amount which gave rise to 1 unit of synthase I in 10 min under the above conditions.

Assay of phosphorylase phosphatase. The enzyme was assayed by conversion of phosphorylase a into phosphorylase b. The standard assay mixture contained 20 mM glycylglycine (pH 7.4), 5 mM $MgCl_2$, 1 mM caffeine, 10% (V/V) glycerol, 0.2 mg of bovine serum albumin, 5 units of substrate (phosphorylase a) and enzyme in a final volume of 0.2 ml. After 10 min-incubation at 30°, 0.08 ml was removed and transferred to 0.12 ml of a phosphorylase test mixture containing 16 μmol of NaF. Phosphorylase activity was then measured as described above. Under the conditions employed, phosphorylase b was inactive; so that

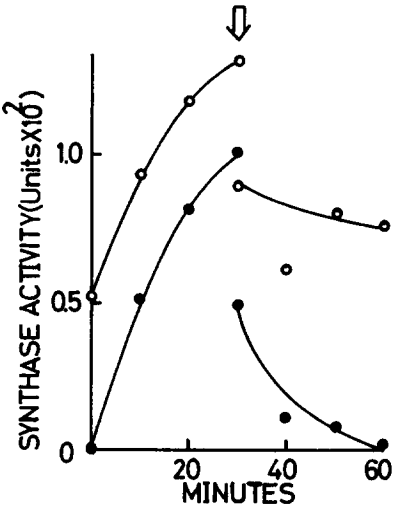

Figure 2. Conversion of synthase D into synthase I and its reconversion into synthase D. Synthase D purified by DE-52 column was incubated at 30° in the presence of 60 mM glycylglycine (pH 7.4), 4 mM $MgCl_2$, 2 mM Na_2SO_4, 1 mM caffeine and the partially purified synthase phosphatase fraction. The reaction was initiated by the addition of synthase phosphatase and at the point indicated by the arrow, ATP and cyclic AMP were added to final concentrations of 15 and 0.1 mM, respectively. At the designated time intervals, aliquots were removed and assayed for glycogen synthase in the presence (○) and absence (●) of glucose 6-phosphate.

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the loss of activity observed in 10 min was due to the conversion of phosphorylase *a* into *b*. One unit of phosphorylase phosphatase was defined as the amount which converted 1 unit of phosphorylase *a* in 10 min.

Chemicals. DE-52 was purchased from Whatman and made CO_2-free before use. [U-^{14}C]glucose 1-phosphate was obtained from New England Nuclear Corp. The sources of other chemicals were described previously (13).

RESULTS

Isolation of glycogen synthase D. In attempts to detach glycogen synthase D from particulate glycogen, the glycogen pellet suspended in an appropriate buffer was applied to columns of DE-52. Although the enzyme became extremely unstable after separation from glycogen, this instability could be overcome by 2% (V/V) glycerol. In a typical experiment shown in Figure 1, the bulk of synthase D was eluted from the column by NaCl as a sharp, single peak, with a recovery from the homogenate of 27% and approximately 1,300-fold purification.

Table 1. Effects of Various Treatments on Synthase Phosphatase and Phosphorylase Phosphatase Activities

Treatments	Phosphorylase phosphatase	Synthase phosphatase
	(%)	
None	100	100
Incubated at 30° with 4 mM Mg^{2+} (A)	126	60
Incubated at 30° with 0.5 M ME* (B)	204	116
Frozen in the presence of 0.5 M ME* (C)	185	22
Precipitated by ethanol at 20° (D)	191	30
Incubated at 30° with trypsin (50 µg/ml) (E)	138	47

*Mercaptoethanol
Partially purified synthase phosphatase fraction was prepared as described in the text except that the buffer contained no mercaptoethanol and glycerol. It was then subjected to various treatments indicated above, with duration of 30 (A-C) or 10 min (E), after which the mixtures were either passed through a Sephadex G-25 column (A-C) or added with soybean trypsin inhibitor (E). For D, the phosphatase fraction was added with 5 volumes of ethanol, centrifuged and the precipitate was extracted with 10 mM glycylglycine (pH 7.4).

Glycogen emerged in the "break-through" and phosphorylase (the a form) came off ahead of synthase.

Experiments were then performed to determine if the synthase D isolated as above could be a "good" substrate for synthase phosphatase. As shown in Figure 2, with Mg^{2+} present, the enzyme was activated by synthase phosphatase while ATP and cyclic AMP reversed the activation. Since Mg^{2+}, ATP and cyclic AMP were all ineffective without synthase phosphatase, except that ATP was slightly inhibitory to synthase D, these results were interpreted as showing that the synthase D first converted by synthase phosphatase into synthase I, which in turn reconverted into synthase D by protein kinase, presumably present in the partially purified synthase phosphatase preparation. Furthermore, the above activation was inhibited by glycogen known as a potent inhibitor of synthase phosphatase reaction (16,17). The synthase D was stable if stored at -20° in the presence of 2% (V/V) glycerol and used as substrate for the experiments described below.

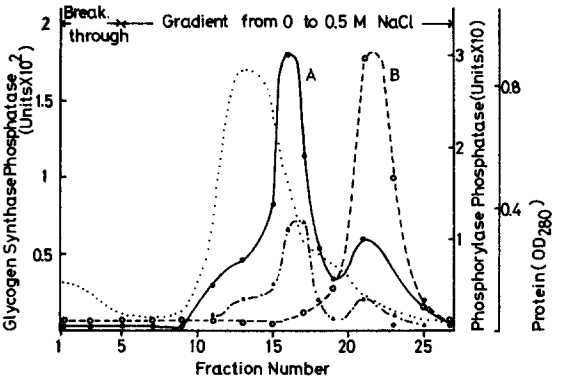

Figure 3. DE-52 column chromatography of rat liver synthase phosphatase and phosphorylase phosphatase. Conditions are as described in the text. Each fraction was assayed for synthase phosphatase in the presence (—●—) and absence (—·▲·—) of glucose 6-phosphate, phosphorylase phosphatase (— —○— —) and protein (·····).

Partially purified synthase phosphatase. In the previous studies (10), synthase phosphatase was partially purified from rat liver homogenates by acid- and $(NH_4)_2SO_4$-fractionation. During the course of these studies, we noted that phosphorylase phosphatase activity was also recovered in the final fraction with 60-70% yields. Although this observation was initially considered to be indicative of a single phosphatase enzyme, the two phosphatase activities responded quite differently to various treatments listed in Table 1. Confirming the results of previous workers (18-20), these treatments produced large activation of phosphorylase phosphatase. But synthase phosphatase was inactivated rather than activated by most of these treatments.

Column chromatography of partially purified synthase phosphatase. We previously reported that the synthase phosphatase activity of the partially purified synthase phosphatase fraction was resolved into 6 peaks on DEAE-cellulose (10). Since this result was obtained with glycogen-bound synthase D as substrate, behavior of the synthase phosphatase was reinvestigated using purified substrate. Moreover, changes were made of the chromatographic conditions so that any possible deterioration of the unstable synthase phosphatase

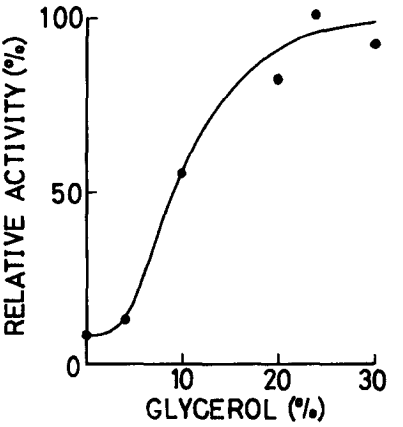

Figure 4. Inactivation of synthase phosphatase by storage and its reactivation by glycerol. Synthase phosphatase eluted from a DE-52 column was subjected to storage at -20° overnight, after which enzyme activity was determined in the presence of varying concentrations of glycerol and expressed as the percentage of that observed prior to the storage.

(Table 1, see also Figure 4) could be avoided. Figure 3 illustrates that under the conditions thus established, the bulk of rat liver synthase phosphatase activity was eluted from a DE-52 column as a sharp, single peak (Peak A). Glucose 6-phosphate, known to stimulate synthase phosphatase reaction (21-23), raised the activity almost 3-fold. The peak was in the same position on rechromatography.

As also shown in Figure 3, Peak A had no phosphorylase phosphatase activity. A sharp, single phosphorylase phosphatase peak, however, followed Peak A and was designated Peak B. Peak B possessed a weak activity against synthase D (Figure 3), which was inseparable from phosphorylase phosphatase activity on rechromatography.

After the DE-52 step, the major synthase phosphatase (Peak A) became particularly unstable. As shown in Figure 4, it lost all the activity after storage at -20° overnight, though the original activity could be restored by addition of 20% (V/V) glycerol. The activity of phosphorylase phosphatase (Peak B), on the other hand, was not diminished by the storage.

DISCUSSION

In tissues such as bovine heart (6) and rabbit muscle (7) and liver (8, 9), synthase phosphatase and phosphorylase phosphatase activities appear to reside in a single protein: the two activities were co-purified during purification and both substrates competed with each other for the phosphatase. In developing rat liver, however, Devos and Hers (24) noted that synthase phosphatase emerged at day 18 of gestation while phosphorylase phosphatase increased rapidly at day 21. Tan and Nuttall (25) recently reported that ethanol precipitation produced a large activation of rat liver phosphorylase phosphatase with no corresponding increase in synthase phosphatase.

Evidence has been presented in this paper to support the view that the two phosphatases may be different enzymes in rat liver. The data reported here show that synthase phosphatase activity (Peak A) was clearly separated from phosphorylase phosphatase activity (Peak B) on DE-52 columns (Figure 3). Furthermore, three lines of evidence suggest that synthase phosphatase is much more labile than phosphorylase phosphatase: i) under chromatographic and analytical conditions less favorable than the present ones, synthase phosphatase (10) but not phosphorylase phosphatase (unpublished result) exhibited heterogenous peaks; ii) several treatments including ethanol precipitation, which activated phosphorylase phosphatase, inactivated synthase phosphatase (Table 1); and iii) after storage at -20° overnight, purified synthase phosphatase but not phosphorylase phosphatase was severely inactivated (Figure 4).

The present results thus differ from the previous ones (6-9) with respect to the nature of the two phosphatase activities. The reason for this difference is unclear. It may simply be attributable to the difference in organ source. But it is also possible that the synthase phosphatase as described above (Peak A) is so unstable that it may not resist some of the purification steps employed in the previous studies (6-9); then the phosphorylase phosphatase (Peak B), also possessing an activity against synthase D, could be an enzyme

corresponding to the phosphoprotein phosphatase described previously in other tissues (6-9). These possibilities are currently under investigation.

Although Segal and Lin (26) reported the inability to separate rat liver glycogen synthase from particulate glycogen on DEAE-cellulose columns, we have been able to detach the enzyme from the glycogen by using DE-52 columns. The procedure is less drastic than those of previous studies, such as enzymatic removal of glycogen (27,28), and has been useful for the preparation of "good" substrate for synthase phosphatase. The procedure may also be useful for the kinetic and molecular studies of rat liver glycogen synthase, which have hitherto been done only very inadequately.

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