Homogeneous Glycogen Synthetase b from Rat Liver*

(Received for publication, April 17, 1973)

DAVID C. LIN AND HAROLD L. SEGAL

From the Department of Biology, State University of New York, Buffalo, New York 14214

SUMMARY
Glycogen synthetase b has been purified about 2,000-fold from a crude extract of rat liver. The purification procedure consisted of the separation of the glycogen pellet by centrifugation, solubilization of the synthetase by phosphorylation of the glycogen by endogenous phosphorylase, and adsorption and elution from Ca(PO_4)_2 gel. The final material appears homogeneous on gel electrophoresis. A subunit molecular weight of 88,000 was obtained from electrophoresis in the presence of sodium dodecyl sulfate. The oligomer appears to have a molecular weight of 260,000 as isolated, suggesting that it is a trimer. On storage in solution in the cold, a species twice this size appears. The peak absorbance of the enzyme was at 278 nm with a value of E_1% at this wave length of 14.5. The spectrum showed no evidence of conjugated chromophores. Determinations of alkali-labile phosphate and reactive sulfhydryl groups gave values of 12.4 and about 6, respectively. Activity decreased linearly to zero with the titration of the first three sulfhydryl groups (or the first two when glycogen was present), indicating that at least one of the most reactive groups is essential for activity. Incubation of the purified synthetase at 0°C led to inactivation which was reversed on rewarming. Glycogen protected against this cold inactivation.

In a recent paper, we described the preparation and some properties of homogeneous glycogen synthetase b from trout liver and pointed out the key role of this enzyme in glycogenesis and the nature of its regulatory mechanisms as they are currently known (1). Highly purified glycogen synthetase preparations have also been obtained from rabbit muscle (2, 3), tadpole liver (4), and swine kidney (5). In this paper, we report the purification from rat liver of homogeneous glycogen synthetase b and some of its properties.

EXPERIMENTAL PROCEDURE

Materials—Male Sprague-Dawley rats weighing 200 to 300 g were obtained from Holtzman and were maintained on Purina lab chow. Guanidine HCl was the product of Mann Research Laboratories. Rabbit muscle pyruvic kinase, beef heart lactic dehydrogenase, the crystalline enzymes used as standards in the SDS-polyacrylamide gel electrophoresis, and all other biochemicals were obtained from Sigma Chemical Co.

Enzyme Assay Glycogen synthetase was assayed by the optical method as previously described (6). Samples were incubated for 10 min at 37°C in a volume of 1 ml, containing 4 μmoles of UDP-glucose, 5 mg of glycogen, 50 μmoles of glycylglycine, pH 7.4, and with and without 4 μmoles glucose-6-P for assay of total glycogen synthetase and glycogen synthetase a, respectively. The reaction was terminated by immersing the tubes in boiling water. To measure the amount of UDP formed, 2.2 ml of a solution was then added containing 200 μmoles of glycylglycine, pH 7.4, 10 μmoles of MgCl_2, 0.6 μmol of phosphaenolpyruvate, and 0.4 mg of NADH. After determination of the initial absorbance at 340 nm, 0.05 ml of a mixture of pyruvic kinase and lactic dehydrogenase, the crystalline enzymes used as standards in the method of Weber and Osborn (7). Protein was stained overnight with 0.25% Coomassie brilliant blue in methanol-acetic acid-water (5:1:5, v/v) and destained with 7% acetic acid.

Phosphate Determination The procedure of Ames (8) was used for the determination of alkali-labile and total phosphate in protein. For the former, the protein was precipitated and washed with 7% trichloroacetic acid followed by incubation in 0.25 M NaOH at 37°C for 20 hours. For the determination of total phosphate, the protein was precipitated as above and ashed with Mg(NO_3)_2 several times.

Sulfhydryl Determination—DTNB was employed for the sulfhydryl determinations (9). The enzyme sample was dialyzed at room temperature in a collodion bag (WWR Scientific) against several changes of a solution of 0.1 M glycylglycine, pH 7.4, 0.25 M sucrose, and 4 mM glucose-6-P. The dialysate was tested with DTNB periodically for the presence of -SH groups, and the dialysis was continued for 2 hours after a negative test was obtained. An excess of DTNB was mixed with a measured amount of synthetase in a cuvette, and the absorbance at 412 nm was determined against a blank containing the dialysis buffer and the same concentration of DTNB but no protein. The absorbance

* The abbreviations used are: SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); 2-ME, 2-mercaptoethanol.
due to protein was subtracted, and an extinction coefficient of 13,600 was used to calculate the moles of sulphydryl reacted.

**Other Determinations—** Refractometry was performed at 20° with a Rayleigh interference refractometer (model 134, Hilger and Watts, England). Protein was measured either by the method of Lowry et al. (10) or from absorbance at 278 nm with a value of E$^{1}%$ of 14.5 for the purified enzyme as determined in this study. Glycogen was determined according to the method of Hassid and Abraham (11).

**RESULTS**

**Enzyme Purification—** Rats were decapitated and drained of blood. Livers were removed and rinsed with 0.14 M NaCl, then homogenized in a blender at 0° with 5 ml per g of liver of a solution of 0.1 M glycylglycine, pH 7.4, 0.25 M sucrose, and 0.1 M NaF. The homogenate was centrifuged at 8,000 × g for 10 min, and the supernatant fluid was filtered through glass wool and cheesecloth. Most of the synthetase activity of the homogenate was recovered in this crude extract. This fraction was then centrifuged at 19,000 rpm for 3 hours at 0° in a Spinco, model L-2, with the type 19 rotor. The glycogen pellet at the bottom of the bottles was resuspended in a volume of the homogenizing buffer equal to one-tenth that of the crude extract and centrifuged at 78,000 rpm for 2 hours. The washed glycogen pellet was then suspended in a solution of 50 mM KPO$_4$, pH 6.9, 0.25 M sucrose, 50 mM NaF, 30 mM 2-ME, and 1 mM AMP. The total volume of this suspension was adjusted to about one-tenth that of the crude extract so that the glycogen synthetase activity was approximately 3 units per ml. The suspension was incubated at room temperature in a dialysis bag against 50 volumes of the same buffer until glycogenolysis was complete as evidenced by clarification of the solution (about 24 hours).

After glycogenolysis, the preparation was centrifuged at 78,000 rpm for 1 hour at 20°, and the sediment was discarded. Calcium phosphate gel was employed as the final step in the purification, which was carried out at room temperature. The gel was washed with a solution of 50 mM KPO$_4$, pH 6.9, 0.25 M sucrose, 50 mM NaF, and 30 mM 2-ME, then suspended at a concentration of 20 mg per ml in the same buffer and added to the enzyme solution with stirring (1 mg dry weight per mg of protein). After further stirring for 5 min, the gel with adsorbed protein was collected by centrifugation at low speed. Essentially all of the glycogen synthetase was bound to the gel, while phosphorylase remained unadsorbed. The gel was washed sequentially with 50 mM KPO$_4$, pH 6.9, containing 5% ammonium sulfate, with 0.1 M KPO$_4$, pH 6.9, and finally with 0.14 M KPO$_4$, pH 6.9. All of these solutions also contained 0.25 M sucrose, 50 mM NaF, and 30 mM 2-ME. The volume of the solution used in the first two washings was equal to that of the original enzyme solution, whereas the volume of the last washing was reduced by one-half. For each washing, the gel was suspended and stirred for 10 min. About 5% of the synthetase activity was removed in the last washing. Glycogen synthetase was eluted from the gel by stirring with a solution of 0.25 M KPO$_4$, pH 8.5, 0.25 M sucrose, and 30 mM 2-ME for 15 min. Usually four elutions were required. The volume of the solution used in each elution was approximately one-tenth that of the original enzyme solution. The eluted enzyme solution was then concentrated to a small volume in a collodion bag. Any calcium phosphate remaining was removed by centrifugation at 27,000 × g for 20 min. The enzyme was then returned to the collodion bag for further dialysis against a solution of 50 mM glycylglycine, pH 7.4, 0.25 M sucrose, 3 mM dithiothreitol, and 4 mM glucose-6-P with three changes of the buffer. The purified enzyme could be stored for at least 2 months in liquid nitrogen with no loss of activity. On polyacrylamide gel electrophoresis, a single protein band was detected which corresponded with the glycogen synthetase activity as previously described (1), except incubation was at 37° for 90 min. Activity is expressed as total absorbance change per segment. The stained gels are shown at the top. A, 8.9 μg of freshly prepared enzyme; B, 178 μg of enzyme aged at 4° for 3 days (see text).

**Fig. 1.** Disc gel electrophoresis of purified glycogen synthetase.$b$. Electrophoresis was at 2 mA per gel for 2 hours. Gels were run in duplicate. One gel was stained for protein, and the other was cut into 2-mm segments which were assayed for glycogen synthetase activity as previously described (1), except incubation was at 37° for 90 min. Activity is expressed as total absorbance change per segment. The stained gels are shown at the top. A, 8.9 μg of freshly prepared enzyme; B, 178 μg of enzyme aged at 4° for 3 days (see text).
Table I

Purification of glycogen synthetase b

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Specific activity</th>
<th>% Form</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>645</td>
<td>232</td>
<td>0.016</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>Glycogen pellet</td>
<td>61</td>
<td>169</td>
<td>1.57</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>Soluble extract</td>
<td>70</td>
<td>95</td>
<td>2.00</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Ca₄(PO₄)₂ gel eluate</td>
<td>1.5</td>
<td>47</td>
<td>35.4</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 2. Subunit molecular weight of glycogen synthetase b. The procedure was that of Weber and Osborn (7). Monomer molecular weights of the reference proteins are: bovine serum albumin (BSA), 68,000 (7); trypsin, 23,000 (7); phosphorylase, 94,000 (12); yeast hexokinase (HK), 62,000 (13); yeast alcohol dehydrogenase (ADH), 37,000 (14). The electrophoretogram of the synthetase (40 μg) is shown in the inset.

dithiothreitol, 5 mM glucose-6-P, and 0.2 mM NaCl before centrifugation. The results of this experiment showed that the preparation was heterodisperse. The weight average molecular weight obtained by extrapolation to the base of the column (15) gave a value of $M_w$ of 500,000. A lower molecular weight species was also present of weight average molecular weight 260,000, as obtained by extrapolation to the meniscus ($c = 0$).

To determine whether the heterogeneity was a result of the handling procedure, a sample similarly treated was analyzed electrophoretically (Fig. 1B). It can be seen by comparison with freshly prepared enzyme (Fig. 1A), that aging produces an aggregated form of the original species (also see below). We can thus tentatively conclude that the enzyme as isolated has a molecular weight of about 260,000, in which case it is composed of 3 subunits of identical size and aggregates to a form double the original size.

Spectral Properties—The purified enzyme has a maximum absorbance at 278 nm (Fig. 3). The spectrum shows no evidence of bound nucleotides or other conjugated chromophores. The average extinction coefficient ($\epsilon_{280}$) at the peak for three different preparations was $14.5 \pm 0.3$, with refractometry as the basis of protein determination.

Phosphate Content—The results of the determination of the phosphate content of the purified synthetase are shown in Table II. The equivalents of alkali-labile and total phosphate content per subunit of $85,000$ daltons were $12.4 \pm 0.6$ and $17.3 \pm 1.1$, respectively. Crystalline rabbit muscle phosphorylase and bovine serum albumin were also included in the determinations as references.

Fig. 3. Absorption spectrum of glycogen synthetase b. The enzyme (1.18 mg per ml) was in the final buffer described under purification. The light path length was 1.0 cm. A Zeiss PMQ II spectrophotometer was used.

Table II

Phosphate content of glycogen synthetase b

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphate equivalents/subunit$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkali-labile</td>
</tr>
<tr>
<td>Glycogen synthetase b</td>
<td>12.4 ± 0.6 (8.9)$^b$</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>0.74 ± 0.1 (2.5)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$^a$ Subunit sizes: glycogen synthetase b, 85,000 (this paper); phosphorylase a, 94,000 (12); bovine serum albumin, 68,000 (7).

$^b$ Numbers in parentheses are the number of preparations assayed and the total number of analyses, respectively.

Sulfhydryl Content and Requirement for Activity—The time course of sulfhydryl titrations with DTNB is shown in Fig. 4. The titers obtained, in sulfhydryl equivalents per subunit ($85,000$ daltons), were 6.5 in the presence of $4$ mM glucose-6-P, 5.4 in the presence of $4$ mM glucose-6-P plus $4$ mM UDP-glucose, and 5.4 in the presence of $4$ mM glucose-6-P plus 0.5% glycogen. With a second synthetase preparation of somewhat lower specific activity, about 1 eq less of -SH was obtained in each case. In the presence of guanidine-HCl or SDS, the reaction was immediate, and the titers obtained were 6.4 with guanidine and 8.3 with SDS. We thus conclude that there are a total of 8 -SH eq per subunit, of which 6 are reactive with DTNB in the native enzyme in the presence of glucose-6-P, and 1 less with UDP-glucose or glycogen present in addition.

Fig. 5 shows the relationship between -SH groups reacted and activity remaining. Activity decreased linearly to zero as the first three -SH groups reacted and activity remaining. Activity decreased linearly to zero as the first three -SH groups reacted when glucose-6-P or glucose-6-P plus UDP-glucose were present, and as the first two groups reacted when glycogen was present. A reasonable interpretation of these results is that three -SH groups react rapidly in the absence of glycogen, at least one of which is essential for activity, and that one of the nonessential ones is protected by glycogen.

Reversible Cold Inactivation and Stability of Glycogen Synthe-
FIG. 4. DTNB titration of glycogen synthetase b. Ten microliters of 10 mM DTNB were added to the enzyme solution. Absorbance was measured as a function of time and converted to sulfhydryl equivalents as described under “Experimental Procedure.” ○, 0.26 mg of enzyme in 0.7 ml containing 4 mM glucose-6-P; △, 0.27 mg of enzyme in 0.6 ml containing 4 mM glucose-6-P and 4 mM UDP-glucose; □, 0.29 mg of enzyme in 0.6 ml containing 4 mM glucose-6-P and 0.8% glycogen.

FIG. 5. Sulphydryl dependence of glycogen synthetase activity. The procedure was as described in Fig. 4 with aliquots removed periodically for assay. Each aliquot was diluted about 20-fold in buffer containing 60 mM 2-ME to remove unreacted DTNB. ○, 0.38 mg of enzyme in 0.6 ml containing 4 mM glucose-6-P; △, 0.39 mg of enzyme in 0.6 ml containing 4 mM glucose-6-P and 4 mM UDP-glucose. □, 0.42 mg of enzyme in 0.6 ml containing 4 mM glucose-6-P and 0.8% glycogen.

tase—Incubation of the purified enzyme at 0° led to a progressive decline in activity to an equilibrium value (Fig. 6). Rewarming at 25° restored the original activity. The presence of glycogen in the enzyme solution prevented this cold inactivation. The existence of an additional form of glycogen synthetase has been reported in chloroma tumors, which was active only in the presence of extraordinarily high concentrations of glucose-6-P (17). The cold-inactivated form described here did not exhibit this property. In fact, there were no kinetic differences from the fully active preparation, except, of course, for the diminished specific activity. Inactive forms of synthetase have also been reported to exist in muscle and other tissues, as indicated by the appearance of increased activity upon incubation of extracts (1, 17–19). Rosell-Pérez (18) has proposed that the inactive species was a hyperphosphorylated form of synthetase and that the conversion reflected the action of a phosphatase. However, the possibility of a conformational change as the basis of the genesis of active forms, as is evidently the case here, does not seem to be ruled out. There was about a 75% loss of activity of glycogen synthetase in 48 hours at 20°, which was slowed but not prevented by 4 mM glucose-6-P, 4 mM UDP-glucose, or 5 mg per ml of glycogen.

With prolonged storage of the purified synthetase at 4°, the degree of reactivatability progressively decreased, and an aggregated form of the enzyme arose (Fig. 1B) which was not reconverted to the original molecular size during the reactivation process.

DISCUSSION

Because of the propensity for aggregation which this enzyme exhibits, the attempt at molecular weight determination yielded less than fully satisfactory results. Yet, a native form of about 250,000 is indicated. This figure is similar to that obtained with the trout liver enzyme (1) by the method of Martin and Ames (20). It also corresponds with the value reported by Brown and Larner (3) for muscle synthetase b. On the other hand, Soderling et al. (2) reported a molecular weight of 400,000 for muscle synthetase (α form), and a figure of 370,000 was obtained with both forms of the kidney synthetase (5). There is general agreement, however, on the size of the subunit of 85,000 to 90,000 daltons, indicating 3 subunits if the molecular weight is 250,000 and 4 subunits if the molecular weight is 370,000 to 400,000.

The sulfhydryl titers reported for the native enzyme from muscle (21) are virtually identical with those obtained here with the liver enzyme. The values for alkali-labile phosphate which we obtained for liver synthetase b, however, are higher (12 versus about 7, corrected to a subunit size of 85,000).
The presence of inactive forms of glycogen synthetase has been noted by several authors, i.e. forms not active even in the presence of glucose-6-P, but which yield active species under certain conditions of incubation (1, 17-19). The demonstration of reversible inactivation and its prevention by glycogen (see Fig. 6) appears to help clarify these observations, since it indicates that the active species is in equilibrium with at least one inactive conformer.

Acknowledgments—We wish to thank Mr. David Adler for skillful assistance in many of the experiments reported here. We also thank Mr. Brian Jordan for the refractometric determinations and Dr. Sara Szuchet for performing the sedimentation equilibrium experiment.

REFERENCES
1. LIN, D. C., SEGAL, H. L., AND MASSARO, E. J. (1972) Biochemistry 11, 4466
9. ELLMAN, G. L. (1959) Arch. Biochem. Biophys. 82, 70
10. LOWRY, O. H., RANDALL, R. J. (1951) J. Biol. Chem. 193, 265
12. ELLMAN, G. L. (1959) Arch. Biochem. Biophys. 82, 70
15. YPHANTIS, D. A. (1964) Biochemistry 3, 297
Homogeneous Glycogen Synthetase $b$ from Rat Liver
David C. Lin and Harold L. Segal


Access the most updated version of this article at http://www.jbc.org/content/248/20/7007

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/20/7007.full.html#ref-list-1