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IDENTIFICATION OF AN Mr 77,000 - 80,000 PRODUCT OF IN VITRO TRANSLATION OF RAT LIVER mRNA USING ANTIBODY TO GLYCOGEN SYNTHASE[1]

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SUMMARY: Rabbit antibody to rat liver glycogen synthase has been used to identify a product of Mr 77,000 - 80,000 from in vitro translation of rat liver mRNA. A comparison of various protease inhibitors on the relative molecular weight of rat liver glycogen synthase suggest that higher molecular weight enzyme forms could arise from incomplete hydrolysis of glycogen before enzyme isolation and enzyme subunit Mr determinations.

A recent report has suggested that liver glycogen synthase is sensitive to proteolytic degradation during the isolation procedures used to purify the enzyme (1). This was based on the observation that rabbit liver synthase, isolated in the presence of an antiprotease "cocktail" composed of PMSF[4], TLCK, leupeptin, p-aminobenzamidine, and EGTA, has a subunit relative molecular weight (Mr) of 90,000 as determined by SDS polyacrylamide gel electrophoresis. Incubation of the nondenatured enzyme with trypsin results in a progressive decrease in subunit Mr from 90,000 to 80,000 which is comparable to a subunit Mr of 77,000-80,000 of isolated rat liver glycogen synthases reported previously (2,3). However, in an earlier report, rat liver synthase isolated in the absence of protease inhibitors had a subunit Mr of 85,000 (4), and in a more recent study (5), synthase also isolated from rat liver, but in the presence of protease inhibitors, showed two components on SDS polyacrylamide gel electrophoresis; one with Mr 80,000 and a second with Mr 85,000.

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[4]The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone

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Since the rat and rabbit liver glycogen synthase subunit Mr has been determined with enzymes isolated using a variety of conditions, it was of interest to see if a glycogen synthase subunit could be synthesized in vitro with an Mr comparable to values reported for isolated enzymes. For this study, poly (A)+mRNA was prepared from rat liver and in vitro translation of the RNA was done. Using rabbit antibody to rat liver glycogen synthase, an RNA translation product of Mr 77,000 - 80,000 was identified.

EXPERIMENTAL PROCEDURES

RNA Preparation - Livers from male adult (200 - 250 g) Sprague-Dawley rats were quickly removed after decapitation, chilled in and blended with 20 volumes of cold (-20°) buffer composed of 20 mM sodium acetate, pH 5.0, 7.0 M guanidine-HCl, 1.0 mM dithiothreitol and 10 mM sodium iodoacetate. The preparation of liver RNA for isolation of poly $(A)^+$mRNA was done as has been described previously (6,7).

Poly $(A)^+$mRNA was isolated by oligo(dT) cellulose affinity column chromatography essentially as has been described (8). Poly $(A)^+$mRNA was eluted from the column as a single fraction with 10 mM Tris-HCl buffer, pH 7.4. The 260/280 A ratio was 1.91 - 2.0, and the RNA was quantitated by $E^{1\%}_{1cm}$ at 260 nm of 222 (9). The RNA was made 0.4 M with NaCl, precipitated with 2 volumes of 95% ethanol (-20°), and centrifuged to collect the precipitate which was dried under a steam of N_2. The dried material was dissolved in 5 ml of 20 mM HEPES, pH 7.4, containing 1 mM EGTA and concentrated to 2-4 µg RNA/ml using an Amicon filtration apparatus fitted with a YM 100 membrane. This was the material used for in vitro translation of poly $(A)^+$mRNA.

In Vitro Translation of Poly$(A)^+$ mRNA - The reticulocyte lysate/L-[3,4,5-^{3}H]-leucine translation kit supplied by New England Nuclear Corporation (NEK-002) was used for in vitro translation of poly $(A)^+$mRNA. The translation tubes were incubated at 37° for one hour. Protein synthesis was stopped by quickly chilling the tubes and adding an equal volume of ice-cold buffer composed of 10 mM Tris-HCl, pH 7.4, 1.0 mM PMSF, 20 mM L-leucine, 0.5% Triton X - 100 and 0.15 M NaCl. Generally, about 7-10 x 10^6 cpm total incorporation into protein was used for product identification which represented a translation efficiency of about 2-3 x 10^5 cpm incorporated into protein per µg mRNA used. The [^{3}H]-leucine incorporation into total protein was linear with time, and the one hour incubation period was chosen for maximal incorporation.

Identification of Poly $(A)^+$mRNA Translation Product - The preparation of rabbit anti-synthase antibody has been described (3). The ammonium sulfate fraction of the antibody preparation (3) was dialyzed against 0.1 M sodium bicarbonate, pH 7.6, and applied to a DEAE-cellulose column (Whatman, DE-52) equilibrated in the same buffer. The "breakthrough" fraction, containing the IgG, was collected as a single fraction, concentrated on a Amicon filtration apparatus to approximately 2 mg gamma globulin/ml and dialyzed against 0.1 M sodium bicarbonate, pH 8.3. Serum from a nonimmunized rabbit was treated the same way and is referred to as nonimmunized rabbit serum (NRS).

Translation products of pooled poly $(A)^+$mRNA incubation tubes were combined into an Beckman airfuge tube and centrifuged at 29-30 pounds in^{-2} for 60 min. The supernatant (total released proteins) was mixed with 125 µl of NRS and incubated at 4° for four hours. One hundred microliter of a 10% solution of Protein A (Miles Co.) was added to the mixture which was incubated at room temperature for 30 min with constant gentle mixing. The preparation was centrifuged, and the supernatant removed. The pellet was saved for subsequent electrophoresis and is referred to as the NRS-pellet.

The supernatant from the NRS-pellet was mixed with 125 µl of antisynthase antibody, and the mixture incubated overnight at 4°. One hundred microliter of Protein A was added, and the mixture incubated as described above. The Protein A pellet was used for electrophoresis and is referred to as the antisynthase-pellet (AS-pellet).

The AS- and NRS-pellets were washed by suspending each in 1.0 ml of 0.5 M sucrose containing 20 mM Tris-HCl, pH 7.4, 1% Triton X-100 and 0.15 M NaCl. After standing for several minutes at room temperature, the suspension was centrifuged for 1 min. This procedure was repeated for a total of five times, with thorough draining of the pellets each time.

Each pellet was suspended in 50 µl water and 75 µl of 83 mM Tris-borate, pH 8.3, containing 2% SDS, 20% glycerol and 20 mM 2-mercaptoethanol. The suspensions were heated in a boiling water bath for 5 min, cooled, centrifuged (1 min), and the supernatants removed for electrophoresis. All of each sample was layered onto an acrylamide stacking gel rod (4%) over a running gel (6%), and SDS electrophoresis was done as has been described (3).

After electrophoresis, each gel was sliced into 2 mm sections, each section placed into a scintillation vial containing 250 µl of Protosol to which was added 9.7 ml of Econofluor. The vials were heated at 40° for 8-10 hrs, allowed to stand overnight to cool and the radioactivity determined. A sample of adult liver glycogen synthase, prepared as described (3), was electrophoresed with the same conditions but stained for protein with Coomassie blue as has been described (3).

Enzyme Isolations - Synthase b from livers of male (200 - 250 g) Sprague-Dawley rats was prepared as described previously (3,10) or with all buffers supplemented with 0.2 mM PMSF, 2.0 mM EGTA, 1 mg/L pepstatin, 10 mg/L TPCK and 2 mg/L aprotinin. Synthase a was prepared essentially as described by Jett and Soderling (11) either in the absence or presence of the protease inhibitors listed above.

Other Assays - The immunospecificity of antisynthase was determined by using an 8,000 x g supernatant fraction of a rat liver homogenate prepared as described (3). Glycogen synthase and phosphorylase activities were assayed as described previously (3,10) in the 8,000 x g fraction of liver after incubation with increasing amounts of added antisynthase.

Proteins were determined by the Bradford (12) and glycogen determined by the anthrone (13) methods.

Materials - The purest quality of reagents available were used and obtained from either Sigma Chemical Company or Fisher Scientific. Standards for the sucrose density gradient centrifugation were obtained from Isolab. UDP-^{14}-C-Glc was obtained from New England Nuclear Corporation, and ^{14}Glc-1-P was obtained from Amersham.

RESULTS AND DISCUSSION

A major product of in vitro translation of liver poly(A)$^+$mRNA was isolated with antisynthase antibody and identified on SDS polyacrylamide gel electrophoresis as having a Mr of about 77,000 - 80,000 (Fig. 1-A). This is similar to the subunit Mr obtained upon SDS polyacrylamide gel electrophoresis of isolated synthase prepared by different methods (2,3).

Although the synthase antibody is heterologous in nature, no mRNA translation products of higher molecular weight were identified. Translation products of higher molecular weight are observed with NRS, as are products with lower molecular weights as well as with Mr of 77,000 - 80,000 (Fig. 1-B); however, the latter are not major components of the translation product identified using the NRS. There were minor, low molecular weight components isolated with synthase antibody which could represent either synthase subunit precursors, caused by premature termination of translation, or products of proteolytic degradation (Fig. 1-A). However, since the in vitro translation was terminated with buffer containing PMSF, proteolysis should be minimal.

To be assured that the antibody has specificity toward synthase in the RNA translation product, the effect of the antibody on glycogen synthase and phosphorylase activities in an 8,000 x g supernatant extract of a rat liver homogenate (3) was determined. The results

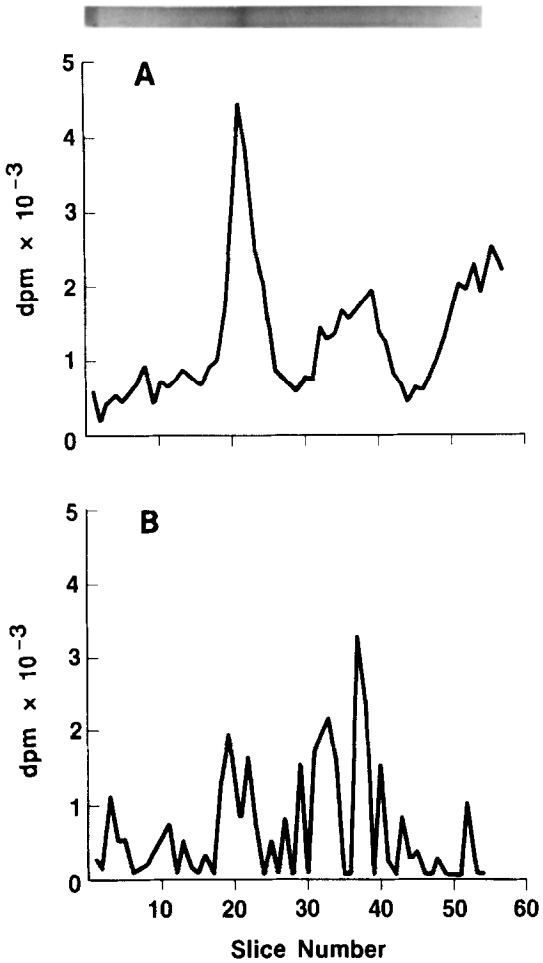

FIG. 1. SDS polyacrylamide gel electrophoresis of the product of in vitro translation of rat liver mRNA. Upper panel, A, the product of the in vitro translation of mRNA identified with antisynthase antibody; lower panel, B, product of the in vitro translation of mRNA identified with serum from a nonimmunized rabbit. The material electrophoresed in A is obtained with antisynthase after the translation product was treated with the nonimmunized rabbit serum. The polyacrylamide gel at the top of the figure is the SDS gel electrophoresis of isolated rat liver synthase b and is stained with Coomassie blue as has been described (9).

(Fig. 2) show that antisynthase has high immunospecificity for synthase with no effect on the activity of phosphorylase. This suggests that synthase antibody would have the same immunospecificity toward synthase formed from the in vitro translation of RNA as toward the enzyme in the 8,000 x g fraction of liver.

The antibody used in this study was developed against rat liver synthase isolated essentially as described by Lin and Segal who reported a subunit Mr of 85,000 for the enzyme (4). Using essentially the same isolation procedures they described, but including PMSF in the isolation buffers, a synthase with subunit Mr of 77,000 - 80,000 has been

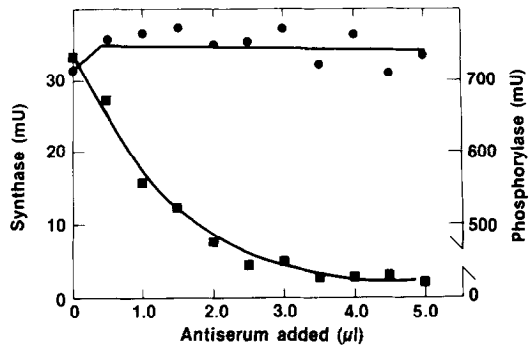

FIG. 2. Immunospecificity of antiglycogen synthase antibody. Liver glycogen synthase and phosphorylase activities in the 8,000 x g supernatant extract of rat liver homogenate, prepared in 0.25 M sucrose containing 0.1 M glycylglycine, pH 7.4, 0.1 M KF and 0.02 M 2-merceptoethanol as described (3). Incubation of the 8,000 x g extract was at 15°for 3 hr. Final volume, after the addition of antibody was 150 μl. Controls done with serum from a nonimmunized rabbit showed no changes in enzyme activities. Symbols are: ●, phosphorylase; ■, synthase.

isolated in this laboratory (3). The use of PMSF in the isolation buffers should not result in a lower synthase subunit Mr, and the discrepancy cannot be easily determined. Isotonic buffers are used for the preparation of the liver homogenate and should maintain the structural integrity of the lysosomes. In addition, sufficient fluoride is present in the buffers which has inhibitory action against metal requiring hydrolases which could include neutral, Ca^{2+}-activated protease (15). Although the pH of the preparation buffers is near neutrality (pH 7.4), this should inhibit the activity of acid hydrolases which could escape the lysosomes (16).

More recent preparations of synthase made in this laboratory have been made with buffers containing PMSF, EGTA, EDTA, and aprotinin (Table 1). These antiproteases have no effect on either the catalytic properties or subunit Mr of the enzyme as compared to the original procedures (3). The addition of pepstatin and TPCK to the above listed antiproteases inhibits phosphorylase-catalyzed hydrolysis of glycogen of the enzyme-glycogen complex which is done before ion-exchange chromatography to isolate the synthase (3,4,10). In the absence of pepstatin and TPCK, the glycogen of the enzyme-glycogen complex is decreased by phosphorylase hydrolysis from 8 - 12 mg/ml of glycogen suspension to 0.3 - 0.5 mg/ml of suspension (96% decrease); however, with pepstatin and TPCK, there is a 67% decrease in glycogen. The presence of residual glycogen associated with the enzyme is reflected by the higher Mr of the nondenatured synthase as determined by sucrose density

TABLE I

Relative Molecular Weight (Mr) of Rat Liver Glycogen Synthase

Enzyme	Mr
Synthase b	
-1	160,000 - 190,000
-2	160,000 - 190,000
-3	210,000 - 250,000
Synthase a	
-1	160,000 - 190,000
-2	160,000 - 190,000
-3	190,000 - 242,000

Sucrose density gradient centrifugation was done according to the method of Martin and Ames (14). Five to 20% linear sucrose gradients (5.0 ml) were formed in 0.1 M glycylglycine, pH 7.4, containing 0.1 M KF, 20 mM 2-mercaptoethanol, 20% glycerol and 5.0 mM Glc-6-P. Synthase and standards in the same buffer (200 µl), made 5% in sucrose, were layered on the tubes and centrifugation done at 4° in a 50.1 SW Rotor (Beckman) at 40,000 rpm for 20 hr. The tubes were pierced at the bottom, 300 µl fractions collected, and synthase activity determined in each fraction by measuring the rate of transfer of ^{14}C-Glc from UDP-^{14}C-Glc to glycogen in the presence of 2.0 mM UDP-Glc and 10.0 mM Glc-6-P as has been described (3,10). Synthase b-1 was prepared as described previously (3). Synthase b-2 was prepared as b-1 except the isolation buffers contained 2.0 mM EGTA, 5.0 m EDTA, and 4 mg/L aprotinin. Synthase b-3 was prepared in buffers containing 0.2 mM PMSF, 2.0 mM EGTA, 1 mg/L pepstatin-A, 2 mg/L aprotinin and 10 mg/L TPCK. Synthase a-1, a-2, and a-3 were prepared essentially as described by Jett and Soderling (11) except the isolation buffers used were the same as for b-1, b-2, and b -3, respectively.

gradient centrifugation (Table I). A representative sucrose density gradient centrifugation of synthase b (Fig. 3-A) and a (Fig. 3-B) shows that the activity of each enzyme is contained in a symmetrical peak suggesting a uniform Mr of the nondenatured enzyme. Using the anthrone method for the glycogen assay, no glycogen could be detected associated with the isolated enzymes used for the Mr studies in Table I. Therefore, the differences in nondenatured enzyme Mr could reflect differences in proteolysis of the enzyme. However, it is observed that the range of Mr of the synthase holoenzyme in this study (Table I) is similar to values reported by others from which the synthase subunit Mr has been reported to be 77,000 - 85,000 (2-4,17). That glycogen associated with the synthase could influence the Mr of the subunit is noted by the observation that synthase completely freed of glycogen by treatment with ethanol has a subunit Mr of 77,000 (2) which is similar to values reported from this laboratory (3).

It is possible that the reported differences in synthase subunit Mr could result from differences in the state of phosphorylation of the enzyme. This is based on the observation that a comparison of Mr of phosphoproteins to non-phosphorylated standards, used to determine Mr by SDS polyacrylamide gel electrophoresis, shows a wide range in Mr of the

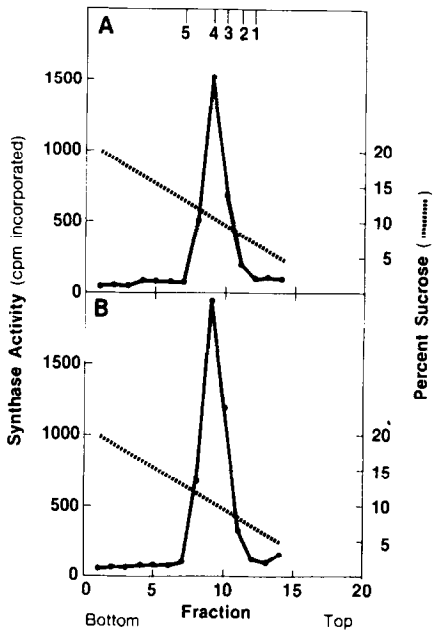

FIG. 3. Sucrose density gradient centrifugation of synthase b and a. Upper panel, A, synthase-b; lower panel, B, synthase-a. The numbers at the top of panel A represent the positions of the standards used and are 1, chymotrypsinogen (MW 25,000); 2, ovalbumin (MW 45,000); 3, aldolase (MW 158,000); 4, phosphorylase b (MW 185,000); 5, catalase (MW 250,000). The recovery of synthase activity ranged from 88-95% of material centrifuged in repeated experiments.

phosphoproteins (18). Recently (19), it has been shown that the state of phosphorylation affects the electrophoretic mobility of skeletal muscle glycogen synthase subunit. An increase in state of phosphorylation causes an apparent increase in Mr of the subunit of synthase. Although most laboratories are cautious to protect the phosphorylated state of isolated synthase b, slight differences in Pi content could account for differences in synthase subunit Mr, which would primarily be a reflection of subunit charge rather than subunit size.

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