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GLYCOGEN-BINDING PROTEIN COMPONENTS OF RAT TISSUES

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SUMMARY A glycogen-adipoyldihydrazide-Sepharose 4B column has been prepared for the analysis of glycogen-binding protein components of rat tissues. Glycogen-metabolizing enzymes; glycogen synthase, phosphorylase, branching enzyme, and debranching enzyme of skeletal muscle and liver have been adsorbed to the column, while those of brain showed very low affinities to it. On SDS gel electrophoresis of the glycogen-binding protein fractions, at least five and nine additional protein components have been detected in skeletal muscle and liver, respectively.

Enzymes participating in glycogen synthesis and glycogenolysis have been extensively studied in mammalian skeletal muscle systems (1-3). It would be useful to analyze and characterize the glycogen-binding protein components by affinity chromatography. Er-el et al.(4) first prepared "glycogen-coated Sepharose 4B". However, the affinity chromatography of rabbit skeletal muscle phosphorylase was unsuccessful due to nonspecific binding of proteins to the hydrophobic spacer arms. In the present study, we have prepared a column of glycogen-adipoyldihydrazide-Sepharose 4B using a nonhydrophobic spacer according to Lamed et al.(5) for the analysis of the glycogen-binding protein components of rat tissues.

MATERIALS AND METHODS

Chemicals Glucose 1-P, UDPglucose, and ATP were obtained from Boehringer. [^{14}C(U)]glucose and UDP[^{14}C(U)]glucose were from New England Nuclear Corp., Boston. All other chemicals were of reagent grade.
Enzyme assays Phosphorylase was assayed in the presence of 1 mM AMP without (skeletal muscle and brain) or with 0.5 M Na_2SO_4(liver) as reported previously (6). Branching enzyme was assayed by the procedure of Brown and Brown(7).

Enzymes. Glycogen synthase(GS, EC 2.4.1.11); phosphorylase(Ph, EC 2.4.1.1); branching enzyme(BE, α-1,4-glucan:α-1,4-glucan 6-glucosyltransferase. EC 2.4.1.18); debranching enzyme(DBE, amylo-1,6-glucosidase plus olio-1,4:1,4-glucantransferase. EC 3.2.1.33 + EC 2.4.1.25); and phosphorylase kinase(EC 2.7.1.38).

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Synthase was assayed with 10 mM glucose 6-P at pH 8.2 by the procedure of Steiner et al.(8) as modified by K. Sato et al.(9). Debranching enzyme was assayed by the method of Hers and Hoof at pH 6.2(10). One unit of the enzyme catalyzes the incorporation of one nmol of glucose into glycogen per min at 37°. Phosphorylase kinase was by the procedure of Cohen at pH 8.6(11). One unit of the enzyme catalyzes the conversion of one unit of rabbit muscle phosphorylase b to a per min at 30°.

Tissue preparations Skeletal muscle, liver, and brain were removed from Wister rats which had been fasted for 48 h and homogenized in 4 volumes of buffer A (10 mM Tris, pH 7.4, and 15 mM 2-mercaptoethanol) in a Teflon or glass homogenizer. For SDS gel electrophoresis, 16 000xg(15 min) supernatants of the crude homogenates of skeletal muscle(5 g), liver(5 g), and brain(3 g), were brought to 70% saturation with ammonium sulfate to avoid unnecessary degradation of the glycogen bound to Sepharose 4B by phosphorolysis. Concentrated protein precipitates were dissolved in buffer B(buffer A + 0.2 M NaCl, 3-5 ml of each) and then subjected to affinity chromatography as in Fig. 1, except that the bound proteins were eluted with buffer B containing 0.5 M maltose. The glycogen-binding proteins were analyzed by SDS gel electrophoresis.

SDS polyacrylamide gel electrophoresis This was performed according to Weber and Osborn(12). The molecular weight of rat skeletal muscle debranching enzyme was taken as 166 000 similar to that of the rabbit enzyme according to Taylor et al. (13), and that of rat skeletal muscle glycogen synthase, 85 000, according to Roach et al.(14). Standard proteins used were bovine serum albumin(mol. wt. 67 000), ovalbumin(45 000), chymotrypsinogen(25 000), and rabbit skeletal muscle actomyosin subunits, the heavy chain of myosin(210 000), actin(42 000), and troponin T(37 000-40 000).

Synthesis of glycogen-adipoyldihydrazide-Sepharose 4B Sepharose 4B(100 ml) was activated with 14 g of BrCN and then reacted with 9 g of adipoyldihydrazide according to Lamed et al.(5). Oyster glycogen(1.8 g) in 10 ml of 0.2% KCl had been oxidized with 142 mg of sodium metaperiodate in the dark at 0° for 1 h. The solution was added to the adipoyldihydrazide-Sepharose 4B suspended in 100 ml of 0.1 M sodium acetate, pH 5.0. The reaction was allowed to proceed overnight at 3-4° and unreacted aldehyde groups were reduced with $NaBH_4$. One ml of the glycogen-adipoyldihydrazide-Sepharose 4B adsorbed 3.2 mg of crystalline rabbit muscle phosphorylase b at 3-4° in buffer B.

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Affinity chromatography of glycogen-metabolizing enzymes of rat tissues As shown in Fig. 1, phosphorylase and debranching enzyme of skeletal muscle and liver were almost quantitatively adsorbed to a column of glycogen-adipoyldihydrazide-Sepharose 4B. Branching enzyme was also quantitatively adsorbed to it irrespective of the tissue of origin. However, the enzyme activity could not be assayed in the presence of glycogen, but was detectable when eluted with 0.5 M maltose. Phosphorylase kinase was not retained on the column. The major part of the unadsorbed phosphorylase fraction of brain was composed of brain type(BB) and hybrid(MB) phosphorylases, and the adsorbed fraction was the muscle type(MM),

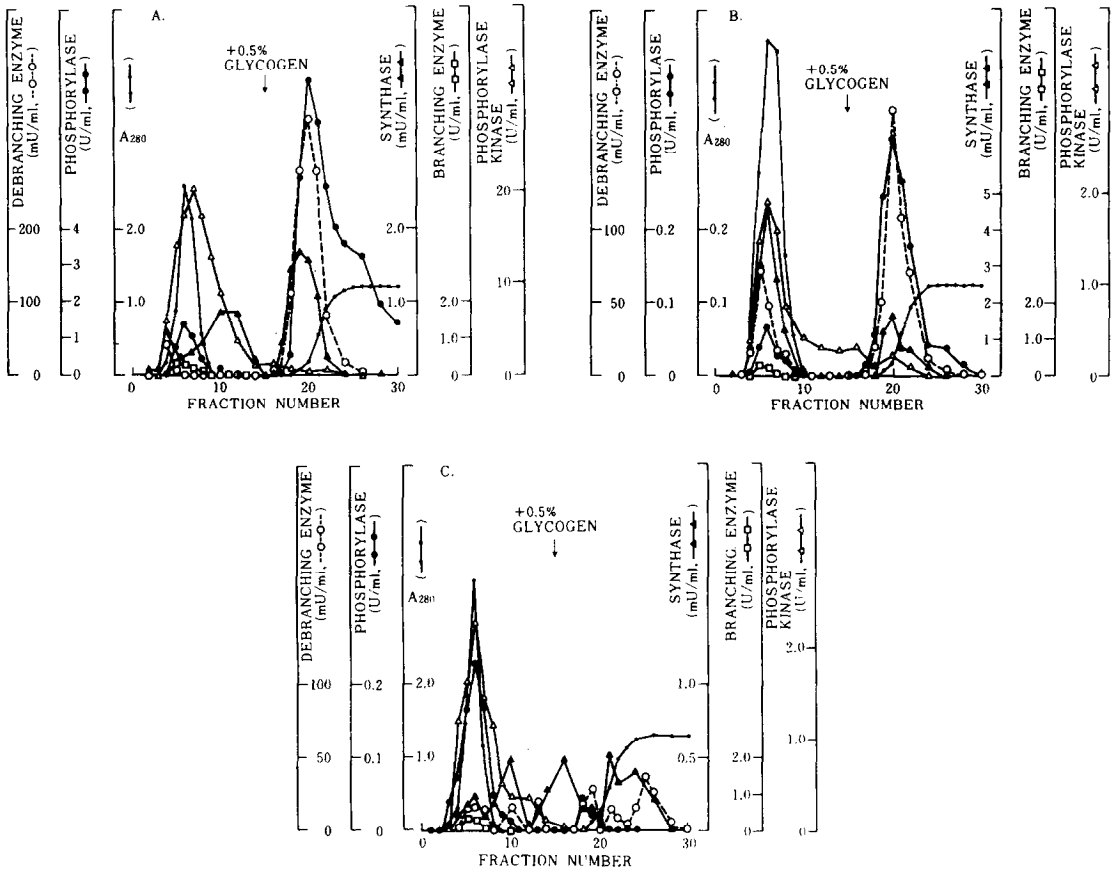

Fig. 1(A, B, and C). Affinity chromatography of glycogen-metabolizing enzymes of rat tissues. One-ml aliquots of 105 000xg(45 min) supernatants of the crude tissue homogenates were applied sequentially to a column(1.2x8 cm) of glycogen-adipoyldihydrazide-Sepharose 4B followed by washing with 20 ml of buffer B, and then eluted with buffer B containing 0.5% of mollusc glycogen. A, B, and C are the elution patterns of skeletal muscle, liver, and brain, respectively. Absorbance at 280 nm of the glycogen solution corresponded to 1.25. The fraction volume was 1.5 ml and the flow rate was approx. 20 ml/h.

when analyzed by the polyacrylamide disc gel electrophoresis(15). Reproducible multiple peaks were observed for both the synthase and debranching enzyme of brain, although their activities were very low.

SDS gel electrophoresis of the glycogen-binding protein components of rat tissues Assignments of enzyme components on gels were made using purified preparations of rat skeletal muscle and liver phos-

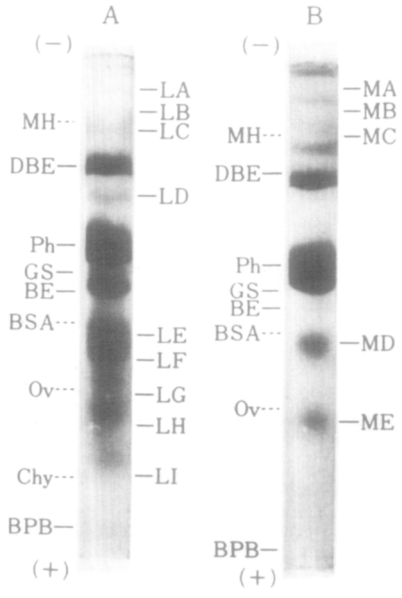

Fig. 2. SDS gel electrophoretic patterns of glycogen-binding protein components of rat skeletal muscle and liver. Glycogen-binding protein fractions of liver(gel A) and skeletal muscle(gel B) were prepared as described in MATERIALS AND METHODS, and analyzed by SDS gel electrophoresis using 5% polyacrylamide gels. Positions of standard proteins are shown by dotted lines; MH(the heavy chain of myosin), BSA(bovine serum albumin), Ov(ovalbumin), and Chy(chymotrypsinogen). LA to LI and MA to ME are the newly detected protein components.

phorylases(subunit mol. wt. 100 000), rat liver branching enzyme (82 000), and rat liver debranching enzyme which will be reported elsewhere. As seen in Fig. 2, a number of glycogen-binding proteins were detected in both liver(gel A) and skeletal muscle(gel B). None was detected in the extracts from brain(data not shown). Phosphorylase was the major protein followed by debranching enzyme, branching enzyme, and synthase in both tissues. It was noteworthy that in addition at least five and nine protein components were observed in skeletal muscle and liver, respectively. Subunit molecular weight values of the skeletal muscle components were; MA (270 000), MB(230 000), MC(200 000), MD(59 000), ME(43 000), and

those of liver components; LA(260 000), LB(220 000), LC(200 000), LD(140 000), LE(60 000), LF(53 000), LG(45 000), LH(37 000), LI (27 000).

DISCUSSION

Based on the high degree of specificity of glycogen, the glycogen-binding protein components of rat tissues have been effectively analyzed by the present affinity chromatography on a column of glycogen-adipoyldihydrazide-Sepharose 4B. Most of the glycogen-metabolizing enzymes; glycogen synthase, phosphorylase, branching enzyme, and debranching enzyme, of skeletal muscle and liver were adsorbed to the column, whereas the corresponding enzymes from brain showed very low affinities to it except for branching enzyme. It is noteworthy that additional protein components have been detected in the glycogen-binding protein fractions of skeletal muscle and liver by SDS electrophoresis. Caudwell et al.(16) analyzed protein components bound to the endogeneous glycogen of rabbit skeletal muscle which were isolated by nonspecific acid precipitation and differential centrifugation. One of the greatest advantages for the affinity chromatography is that nonspecific proteins can be washed out at breakthrough fractions with appropriate buffer, and then the bound proteins are eluted specifically with substrates or their analogues. In control experiments, we found that no detectable quantities of proteins from the three tissues were recovered from a column of unsubstituted adipoyldihydrazide-Sepharose 4B after washing with buffer B and elution with glycogen. A number of enzymes and proteins have been reported to participate in glycogen metabolism(1-3,17,18). The 14 additional protein components detected in rat skeletal muscle and

liver must be purified and characterized individually in order to identify their possible role in the fine control mechanisms of glycogen metabolism in mammalian tissues.

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