Isozymes of glycogen synthase

Harvey R. Kaslow and David D. Lesikar

Department of Physiology and Biophysics, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033, USA

Received 26 April 1984

Antisera to rat skeletal muscle glycogen synthase failed to recognize liver glycogen synthase by electroblot analysis. The antisera recognized the enzyme in skeletal muscle, heart, fat, kidney, and brain. The results support the hypothesis that there are at least two isozymes of glycogen synthase, and that most tissues contain a form similar or identical to the skeletal muscle type. There is a virtual absence of the muscle-type enzyme in adult rat liver.

Glycogen synthase *Isozyme* *Electrophoretic mobility*

1. INTRODUCTION

Although glycogen is found in nearly all tissues, the glycogen in liver can serve a unique purpose: to buffer blood glucose. This buffering function depends on the presence of glucose-6-phosphatase, which allows the products of glycogenolysis to be converted to glucose and released from the cell. In other tissues, which lack glucose-6-phosphatase, the glucose phosphate produced by glycogenolysis is retained for intracellular metabolism. This buffering function of the liver has also led to the unique expression of enzymes involved in gluconeogenesis, and to the expression of glycolytic enzymes different from those found in many other tissues. These different enzymes are considered to be isozymes. If the buffering function of the liver is related to this expression of different enzymes, it would not be surprising to find that the enzymes of glycogen metabolism in liver are also different from those found in other tissues.

Evidence is accumulating that these enzymes are different. For example, phosphorylase kinase from liver is kinetically different from the enzyme found in muscle (for references see [1]). Another example is glycogen synthase: treating this enzyme from rabbit skeletal muscle and liver with CNBr produced peptides with different electrophoretic mobilities [2,3]. Thus, it has been proposed that muscle and liver glycogen synthase are isozymes, and that primary structural differences may account for kinetic differences that distinguish these two forms [2,3]. Here we present immunological evidence that supports this proposal, and extends it to other tissues in another species: the rat.

Abbreviations: *p*ABAD, *p*-aminobenzamidine; PMSF, phenylmethylsulfonyl fluoride; Con-A, concanavalin A

2. EXPERIMENTAL

Glycogen synthase was assayed in 50 mM Tris–HCl (pH 7.8), 15 mg/ml bovine liver glycogen, 5 mM EDTA, 2 mM DTT, 30 mM NaCl, 0.1 mg/ml heat-treated bovine serum albumin, 10 (fig.1) or 4 (fig.2) mM UDPG, with or without 25 mM glucose-6-P at 30°C for 15 min. The reaction was terminated by heating at 95°C for 1 min, and the [^{14}C]glycogen was purified as in [4]. The activity in the absence of glucose-6-P was divided by the activity in its presence to obtain the $-/+$ glucose-6-P activity ratio. A unit of activity is defined as 1 μmol/min at 30°C.

Rat skeletal muscle glycogen synthase was purified by modifying procedures in [5–8]. All steps

Published by Elsevier Science Publishers B.V.

were at 0–4°C and pH 7.6, unless stated otherwise. Buffer A is 100 mM NaF, 5 mM EDTA, 2 mM DTT, and 1 μg/ml leupeptin. Buffer B is buffer A with the addition of 1 mM *p*ABAD and 0.5 mM PMSF. One part (approx. 400 g) hindlimb, thoracic, and abdominal muscle from 8 250-g fed Sprague-Dawley rats was homogenized (Waring blendor) in 4 parts buffer B without DTT and centrifuged at 23000 × *g* for 20 min. The filtered supernatant was adjusted to pH 5.5 with 1 N acetic acid, stirred for 10 min, and centrifuged again at 23000 × *g* for 20 min. The glycogen particle was resuspended in buffer B plus 50 mM β-glycerophosphate and stored at −70°C. The purification was continued by incubating the glycogen particle at 30°C for 1 h after adding 10 units/ml α-amylase (Sigma A-0521), 10 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 5% sucrose, and 0.5 mM PMSF, and centrifuging at 78000 × *g* for 30 min. The filtered supernatant was applied to a 30 ml DEAE–cellulose (Whatman DE-52) column equilibrated in buffer B with 50 mM Tris–HCl, the column washed with 300 ml of the same buffer, and the enzyme eluted with a 250 ml gradient from 0 to 350 mM NaCl in buffer A with 50 mM Tris– HCl, 1 mg/ml bovine glycogen, 5 mM glucose-6-P, and 0.1 mM UDPG. Fractions containing glycogen synthase activity were then applied to a 60 ml Con-A–Sepharose column (Pharmacia) equilibrated in buffer A omitting leupeptin, with 50 mM Tris–HCl, 400 mM NaCl, but without EDTA which can cause release of Con-A from the column. The column was washed with a 600-ml gradient from the equilibration buffer to buffer A without EDTA and leupeptin, but with 50 mM β-glycerophosphate and 10% (v/v) glycerol. The purified enzyme (approx. 500 μg, 5% yield) is eluted in the latter buffer with 200 mM glucose, and EDTA is added to the eluted enzyme to give 5 mM. At this point the enzyme may be contaminated with Con-A. The enzyme can be concentrated and separated from Con-A by centrifuging at 220000 × *g* for 2 h. The pelleted enzyme was resuspended in 50 mM β-glycerophosphate, 5 mM EDTA, 2 mM DTT, and 25% glycerol, and stored at −70°C. Specific activities ranged from 15–50 units/mg protein. Antisera to the purified enzyme were raised in rabbits and guinea pigs at the Pocono Rabbit Farm by injecting 50–100 μg enzyme at 2–6 week intervals.

Electroblot analysis was performed essentially as in [9]. Tissue samples were electrophoresed in 7.5 or 10% bisacrylamide gels [10], gels equilibrated first with 0.5 M and then 50 mM Na_xPO_4 (pH 6.5), and proteins electroblotted to DBT paper [11] in 25 mM Na_xPO_4 (pH 6.5). The paper was blocked with ethanolamine, incubated with antisera, washed, incubated with ^{125}I-labelled protein A (Amersham), washed, and used to expose Kodak XAR-5 film with a Cronex (DuPont) screen at −70°C [9].

For electroblot analysis, tissues were homogenized in 25 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM *p*ABAD, 0.5 mM PMSF, 1.0 μg/ml leupeptin either with (kidney, liver, brain) or without (muscle, heart, fat) 250 mM sucrose. NaF (100 mM) was included in the homogenizing buffer, unless the extract was to be incubated to allow endogenous phosphatases to act on glycogen synthase. The homogenates were spun at 35000 × *g* for 20 min, and the supernatants either used directly (fig.1) or incubated and additionally purified. Incubations were for 1 h at 30°C with 14 mM 2-mercaptoethanol added. Kidney and brain extracts were purified by adsorption to DEAE–cellulose (Whatman, DE-52), and elution with an NaCl gradient in the homogenization buffer with glycerol added to 10% (w/w). Liver supernatants were purified by pelleting the glycogen particle by centrifugation at 78000 × *g* for 2 h. The glycogen particle was resuspended in 50 mM β-glycerol phosphate (pH 7.6), 5 mM EDTA, 2 mM DTT, 1 mM *p*ABAD, and 1 μg/ml leupeptin, with glycerol added at 20% (w/w). Extracts were stored at −70°C. Samples were prepared for electrophoresis by heating in 2% SDS, 5% 2-mercaptoethanol, and 15% glycerol, in electrophoresis running buffer [10].

Protein was determined as in [12].

Buffers, protease inhibitors, and assay materials were from Sigma, electrophoresis materials from Bio-Rad, [^{14}C]UDPG from ICN, and [^{14}C]protein standards from BRL.

3. RESULTS

Antibodies can be used to study protein structure, e.g., electroblot analysis is a method of using antibodies to determine the electrophoretic mobility of proteins without requiring their purification. Thus, to study the electrophoretic mobility of unpurified rat glycogen synthase we raised antisera

against the purified rat skeletal muscle enzyme. When we used these antisera to determine the mobility of the unpurified rat enzyme, we found that antisera from 3 separate animals (2 rabbits and 1 guinea pig) failed to detect the enzyme in rat liver extracts.

Fig.1 shows the autoradiogram resulting from the analysis of relatively crude extracts. In this experiment tissues were homogenized and centrifuged, the supernatant assayed for enzyme activity and protein, and an aliquot prepared for electrophoresis (see section 2). Equal amounts of enzyme activity were applied to each lane of the gel. The antibody failed to detect any antigenic material in the liver sample. Because these samples were relatively crude, substantial amounts of protein were electrophoresed. To demonstrate that saturation of the DPT paper with other proteins in the liver sample did not prevent the binding of glycogen synthase to the paper, or sterically hinder antibody recognition of bound protein, one lane contained a mixture of heart and liver samples. The antibody readily detected glycogen synthase in this mixture. In contrast to the liver sample, the antibody recognized a protein in crude extracts with a mobility similar to that for the purified rat muscle enzyme (not shown) in every other tissue examined: skeletal muscle (from which the immunogen was derived), heart, fat, kidney, and brain.

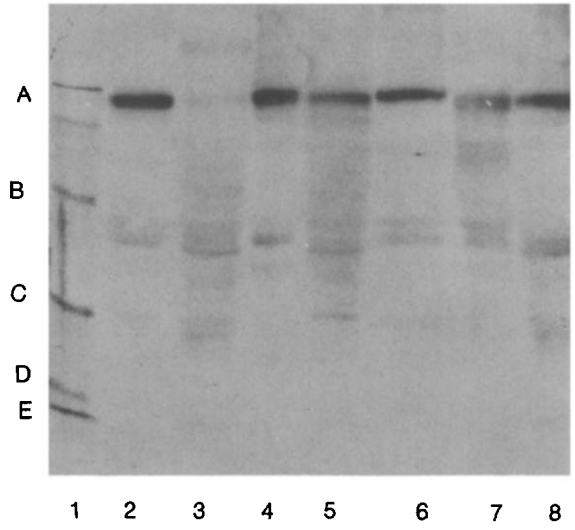

Fig.1. Electroblot analysis of rat tissues with antisera (A-1/27/84) against rat skeletal muscle glycogen synthase; 35000 × *g* supernatant fractions were electrophoresed (10% gel), electroblotted, and immunolabeled (see section 2). Lane 1, ^{14}C-labeled proteins: (A) phosphorylase *b*, M_r 92500; (B) bovine serum albumin, M_r 68000; (C) ovalbumin, M_r 43000; and (D) α-chymotrypsinogen, M_r 25700; (E) β-lactoglobulin, M_r 18400 and cytochrome *c*, M_r 12300. Lanes 2–8, supernatants containing 1 munit glycogen synthase activity. Tissues, with activity expressed as (munits/mg protein), are: 2, skeletal muscle (27); 3, liver (4.5); 4, heart (47); 5, kidney (5.7); 6, fat (8.1); 7, brain (6.5); 8, liver (4.5) and heart (47).

Although equal amounts of activity were applied to each lane of the gel, it is clear that the ^{125}I signal varies from sample to sample. It is possible that antigenically distinct forms of the enzyme produced this variation. An alternative explanation is that differences in phosphorylation of the enzyme lead to differences in antibody recognition. For example, while the purified rabbit skeletal muscle enzyme has been reported to contain up to 6 mol PO_4 per subunit (review [13]), the liver enzyme may contain up to 17 mol [14]. To examine the effect of phosphorylation on antibody recognition, we incubated crude extracts in the absence of NaF at 30°C for 1 h to allow endogeneous phosphatases to reduce the phosphate content of the enzyme. We then purified the dephosphorylated enzyme (see section 2) to reduce background binding of ^{125}I-labelled protein A, and analysed the protein. In fig.2 is the autoradiogram of the resulting electroblot. Although incubating the liver extract increased the −/+ glucose-6-P activity ratio from 0.12 to 0.45 there was still no substantial increase in binding of antibody. When kidney or brain extracts were incubated the activity ratio rose from 0.07 to 1.01 (kidney) and from 0.32 to 0.99 (brain). There was no alteration in the detection of antibody binding. It thus appears that this antiserum recognizes some sites on these proteins regardless of phosphorylation state.

The original goal of our investigation was to examine the electrophoretic mobility of glycogen synthase prior to purification. Some controversy surrounds the mobility of the native enzyme, particularly from liver [7,14]. This controversy may arise from the sensitivity of the enzyme to proteolytic degradation. The relationship between the electrophoretic mobility and the molecular mass of the native enzyme is further complicated by the

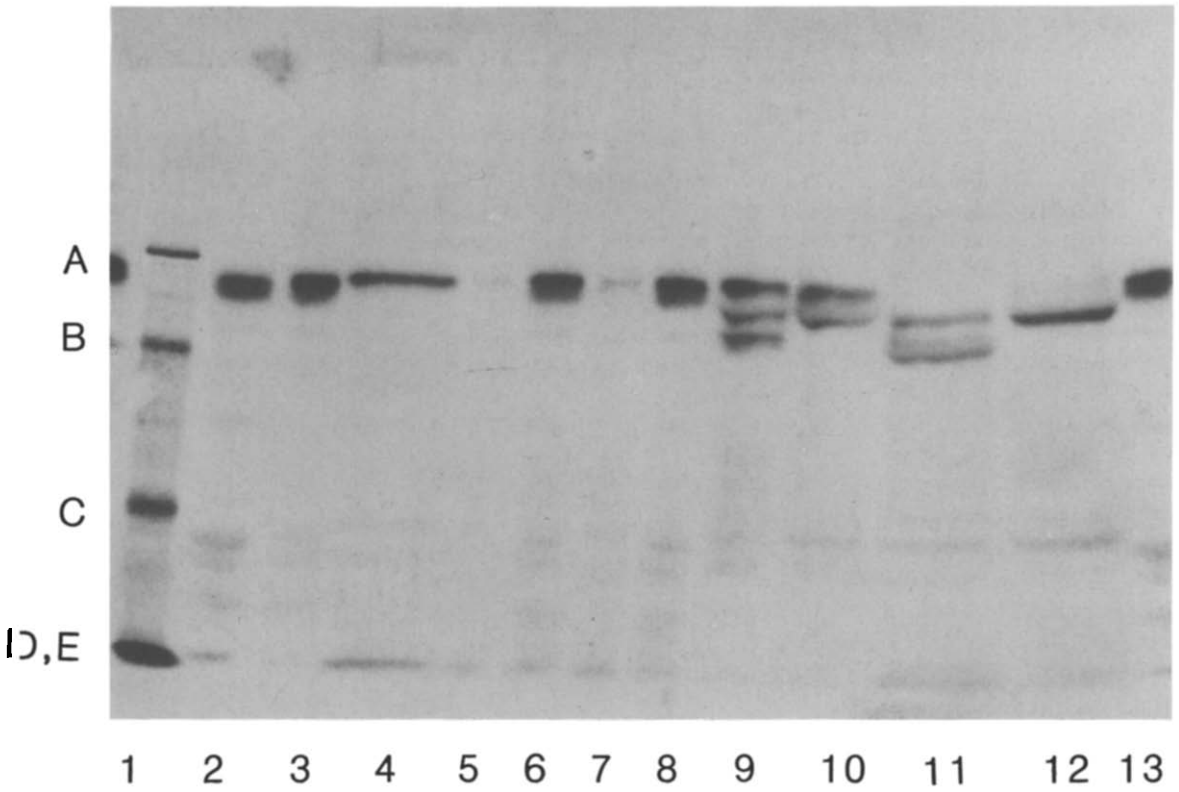

Fig.2. Electroblot analysis (7.5% gel) of rat tissues with antisera (A-1/27/84) against rat skeletal muscle glycogen synthase. Extracts were either 35000 × *g* supernatants (muscle, heart and fat), 78000 × *g* glycogen particles (liver), or DEAE–cellulose purified extracts (brain and kidney) (see section 2). Lane 1, ^{14}C-labeled proteins: (A) phosphorylase *b*, M_r 92500; (B) bovine serum albumin, M_r 68000; (C) ovalbumin, M_r 43000; (D) α-chymotrypsinogen, M_r 25700; (E) β-lactoglobulin, M_r 18400 and cytochrome *c*, M_r 12300. Lanes 2–15, extracts containing 0.5 munit glycogen synthase activity. Tissues designated with '(−NaF)' were incubated to allow endogenous phosphatases to dephosphorylate glycogen synthase (see section 2). Tissues with activity expressed as (munit/mg protein, −/+ glucose-6-P activity ratio) are: 2, skeletal muscle (26, 0.38); 3, heart (48, 0.26); 4, fat (7.2, 0.16); 5, liver (75.7, 0.12); 6, liver and heart; 7, liver(−NaF) (27.7, 0.45); 8, liver(−NaF) and heart; 9, kidney (21.1, 0.07); 10, brain (9.9, 0.32); 11, kidney(−NaF) (7.9, 1.01); 12, brain(−NaF) (6.4, 0.99); 13, skeletal muscle.

observation that phosphorylation can decrease the mobility of both purified rabbit skeletal muscle [15] and liver [2,3] glycogen synthase. This phenomenon may account for the increased electrophoretic mobility reported for the enzyme produced during an in vitro translation of rat liver mRNA [16]. This form of the enzyme is probably not phosphorylated.

We have found (not shown) that the purified rat skeletal muscle enzyme migrates in SDS gels [10] in multiple bands, usually as a distinct triplet. Electroblot analysis of the pure enzyme shows a similar pattern. Heterogeneity in phosphorylation of the enzyme may produce this triplet. It is therefore interesting to note that incubation, and concomitant activation, of the enzyme in crude kidney and brain extracts led to marked increases in the mobility of the enzyme detected by the antibody (fig.2). The increase in mobility may be the result of dephosphorylation, proteolysis, or both. Studies designed to show that rephosphorylation of the enzyme reverses this change in mobility should resolve this issue. The ability to conduct such studies, without the need for enzyme purification, points out the usefulness of this approach.

4. DISCUSSION

The failure of our antisera to skeletal muscle glycogen synthase to recognize liver glycogen synthase in electroblot analysis strongly supports the hypothesis [2,3] that these two enzymes are isozymes. In keeping with nomenclature used for other isozymes we will refer to the skeletal muscle enzyme as 'M-type' glycogen synthase, and the liver enzyme as 'L-type' glycogen synthase.

Because our antisera recognize the enzyme in heart, fat, kidney, and brain under these conditions, we conclude that these tissues contain an enzyme that resembles the M-type enzyme. Whether these enzymes are identical to the skeletal M-type enzyme remains to be determined. In addition, these tissues may contain L-type glycogen synthase, or another form of the enzyme, not recognized by our antisera. We can conclude, however, that in livers from adult rats there is a virtual absence of M-type glycogen synthase.

ACKNOWLEDGEMENTS

Dr Alicia McDonough gave valuable advice and generous gifts of supplies in establishing electroblot analysis in our laboratory. The excellent technical contributions of Sharon DeLaHunt are also appreciated. NIH grant AM-31116 and a grant from the Los Angeles Affiliate of the American Diabetes Association supported this work.

REFERENCES

[1] Chrisman, T.D., Sobo, G.E. and Exton, J.H. (1984) FEBS Lett. 167, 295–300.
[2] Camici, M., Ahmad, Z., DePaoli-Roach, A.A. and Roach, P.J. (1984) J. Biol. Chem. 259, 2466–2473.
[3] Camici, M., DePaoli-Roach, A.A. and Roach, P.J. (1984) J. Biol. Chem. 259, 3429–3434.
[4] Thomas, J.A., Schlender, K.K. and Larner, J. (1968) Anal. Biochem. 25, 486–499.
[5] Solling, H. and Wang, P. (1973) Biochem. Biophys. Res. Commun. 53, 1234–1239.
[6] Uhing, R.J., Shikama, H. and Exton, J.H. (1981) FEBS Lett. 134, 185–188.
[7] Camici, M., DePaoli-Roach, A.A. and Roach, P.J. (1982) J. Biol. Chem. 257, 9898–9901.
[8] Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A. and Krebs, E.G. (1971) J. Biol. Chem. 245, 6317–6328.
[9] Renart, J., Reiser, J. and Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3116–3120.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Seed, B. (1982) Nucleic Acids Res. 10, 1799–1810.
[12] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[13] Roach, P.J. (1982) Curr. Top. Cel. Regul. 20, 45–105.
[14] Tan, A.W.H. and Nuttal, F.Q. (1983) J. Biol. Chem. 258, 9624–9630.
[15] DePaoli-Roach, A.A., Ahmad, Z., Camici, M., Lawrence, J.C. and Roach, P.J. (1983) J. Biol. Chem. 258, 10702–10709.
[16] Bahnak, B.R. and Gold, A.H. (1983) Biochem. Biophys. Res. Commun. 117, 332–339.