Biochimica et Biophysica Acta, 612 (1980) 344—351

BBA 68958

MICROHETEROGENEITY OF RAT GLYCOGEN PHOSPHORYLASE LIVER-TYPE ISOZYME

TSUYOSHI SATO and KIYOMI SATO

Second Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki 036 (Japan)

(Received September 10th, 1979)

Key words: Glycogen phosphorylase isozymes; Microheterogeneity

Summary

We devised a method of polyacrylamide gel electrophoresis at pH 7.3, modified by omitting base catalyst, *N,N,N',N'*-tetramethylethylenediamine, in the preparation of separating gels. Using this method, both liver and liver-like types of rat glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) were resolved into multiple forms, about 6—10, although either of them was purified to a single protein with the same molecular size on sodium dodecyl sulfate gel electrophoresis. The microheterogeneity of these two types was also confirmed by isoelectric focusing in polyacrylamide gels (pH 5—8). The major isoelectric points of the liver type phosphorylase were between 5.72 and 5.86, but those of the liver-like type were between 5.86 and 5.92, and so the former had slightly but significantly lower isoelectric points than the latter. However, the both types were not distinguished immunologically. The brain and muscle types of rat phosphorylase did not show such a distinct heterogeneity by the same electrophoresis methods.

Introduction

It is well established that there are at least three types of glycogen phosphorylase isozyme, namely muscle, brain (or fetal) and liver types in mammalian organs in common [1—7]. One of the great differences among them is evident in catalytic properties of their dephosphorylated form (*b* or inactive form). Muscle and brain phosphorylase *b* forms exhibit their activities in the presence of AMP, whereas liver phosphorylase *b* is inactive even in the presence

Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate.

of this nucleotide and requires further sulfate ion for the full activity [8]. The liver type isozyme is located predominantly in the liver, but it is also present in other adult tissues such as ovary, spleen, kidney, testis, lung, stomach, and small intestine, and also in fetal tissues and transplantable hepatomas [9—11]. This type in these tissues is similar to the type in the adult liver immunochemically and kinetically in requiring sulfate ion for activities, but it has the slightly smaller mobility on polyacrylamide gel electrophoresis [9,10] by the method of Takeo and Nakamura (pH 8.9) [12]. We designated previously this type as liver-like type [9,10]. In this study we investigated the differences between liver and liver-like types on polyacrylamide gel isoelectric focusing (pH 5—8) as well as on polyacrylamide gel electrophoresis by the method of Yonezawa and Hori [13], modified by omitting base catalyst TEMED in the separating gels (pH 7.3). Both enzymes were purified to a single protein, respectively, and compared with each other. We found different microheterogeneities between them.

Materials and Methods

Assay of glycogen phosphorylase. Glycogen phosphorylase was assayed in the direction of glycogen synthesis by measuring P_i liberated from glucose 1-phosphate determined by the method of Fiske and SubbaRow [14]. The assay mixture was the same as reported previously [6]. The activities of the *b* forms of the liver and liver-like type phosphorylases were assayed in the presence of 0.5 M Na_2SO_4 together with 1 mM AMP. Protein was determined by the method of Lowry et al. [15].

Enzyme sources. Sprague-Dawley rats weighing about 250 g were used and the Yoshida ascites hepatoma AH66F [10] was propagated by intraperitoneal implantation and was harvested within 6—7 days after inoculation.

Polyacrylamide gel electrophoresis. This was performed according to the method of Yonezawa and Hori [13], slightly modified as follows. The separating gels without TEMED (5 × 50 mm) were polymerized by incubating them at 37°C overnight (15—20 h). Samples with 80 munits in 15% sucrose solution were directly applied to large pore gels without making sample gels. Electrophoresis was carried out at constant current 0.5 mA/column at 4°C until bromophenol blue moved 1 cm into the separating gels, and then the current was raised to 1 mA/column.

Polyacrylamide gel isoelectric focusing. This was performed according to the method of Drysdale et al. [17] with slight modification at pH 5 to 8 in 6% polyacrylamide gel (2.5 × 50 mm) using 0.02 N H_2SO_4 as anolyte. Samples with 10—20 munits activities were applied to separate columns. After isoelectric focusing at 250 V for 5 h at 4°C, the gels were incubated for 30 min to 1 h in 40 mM Tris-maleate, pH 6.1, containing 40 mM glucose 1-phosphate and 2% hydrolyzed glycogen with and without 2 mM AMP and/or 0.75 M Na_2SO_4. At the end of the incubation time the gels were stained by immersing them in an iodine solution as reported previously [9,10].

SDS polyacrylamide gel electrophoresis. This was performed according to the method of Weber and Osborn [16] at 7.5% acrylamide concentration.

Specific antibodies to muscle, brain, liver, and liver-like type phosphorylases.

They were prepared in rabbits as reported previously [6].

Chemicals. Glucose 1-phosphate, AMP and glycogen (from shell fish) for phosphorylase assay were obtained from Boehringer Mannheim, F.R.G.; Ampholine carrier ampholites from LKB Produkter AB, Sweden; glycogen (from oyster) for enzyme activity staining after isoelectric focusing and polyacrylamide gel electrophoresis from Nakarai Chemicals, Kyoto, Japan; Freund's complete adjuvant from Iatron Laboratories, Tokyo, Japan, and other reagents from Wako Pure Chemical Industries, Osaka, Japan. The hydrolyzed glycogen for staining of phosphorylase in gels was prepared as follows: 10% of the glycogen suspension was hydrolyzed in 0.05 N HCl at 100°C for 30 min, then cooled and neutralized with 2 M Tris.

Results

Purification of each type of phosphorylase

The muscle type isozyme from the skeletal muscle was recrystallized 3 times according to the method of Sevilla and Fischer [18]. The liver type isozyme from the rat liver was prepared according to the method of Appleman et al. [8], slightly modified as follows: 105 000 × *g* precipitate was suspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 15 mM 2-mercaptoethanol (buffer A) and this suspension without amylase digestion was directly applied to a column of DEAE-cellulose equilibrated with the above buffer and eluted with 0—0.4 M NaCl gradient. The brain type and liver-like type isozymes were prepared from the rat brain and AH66F cells, respectively. The rat brain was homogenized with a Teflon glass homogenizer in 4 volumes of 62.5 mM Tris-HCl, pH 7.4, containing 25 mM 2-mercaptoethanol and 6.25 mM EDTA, and AH66F cells were sonicated at 9 KHz, 200 W, with a Kubota Insonator model 200 M (Japan). The homogenate was centrifuged at 5000 × *g* for 10 min, and the supernatant was incubated at 30°C for 45 min to convert *a* form phosphorylases into *b* forms, and centrifuged at 105 000 × *g* for 45 min. The supernatant was brought to 0.5 saturation with $(NH_4)_2SO_4$ and the precipitate was dissolved in the buffer A, and then to this medium was added the suspension of glycogen purified from rabbit liver [10], which contained no detectable protein on polyacrylamide gel electrophoresis, to final concentrations of 1.5—2.0%. The suspension was left at 4°C for 20 min, and then centrifuged at 105 000 × *g* for 45 min. This glycogen precipitation step was repeated 2—3 times. The specific antibody to the muscle or brain isozyme prepared in rabbits was added to the brain or AH66F glycogen precipitate fraction, respectively, to absorb the muscle type and its hybrid with the brain type in the brain tissue, or the brain type and its hybrid with liver-like type in AH66F cells [10]. This mixture was left at 4°C for 20 h and centrifuged at 10 000 × *g* for 10 min and the supernatant was applied to a column of DEAE-cellulose, and eluted with 0—0.4 M NaCl gradient. The fractions containing the phosphorylase activity were concentrated in a collodion bag and dialyzed against the buffer A. When phosphorylase activity was detectable in the 105 000 × *g* precipitate fraction from AH66F homogenate, the fraction was suspended in the buffer A and digested with human salivary α-amylase at 4°C for 20 h, and centrifuged at 105 000 × *g* for 45 min. The supernatant was applied to a column of DEAE-cellulose

TABLE I

PURIFICATION OF LIVER-LIKE TYPE PHOSPHORYLASE FROM AH66F CELLS

Details of procedure are given in the text. The staining material was 125 ml of AH66F packed cells.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude extract	9564	359	0.038	100	1
105 000 × *g* supernatant *	6760	192	0.028	53.5	0.74
$(NH_4)_2SO_4$ (0—50%) fraction	4373	179	0.041	49.9	1.08
Glycogen precipitate, absorbed by antibody to brain type	23	120	5.217	23.4	137.3
Eluate from DE-52 chromatography	9.9	82	8.282	22.8	218.0

* In 105 000 × *g* precipitate fraction, 34.7% of phosphorylase activities were recovered, and this fraction was also used for purification of the liver-like isozyme, as described on the text. Finally, recovery was 13.3%, and the specific activity was 7.83 units/mg protein.

equilibrated with the buffer A, and eluted step-wisely with 0.4 M NaCl. To the fractions containing the activity the rabbit liver glycogen was added and the same glycogen precipitation procedure described above was performed. The typical purification procedure for liver-like type phosphorylase from AH66F cells is shown in Table I.

Microheterogeneity of liver and liver-like type phosphorylases

To the respective purified enzymes 1.5 volumes of 6.5 mM HCl-imidazole solution, pH 6.8, containing 25% sucrose were added and 0.1 ml of the solution

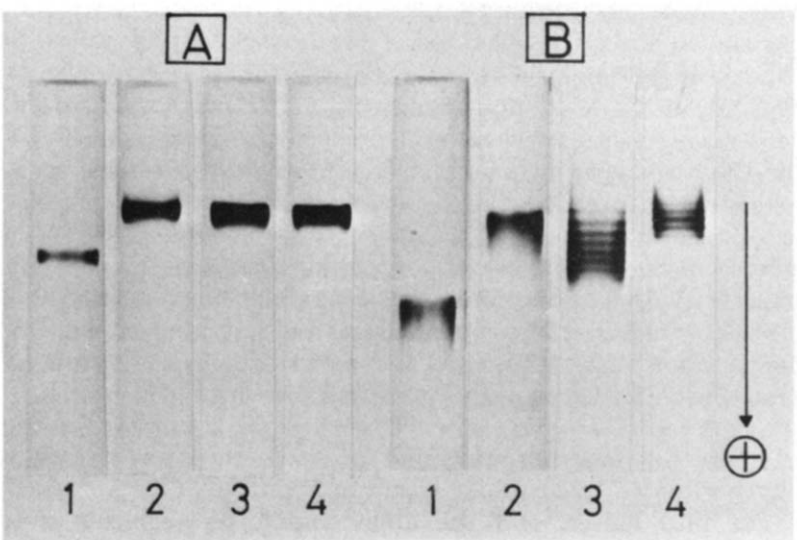

Fig. 1. Electrophoretic patterns of purified four types of rat phosphorylase *b*-form in polyacrylamide gel polymerized with (A) and without TEMED (B). 1, brain; 2, muscle; 3, liver; 4, liver-like types. The purification procedure of each type is described in the text. Gel 1 and 2 were stained in the presence of 2 mM AMP, and gel 3 and 4 were stained by further addition of 0.75 M Na_2SO_4.

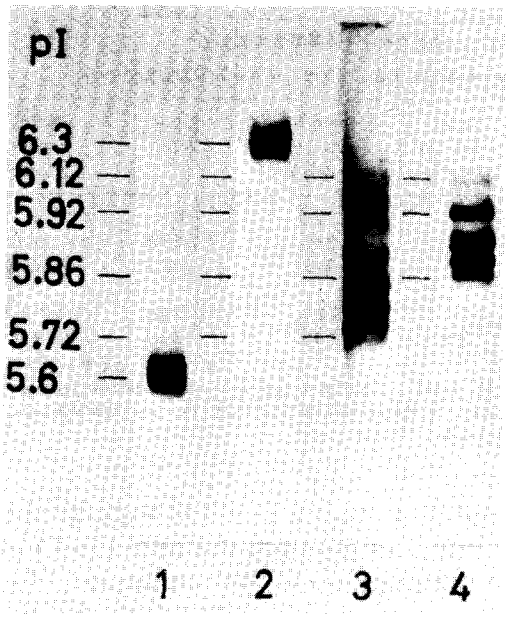

Fig. 2. Isoelectric focusing patterns of four types of phosphorylase in polyacrylamide gels. 20 munits of a respective phosphorylase was applied to a gel containing pH 5—8 Ampholines. The sample numbers are the same as those in Fig. 1.

applied to polyacrylamide gel electrophoresis with or without TEMED in separating gels. As shown in Fig. 1A, all types of phosphorylase were stained as a single sharp band in separating gels polymerized with TEMED. However, as can be seen in Fig. 1B, in the gels polymerized without TEMED, the liver and liver-like types were resolved into active multiple molecular forms, about 6—10, while the brain and muscle types were stained as a broad but still single band, respectively. The multiple forms of the liver and liver-like types were also observed on the gel isoelectric focusing with pH 5—8 gradient, as shown in Fig. 2. The patterns were similar to those of polyacrylamide gel electrophoresis. The brain and muscle types had only one isoelectric point at p*I* 5.6 and 6.3, respectively, but the liver and liver-like types had the multiple isoelectric points over the wide range from 5.72 to 6.12, as about ten forms. The difference between the liver and liver-like types was in the dominant forms. The dominant forms of the liver type had p*I* 5.72—5.86, while those of the liver-like type had p*I* 5.86—5.92. To confirm the reproducibility of these multiple molecular forms, the activity portion of the liver-like type containing about 6—10 molecular forms was cut into halves, and the upper and lower segments were repacked in separate columns and reelectrophoresis was performed. The mobilities to the anode and number of phosphorylase forms were identical with those in the original segments, respectively, and therefore the microheterogeneity was quite reproducible (not shown).

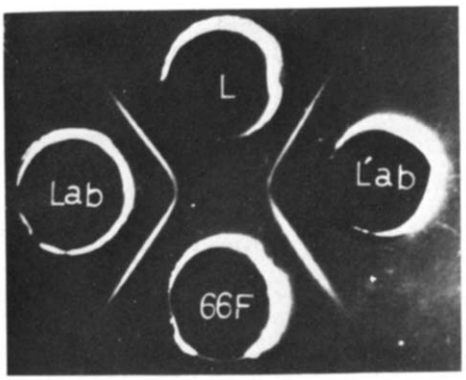

Fig. 3. Ouchterlony double diffusion test of liver and liver-like type phosphorylases. L, liver type; L', liver-like type; Lab, antibody to the liver type; L'ab, antibody to the liver-like type obtained from AH66F cells.

Immunological identity of liver and liver-like type phosphorylases

In Ouchterlony double diffusion tests in agar the antibody to liver enzyme made a single precipitin line with both liver and liver-like types, respectively, and the lines fused smoothly with each other. The same result was obtained by using the antibody to liver-like enzyme, as shown in Fig. 3.

Each type isozyme used for the above examination of heterogeneity was a single protein having the same molecular weight of a monomer at 100 000 of the muscle type and no microheterogeneity was observed.

Discussion

Yonezawa and Hori [13] divised a new method of polyacrylamide gel electrophoresis modified from that of Davis et al. [3], and could clearly separate the muscle-brain and liver-brain hybrids from their parent isozymes in various tissues. We eventually modified their method by omitting TEMED in the preparation of separating gels and found multiple forms in both liver and liver-like type isozymes.

It is unlikely that the use of separating gels without TEMED caused the artifactural microheterogeneity, because (1) the pattern of microheterogeneity was constant and reproducible for each individual isozyme preparation and through purification steps, (2) the microheterogeneity and the patterns were confirmed to be reproducible by reelectrophoresis, (3) the heterogeneity was also observed on the polyacrylamide gel isoelectric focusing (Fig. 2) and further on the cellulose acetate membrane electrophoresis (Cellogel, Chemetron) as observed by Kobayashi et al. [19] and confirmed by us (not shown), and (4) the patterns of microheterogeneity seen by this method was well correlated with that found on isoelectric focusing; that is, the most electronegative form of the liver type seen on disc electrophoresis showed the lowest isoelectric point, as expected.

The advantage of this method in comparison with gel isoelectric focusing is that the multiple forms in a crude extract can be clearly separated in a shorther time (1.5—2 h).

By means of the SDS gel electrophoresis and of the usual polyacrylamide gel electrophoresis using TEMED in the separating gels, we have been unable to detect the microheterogeneity of any type of isozymes.

So far we do not known why the microheterogeneity can be observed in the separating gel polymerized without TEMED, and we also are not sure that this new method is useful for detection of microheterogeneity of other proteins. It has been demonstrated that many other proteins, such as albumin [20,21], α-fetoprotein [22—26], creatine kinase [27], aldolase [28], lactate dehydrogenase [29], alkaline phosphatase [30], γ-glutamyltransferase [32], amylase [33], and so on, have the microheterogeneity observed on isoelectric focusing in gels or in sucrose gradient, on polyacrylamide gel electrophoresis and on cellulose acetate membrane electrophoresis. Many factors may cause microheterogeneity of a protein, as discussed by Williamson et al. [34]. One of the most possible factors among them as known on alkaline phosphatase, γ-glutamyltransferase, β-glucuronidase, α-fetoprotein, and galactosyltransferase [35], is the difference in the sialic acid content. However, liver and liver-like types showed no change in heterogeneity by neuraminidase treatment. The possibility of protease digestion such as by trypsin in the purification steps was not believable, because microheterogeneity was observed in freshly prepared crude extract as well as in purified samples. No significant difference between phosphorylated (*a*) and dephosphorylated (*b*) forms was observed for explanation of these heterogeneities.

Recently Ogawa et al. [36] reported that purified human pancreatic amylase was converted into several different minor molecular forms due to enzymic deamidation by peptidoglutaminase. The heterogeneity of phosphorylase reported here should be also confirmed to be due to the deamidation of asparagine or glutamine in the phosphorylase molecule.

The liver-like type has been also distinguished from the liver type by the polyacrylamide gel electrophoresis which used glycogen in the separating gels [9,10]. We purified these two types and compared with each other, and were able to discriminate them more clearly not only by polyacrylamide gel electrophoresis without TEMED in the separating gels but also by isoelectric focusing. On polyacrylamide gel electrophoresis, more electronegative forms were predominant in the liver type, while less electronegative forms were predominant in the liver-like type. On isoelectric focusing, the liver type was separated into the forms having the low isoelectric points, while liver-like type had higher isoelectric points. This result suggests that the liver type may have a higher amount of electronegative groups in the molecular than the liver-like type.

By polyacrylamide gel isoelectric focusing (pH 3—10), Koster et al. [37] reported that human liver and white blood cells had three forms of liver type enzyme, but the white blood cells and fibroblasts cultured from a patient with liver type phosphorylase deficiency (glycogen storage disease Type VI) defected one or two forms among them. These results together with our data reported here suggest that the liver-like type phosphorylase is controlled genetically differently from the liver type isozyme. The liver-like type was observed in fetal tissues as well as in ascites hepatomas [9,10] and further even in cultured human skeletal muscle cells [38] and in primary cultured rat myo-

blasts (Hatayama, I. and Sato, K., unpublished data) and therefore the liver-like type may be another prototypic phosphorylase other than the brain type.

Acknowledgement

This work was supported in part by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture of Japan.

References

1 Henion, W.F. and Sutherland, E.W. (1957) J. Biol. Chem. 224, 477—488
2 Yunis, A.A., Fischer, E.H. and Krebs, E.G. (1962) J. Biol. Chem. 237, 2809—2815
3 Davies, C.H., Schliselfeld, L.H., Wolf, W.P., Leavitt, C.A. and Krebs, E.G. (1967) J. Biol. Chem. 242, 4824—4833
4 Schliselfeld, L.H. (1974) Ann. N.Y. Acad. Sci. 210, 181—191
5 Sato, K., Morris, H.P. and Weinhouse, S. (1972) Science 178, 879—881
6 Sato, K., Morris, H.P. and Weinhouse, S. (1973) Cancer Res. 33, 724—733
7 Sato, K. and Weinhouse, S. (1973) Arch. Biochem. Biophys. 159, 151—159
8 Appleman, M.M., Krebs, E.G. and Fischer, E.H. (1966) Biochemistry 5, 2101—2107
9 Sato, K., Sato, T., Morris, H.P. and Weinhouse, S. (1975) Ann. N.Y. Acad. Sci. 259, 273—286
10 Sato, K., Satoh, K., Sato, T., Imai, F. and Morris, H.P. (1976) Cancer Res. 36, 487—495
11 Proux, D. and Dreyfus, J. (1973) Clin. Chim. Acta 48, 167—172
12 Takeo, K. and Nakamura, S. (1972) Arch. Biochem. Biophys. 153, 1—7
13 Yonezawa, S. and Hori, S.H. (1975) J. Histochem. Cytochem. 23, 745—751
14 Fiske, C.H. and SubbaRow, Y. (1929) J. Biol. Chem. 81, 629—679
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Drysdale, J.W., Righetti, P. and Bunn, H.F. (1971) Biochim. Biophys. Acta 229, 42—50
18 Sevilla, C.L. and Fischer, E.H. (1969) Biochemistry 8, 2161—2171
19 Kobayashi, M., Taketa, K. and Nagashima, H. (1978) The Physico-Chemical Biology (in Japanese) Japan 22, 64
20 Oda, K., Ikehara, Y. and Kato, K. (1978) Biochim. Biophys. Acta 536, 97—105
21 Quinn, P.S., Gamble, M. and Judah, J.D. (1975) Biochem. J. 146, 389—393
22 Lester, E.P., Miller, J.B., Baron, J.M. and Yachnin, S. (1978) Immunology 34, 189—198
23 Alpert, E., Drysdale, J.W., Isselbacher, K.J. and Schur, P.H. (1972) J. Biol. Chem. 247, 3792—3798
24 Yachnin, S., Hsu, R., Heinrikson, R.L. and Miller, J.B. (1977) Biochim. Biophys. Acta 493, 418—428
25 Alpert, E. and Perencevich, R.C. (1975) Ann. N.Y. Acad. Sci. 259, 131—135
26 Lester, E., Miller, J. and Yachnin, S. (1978) Biochim. Biophys. Acta 536, 165—171
27 Wevers, R.A. Wolters, R.J. and Soons, J.B.J. (1977) Clin. Chim. Acta 78, 271—276
28 Schapira, F., Gregori, C. and Hatzfeld, A. (1977) Clin. Chim. Acta 78, 1—8
29 Markert, C.L. (1963) Science 140, 1329—1330
30 Crofton, P.M. and Smith, A.F. (1978) Clin. Chim. Acta 86, 81—88
31 Tate, S.S. and Meister, A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2599—2603
32 Owens, J.W., Gammon, K.L. and Stahl, P.D. (1975) Arch. Biochem. Biophys. 166, 258—272
33 Malacinski, G.M. and Rutter, W.J. (1969) Biochemistry 8, 4382—4390
34 Williamson, A.R., Salaman, M.R. and Kreth, H.W. (1973) Ann. N.Y. Acad. Sci. 209, 210—222
35 Gerber, A.Ch., Kozdrowski, I. Wyss, S.R. and Berger, E.G. (1979) Eur. J. Biochem. 93, 453—460
36 Ogawa, M., Kosaki, G., Matsuura, K., Fujimoto, K., Minamiura, N., Yamamoto, T. and Kikuchi, M. (1978) Clin. Chim. Acta 87, 17—21
37 Koster, J.F., Slee, R.G., Daegelen, D., Meienhofer, M.C., Dreyfus, J.C., Niermeyer, M.F. and Fernandes, J. (1976) Clin. Chim. Acta 69, 121—125
38 Sato, K., Imai, F., Hatayama, I., and Roelofs, R.I. (1977) Biochem. Biophys. Res. Commun. 78, 663—668