Purification of Two Forms of Bovine Liver Glycogen Phosphorylase b with Distinct Subunit Composition

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The procedures for the purification of two forms of bovine liver glycogen phosphorylase b are described. Both forms showed a single band in nondenaturing gel electrophoresis. Gel electrophoresis in the presence of sodium dodecyl sulfate produced a single-band pattern for one of the enzyme forms (phosphorylase b1) and a triple-band pattern for the other (phosphorylase b3). Molecular weights associated with these bands were 97 kDa in the first case and 97, 55, and 40 kDa in the second. The yield from 1 kg of liver was approximately 10 mg for phosphorylase b1 and 140 mg for phosphorylase b3. The specific activity was 40–44 U/mg in both cases. As phosphorylase b1 is composed of just one kind of monomer, it is a novel bovine liver phosphorylase b structure. © 1994 Academic Press, Inc.

Glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) catalyzes glycogen degradation according to the reaction

\[(\text{glucose})_n + \text{phosphate} \rightleftharpoons (\text{glucose})_{n-1} + \text{glucose 1-phosphate}.

Liver glycogen phosphorylase plays an important role in the maintenance of correct blood glucose levels in higher animals. The enzyme's activity is subject to a covalent control via a phosphorylation mechanism. The dephosphorylated b form is inactive under the conditions prevailing in the liver cell, whereas the phosphorylated a form is active. Conversion from the b to the a form is catalyzed by phosphorylase kinase and the reverse by phosphorylase phosphatase. The enzyme's activity is also regulated by the presence of small metabolites such as AMP,2 IMP, and glucose, among others. High saline concentrations enhance the enzymatic activity of the b form in vitro. [For reviews see Refs. (1) and (2)].

Liver glycogen phosphorylase together with the muscle and brain enzymes make up the family of three isoenzymes of glycogen phosphorylase, named for the tissues in which they are preferentially expressed. The three forms are encoded by different genes and their amino acid sequences have 80–83% homology (2). Extensive studies have been made into the structure, thermodynamics, and kinetics of the muscle enzyme and detailed knowledge of its X-ray structure provides the grounds for an accurate description of the molecular basis of its biological function (2). Physical–chemical studies with the purified liver enzyme are scarce on the other hand, and it has not yet been possible to elucidate structure–function relationships to the same degree. In spite of the high homology of the two isoenzymes there are significant differences between them, both in their overall physiological role and in their allosteric response to small metabolites, particularly to AMP. Thus it seems that more detailed biochemical and physical–chemical studies of the liver enzyme might greatly help in our understanding of the structure–function relation.

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2 Abbreviations used: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; IEF, isoelectric focusing; AMP, 5'-adenosine monophosphate; IMP, 5'-inosine monophosphate; DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
tionships in glycogen phosphorylases. Furthermore, these studies are now very necessary in light of the fact that the first glycogen phosphorylase has been engineered based on human liver enzyme (3). In any case appropriate schemes for the purification of liver phosphorylases are to be set. Fischer, Krebs, and co-workers (4,5) were the first to describe a method of purifying glycogen phosphorylase from rabbit and pig livers. Since then, most of the procedures described have been for the enzyme from the same sources (for example, Refs. 6 and 7) with very little if any attempts to further characterize the purified protein. Hwang et al. (8) have described a procedure for the purification of bovine liver glycogen phosphorylase α, but which is liable to proteolysis of one specific peptide bond in the molecule.

We describe here a purification procedure for bovine liver glycogen phosphorylase β. In planning physical-chemical studies, we chose bovine liver as an abundant source of the enzyme. We consider two stages in the process; in the first we record the purification of an enzyme showing a triple-band pattern in SDS–PAGE, which we call phosphorylase b3. The second stage involves the purification from phosphorylase b3, by preparative isoelectric focusing, of a fully active SDS–PAGE homogenous enzyme, which we denominated phosphorylase b1.

MATERIALS AND METHODS

Materials

All chemicals were bought from either Sigma or Merck. Standard liquid chromatographic materials used were DEAE-cellulose DE-52 from Whatman and Blue Sepharose 6B and Sephacryl S-300 from Pharmacia. Analytical isoelectric focusing was performed on precast polyacrylamide gels (with ampholines in the pH ranges 3.5–9.5 and 5–6.5) from Pharmacia. For preparative isoelectric focusing, ampholines solutions from Pharmacia (pH 5–6.5) and Serva (pH 5–6) were used in Ultrodex gel from Pharmacia. Veal livers were obtained from a local slaughterhouse immediately after the animals were killed.

Measurement of Phosphorylase b Activity

Enzyme activity was determined by measuring the inorganic phosphate produced in the phosphorylase-catalyzed reaction of glycogen with glucose-1-phosphate, according to the method of Hedrick and Fischer (12), as modified by Appleman et al. (4). This modification takes into account the need for a high saline concentration (0.7 M Na₂SO₄) for the enzyme to remain active (in our assays in the presence of 1 mM AMP).

Protein Electrophoresis

Non-denaturing Conditions

Gel electrophoresis of the native enzyme was performed in 6 and 10% acrylamide gels according to Davis (13), in a discontinuous system with stacking gel buffer Tris–HCl (pH 6.8), separating gel buffer Tris–HCl (pH 8.8), and running buffer Tris–glycine (pH 8.3).

Denaturing Conditions

SDS–PAGE of the enzyme was performed according to Laemmli (14). For routine checks of the purity of our preparations Coomassie brilliant blue R was used for staining the protein bands. Densitometric analyses were performed with a Beckman DU70 spectrophotometer using the appropriate accessory.

Isoelectric Focusing

Analytical and preparative isoelectric focusing were performed using a Pharmacia Multiphor II system, following the methods described by Radola (15).

Bovine Liver Glycogen Purification

The first glycogen pellet obtained in step 3 of the purification of phosphorylase b3 detailed above shows two clearly distinct parts: one glowing white and consistent at the bottom of the centrifuge tube, and another soft, brownish one above it, which could easily be washed away with water. Once the brown part has been eliminated, the remaining white fraction was resuspended with 10 vol of water and showed a very low protein content. It was then centrifuged at 70,000g for 2 h. The pellet was resuspended and centrifuged twice more under the same conditions. The final pellet was lyophilized and stored at −20°C.

Isolation Procedures

Purification of Phosphorylase b3

Step 1. Up to 1.5 kg of fresh veal liver was purchased from a local slaughterhouse and immediately taken to our laboratory in crushed ice. From this moment on the
entire procedure was carried out at 4°C. Small portions of the liver were homogenized in a blender with 5 mM EDTA, pH 7.4, at a 3:1 v/w ratio. The homogenate was centrifuged at 4000g for 30 min and the supernatant, carefully filtered through glass wool, constituted the extract. The extract was reddish and very turbid, mainly due to glycogen. No loss of phosphorylase activity was detected in it after 1 week when kept at 4°C.

**Step 2.** The extract was centrifuged at 70,000g for 30 min. After centrifugation, the supernatant retained its reddish color but most of the turbidity was gone, the glycogen having sedimented in the pellet with most of the phosphorylase bound to it. This pellet formed the glycogen–protein complex. The supernatant was separated and the pellet was weighed and resuspended with 25 mM Hepes, 2 mM EDTA, 30% maltose at pH 7.6 with a v/w ratio of 4:1.

**Step 3.** The solution was kept overnight under continuous stirring. Phosphorylase is separated from glycogen in the presence of maltose because of its competition for the glycogen site in the enzyme (16). The solution was then centrifuged at 70,000g for 2 h and the supernatant was separated and centrifuged again under the same conditions. The pellet from the first centrifugation, made of glycogen devoid of phosphorylase, was kept at −20°C to be further purified when necessary (see below) and the supernatant of the second centrifugation was dialyzed against several changes of 5 mM Hepes, 1 mM EDTA, 1 mM β-mercaptoethanol at pH 7.0 (buffer A). After dialysis, with the maltose eliminated from the solution, if any turbidity was still present a final 70,000g centrifugation for 30 min eliminated it.

**Step 4.** The dialyzed solution was applied to a DEAE-cellulose column equilibrated in buffer A. Approximately 200 ml of the exchanger in a 5 × 10-cm column was used for a purification starting from 1 kg of liver. The flow rate during chromatography was approximately that produced by a hydrostatic pressure of 1 m. Phosphorylase bound to the column, which was washed with 3 column vol of buffer A; the enzyme was then eluted with a linear gradient of NaCl (from 0 to 0.2 M) in the same buffer. Ten column volumes of buffer A plus NaCl was used to make the gradient. Fractions were collected for monitoring 280 nm absorbance and measuring enzyme activity: a phosphorylase peak was usually detected in the second third of the gradient elution. Fractions of this peak were pooled and concentrated to approximately 50 ml in an Amicon cell using YM 30 membranes. NaCl was then eliminated by dialysis against buffer A.

**Step 5.** The phosphorylase solution was finally chromatographed in an affinity Blue Sepharose column. Approximately 250 ml of Blue Sepharose in a 2.5 × 50-cm column was used for a purification starting from 1 kg of liver. The solution coming from DEAE-cellulose chromatography was applied at a flow rate of 50 ml per hour to the Blue Sepharose column equilibrated in buffer A. Once the entire solution had been applied the flow was stopped for 2 h and the column was washed with 2 column vol of buffer A. Phosphorylase was then eluted with a linear gradient (from 0 to 0.5 M) of NaCl in 4 column vol of the same buffer. The flow rate of 50 ml per hour was maintained for both the washing of the column and the gradient elution. Fractions of the gradient elution were collected and those with phosphorylase activity were pooled, this pool constituting our glycogen phosphorylase b3 preparation. It was usually concentrated with an Amicon cell using a YM30 membrane to a protein concentration of approximately 5 mg/ml. In this solution the enzyme retains its full activity for at least 6 months.

**Purification of Phosphorylase b1**

Phosphorylase b1 was purified by preparative isoelectric focusing with a Multiphor II electrophoresis system. A purified phosphorylase b3 solution was mixed with 100–200 ml of Ultradex gel and ampholytes in the pH range 5–6.5. The slurry was put into a rectangular (24 × 11 cm) mold on the surface of the electrophoresis apparatus, and excess solvent was allowed to evaporate. Electrofocusing was performed during 24 h at 8 W, with the system refrigerated by running water from a thermostatic bath at 4°C. Once the isoelectric focusing was over the gel was fractionated in 13 portions with the aid of a special grid and each of the different portions was transferred to a column from which the protein was eluted with 5 mM Hepes, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.0.

**RESULTS AND DISCUSSION**

The main features of the bovine phosphorylase b3 purification procedure described in the previous section are shown in Table 1. As can be seen, 140 mg of phosphorylase b with a specific activity of 44 U/mg was obtained from 1 kg of liver.

Like most purification procedures of liver phosphorylases, from whatever the source, our technique follows the general lines established by Appleman et al. (4) with rabbit enzyme, modified by Hwang et al. (8), who replaced amylase digestion of the glycogen pellet with digestion with maltose as a means of obtaining the free enzyme in solution. We have followed this process up to the first DEAE-cellulose chromatography, at which a partially purified phosphorylase b solution is obtained. We get values similar to those of Hwang et al. (8) for the yield in all steps, but somewhat different ones for the
purification after both the maltose exchange and the DEAE chromatography steps. The specific activity in our phosphorylase b3 solution after the chromatography (at least 25 U/mg) is the highest one reported at that point for any liver phosphorylase from any source.

After the DEAE-cellulose step, Hwang et al. (8) further purify the enzyme by converting it to the a form with the aid of phosphorylase kinase, followed by a second DEAE-cellulose chromatography. In our case, we have been able to purify the b form from the same first DEAE cellulose eluate with a Blue Sepharose chromatography. This material has been described (17,18) as being able to bind the nucleoside site in the rabbit muscle enzyme. Our results show that it is also able to bind liver phosphorylase and thus can be used in its purification. Figure 1 shows a typical profile of the Blue Sepharose elution. There was a first peak corresponding to impurities in which no phosphorylase activity was measured. Once the gradient in ionic strength began, a protein peak running closely parallel to phosphorylase activity was obtained.

At this point we should mention that we have tried to eliminate DEAE-cellulose chromatography from the purification procedure by using just the Blue Sepharose step, but have had unsatisfactory results as far as the purity of the final phosphorylase b sample is concerned. Thus, we deem both chromatographies to be necessary and have kept them in the procedure.

Gel electrophoresis of the purified enzyme after the Blue Sepharose step is shown in Fig. 2. The results of electrophoresis under native conditions at two different acrylamide concentrations are shown in Fig. 2A. Only one band is seen in both cases, which demonstrates the purity of our enzyme. When electrophoresis is performed under denaturing conditions, on the other hand,

<table>
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<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<tr>
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<td>31</td>
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<td>44</td>
<td>6,200</td>
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FIG. 1. Elution profile in the Blue Sepharose 6B chromatography; 230 mg of protein with a specific activity of 26 U/mg was applied on a 2.5 × 25-cm column and 10-ml fractions were collected. Phosphorylase b3 was eluted with a linear NaCl gradient, shown by the straight line. The enzymatic activity is shown with the open circles, and the specific activity in the pooled solution, containing 140 mg of protein, was 44 U/mg. The chromatography was performed at a flow rate of 50 ml/h at 4°C.

FIG. 2. Gel electrophoresis of phosphorylase b3. In A the electrophoresis under nondenaturing conditions at pH 8.9 and at 6% (lane 1) and 10% (lane 2) acrylamide concentration are shown. Slab SDS-PAGE is shown in B. Phosphorylase b3 is shown in lane 3. In lane 1 different molecular weight standards were run, from top to bottom: rabbit muscle phosphorylase monomer (97 kDa), bovine serum albumin (67 kDa), egg albumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). Rabbit muscle phosphorylase monomer is shown in lane 2.
three distinct bands can be seen, as shown in Fig. 2B, lane 3. It is important to state that the gels were overloaded to bring out the presence of impurities. From a qualitative point of view, our results with the SDS gels of phosphorylase $b$ were the same as those obtained by Hwang et al. (8) with the $a$ form. Because of the presence of three polypeptides we call the enzyme phosphorylase $b_3$. On reviewing the literature for liver phosphorylases from other sources we have only been able to find a couple of SDS gels from rabbit enzyme (5,6) and in both cases only one band is apparent; nevertheless, the presence of proteolytic activity cannot be ruled out in either case.

We have checked our purification procedure starting from livers kept at $-20^\circ$C for up to 1 year and have obtained the same results as above. It is also possible to store the protein–glycogen complexes obtained as pellets after the first centrifugation at 70,000g (in step 2) at $-20^\circ$C and start purification from them after a period of 2 months with no change in the final results.

It should be mentioned as well that the phosphorylase yield in the protein–glycogen complex was related to the glycogen content of the extract. This parameter is out of our control as it depends on how long the animal has been fasted before being slaughtered. Nevertheless, when a low yield is obtained it can be significantly increased by adding liver glycogen to 0.2 mg/ml to the supernatant after the first 70,000g centrifugation. Liver glycogen was prepared from enzyme purifications as described under Materials and Methods. When the solution was centrifuged again at the same speed for 30 min, most of the enzyme that did not sediment in the first centrifugation was recovered in the pellet.

As far as the polypeptides in the SDS gels are concerned, the molecular weight for the slowest migrating band was 97 kDa, the same as that of rabbit muscle glycogen phosphorylase monomer. The molecular weights for the other two bands were determined with the aid of a representation of mobility vs 1 g (molecular weight) of known protein standards (19). The values measured were 55 and 40 kDa. That the sum of the molecular weights of the two smaller polypeptides is very close to that of the largest one strongly suggests that the former two result from the rupture of the latter. We have named the three polypeptides $F_40, F_55$, and $M_97$, where $F$ stands for fragment and $M$ for monomer.

When purified phosphorylase $b_3$ was subjected to analytical isoelectric focusing, a diffuse pattern showing three main bands was obtained. This can be seen in Fig. 3A. When the focused sample is subjected to a second SDS–PAGE dimension the pattern shown in Fig. 3B is obtained. As can be seen, the portion of phosphorylase

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**FIG. 3.** Isoelectric focusing and second-dimension SDS–PAGE of phosphorylase $b_3$. Analytical isoelectric focusing in the pH range 5–6.5 of the purified enzyme is shown in A. After the isoelectric focusing was over, the gel was cut and placed on a standard SDS–PAGE slab. The electrophoresis was run in a direction perpendicular to that of the isoelectric focusing, and the result is shown in B.

**FIG. 4.** Preparative isoelectric focusing of phosphorylase $b_3$ and purification of phosphorylase $b_1$. Two milliliters of a 5 mg/ml solution of phosphorylase $b_3$ (40 U/mg) was dialyzed against 1% glycine and mixed with 100 ml granulated gel with ampholines in the pH range 5–6.5. The mixture was placed in a 125 $\times$ 250-mm tray and the isoelectric focusing was carried out at 8 W during 20 h keeping the temperature at 4°C with a thermostatic bath. The distribution of the protein was detected staining with Commassie brilliant blue R250 with a print paper placed on top of the gel. This was fractionated by cutting with blades with the aid of a grid frame. Each gel fraction was transferred to a small column from which the protein was eluted passing through 1.5 ml of 5 mM Hepes, 1 mM EDTA, 1 mM $\beta$-mercaptoethanol. In A, the activity measurements (C) and protein concentrations (O) of the different fractions are shown. Part I of B shows the SDS–PAGE of starting phosphorylase $b_3$ (left lane) and fraction 3 in A after isoelectric focusing (right lane). Part II of B shows the isoelectric focusing of a phosphorylase $b_1$ preparation.
b3 with the lowest isoelectric point is enriched in the 97-kDa monomer, whereas the portion with the highest isoelectric point is enriched in the 55- and 40-kDa monomers.

It is possible to scale out these results by performing the isoelectric focusing both on a semipreparative or a preparative scale. Thus, the results obtained after the isoelectric focusing of 10 mg of phosphorylase b3 in 100 ml of pH 5.0–6.5 ampholines gel are shown in Fig. 4A. After focusing was completed the gel was fractioned and the protein collected with buffer. The measurements of both concentration and phosphorylase specific activity on the resulting solutions are shown in the figure. As can be seen, there is one protein peak, approximately in the middle of the fractionation range. When SDS–PAGE was run for the different fractions the same type of results as that shown in Fig. 3B was obtained. The fractions corresponding to the lower isoelectric points, i.e., those with the lower number in the figure, are enriched in the 97-kDa monomer and those with the higher isoelectric point in both the 55- and 40-kDa fragments. The SDS–PAGE electrophoresis of fraction 3 is shown in Fig. 4B (right lane, part I). In order to compare, the left lane of part I of the same figure shows the SDS–PAGE electrophoresis of the starting phosphorylase b3 preparation. Fractions from the lowest isoelectric point side are pooled up to 10% of the protein content of the whole protein peak. This is our phosphorylase b1 preparation. When SDS–PAGE was run on it, more than 90% of the area was measured beneath the 97-kDa peak after direct densitometric analysis of the Coomassie brilliant blue-stained gels. Analytical isoelectric focusing of this protein is also shown in Fig. 4B (part II). The isoelectric point, measured by comparison with standards, is 5.3–5.4. The specific activity of the enzyme was the same as that of the phosphorylase b3 from which the purification was made, i.e., 40 U/mg. Thus, both SDS–PAGE electrophoresis and isoelectric focusing show a highly purified enzyme which is fully active and mainly composed of M97 monomers; this is a novel bovine liver phosphorylase b structure. We got the same results after scaling up this procedure to an isoelectric focusing of 100 mg of phosphorylase b3 in 200 ml of gel. No further scaling-up was attempted. The yield of this purification was low: between 5 and 10%. In addition to the fact that only around 10% of the protein peak was pooled, it must be borne in mind that only 60–70% of the starting protein was recovered after isoelectric focusing. This constitutes a limitation to the technique itself (20).

At this point we must say that attempts were made to separate both phosphorylase isoforms by high-performance liquid chromatography on a Pharmacia Mono Q column, with no success.

As previously stated, the molecular weight complementarity of F55 and F40 fragments to add up to approximately the molecular weight of M97 monomers, strongly suggesting that the former two come from a rupture of the latter. Moreover, all phosphorylases b from other sources are known to be dimers composed of equal monomers (2). Some experiments were performed to check whether the existence of the F55 and F40 fragments are due to proteolysis during purification. From the same batch of liver a couple of purifications were run simultaneously, introducing significant differences between them during the first two steps. In one of them we used a protease-inhibitor cocktail (1 μM leupeptin, 1 μM pepstatin, 200 μM PMSF, freshly prepared and added to those final concentrations immediately before use) in the buffers, both to obtain the homogenate and to resuspend the protein–glycogen complex. All the operations up to this resuspension were made as quickly as possible at 4°C. In the other preparation no inhibitors were used in the buffers and the operations were made at room temperature. The procedure was also carried out more slowly: thus, the homogenate was left at room temperature for 30 min prior to centrifugation to obtain the extract, and this was left for 2 h, again at room temperature, prior to the ultracentrifugation to obtain the protein–glycogen complex. From the resuspension of the protein–glycogen pellet onward, both preparations were carried out in parallel in the cold room following the standard procedure. In both cases the purification and yield for the different steps of the purification were identical, as were the final specific activities of the enzymes, their electrophoresis under non-denaturing and denaturing conditions, and the densitometric analysis of the latter. These results seem to indicate that proteolytic action does not take place during the purification.

Moreover, preliminary experiments indicate that there is a constant 1:1:1 stoichiometry between M97, F55, and F40 in phosphorylase b3. This result suggests that we could be dealing with a specific proteolytic action that might have significance in vivo. To see if this is the case the availability of unnicked phosphorylase b1 is a valuable tool. Studies in the molecular characterization of both liver phosphorylase isoforms described in this paper are now in progress in our laboratory.

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