Purification and Characterization of the Glycogen-bound Protein Phosphatase from Rat Liver*

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Glycogen-bound protein phosphatase G from rat liver was transferred from glycogen to β-cyclodextrin (cycloheptaamylose) linked to Sepharose 6B. After removal of the catalytic subunit and of contaminating proteins with 2 M NaCl, elution with β-cyclodextrin yielded a single protein on native polyacrylamide gel electrophoresis and two polypeptides (161 and 54 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Several lines of evidence indicate that the latter polypeptides are subunits of the protein phosphatase G holoenzyme.

First, these polypeptides were also present, together with the catalytic subunit, in the extensively purified holoenzyme. Also, polyclonal antibodies against these polypeptides were able to bind the holoenzyme. Further, while bound to cyclodextrin-Sepharose, the polypeptides were able to recombine with separately purified type-1 (AMD) catalytic subunit, but not with type-2A (PCS) catalytic subunit.

The characteristics of the reconstituted enzyme resembled those of the nonpurified protein phosphatase G. At low dilutions, the spontaneous phosphorylase phosphatase activity of the reconstituted enzyme was about 10 times lower than that of the catalytic subunit, but it was about 1000-fold more resistant to inhibition by the modulator protein (inhibitor-2). In contrast with the free catalytic subunit, the reconstituted enzyme cosedimented with glycogen, and it was able to activate purified liver glycogen synthase b. Also, the synthase phosphatase activity was synergistically increased by a cytosolic phosphatase and inhibited by physiological concentrations of phosphorylase a and of Ca²⁺.

Type-1 or ATP,Mg-dependent protein phosphatases comprise a group of widely distributed enzymes that regulate various aspects of cellular metabolism (1–3). They share the same catalytic subunit and are specifically inhibited by two heat-stable proteins, termed inhibitor-1 and modulator protein or inhibitor-2. One particular type-1 protein phosphatase is tightly associated with glycogen. It is essential for the dephosphorylation of glycogen synthase and phosphorylase, the rate-limiting enzymes of glycogen metabolism (3, 4). The glycogen-bound phosphatase from rabbit skeletal muscle has been purified to homogeneity and is now fairly well characterized (3, 5). It is a heterodimer consisting of the 37-kDa catalytic subunit and a 161-kDa glycogen-binding "G-subunit" which contains three identified phosphorylation sites.

The corresponding liver enzyme, called protein phosphatase G (6), has not yet been isolated. Nevertheless, studies of the partially purified enzyme from rat liver provided evidence for a glycogen-binding subunit (6, 7) and possible additional subunits (8, 9) that should account for various regulatory properties. Since the free type-1 catalytic subunit cannot activate hepatic glycogen synthase b, protein phosphatase G must indeed contain a subunit that enables it to recognize glycogen synthase as a substrate (9). Also in the liver, but not in skeletal muscle, the synthase phosphatase activity of the glycogen-bound enzyme is allosterically inhibited by physiological concentrations of phosphorylase a (7, 8, 10) and of Ca²⁺ (11), and it acts synergistically with a cytosolic synthase phosphatase (12, 13). Specific for the liver enzyme is also the extreme resistance to inhibition by the modulator protein (9) and the dissociation of the catalytic subunit upon interaction with the modulator protein (14). Finally, compared to skeletal muscle, the phosphorylase-phosphatase activity of the liver enzyme is much more suppressed by interaction with a trypsin-sensitive noncatalytic subunit(s) (9, 15).

The purification of hepatic protein phosphatase G has been hampered by its spontaneous dissociation during chromatography (9, 10). We have now overcome this problem by the separate purification of the noncatalytic subunits and of the catalytic subunit, with subsequent recombination. The reconstituted enzyme contains three polypeptides and has the regulatory characteristics of the enzyme present in the protein-glycogen complex.

EXPERIMENTAL PROCEDURES

Materials and Buffer—Heparin-Sepharose CL-6B, CNBr-activated Sepharose 4B, and epoxy-activated Sepharose 6B were purchased from Pharmacia LKB Biotechnology Inc., nitrocellulose membranes from Du Pont-New England Nuclear, and (3-cyclodextrin from ICT (Frankfurt, Federal Republic of Germany). Peroxidase-labeled antibody IgG were obtained from DAKO-immunoglobulins (Copenhagen, Denmark). The modulator protein (16), the catalytic subunits of type-1 (17) and of type-2A protein phosphatases (18), as well as phosphorylase b (19) were purified from dog skeletal muscle. Phosphorylase was converted to the active a-form by purified muscle phosphorylase kinase (20). Glycogen synthase b and particulate glycogen were prepared from dog liver as described (12). Cyclodextrin-Sepharose and antibody-Sepharose were prepared according to the recommendations of Pharmacia. 0.3 g of β-cyclodextrin were dissolved in water at pH 13 and mixed with 1 g of epoxy-activated Sepharose 6B. Purified immunoglobulins (3 mg) were coupled to 1 g of CNBr-activated Sepharose 4B at pH 7.4.

The standard buffer contained 50 mM glycylglycine, 3 mM EGTA;

The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrito)]tetraacetic acid; PEG: polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
0.5 mM dithiothreitol, 5% glycerol, 2 mM mercaptoethanol, 0.5 mM benzamidine, and 0.3 mM phenylmethylsulfonyl fluoride, at pH 7.4, unless stated otherwise.

Preparation of Subcellular Liver Fractions—Overnight fasted Wistar rats of about 250 g were injected intraperitoneally with 0.3 mg of glucagon to induce maximal depletion of hepatic glycogen (6, 12). 50 min later, the animals were decapitated, and the livers were homogenized in a Potter-Elvehjem tube in 2 volumes of ice-cold standard buffer plus 0.25 M sucrose. The homogenate was centrifuged for 35 min at 230,000 × g. Particulate liver glycogen (5 mg/ml) was added to the resulting cytosol, and a second high speed centrifugation yielded the post-glycogen supernatant (used for the experiment shown in Fig. 7A) and a tiny glycogen pellet. This pellet was resuspended in standard buffer until the initial volume and recentrifuged. The washed glycogen pellet was the starting material for the purification of protein phosphatase G and of its noncatalytic subunits.

The concentration of the liver fractions is expressed with respect to the liver tissue from which they were derived; for example, a 1% cytosol corresponds to the cytosol of 1 mg of liver in a volume of 100 μl.

Purification of the Holoenzyme—It was found necessary to finish the purification in 1 day. The freshly prepared glycogen fraction from about 70 g of liver was resuspended in 10 ml of standard buffer at room temperature and at pH 7.0. The suspension was slowly applied (0.5 ml/min) to a 10-ml column of heparin-Sepharose, equilibrated in the same buffer, and the flow-through was immediately reapplied to the column. After recirculation of the sample for 30 min the column was moved to the cold room and rinsed with 30 ml of ice-cold standard buffer plus 50 mM NaCl. The phosphatase was eluted with standard buffer plus 600 mM NaCl. PEG was slowly added to the pooled fractions until a concentration of 10% (w/v) was reached, and 30 min later the flocculated proteins were sedimented (15 min at 15,000 × g). The pellet was dissolved in 1 ml of standard buffer and slowly applied (0.5 ml/min) to a 10-ml column of β-cyclodextrin-Sepharose. The column was washed with 30 ml of the buffer plus 50 mM NaCl, and the phosphatase was eluted with standard buffer containing 1 mg/ml of PEG. The fractions containing phosphatase activity were pooled and supplemented with PEG to a concentration of 25%. The mixture was kept at 0°C overnight, or for 30 min with more protein (0.1 mg/ml hemoglobin) was added. The precipitated proteins were collected by centrifugation (35 min at 220,000 × g), dissolved in 0.5 ml of standard buffer, and stored at −20°C.

Purification of the Noncatalytic Subunits—The washed glycogen pellet from 10 rat livers was resuspended in 5 ml of standard buffer at room temperature and applied (1 ml/min) to a 10-ml column of β-cyclodextrin-Sepharose, equilibrated in the same buffer. For 30 min the column was washed with the buffer until the supernatant was free of glycogen. After the washing step, the column was transferred to the cold room, washed first with 30 ml of ice-cold standard buffer plus 50 mM NaCl, and then with 30 ml of standard buffer plus 2 M NaCl to release the phosphatase-phosphatase activity. The noncatalytic subunits of protein phosphatase G were subsequently eluted with 30 ml of standard buffer containing β-cyclodextrin (1 mg/ml) and concentrated by dialysis against standard buffer plus 50% (w/v) PEG or by ultrafiltration (Amicon Centrprep 30).

Preparation of Reconstituted Protein Phosphatase G—Before elution from the β-cyclodextrin-Sepharose column (see above), the noncatalytic subunits of protein phosphatase G could be recombined with the type-1 catalytic subunit. For that purpose, the column was re-equilibrated with standard buffer after the wash procedure with 2 M NaCl. Homogeneously purified type-1 catalytic subunit (200–400 units in 1 ml of standard buffer) was slowly applied to the column (0.5 ml/min). The column was then washed with 30 ml of buffer plus 50 mM NaCl, and the reconstituted protein phosphatase G was eluted with standard buffer containing 1 mg/ml β-cyclodextrin. The fractions containing phosphorylase-phosphatase activity were pooled and concentrated as described above for the holoenzyme.

Polyclonal Antibodies—300 μg of the purified noncatalytic subunits, containing about equal amounts of the 161-kDa and 54-kDa polypeptides, were mixed with 1 ml of complete Freund’s adjuvant and injected subcutaneously into a goat. Additional injections of one-third volume of this mixture were given 3 and 6 weeks later. The immunoglobulin fraction was isolated (21) from blood collected before as well as after immunization. Western blotting was performed as described (21), except that the incubation with primary antiserum was done overnight at a final dilution of 1:2000.

Electrophoresis—Unless otherwise stated, SDS/PAGE (22) was performed on 10% slab gels. The apparent Mr, was calculated by comparison with the following standard proteins: myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The Phast system (Pharmacia) was used for native polyacrylamide gel electrophoresis. Silver staining (23) was done according to the protocol of Bio-Rad.

Assays—The synthease phosphatase activity was determined from the rate of activation of dog liver synthase b (12). One unit of synthease phosphatase activates 1 unit of glycogen synthase b per min at 25°C. Phosphorylase phosphatase (24) was assayed with 32P-labeled phosphorylase a as substrate on samples that were either used as such (spontaneous activity) or after preincubation with trypsin (0.1 mg/ml) for 5 min at 25°C (total activity). The proteolysis was arrested by the addition of soybean trypsin inhibitor (1 mg/ml). During trypticinolysis, the noncatalytic subunit(s) of protein phosphatase G are destroyed, resulting in the release of fully active catalytic subunit (9, 15). One unit of phosphorylase phosphatase dephosphorylates 1 unit of phosphorylase a per min at 25°C. Except when specified otherwise, the phosphatases were assayed at a concentration corresponding to a glycogen fraction of 10–30%. Glycogen synthase (12) and phosphorylase (25) were assayed as described. One unit of either enzyme converts 1 unit of substrate into product per min at 25°C. The amylo-1,6-glucosidase activity of debranching enzyme was assayed by the ability of the enzyme to incorporate 3H-labeled glucose into glycogen (26). Protein was measured with bovine serum albumin as a standard (27).

Results are means ± S.E. for the indicated number (n) of observations.

RESULTS

Purification of Intact Protein Phosphatase G—The holoenzyme was extensively purified from a freshly prepared protein-glycogen complex by successive affinity chromatographies on heparin-Sepharose and on β-cyclodextrin-Sepharose (see “Experimental Procedures”). Binding of the phosphatase to heparin-Sepharose was time- and temperature-dependent; it was optimal after recirculation during 30 min at room temperature (not shown). The purification was about 6-fold, and the recovery of the synthease phosphatase activity and of the total (i.e. trypsin-revealed) phosphorylase-phosphatase activity amounted to about 75% (Table I). At this stage, the spontaneous phosphorylase-phosphatase activity was more than 2-fold higher, and the activity was stimulated to a lesser extent by trypsin, probably as a result of a partial dissociation of the catalytic subunit from the inhibitory subunit(s). However, the stimulation by trypsin was partially restored after precipitation of the phosphatase with 10% PEG. The preparation was then applied to a column of β-cyclodextrin (cyclodeptaamylose) covalently linked to Sepharose 6B (Table I). About 20% of the phosphatase was retained and could be eluted with free β-cyclodextrin. Finally, the enzyme was precipitated with 25% PEG. Again, this concentration increased the stimulation of the phosphorylase-phosphatase activity by trypsin.

After overall recovery, estimated from the phosphorylase-phosphatase activity after trypsin, amounted to 3%. This represented a 30-fold purification over the initial protein-glycogen complex. The recovery of the synthease-phosphatase activity could not be correctly assessed because it is strongly inhibited by free β-cyclodextrin, a 50% inhibition being obtained at about 0.2 mg/ml. In contrast, even 1 mg/ml did not affect the spontaneous phosphorylase-phosphatase activity.

When subjected to SDS-PAGE, a crude glycogen fraction showed two major bands (Fig. 1A) corresponding to about 160 and 100 kDa, which probably represent debranching enzyme and phosphorylase. The 100-kDa band was also the major polypeptide after heparin-Sepharose (Fig. 1B). After heparin-Sepharose plus β-cyclodextrin-Sepharose, there were two major bands corresponding to molecular weights of
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If not indicated otherwise, the activities are expressed as a percentage of the activity in the glycogen fraction and represent the means for the indicated number (n) of preparations. The values between square brackets represent phosphatase activities in units/g liver.

Table I
Partial purification of the protein phosphatase G holoenzyme

| Phosphorylase phosphatase | Specific activity after trypsin | Synthase phosphatase | Protein |
|---------------------------|--------------------------------|----------------------|---------|
| Glycogen fraction (7)     | [5.0 ± 0.1]                     | 5.4 ± 1.2            | 0.25 ± 0.01 |
| Heparin-Sepharose (7)     | 100                            | 0.07                 | 100     |
| First PEG precipitation (4)| 218 ± 43                       | 2.0 ± 0.3            | 74 ± 10 |
| β-Cyclodextrin-Sepharose (4)| 76 ± 16                       | 3.9 ± 0.7            | 47 ± 14 |
| Second PEG precipitation (4)| 25 ± 2                        | 1.7 ± 0.2            | --      |
|                           |                                 | 2.9 ± 0.4            | --      |

*β-Cyclodextrin reacts with the Bradford reagent.

**β-Cyclodextrin inhibits synthase phosphatase (see text).**

![Fig. 1. SDS/PAGE of protein phosphatase G at different stages of purification. Washed glycogen fraction, lane A; after chromatography on heparin-Sepharose, lane B; and after subsequent chromatography on β-cyclodextrin-Sepharose, lanes C and D. All the gels were Coomassie-stained, and gel D was additionally silver-stained.](image)

161,000 and 54,000 (Fig. 1C). Additional silver staining revealed bands corresponding to 32,000 and 36,000, which probably represent the catalytic subunit (Fig. 1D). Other minor bands were usually present in the final preparations, e.g. bands of 105 kDa (lane C) and 132 kDa (lane D).

Efforts to achieve additional purification by gel filtration or ion exchange chromatography failed because the characteristics of the enzyme change upon dilution (7) and in the presence of salt (not shown). The most striking changes were a loss of the synthase phosphatase activity and an increase of the spontaneous phosphorylase-phosphatase activity. These changes result probably from dissociation of the catalytic subunit. For the same reason, it was not possible to identify directly the noncatalytic subunits of protein phosphatase G, since they failed to co-migrate with the catalytic subunit during native gel electrophoresis, gel filtration, and centrifugation in glycerol or sucrose gradients (not shown).

Reconstitution of Protein Phosphatase G—Because of these problems, we adopted a different strategy, aimed at the initial isolation of the noncatalytic subunits and their subsequent recombination with separately purified catalytic subunit. The high affinity of the noncatalytic subunits for β-cyclodextrin allowed us to obtain them essentially pure in one step, starting from a freshly prepared glycogen fraction which was recirculated through a column of β-cyclodextrin-Sepharose (see "Experimental Procedures"). None of the more abundant enzymes of glycogen metabolism, i.e. phosphorylase, debranching enzyme, and glycogen synthase, was retained on the column (not shown). However, about one-third (37 ± 6%; n = 4) of the total phosphorylase-phosphatase activity was retained and could be eluted with 2 M NaCl (Fig. 2). The eluted enzyme did not represent intact protein phosphatase G, since its phosphorylase-phosphatase activity was not stimulated by trypsin (Fig. 2). Furthermore, it did not display any synthase-phosphatase activity and could not be rebound to glycogen (not shown). It appeared that the noncatalytic subunits of protein phosphatase G were still present on the affinity column after the wash with 2 M NaCl; indeed, at this stage, free catalytic subunit (purified from rabbit skeletal muscle) could be applied and was quantitatively retained (Fig. 2). In contrast, no catalytic subunit was retained by freshly prepared β-cyclodextrin-Sepharose (not shown).

The reconstituted phosphatase could then be eluted with free β-cyclodextrin (Fig. 2). The phosphorylase-phosphatase activity of the freshly eluted enzyme was only marginally stimulated by preincubation with trypsin (1.6 ± 0.1-fold; n = 4), as compared to the enzyme in the initial protein-glycogen complex (5.3 ± 0.4-fold; n = 4). However, after precipitation with 25% PEG, the stimulation by trypsin was largely recovered (4.3 ± 0.7-fold; n = 4).

After the wash with 2 M NaCl, a small amount of protein could be eluted directly from the column with free β-cyclodextrin (not shown). As a mean, 1.2 ± 0.3 μg (n = 3) of this protein was obtained from 1 g of liver. Native gel electrophoresis indicated the presence of a single protein only (Fig. 3A). However, on SDS/PAGE, two polypeptides appeared (Fig. 3B), with molecular masses of 161 ± 3 and 54 ± 1 kDa (n = 5). The same picture was obtained when the column was eluted with 8 M urea instead of β-cyclodextrin (not shown). In addition to these polypeptides, a less abundant 36-kDa polypeptide was observed in a preparation where reconstitution had taken place with purified catalytic subunit from rabbit skeletal muscle, prior to elution with β-cyclodextrin (Fig. 3C). The latter polypeptide migrated identically with the free catalytic subunit that was used for the reconstitution (Fig. 3D).

Immunological Identification of the Noncatalytic Subunits—Since the polypeptides of 161 kDa and 54 kDa were able to recombine with free catalytic subunit, they represent presumably subunits of protein phosphatase G. However, such a recombination could not be obtained after the noncatalytic subunits had been eluted from the β-cyclodextrin-Sepharose, not even after prolonged incubation in the presence of PEG (not shown). In order to identify unequivocally the noncatalytic subunits, polyclonal antibodies were raised in a goat
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**FIG. 2.** Binding of protein phosphatase G to β-cyclodextrin-Sepharose. A liver glycogen fraction from 10 rats was slowly applied to 10 ml of β-cyclodextrin-Sepharose as described under "Experimental Procedures." The column was successively washed with 30 ml of standard buffer plus 50 mM NaCl and 30 ml of standard buffer plus 2 M NaCl. After re-equilibration of the column in regular standard buffer, about 300 units of purified type-1 phosphatase catalytic subunit were applied, followed by standard buffer plus 50 mM NaCl. The column was then eluted with standard buffer plus 1 mg/ml β-cyclodextrin. Fractions of 3 ml were collected and assayed for spontaneous (○) and total (●) phosphorylase-phosphatase activity.

**FIG. 3.** Electrophoresis of the noncatalytic subunits of protein phosphatase G before and after reconstitution with the catalytic subunit. The noncatalytic subunits were prepared by chromatography of a glycogen fraction on β-cyclodextrin-Sepharose as described under "Experimental Procedures." The cyclodextrin eluate was concentrated and submitted to native (lane A) or denaturing (lane B) polyacrylamide gel electrophoresis. Lane C shows a SDS/PAGE of a sample that was reconstituted with type-1 catalytic subunit prior to elution with β-cyclodextrin, as shown in Fig. 1. Lane D shows a SDS/PAGE of the catalytic subunit that was used for recombination. The gels were successively stained with Coomassie Brilliant blue and with silver.

**FIG. 4.** Western blots of the noncatalytic subunits of protein phosphatase G. Lane A, the proteins in a washed glycogen fraction were separated by SDS/PAGE (7.5%), transferred to nitrocellulose, and immunostained with antibodies raised against a mixture of the 161-kDa and 54-kDa polypeptides. Lanes B and C, the purified noncatalytic subunits of protein phosphatase G were similarly treated and stained (lane B) or immunostained with an IgG preparation depleted of antibodies against the 161-kDa polypeptide (lane C). Lane D shows a Coomassie-stained SDS/PAGE of the protein that was transferred from a washed glycogen fraction to an antibody-Sepharose column and subsequently eluted with 3 M KSCN.

Western blotting showed that the purified immunoglobulin fraction of the antiserum recognized either polypeptide in a crude glycogen fraction (Fig. 4A), as well as after purification on β-cyclodextrin-Sepharose (Fig. 4B). In the protein-glycogen complex there was sometimes also a minor reaction with polypeptides of 105 and 66 kDa. The 105-kDa polypeptide may represent a proteolytic degradation product of the 161-kDa polypeptide, since its concentration increased with the age of the protein-glycogen complex (not shown). An important point is that the 54-kDa band did not originate from the 161-kDa polypeptide; indeed, a reaction of the smaller polypeptide still occurred with an immunoglobulin fraction that had been depleted of antibodies against the 161-kDa polypeptide (Fig. 4C).

The polyclonal antibodies have allowed us to identify directly the 161-kDa and 54-kDa polypeptides as subunits of the native protein phosphatase G. Upon passage of a protein-glycogen complex through a column with covalently bound antibodies, 75% of the phosphorylase-phosphatase activity was retained (not shown), together with the 161-kDa and 54-kDa polypeptides (Fig. 4D). In addition, a protein of 105 kDa was sometimes bound. Similar results were obtained with protein phosphatase G purified on heparin-Sepharose (not shown). In contrast, no binding of phosphorylase phosphatase occurred after preincubation of the holoenzyme with trypsin. This confirms that the binding of the phosphorylase phosphatase activity to the antibody-Sepharose was mediated by subunit(s) other than the catalytic subunit.

In agreement with the data illustrated in Fig. 2, a β-cyclodextrin-Sepharose column with bound 161-kDa and 54-kDa polypeptides was able to retain free catalytic subunit (Fig. 5). In contrast, when the column was first saturated with the polyclonal antibodies, its capacity to bind catalytic subunit was largely lost (Fig. 5). Since the antibodies were not...
able to dissociate the 161-kDa or the 54-kDa subunit from the affinity column (not illustrated), this result strongly indicates that the reconstitution with the catalytic subunit results from a specific interaction with one or either polypeptide. The specificity of the recognition is also illustrated by the fact that the type-2A catalytic subunit was not at all retained on the column that contained the noncatalytic subunits (not shown).

**Binding of Protein Phosphatase G to Glycogen**—In this and the following sections, the enzymatic characteristics of the purified protein phosphatase G are described. All the experiments were performed with the extensively purified holoenzyme (cf. Fig. 1D) and with the homogeneous reconstituted enzyme (cf. Fig. 3C). Since no differences were found between the preparations, only the characteristics of the latter are illustrated.

In contrast with the catalytic subunit, the reconstituted protein phosphatase G co-sedimented with added particulate glycogen (Table II). However, one-third of the phosphatase activity did not bind, although the adopted glycogen concentration should be saturating (6). This feature can be largely explained by competition between glycogen and β-cyclodextrin that contaminated the phosphatase preparation; indeed, renewed addition of glycogen caused again 60% of the unbound enzyme to sediment (Table II).

Incubation of the glycogen-associated protein phosphatase from rabbit skeletal muscle with cAMP-dependent protein kinase results in phosphorylation of the G-subunit at 2 serine residues, and the phosphorylation of site 2 parallels the release of the catalytic subunit (28). However, in similar conditions, this protein kinase failed to phosphorylate either noncatalytic subunit of the reconstituted rat liver enzyme (not shown).

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**TABLE II**

| Glycogen Centrifugation | Phosphorylase phosphatase after trypsin |
|-------------------------|---------------------------------------|
| mg/ml                   | % total recovered activity             |
| 0                       | First 89 ± 4                           |
| 50                      | First 37 ± 10                           |
| 50                      | Second 40 ± 10                          |

Effect of Dilution and of a Preincubation with Trypsin—The specific phosphorylase-phosphatase activity of the reconstituted enzyme increased 8-fold when the concentrated preparation was diluted 250-fold. At the latter dilution, the spontaneous phosphorylase-phosphatase activity reached 80% of the trypsin-revealed activity. These properties indicate a trypsin-sensitive inhibition, conferred by one or either noncatalytic subunit.

**Inhibition by the Modulator Protein**—Protein phosphatase G differs from other type-1 protein phosphatases by its extreme resistance to inhibition by the modulator protein (9). Fig. 6 illustrates the difference in sensitivity between the reconstituted enzyme and the free catalytic subunit, obtained by treatment of the former enzyme with trypsin. At the lowest concentration of modulator (0.6 nM), the reconstituted enzyme and the free catalytic subunit were inhibited by about 40% and 60%, respectively. This may indicate that the reconstituted enzyme contained some free catalytic subunit or an incompletely reconstituted species. However, at higher modulator concentrations, the resistance of the reconstituted enzyme became increasingly apparent; e.g. there was a 1000-fold difference in the modulator concentration required to achieve a 70% inhibition. This corresponds closely to the modulator resistance of the native glycogen-bound phosphatase G (9).
Synthase-Phosphatase Activity—When matched for the same spontaneous phosphorylase-phosphatase activity, the synthase-phosphatase activity of purified protein phosphatase G was only 24 ± 4% (n = 3) of the activity in a crude 30% protein-glycogen fraction. Nevertheless, as illustrated in Fig. 7, the reconstituted enzyme possessed the prominent regulatory properties of crude synthase phosphatase G that were earlier described by this laboratory (see introduction to the text).

It was shown that the rate of activation of hepatic glycogen synthase by a diluted protein-glycogen complex was synergistically increased by a cytosolic phosphatase (12). This cooperative effect was also observed with purified protein phosphatase G (Fig. 7A). A possible explanation for this synergism is that the activation of hepatic glycogen synthase requires the dephosphorylation of distinct sites that are specifically recognized by protein phosphatase G and by a cytosolic protein phosphatase (13).

In the presence of small concentrations of glycogen and salt, phosphorylase a is a potent allosteric inhibitor of the glycogen-associated synthase phosphatase from liver (6-8, 10). Likewise, the addition of phosphorylase a induced a lag period before the reconstituted protein phosphatase G started to activate purified liver synthase b (Fig. 7B). This lag was apparent with as little as 0.1 unit/ml (40 nM) phosphorylase a, and its length increased with higher concentrations. It has been shown earlier that the lag period corresponds to the time required to convert phosphorylase a into the noninhibitory b-form (10).

Crude synthase phosphatase G is inhibited by micromolar concentrations of Ca²⁺, especially in the presence of glycogen (11). Fig. 7C shows that 0.3 µM free Ca²⁺ inhibited the synthase phosphatase activity of the reconstituted protein phosphatase G by more than 50% in the presence of 10 mg/ml glycogen. The magnitude of the inhibition by Ca²⁺ increased further with higher glycogen concentrations (not shown), in agreement with earlier data on the crude enzyme (11).

**DISCUSSION**

**Purification of Protein Phosphatase G**—Purification of the holoenzyme from the glycogen fraction on heparin-Sepharose and β-cyclodextrin-Sepharose gave a yield of about 3% (Table I). This preparation was not homogeneous (Fig. 1, C and D), but various trials to purify the holoenzyme beyond the latter stage ended invariably with extremely low recoveries, apparently due to dissociation. The problem was finally overcome by the one-step purification of the noncatalytic subunits on β-cyclodextrin-Sepharose and subsequent recombination with separately purified catalytic subunit (Fig. 2), which yielded homogeneous reconstituted enzyme (Fig. 3C) with an apparent recovery of 6–10%. Table II supports the assumption that the transfer of protein phosphatase G from glycogen to β-cyclodextrin involves competition for the glycogen-binding site.

**Identification of the Noncatalytic Subunits**—The 161-kDa and 54-kDa polypeptides behave like noncatalytic subunits of protein phosphatase G. The two polypeptides co-migrated during native gel electrophoresis (Fig. 3A), and they were also the prominent polypeptides in the extensively purified holoenzyme (Fig. 1, C and D). The type-1 catalytic subunit (but not type-2A) could be recombined with these noncatalytic subunits (Fig. 2), but the recombination was prevented by antibodies against the polypeptides (Fig. 5). These antibodies were also able to bind the protein phosphatase G holoenzyme.

**Role of the Subunits**—The reconstituted protein phosphatase G and the extensively purified holoenzyme displayed the general regulatory properties that are characteristic of the enzyme in a crude glycogen fraction. Therefore, one or either noncatalytic subunit confers to the catalytic subunit a series of properties, including affinity for glycogen (Table II), resistance to inhibition by modulator (Fig. 6), activation by trypsin, and recognition of glycogen synthase as substrate.
The synthase-phosphatase activity furthermore displayed regulatory properties (Fig. 7) which are specific for hepatic phosphatase G. Since the reconstitution of the holoenzyme was performed with the catalytic subunit from skeletal muscle, the different properties of the holoenzymes from muscle and liver cannot be conferred by the catalytic subunit. This confirms the conclusion reached by Cohen et al. (29) on the basis of the identity of cDNA clones encoding the catalytic subunits from rabbit liver and skeletal muscle.

Since the glycogen-bound protein phosphatase from rabbit skeletal muscle contains only the catalytic subunit and a 161-kDa G-subunit, it is highly probable that the glycogen binding of hepatic protein phosphatase G is mediated by the 161-kDa subunit. It may be recalled that the G-subunit from skeletal muscle was initially described as a 103-kDa polypeptide (31), our purification procedure also allows one to isolate intact 161-kDa subunit from rat liver (Fig. 4). Since current preparations of holoenzyme from skeletal muscle contain both 161-kDa and 103-kDa polypeptides (31), affinity chromatography on β-cyclodextrin-Sepharose may be useful in obtaining undegraded enzyme from skeletal muscle as well.

It has not been possible yet to separate the 161-kDa subunit from the 54-kDa polypeptide in nondenaturing conditions. Thus, we can only conclude with certainty that the distinctive regulatory properties of hepatic protein phosphatase G must reside in one or the other polypeptide. Further work is required to establish their exact functions.

Acknowledgments—M. Evens and N. Sente provided expert technical assistance.

Note Added in Proof—The sequence of a tryptic peptide from the 54-kDa polypeptide has been determined by Dr. J. Vandekerckhove (Gent, Belgium) and proved identical with the cDNA-derived sequence 286-297 of the α-amylase precursor from rat pancreas (MacDonald, R. J., Crerar, M. M., Swain, W. F., Pictet, R. L., Thomas, G., and Rutter, W. J. (1980) Nature 287, 117–122). Antibodies raised against pancreatic α-amylase bind to the 54-kDa polypeptide, and α-amylase activity can be demonstrated in the complex of 161+54 kDa. It remains to be established whether or not α-amylase is a regulatory subunit of protein phosphatase G.

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