The modulator protein dissociates the catalytic subunit of hepatic protein phosphatase G from glycogen

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1. The phosphorylase phosphatase and glycogen-synthase phosphatase activities associated with the glycogen particles from rat liver were progressively inhibited by incubation with modulator protein. However, the phosphorylase phosphatase activity of the catalytic subunit was entirely recovered after destruction of the modulator and the regulatory subunit(s) by trypsin. 2. Inhibition of protein phosphatase G by modulator was associated with a translocation of the phosphorylase phosphatase activity (measured after incubation with trypsin) from glycogen to the soluble fraction. The degree of inhibition of phosphatase G corresponded closely to the extent to which the phosphorylase phosphatase activity was released from the glycogen particles. Incubation of glycogen-free protein phosphatase G with modulator did not change the affinity of the enzyme for added glycogen, but decreased the amount of phosphatase that could be bound to glycogen. 3. The phosphorylase phosphatase activity that was released from the glycogen particles by modulator migrated on gel filtration as a complex (M_r 106000) of the catalytic subunit with modulator. Phosphorylase phosphatase activity could be transferred from glycogen-bound protein phosphatase G to modulator that was covalently bound to Sepharose. After elution from the column, the enzyme was identified as the free catalytic subunit (M_r 37000).

INTRODUCTION

Protein phosphatases have been classified on the basis of their substrate specificities and the effect of certain stimulators and inhibitors [1,2]. Type-1 (ATPMg-dependent) phosphatases have the unique characteristic of being inhibited by two heat-stable trypsin-sensitive proteins, called inhibitor-1 and modulator (or inhibitor-2). All described type-1 phosphatases share the same catalytic subunit, of M_r 37000. The activity of this subunit is little affected by limited proteolysis [3,4]. In contrast, the other subunit(s) are destroyed by trypsin. This results in a markedly increased phosphorylase phosphatase activity and a loss of synthase phosphatase activity [4,5].

In the liver, the glycogen-bound protein phosphatase plays a major role in the dephosphorylation of glycogen synthase and phosphorylase [4–6]. We have previously referred to this enzyme as the G-component of glycogen-synthase phosphatase, and recently renamed it 'protein phosphatase G' [7]. It is under tight hormonal and metabolic control (see [8]), and binds to glycogen with an unusually high affinity [7]. Protein phosphatase G is a type-1 phosphatase, since it can be completely inhibited by the modulator protein. However, the inhibition characteristics are quite different from those of the free catalytic subunit. Indeed, the inhibition of phosphatase G is time-dependent [4], and it requires an up to 1000-fold higher concentration of modulator [5].

Hepatic protein phosphatase G has not been purified to homogeneity. In contrast, the glycogen-bound enzyme from skeletal muscle has been purified and characterized. It consists of the catalytic subunit and a glycogen-binding subunit [9]. Phosphorylation of the latter promotes the release of the enzyme from glycogen [10]. We report here that the modulator protein binds to the catalytic subunit of the hepatic protein phosphatase G and that the complex dissociates from the glycogen particles.

EXPERIMENTAL

Materials and buffer

Hepatic particulate glycogen [11] and homogeneous modulator protein [12] were purified as described in the references. The catalytic subunit of protein phosphatase G was prepared from the glycogen fraction of dog liver [13]. CNBr-activated Sepharose 4B was purchased from Pharmacia LKB. The α -amylase inhibitor Bay e 4609 was a gift from Bayer A.G. The source of other relevant biochemicals has been previously reported [14].

The buffer used throughout this work contained 50 mM-imidazole, 0.5 mM-dithiothreitol, 0.25 M-sucrose, 1 mM-magnesium acetate, 0.5 mM-benzamidine, 0.3 mM-phenylmethanesulphonyl fluoride and 3 mM-EGTA, and was adjusted at room temperature to pH 7.4.

Preparation of liver subcellular fractions

The post-microsomal supernatant and the glycogen fraction were prepared from glycogen-depleted rat livers as described [14], except for the use of a more complex buffer (see above) and for the homogenization of the liver in only 1 vol. of buffer. Briefly, to the post-microsomal supernatant was added 0.5% particulate glycogen, before centrifugation at high speed, which separated the glycogen fraction from the supernatant. For the sedimentation of glycogen in small samples, a Beckman Airfuge was used (10 min at 122000 $g_{av.}$ at room temperature).

Chromatographic procedures

Purified modulator (2 mg) was covalently bound to 10 ml of CNBr-activated Sepharose 4B according to the recommendations of Pharmacia LKB. The glycogen fraction from one rat liver was dissolved in 2 ml of standard buffer and applied to the modulator–Sepharose column, equilibrated in the same buffer. The effluent was recirculated through the column at room temperature for

Fig. 1. Effect of modulator on the activity of protein phosphatase G

A glycogen fraction (150 %) was incubated at 25 °C with the indicated concentrations of modulator. At the indicated times, samples were taken for the assay of phosphorylase phosphatase before (a) or after (b) incubation with trypsin. During the assay the glycogen fraction was diluted 10-fold (a) or 50-fold (b). Results are expressed as a percentage of the spontaneous activity in the control condition at 0 min.

30 min at 0.7 ml/min. The column was then washed with 20 ml of standard buffer before the retained phosphatase (about 50 % of the total applied phosphorylase phosphatase activity) was eluted with 1 M-NaCl in the buffer. The active fractions were pooled and dialysed against standard buffer containing 0.1 M-NaCl and 1 % propan-2-ol, before gel filtration (see below). The affinity column retained almost no phosphatase when it was operated in the cold-room. This may be explained by the slow interaction of modulator with protein phosphatase G, even at 25 °C (see the Results section).

Gel filtration was performed on a Superose 12 column (30 cm × 1 cm) connected to a fast-protein-liquid-chromatography system (Pharmacia LKB). Samples and marker proteins were applied in 0.2 ml of standard buffer containing also 0.1 M-NaCl and 1 % propan-2-ol, and eluted with the same solution at 0.3 ml/min. The column was calibrated with ferritin (M_r 440 000), catalase (232 000), aldolase (158 000), bovine serum albumin (67 000), ovalbumin (45 500) and soya-bean trypsin inhibitor (20 000). The linear relationship between elution time and $\log M_r$ of the markers yielded a correlation coefficient $r = 0.994$.

Assays

The synthase phosphatase activity was calculated from the rate of activation of purified dog liver synthase b_2/b_3 [14]. Phosphorylase phosphatase was measured with ^{32}P -labelled phosphorylase a as substrate [15]; the assays were performed as such or on samples that had been preincubated with trypsin (2 mg/ml) for 20 min at 25 °C, followed by addition of soya-bean trypsin inhibitor (5 mg/ml). Trypsin-treated samples were always assayed at a 5-fold lower concentration than were the non-treated samples.

The concentrations of the liver fractions during incubations and assays are expressed relative to the liver from which they were prepared; e.g. a glycogen fraction (150 %) means that the enzyme-glycogen complex from 1.5 g of liver was dissolved in a final volume of 1 ml.

RESULTS

Characteristics of the inhibition of phosphatase G by modulator

The phosphorylase phosphatase activity in a protein-glycogen complex from rat liver remained essentially constant during incubation for 30 min at 25 °C (Fig. 1a). However, in the presence of modulator the activity was gradually lost. The final extent of inhibition was dependent on the concentration of modulator. Some 8 μM -modulator was required to obtain a final inhibition of 80 %. The high concentration of the protein-glycogen complex (150 %) in the present experiment explains the low sensitivity of phosphatase G to modulator. Indeed, the modulator-sensitivity of protein phosphatase G increases considerably with dilution, presumably as a result of dissociation of the enzyme [5].

Incubation of the protein-glycogen complex with trypsin increased the phosphorylase phosphatase activity about 5-fold (Fig. 1b). When the protein-glycogen complex had been preincubated with modulator, the treatment with trypsin increased the phosphorylase phosphatase activity to the same final value.

Translocation of phosphorylase phosphatase activity by modulator

Fig. 1 illustrated that the activity of phosphorylase phosphatase G remained stable during incubation for up

Table 1. Effect of modulator on the binding of phosphatase G to glycogen

A glycogen fraction (150%) was incubated either as such or in the presence of 6 μM -modulator. Also included was 0.1 mg of Bay e 4609/ml, in order to prevent α -amylolytic breakdown of glycogen. After 20 min at 25 °C a sample was removed for the assay of phosphorylase phosphatase ('total fraction'), and the remainder was centrifuged at high speed, yielding a glycogen 'pellet' and a 'supernatant'. The pellet was resuspended in the standard buffer. The final concentrations of the fractions in the phosphorylase phosphatase assays were 15% and 3% respectively, before and after treatment with trypsin. The results are the means of two experiments; they are expressed as a percentage of the 'total' spontaneous activity in the absence of modulator.

Part of glycogen fraction	Preincubation with modulator	Phosphorylase phosphatase activity (%)	
		Spontaneous	After trypsin
Total	—	100	450
	+	31	452
Supernatant	—	12	62
	+	4	327
Pellet	—	82	304
	+	17	89

to 30 min at 25 °C. The data in Table 1 show, moreover, that during subsequent high-speed centrifugation about 85% of the recovered enzyme activity (before as well as after treatment with trypsin) remained associated with glycogen. The distribution of the activity was also

measured after preincubation of the protein-glycogen complex with an amount of modulator that had inhibited the phosphatase by 70% (Table 1). When the assays were performed without trypsin pretreatment, the major part of the residual activity co-sedimented with the glycogen. However, after abolition of the inhibition by trypsin, most of the phosphatase activity was recovered in the soluble fraction. This indicates that during incubation with modulator the catalytic activity had been dissociated from the glycogen particles. Assay of synthase phosphatase yielded similar results, inasmuch as the spontaneous activity is concerned (not shown). However, the complete destruction of the synthase phosphatase activity by trypsin [5] precluded further investigation.

The relationship between the inhibition of phosphatase G by modulator and the translocation of the phosphorylase phosphatase activity from the glycogen particles to the soluble fraction is illustrated in Fig. 2. Clearly (Fig. 2a) the activity in the soluble fraction (as revealed by trypsin treatment) increased linearly with the degree of inhibition of phosphatase G (as shown by the decrease in spontaneous activity before centrifugation). Conversely, the more the enzyme was inhibited, the less activity remained associated with the glycogen particles (Fig. 2b).

Effect of modulator on the affinity of phosphatase G for glycogen

In the liver of an overnight-starved glucagon-treated rat, protein phosphatase G is essentially cytosolic [7]. However, the phosphatase binds to added glycogen, and the complex can be easily sedimented. The effect of the glycogen concentration on this process of association is illustrated in Fig. 3. About 0.1 mg of glycogen/ml was

Fig. 2. Correlation between the degree of inhibition of phosphatase G by modulator and its translocation from glycogen to the soluble fraction

Three preparations of enzyme-glycogen complex were incubated and assayed as described in the legend to Table 1, except for the use of various concentrations of modulator (0–9 μM). The ordinate shows the spontaneous phosphorylase phosphatase activity after incubation with modulator (filled and half-filled symbols), expressed as a percentage of the activity recorded after incubation without modulator (open symbols). These percentages are individually related on the abscissa to the amounts of phosphorylase phosphatase (measured after preincubation with trypsin) recovered in the supernatant (a) and the pellet (b) after high-speed centrifugation. The latter activities are expressed as a percentage of the total trypsin-revealed activity before centrifugation.

Fig. 3. Effect of incubation of a post-microsomal supernatant with modulator on the subsequent binding of phosphatase G to glycogen

A cytosolic fraction from a glycogen-free liver, at a final concentration of 35 %, was incubated for 20 min at 25 °C in the presence of the indicated concentrations of modulator. Samples were then mixed with Bay e 4609 (0.1 mg/ml) and with glycogen to the indicated final concentrations, and centrifuged at high speed. The glycogen pellets were resuspended in standard buffer and assayed at a final concentration of 7 % for spontaneous phosphorylase phosphatase activity. The results are expressed as a percentage of the phosphorylase phosphatase activity recovered in the pellet at the highest glycogen concentration without modulator.

sufficient for half-maximal binding of phosphorylase phosphatase G, in good agreement with a previous report on the binding of synthase phosphatase G to glycogen [7]. After preincubation with various concentrations of modulator, the affinity of the protein phosphatase G for glycogen was unchanged, but the amount of enzyme that could be sedimented at saturating concentrations of glycogen (above 0.5 mg/ml) was progressively decreased and became ultimately non-existent (Fig. 3). Similar results were obtained when the samples were treated with trypsin before the assay, or when synthase phosphatase activity was measured (not shown). We conclude from these results that interaction with the modulator causes phosphatase G to lose completely its capacity to bind to glycogen.

Molecular size of the translocated phosphorylase phosphatase

By means of gel filtration we have investigated whether it is the holoenzyme or only a part of protein phosphatase G that is released by modulator from the glycogen particles. As shown in Fig. 4, the elution profile of the released enzyme coincided with that of purified catalytic subunit that had been complexed with modulator. The

Fig. 4. Gel filtration on Superose 12 of phosphorylase phosphatase released from glycogen by interaction with the modulator

The purified catalytic subunit (Δ) and the enzyme that had been transferred from the enzyme-glycogen complex to modulator-Sepharose ($\blacktriangle$) were assayed in the column fractions without further treatment. In the other experiments the activity was determined after preincubation of the fractions with trypsin: $\bullet$, enzyme released from the enzyme-glycogen complex by incubation for 20 min with modulator (6 μ M); $\circ$, purified catalytic subunit (10 pmol), after incubation for 5 min with a large excess (250 nmol) of modulator. The thin arrows locate the elution positions of the marker proteins ($M_r \times 10^{-3}$). The bold arrows show the calculated apparent M_r values of the protein phosphatases.

apparent M_r of the latter complex (106000) is higher than a previous estimate (87000) of that of the ATPMg-dependent enzyme-modulator complex from skeletal muscle [16].

The phosphorylase phosphatase activity could be transferred from the glycogen particles to modulator that was covalently bound to Sepharose (see the Experimental section). After elution from the affinity column with NaCl, the migration of the enzyme on Superose 12 was identical with that of the purified catalytic subunit, with an apparent M_r of 37000 (Fig. 4).

These results show that the catalytic subunit is displaced from the holoenzyme upon interaction with modulator. However, this does not exclude that other subunits may also be released from the glycogen particles.

DISCUSSION

Our results show that the modulator protein not only inhibits protein phosphatase G, but it disrupts the

subunit structure of the enzyme and causes the release of catalytic subunit bound to modulator. The latter point stands in contrast with the findings by Strålfors *et al.* [9]. They reported that the glycogen-bound phosphatase from skeletal muscle was eluted from a Sephadex G-100 column with an increased molecular size after interaction with modulator. Their observations argue against a dissociation of the muscle enzyme. This discrepancy may be explained by the important structural differences between the liver and muscle enzymes [4,17]. Subunits of the hepatic enzyme dissociate readily upon dilution [5].

The present work has only been possible because under our experimental conditions the inhibition of phosphatase G by modulator was reversed by trypsin. This means that the catalytic subunit was not converted into an inactive conformation, which requires ATPMg and the kinase F_A for re-activation (see [18] for a detailed discussion). Yet such an inactivation has been described for the purified glycogen-bound phosphatase from skeletal muscle [9] and for partially purified liver enzyme [19]. The reason for this discrepancy requires investigation.

Because of the rather low sensitivity of protein phosphatase G to inhibition and translocation by modulator, it will be important to obtain information on the concentration of modulator in the liver. In skeletal muscle its concentration has been estimated at $0.35 \mu\text{M}$ [20]. But most of the modulator appears to be associated with other polypeptides, and unavailable for inhibition of phosphatases [21]. Therefore protein phosphatase G, as it is present in the liver, could be largely protected from modulator. Yet it cannot be excluded that under other conditions the phosphatase might become sensitive to physiological concentrations of modulator. Hiraga & Cohen [10] have shown that phosphorylation of the muscle enzyme promotes the release of the phosphatase from glycogen into the cytosol, a process which in turn may facilitate the inactivation of the enzyme by inhibitor-1. However, we have been unable until now to find evidence for an altered sensitivity to modulator, or a different affinity for glycogen, after incubation of hepatic protein phosphatase G with cyclic-AMP-dependent protein kinase in phosphorylation conditions.

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