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Native cytosolic protein phosphatase-1 (PP-1S) containing modulator (inhibitor-2) is an active enzyme

Mathieu Bollen[a,*], Anna A. DePaoli-Roach[b], Willy Stalmans[a]

[a]*Afdeling Biochemie, Fakulteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*
[b]*Department of Biochemistry and Molecular Biology, Indiana University School of Medecine, Indianapolis, IN 46202-5122, USA*

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Abstract

In vitro, the modulator protein (inhibitor-2) slowly converts the catalytic subunit of protein phosphatase-1 (PP-1_C) into an inactive 'MgATP-dependent form' that can be reactivated by the transient phosphorylation of modulator with GSK-3/F_A. We report here that this modulator-induced inactivation of PP-1_C can be blocked by addition (at pH 7.5) of either 0.3 mM NaF or 150 mM NaCl, or by raising the pH to 8.5. Making use of a combination of the latter conditions, we have partially purified a soluble modulator-associated form of PP-1 (PP-1S) from rabbit skeletal muscle as a spontaneously active enzyme that cannot be further activated by kinase GSK-3/F_A. These observations argue against a role for the 'MgATP-dependent' form of PP-1S as an inactive reservoir of PP-1_C. PP-1S was separated on aminohexyl Sepharose from another active, cytosolic species of PP-1, which appears to be a proteolytic product of the glycogen-bound PP-1G.

Key words: Protein phosphatase; Inhibitor-2; Chaperone; Skeletal muscle

1. Introduction

Within the rapidly expanding group of Ser/Thr-specific protein phosphatases, the type-1 enzymes (PP-1) are easily identified by the unique structure and enzymic properties of their catalytic subunit (reviewed in [1]). For example, only the catalytic subunit of PP-1 is inhibited by specific cytoplasmic (inhibitor-1 and inhibitor-2 or modulator) and nuclear (NIPP-1) polypeptides [1,2].

The type-1 catalytic subunit (PP-1_C) appears to contain two binding sites for the modulator protein [1]. Binding of modulator to a low-affinity binding site causes an instantaneous inhibition of the catalytic subunit, while the association of modulator with a high-affinity binding site results in a slow ($t_{1/2}$ of 10–30 min at 30°C) transition of the catalytic subunit into an inactive conformation. One can differentiate between inhibition and inactivation of the catalytic subunit by appropriate phosphatase assays: inhibition is released upon destruction of modulator by trypsin, which removes also the carboxyterminal part of the catalytic subunit with little effect on its activity. On the other hand, the inactive 'MgATP-dependent' phosphatase, consisting of modulator and inactive PP-1_C, can only be reactivated either by trypsin in the presence of Mn^{2+} or by phosphorylation of modulator with GSK-3/F_A.

The 'MgATP-dependent' phosphatase has also been purified from muscle and brain cytosol [1]. Combined with findings that some isoforms of PP-1_C are produced in bacteria in their inactive conformation, these data have been taken as evidence that the association of inactive PP-1_C with modulator represents an intermediate step in the generation of different PP-1 holoenzymes [3]. After reactivation by GSK-3/F_A, the catalytic subunit would then become available for association with other targeting subunits of PP-1.

The above hypothesis implies that the cytosolic (soluble) species of PP-1, which we termed PP-1S [1], is at least partially present in its inactive, MgATP-dependent form in vivo. Previous studies have indicated that inactive enzyme is indeed present to a measurable extent in muscle extracts [4,5]. However, since PP-1S is gradually inactivated after tissue homogenization [6–8], it is difficult to assess the amount of PP-1S in its MgATP-dependent form in vivo. A detailed study of agents and conditions that interfere with the modulator-induced inactivation of purified PP-1_C has enabled us to select homogenization conditions that should virtually freeze the modulator-associated phosphatase in its native state of activation. Using these tools, we were unable to detect a cytosolic pool of inactive, MgATP-dependent protein phosphatase in skeletal muscle. The modulator protein emerges from our studies as a (noninhibitory) subunit of a spontaneously active phosphatase.

*Corresponding author. Fax: (32) (16) 345995.

Abbreviations: GSK-3/F_A, glycogen synthase kinase-3, also termed protein kinase F_A; PP-1, protein phosphatase-1; PP-1S, soluble (cytosolic) PP-1; PP-1G, glycogen-bound protein phosphatase-1; PP-1_C, catalytic subunit of protein phosphatase-1.

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2. Experimental

2.1. Materials and assays

The catalytic subunit of PP-1 [9], modulator [10] and GSK-3/F_A [11] were purified from rabbit skeletal muscle. Phosphorylase, phosphorylase kinase and particulate glycogen were prepared as described previously [12]. Antibodies against rabbit skeletal muscle modulator were raised in a goat [13]. A synthetic peptide encompassing the protein kinase A phosphorylation site of the G-subunit of PP-1G (R_{Gi}) was used to raise antibodies in guinea pigs [14].

Buffer A contained 50 mM Tris (pH 8.0 at 4°C), 0.5 mM dithiothreitol, 15 mM 2-mercaptoethanol, 0.3 mM NaF and 100 mM NaCl. Buffer B contained 50 mM Tris (pH 7.0 at 4°C), 0.5 mM dithiothreitol and 15 mM 2-mercaptoethanol.

The phosphorylase phosphatase activity was measured either as such ('spontaneous' activity) or after preincubation with trypsin, which reveals also the activity of catalytic subunit that is inhibited by modulator or other subunits; or still after a preincubation with GSK-3/F_A followed by a trypsin treatment, which reveals, in addition, the activity of the MgATP-dependent enzyme [15].

2.2. Partial purification of cytosolic phosphorylase phosphatase

About 500 g of muscle from the back and hind legs of a rabbit were homogenized in 3 volumes of either buffer A or buffer B, containing in addition 250 mM sucrose, 0.5 mM benzamidine, 0.3 mM phenylmethanesulfonyl fluoride, 0.3 mM EGTA, 1 mM EDTA, 0.1 mM L-1-4′-tosylamino-2-phenylethyl-chloromethyl ketone, 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanonehydrochloride, 4 μg/ml leupeptin and 4 μg/ml pepstatin. Successive centrifugations at 8,000 × *g* (10 min) and 140,000 × *g* (60 min) yielded a 'cytosolic' fraction, which was stirred for 60 min at 4°C with 200 ml DEAE Sephadex A50, equilibrated with either buffer A or B. After washing with 5 vols. buffer on a glass funnel, the gel was poured into a column. All the cytosolic phosphorylase phosphatase activity was retained by the resin and was subsequently eluted with a linear 800-ml gradient of 0–0.5 M NaCl in either buffer A or buffer B. The phosphatase-containing fractions were pooled, diluted with 1 volume 0.3 mM NaF, and applied to a 30-ml column of aminohexyl Sepharose 4B, equilibrated in the corresponding buffer. The column was washed with 30 ml buffer, and developed with a 300-ml gradient of 0–0.7 M NaCl in either buffer A or buffer B (Fig. 4).

3. Results

3.1. Inhibitors of the modulator-induced inactivation of PP-1_C

We have previously shown that the conversion of type-1 protein phosphatases to the inactive, MgATP-dependent form is blocked by low concentrations of fluoride (0.3 mM), that are insufficient to inhibit the phosphatase activity [15]. However, fluoride was unable to prevent totally the gradual inactivation of the modulator-associated phosphatase (PP-1S) that is observed during purification from skeletal muscle cytosol (not shown). This prompted us to investigate in more detail the conditions that offer protection against inactivation by modulator.

While the addition of 0.3 mM NaF blocked completely the modulator-induced inactivation of PP-1_C at pH 7.5 (Fig. 1B), fluoride did not display this effect at the acidic pH (Fig. 1A) that can be observed in skeletal muscle extracts prepared in weak neutral buffers. Unexpectedly, the pH itself also affected the modulator-mediated inactivation of PP-1_C. While an incubation in the absence of fluoride for 60 min at pH 6.5 (Fig. 1A) or 7.5 (Fig. 1B) resulted in 80% inactivation of the catalytic subunit, virtually no inactivation occurred at pH 8.5 (Fig. 1C). The pH also interfered with the sensitivity of the free catalytic subunit to inhibition by fluoride. Thus, the concentration of fluoride that caused 50% inhibition increased from about 0.5 mM at pH 6.5 to 3 mM at pH 7.5, and to about 20 mM at pH 8.5 (Fig. 1).

Fig. 2 shows the effect of the pH on the time-dependent inactivation of PP-1_C by modulator. While the inactivation rate was similar at pH 6.5 and 7.5, a slight further increase of the pH to 8.0 blocked the modulator-induced inactivation nearly completely. On the other hand, the activity of the free catalytic subunit did not change significantly during incubation for 60 min at any of these pH values (not shown).

In Fig. 3 it is demonstrated that KCl also protected PP-1_C against inactivation by modulator at pH 7.5. The addition of 30 mM KCl decreased the inactivation rate about 3-fold and 150 mM caused a complete block. A similar protection against inactivation was obtained with the same concentrations of NaCl, NaBr or NaI (not shown). Addition of 150 mM KCl after 30 min of incubation with modulator also resulted in an instantaneous block of further inactivation of the phosphatase (Fig. 3). It appears therefore that salt does not interfere with the rapid association of catalytic subunit with modulator, but blocks the subsequent inactivation, which is a very slow process [15]. In gel filtration experiments, we have checked directly that this conclusion also applies to the composite effect of buffer A, which includes 0.1 M NaCl and 0.3 mM NaF at pH 8.0 (not shown).

3.2. Activity of native PP-1S

When the cytosolic fraction of rabbit skeletal muscle was prepared in a 'classical' buffer (i.e. at pH 7.0 without salt), only one spontaneously active species of phosphorylase phosphatase could be identified after consecutive chromatographies on DEAE Sephadex A50 (not shown) and aminohexyl Sepharose 4B (Fig. 4A). However, an additional species of phosphorylase phosphatase, which was eluted earlier, was revealed after preincubation of the fractions with GSK-3/F_A under phosphorylating conditions (Fig. 4A), or after preincubation with trypsin in the presence of Mn^{2+} (not shown). Since this additional species was not revealed by a preincubation of the fractions with trypsin alone, it must contain PP-1_C in its inactive, MgATP-dependent conformation. Moreover, the activity profile of the MgATP-dependent phosphatase overlapped perfectly with the distribution of modulator, measured either immunologically or as heat-stable inhibitor of PP-1_C (not shown).

After partial purification of the cytosolic phosphatases from skeletal muscle in a buffer at pH 8.0, and in the presence of 0.3 mM NaF and 100 mM NaCl, two spontaneously active species of phosphorylase phosphatase could be detected (Fig. 4B). The first peak of activity could only be increased about 30% by trypsin, whether or not the fractions had been pretreated with GSK-3/F_A

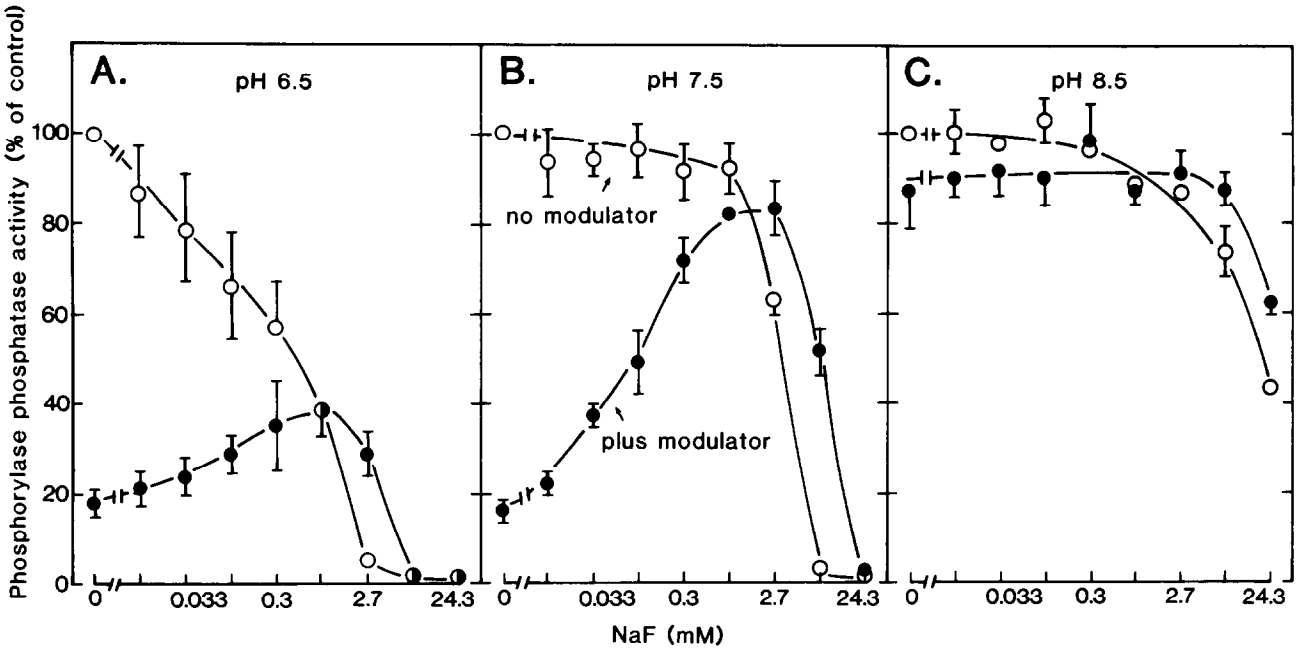

Fig. 1. Dependence on the pH of the interaction of fluoride with PP-1_C and with its inactivation by modulator. The type-1 catalytic subunit (2 nM) was incubated for 60 min at 25°C in a buffer containing 10 mM MES, 10 mM imidazole, 10 mM Tris and 1 mM dithiothreitol, at pH 6.5 (left panel), or at pH 7.5 (middle panel), or else at pH 8.5 (right panel). Modulator (0.2 μM) and NaF were added as indicated. After this incubation, additions were made for the assay of the trypsin-resistant phosphorylase phosphatase activity, causing an additional dilution of 1.7-fold. The results represent the means ± S.E.M. for 4 experiments. In comparison with pH 7.5, the activity of the free catalytic subunit amounted to 86 ± 17% at pH.6.5 and 69 ± 2% at pH 8.5.

under phosphorylating conditions. This small increase by trypsin can be explained by proteolysis of the carboxyterminal domain of the catalytic subunit [1]. Following dialysis against buffer at pH 7.0 without salt or fluoride (buffer B), the firstly eluted phosphatase could be converted to the inactive, MgATP-dependent form by incubation at 30°C (not shown). Moreover, the distribution of modulator coincided perfectly with the first peak of phosphatase activity (not shown). Taken together these data indicate that the first eluted phosphatase represents an active form of the modulator-associated enzyme. The properties of the second peak of phosphorylase phosphatase were similar to the corresponding peak obtained after purification at pH 7.0.

3.3. Identification of a soluble form of PP-1G

Several lines of evidence suggest that the phosphatase eluted from aminohexyl Sepharose after the modulator-associated phosphatase represents a form of PP-1G that has lost its affinity for glycogen and/or membranes. First, the enzyme could be affinity-purified by chromatography on β-cyclodextrin Sepharose, which also specifically binds the glycogen-bound enzyme from rat liver [12] and from rabbit skeletal muscle (Wera, Bollen and Stalmans, unpublished). In contrast to PP-1G, however, the soluble enzyme did not co-sediment with glycogen, and remained also soluble when extra α-particulate glycogen (1%) was added before high-speed centrifugation (not shown). Second, as for muscle PP-1G [16], incubation of the enzyme with protein kinase A under phosphorylating conditions resulted in a dissociation of the catalytic subunit from the holoenzyme, as measured by a shift of the phosphatase during gel filtration to 35 kDa (not shown). Third, consistent with previous findings [17], the phosphatase purified by chromatography on β-cyclodextrin Sepharose contained multiple polypeptides that could be phosphorylated by protein kinase A. These polypeptides (30–100 kDa during SDS-PAGE) are likely to be proteolytically derived fragments of the G-subunit, since they were recognized by polyclonal antibodies raised against a synthetic peptide encompassing the protein kinase A phosphorylation site-1 of the G-subunit (not shown). Moreover, phosphopeptide mapping by reverse-phase chromatography indicated that the same two phosphopeptides were generated from each polypeptide by proteolysis with trypsin. These phosphopeptides migrated identically as previously described [17] for similarly isolated phosphopeptides from the G-subunit of PP-1G (not shown).

Taken together, the above data strongly indicate that the type-1 phosphatase eluting after PP-1S on aminohexyl Sepharose represents an artefact generated by proteolysis of the glycogen-binding domain of PP-1G. In agreement with this view, we found that the presence of 'soluble' PP-1G was remarkably species-dependent: the enzyme could barely be detected after chromatography of a rat skeletal muscle cytosol on aminohexyl Sepharose (3 experiments; not shown).

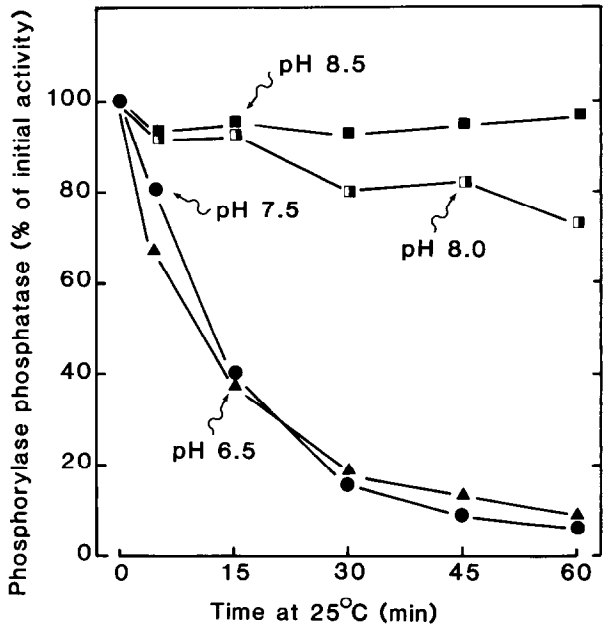

Fig. 2. Effect of the pH on the time-dependent inactivation of PP-1_C by modulator. The type-1 catalytic subunit was incubated with modulator at the indicated pH values, as detailed in the legend to Fig. 1. At the indicated time points samples were taken for the assay of trypsin-resistant phosphorylase phosphatase activity.

4. Discussion

4.1. The interaction of fluoride with PP-1

The present data confirm that the effect of fluoride on the modulator-mediated inactivation of PP-1 is unrelated to the inhibition of the phosphatase activity by fluoride. As was noted previously [1,15], there was a large difference in IC_{50} between the two fluoride effects at pH 7.5 (Fig. 1). Further, the two phenomena were very differently affected by pH. The large variations with pH suggest that the fluoride effects are critically dependent on the state of ionization of specific groups. The increased sensitivity of the phosphatase to inhibition by fluoride at lower pH values may suggest that the actual phosphatase inhibitor is HF or HF_2^-, rather than F^- [18]. In contrast, the sensitivity to fluoride of the modulator-mediated inactivation did not increase at lower pH and is therefore most likely due to F^-.

4.2. The activation state of PP-1S in vivo

In keeping with the general agreement to name type-1 protein phosphatases according to their cellular location, we have previously proposed the name PP-1S for the 'soluble' (cytosolic) species of PP-1 [1]. Our present results (section 3.3) suggest that one of the cytosolic species of PP-1 in rabbit skeletal muscle actually represents a proteolytically degraded form of the glycogen-associated phosphatase (PP-1G). Thus, the name PP-1S solely refers to the modulator-associated phosphatase.

The presence of spontaneously active PP-1S in vivo may be explained by the presence of inhibitors of the inactivation like salts (Fig. 3), inhibitor-1 or Mg^{2+} [19], and/or by the continuous reactivation of the MgATP-dependent form by GSK-3/F_A. Using high concentrations of Mg^{2+} to counteract the gradual inactivation of PP-1S after tissue homogenization, Vandenheede et al. [20] previously also concluded that the catalytic subunit of this enzyme exists in the cell in an active conformation. In contrast to their findings, however, we observed only a mild increase in the activity of PP-1S by a pretreatment with trypsin (Fig. 4). Although the reason for this discrepancy is not clear, our results show that modulator can form an active complex with the catalytic subunit, and does not function as a simple phosphatase inhibitor in vivo.

The present findings also call for a revision of the physiological role of PP-1S. Recently, it has been proposed that modulator acts like a 'chaperone' that is required for the GSK-3/F_A-dependent activation of nascent PP-1_C isoforms [3]. PP-1S would then serve as a cytosolic reservoir of inactive PP-1_C which, following reactivation by F_A/GSK-3, would be directed to other cellular locations by transfer to specific targeting subunits. However, under conditions that prevent inactivation we could not detect a pool of cytosolic inactive PP-1S. As further support of the 'chaperone' hypothesis was invoked the finding that the glycogen-binding subunit and the myosin-binding subunit from muscle were able to displace the catalytic subunit from an active complex between modulator and PP-1_C [3]. It should be noted, however, that the opposite is true for liver, where

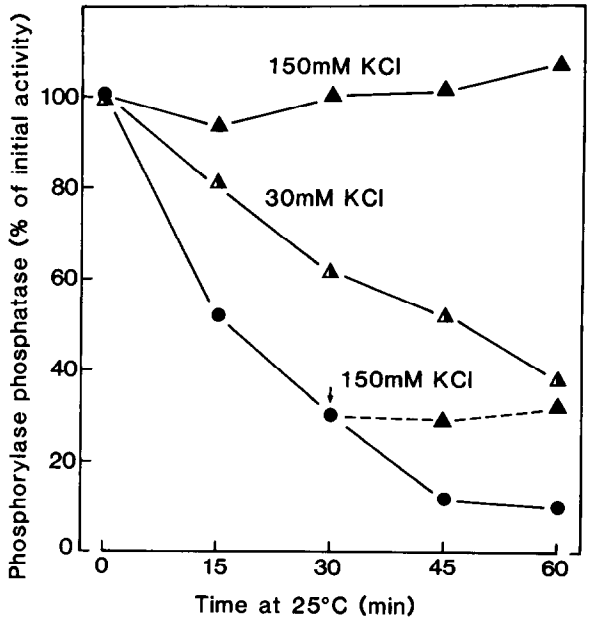

Fig. 3. Effect of KCl on the modulator-induced inactivation of PP-1_C. The type-1 catalytic subunit was incubated at pH 7.5 with modulator in the presence of the indicated concentrations of KCl, as explained in the legend to Fig. 1. At the indicated time points, aliquots were taken for the assay of trypsin-resistant phosphorylase phosphatase activity.

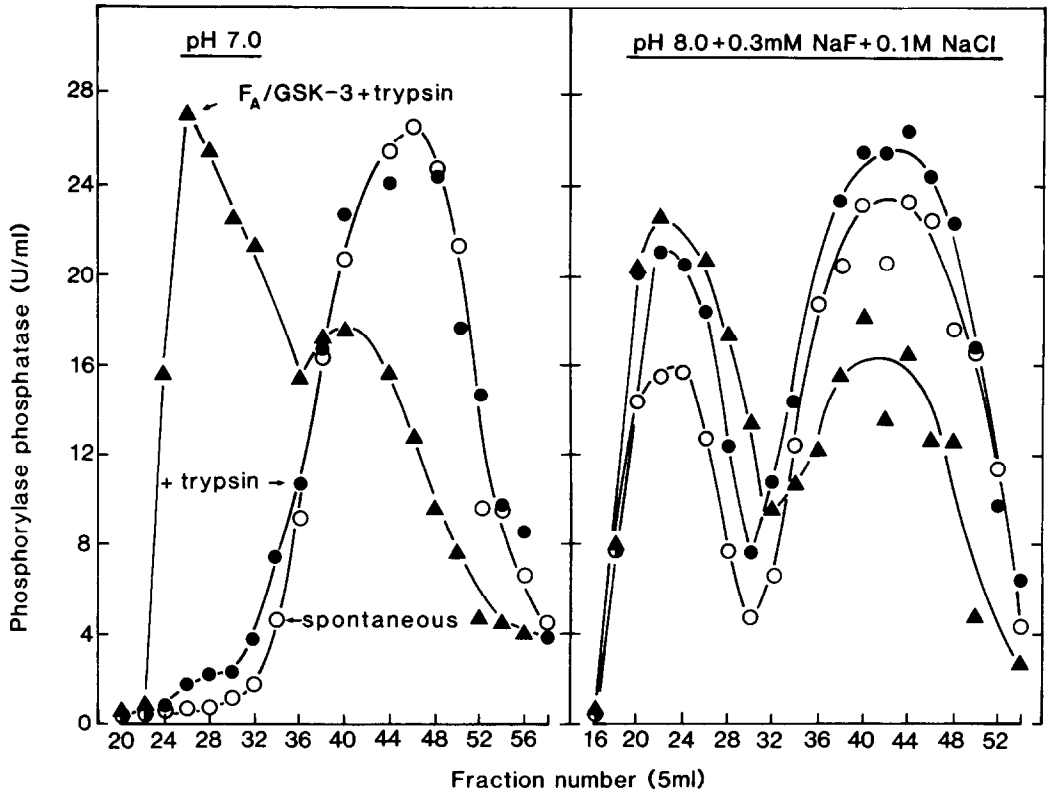

Fig. 4. Chromatography of cytosolic PP-1 on aminohexyl Sepharose. The cytosolic species of PP-1 were partially purified in buffer A (left panel) or buffer B (right panel), as described in the experimental section. The figure shows the elution of the phosphorylase phosphatase activity from aminohexyl Sepharose. The phosphorylase phosphatase activity was measured as such (○), after a preincubation with trypsin (●), and after phosphorylation by GSK-3/F_A followed by a trypsin treatment (▲).

modulator has been shown to dissociate the catalytic subunit from the glycogen-bound phosphatase [21]. In addition, Cohen and co-workers [22] reported that modulator can form with muscle PP-1G a ternary complex which can be reactivated by F_A/GSK-3. The formation of such a complex is difficult to reconcile with the proposed chaperone role of the modulator.

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