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Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography

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Abstract A microcystin (MC)-Sepharose column was prepared by addition of 2-aminoethanethiol to the α,β-unsaturated carbonyl of the *N*-methyldehydroalanine residue of MC-LR, followed by reaction of the introduced amino group with *N*-hydroxysuccinimide-activated CH-Sepharose. The MC-Sepharose bound protein phosphatase-1 (PP1) with high capacity and purified human PP1γ in one step from *E. coli* extracts. It was also used to purify forms of PP1 bound to myofibrils from skeletal muscle. Two of these comprised PP1 complexed to N-terminal fragments of the M-subunit which enhance its myosin phosphatase activity, while the third comprised PP1 and an N-terminal fragment of the glycogen-binding (G)-subunit.

Key words: Microcystin; Affinity chromatography; Protein phosphatase 1; Skeletal muscle

1. Introduction

The protein phosphatases (PPs) which dephosphorylate Ser/Thr residues play critical roles in determining the level of phosphorylation and hence the biological activities of many proteins, which may explain why their structures have been more conserved during evolution than any other enzyme. One of the two protein (Ser/Thr) phosphatase gene families comprises PP1, PP2A, PP2B and many related enzymes [1] which are the intracellular targets for a number of drugs, toxins and tumour promoters [2]. For example, the calcium/calmodulin-dependent phosphatase (PP2B) is inhibited specifically by the immunosuppressant cyclosporin when the latter is complexed to a protein called cyclophilin [3], while the regulatory A-subunit of PP2A interacts with the small T-antigen of sarcoma virus 40 and the small and medium T-antigens of polyoma virus. Interaction with these T-antigens appears to compromise the ability of PP2A to inactivate MAP kinase kinase explaining the helper function of T-antigens in cell transformation by these viruses [4]. PP1, PP2A and other members of this family are also inhibited by several naturally occuring toxins, such as okadaic acid, the substance responsible for diarrhetic seafood poisoning [5], and microcystins, the cyclic heptapeptides produced by blue-green algae which are hepatotoxins and a potential threat to water supplies [2, 6]. Okadaic acid is also a powerful tumour promoter [7] and microcystin a potent liver carcinogen [8].

Many protein phosphatases do not exist in vivo as the free catalytic subunit, but are complexed to other proteins which modify their specificities and/or target them to particular subcellular locations. For example, all forms of PP2A are complexed to the A-subunit, which interacts with many proteins in normal cells [9]. One of these, termed the B-subunit, greatly increases the activity of PP2A towards some proteins, while decreasing its activity towards others [10]. The catalytic subunit of PP1 also interacts with many proteins in vivo, which direct it to particular subcellular locations and have therefore been termed 'targetting' subunits. Targetting subunits may also modify the substrate specificity of PP1 and/or be subject to regulation by phosphorylation or allosteric effectors, allowing different forms of PP1 to be controlled selectively by extracellular signals [11–13].

The analysis of protein Ser/Thr phosphatases and their regulatory subunits has been hampered by the difficulty in isolating these enzymes. This is due to their low concentrations in cells, the complexity of their multiple molecular forms, the susceptibility of regulatory subunits to proteolysis [14–16] and their tendency to dissociate from the catalytic subunit during purification. The development of a simple affinity purification method would therefore greatly facilitate the analysis of these enzymes. Here we describe an improved microcystin-affinity column which has a high capacity for PP1 and PP2A, and demonstrate its potential for purifying the catalytic subunit of PP1 and forms of PP1 which are complexed to targetting subunits.

2. Materials and methods

2.1. Materials

Microcystin containing leucine and arginine at positions X and Y in the ring (MC-LR; Fig. 1) was purified [17] from *Microcystis aeruginosa* harvested from Rutland Water (Leicestershire, UK). MC-YR (containing tyrosine and arginine at positions X and Y) was a generous gift from Dr. P. Thiel (Research Institute for Nutritional Diseases, Tygerberg, South Africa) and Drs. P. Cruz and W. Carmichael (Wright State University, Dayton, OH, USA). Both MCs were quantified by amino acid analysis [6]. Inhibitor-1 [18] and bacterially expressed inhibitor-2 [19] were purified by Dr. M. Hubbard and Dr. N. Helps, respectively, in this laboratory. *N*-Hydroxysuccinimide-activated CH-Sepharose 4B, Mono Q (HR 5/5) and Superose 12 (HR 10/30) columns were from Pharmacia G.B. Ltd. (Milton Keynes, UK) and 2-aminoethanethiol hydrochloride from Aldrich Chemicals Ltd., Gillingham, UK.

2.2. Preparation of ^{32}P-labelled substrates and protein phosphatase assays

^{32}P-labelled phosphorylase *a* (containing 1.0 mol phosphate per mol subunit) was prepared by phosphorylation with phosphorylase kinase [20] and heavy meromyosin (labelled to 0.8–1.0 mol phosphate per mol enzyme (360 kDa)) with skeletal muscle myosin light chain kinase [14].

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The dephosphorylation of phosphorylase *a* (10 μM), and heavy meromyosin (0.3 μM) were assayed by standard procedures [20]. One unit of activity (U) was that amount of enzyme which catalysed the release of 1 μmol of phosphate from each substrate in one minute. Unless stated otherwise, units refer to activity towards phosphorylase *a*.

2.3. Preparation of iodinated microcystin-YR

MC-YR (10 μg) was dissolved in 0.02 ml of methanol, diluted to 0.1 ml with 0.125 M sodium phosphate buffer, pH 7.4, and added to a tube containing 0.01 mg of dried Iodogen.

5 μl (0.5 mCi) of ^{125}I sodium iodide was added, and after standing for 15 min at 0°C, 0.01 ml of 20 mM tyrosine was added followed by 0.01 ml of 0.5 M (unlabelled) sodium iodide. The solution was diluted to 1.0 ml with 0.1% (v/v) trifluoroacetic acid (TFA) and applied to a Waters C_{18} Sep-pak cartridge equilibrated in 0.1% (v/v) TFA. After washing with 0.1% (v/v) TFA in 10% (v/v) acetonitrile to remove sodium iodide and tyrosine, the ^{125}I-MC-YR was eluted with 0.1% TFA/100% (v/v) acetonitrile, dried and redissolved in 0.02 ml of 100% (v/v) methanol.

2.4. Labelling of the PP1 catalytic subunit with ^{125}I-labelled microcystin

Fractions containing PP1 (20 μl) were made 0.5% (v/v) in 2-mercaptoethanol, incubated for 30 min at room temperature, diluted to 100 μl with 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.03% (w/v) Brij-35 and 5 μl ^{125}I-labelled MC-YR (200,000 cpm/μl) was added. After 2 h, aliquots were heated for 2 min at 100°C in 1% SDS, subjected to electrophoresis on SDS/10% polyacrylamide gels and autoradiographed for three days to visualise PP1.

2.5. Preparation of aminoethanethiol-microcystins

Water, DMSO and 5 N NaOH were purged separately with N_2 gas. 1 vol. MC-LR (1–5 mg/ml in ethanol) was then added to a solution comprising 1.5 vol. water, 2 vol. DMSO, 0.67 vol. 5 N NaOH and 1 vol. aminoethanethiol hydrochloride (1 g/ml). After incubation for 30 min at 50°C under N_2 gas, the solution was cooled, mixed with an equal volume of glacial acetic acid, then diluted 5-fold with 0.1% (v/v) TFA and the pH reduced to 1.5 by dropwise addition of 100% (v/v) TFA. The sample was applied to a C_{18} Sep-pak cartridge equilibrated in 0.1% (v/v) TFA, and after washing with 0.1% (v/v) TFA in 10% (v/v) acetonitrile, the aminoethanethiol-MC-LR was eluted from the cartridge with 0.1% (v/v) TFA/100% (v/v) acetonitrile, dried by rotary evaporation and dissolved in 0.02 ml of methanol. Chromatography of an aliquot on a Vydac C_{18} column developed with a gradient of water/acetonitrile in 0.1% TFA showed that 95% of the MC-LR had been converted to the aminoethanethiol derivative which was eluted at 33.7% acetonitrile, compared with 35.6% acetonitrile for unmodified MC-LR. The aminoethanethiol-derivative of ^{125}I-MC-YR was prepared in an identical manner.

2.6. Preparation of microcystin-thioethaneamino-Sepharose

Aminoethanethiol-MC-LR (1.0 mg), spiked with ^{125}I-aminoethanethiol-MC-YR (120,000 cpm), was reacted for 3–4 h at room temperature with 6 ml of swollen *N*-hydroxysuccinimide-activated CH-Sepharose 4B in 50 mM sodium bicarbonate, pH 9.2. The columns were washed alternately with 50 mM Tris-HCl, pH 8, 0.5 M NaCl and 50 mM sodium acetate pH 4, 0.5 M NaCl (five times with each buffer) and, based on the recovery of ^{125}I-radioactivity, 97% of the aminoethanethiol-MC bound covalently to the Sepharose compared to only 3% when unmodified MC-YR was used. This confirmed that the linkage to Sepharose was almost entirely through the introduced amino group. This MC-thioethaneamino-Sepharose is referred to hereafter as MC-Sepharose.

2.7. Purification of PP1γ from Escherichia coli *extracts by microcystin affinity chromatography*

Escherichia coli DH5α carrying a pCW-PP1γ construct was incubated for 16 h at 28°C with 0.3 mM isopropyl-thio-β-D-galactopyranoside to induce PP1γ expression [21]. The cells were then lysed by two passes through a French Press in 3 vols of ice-cold 50 mM HEPES-NaOH pH 7.5, 100 mM KCl, 5% (v/v) glycerol, 1 mM EDTA, 2 mM $MnCl_2$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine. The homogenate was centrifuged for 30 min at 14,000 × *g* and the supernatant (termed the bacterial extract) filtered through a 0.45 μM filter. Five ml of the filtrate (containing 50 mg protein and 3,000 mU of PP1 activity) was loaded on to a 2 ml (0.3 mg of ligand) column of MC-Sepharose and the column washed with 100 ml of 50 mM triethanolamine-HCl, pH 7.5, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (Buffer A) containing 0.5 M NaCl and 1 mM $MnCl_2$ and Buffer A containing 3 M sodium isothiocyanate (NaSCN) and 1 mM $MnCl_2$ (5 ml) was passed through the column and the flow stopped for 30 min. The flow was restarted and 1 ml fractions were collected. The active fractions (6 ml) were pooled, dialysed into Buffer A containing 55% (v/v) glycerol plus 1 mM $MnCl_2$ and stored at −20°C.

2.8. Purification of forms of PP1 from skeletal muscle myofibrils

The myofibrillar fraction from one New Zealand White rabbit was extracted with Triton X-100, and the associated PP1 solubilised in 0.6M NaCl and fractionated from 3% to 12% poly(ethylene glycol) as described [14]. The 12% poly(ethylene glycol) pellets were resuspended in 30 ml of 50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1 mM EGTA, 0.6 M NaCl, 0.1% (v/v) 2-mercaptoethanol, 0.03% (w/v) Brij 35, 10% glycerol, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 0.04 mg/ml leupeptin, 0.1 mg/ml lima bean trypsin inhibitor and 0.5 mM tosylphenylchloromethyl ketone using a hand homogeniser, incubated on ice 15 min and centrifuged for 15 min at 100,000 × *g*. The supernatant, containing >90% of the activity, was loaded on to a 2.5 ml column of MC-Sepharose at a flow rate of 1 ml/min. The column was washed with 25 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol (Buffer B) containing 0.6 M NaCl until the protein concentration in the eluate was <0.005 mg/ml. Buffer B containing 3 M NaSCN (0.75 ml, one void volume) was then passed through the column and the flow stopped for 30 min. The flow was then restarted and the next 15 ml of Buffer B plus 3 M NaSCN (containing the PP1 activity) was collected, dialysed against Buffer B and chromatographed on Mono Q and Superose 12 as described in section 3.

3. Results

3.1. Design of the microcystin affinity column

The α,β-unsaturated carbonyl group of the *N*-methyldehydroalanine (Mdha) residue in MC-LR is arranged with the π-electrons polarised so that nucleophilic addition of thiolates to the β position is favoured at high pH ([22]; Fig. 1). Reacting MC-LR at this position with either ethanethiol or butanethiol at pH 11 gave >95% conversion to products which inhibited PP1 and PP2A with similar potency to the parent toxin (not shown), suggesting that thiol addition would provide a suitable spacer to immobilise MC for affinity purification of protein phosphatases. Immobilisation was achieved by addition of

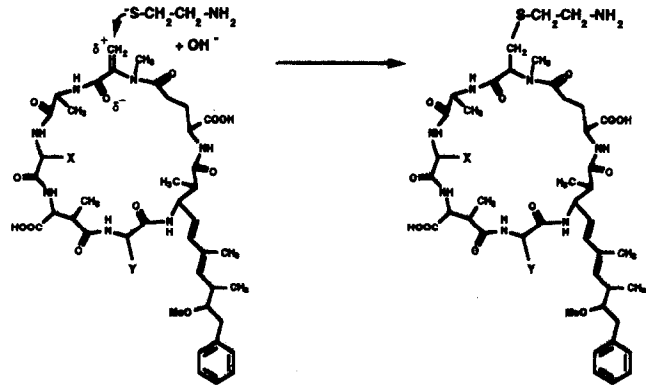

Fig. 1. Nucleophilic addition of aminoethanethiol to the β carbon of the *N*-methyldehydroalanine residue of microcystin. In microcystin-LR, residue X is leucine and Y is arginine. In microcystin-YR, residue X is tyrosine and Y is arginine.

2-aminoethanethiol to MC and subsequent reaction of aminoethanethiol-MC with *N*-hydroxysuccinimide-activated-Sepharose to form MC-thioethaneamino-Sepharose (MC-Sepharose, Fig. 1).

3.2. *Isolation by microcystin affinity chromatography of human PP1γ from* Escherichia coli *extracts*

PP1γ was purified to homogeneity from an *E. coli* extract in a single step by MC-Sepharose chromatography (Fig. 2). This represented a 130-fold purification from the extract to a final specific activity of 38 U/mg, with an apparent recovery of 110%. MC-Sepharose chromatography did not change the sensitivity of the enzyme to inhibitor-2 (IC_{50} = 2 nM) and microcystin-LR (IC_{50} = 0.4 nM). By titration of MC-Sepharose with purified PP1γ, the column capacity was found to be 5 mg enzyme/mg bound ligand.

3.3. *Isolation by microcystin affinity chromatography of forms of PP1 bound to skeletal muscle myofibrils*

A substantial proportion of the PP1 in skeletal muscle is specifically associated with Triton X-100-washed myofibrils [14]. In the present study we solubilised PP1 activity from this fraction with 0.6 M NaCl, purified it about 30-fold by fractionation with poly(ethylene glycol) 8000 as described [14] and a further 1,200-fold by chromatography on MC-Sepharose (see section 2). The recovery of activity from MC-Sepharose was 70% and the eluate showed a major 37 kDa band on SDS/PAGE which comigrated with the catalytic subunit of PP1. There were also a number of other bands, most of which were identified subsequently as regulatory subunits.

The MC-Sepharose eluate was chromatographed on Mono Q and the fractions assayed for myosin phosphatase as well as phosphorylase phosphatase activity. Four peaks of activity were resolved, termed PP1C, PP1G′, PP1Mα and PP1Mβ (Fig. 3). PP1C and PP1G′ had low myosin phosphatase:phosphorylase phosphatase activity ratios of <0.005 and PP1Mα and PP1Mβ high activity ratios of about 0.1. The dephosphorylation of phosphorylase or myosin by all four peaks was completely inhibited by preincubation for 15 min with 200 nM inhibitor-1 or inhibitor-2, thermostable proteins which are specific PP1 inhibitors (data not shown).

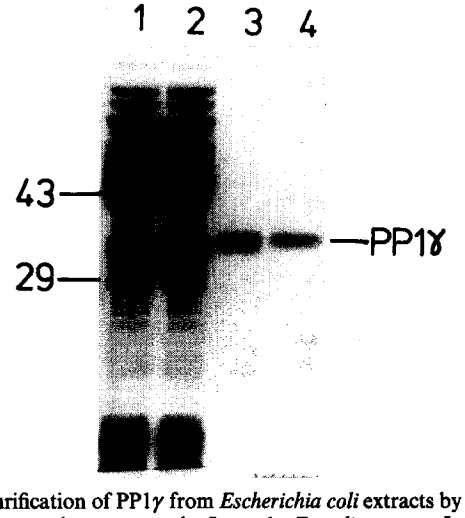

Fig. 2. Purification of PP1γ from *Escherichia coli* extracts by microcystin-Sepharose chromatography. Lane 1, *E. coli* extract; Lane 2, the MC-Sepharose flow-through; Lane 3, MC-Sepharose eluate; Lane 4, PP1γ purified by the procedure of Alessi et al. [21]. Samples were denatured in SDS, heated at 100°C for 2 min and subjected to electrophoresis on a SDS/12% polyacrylamide gel and stained with Coomassie blue. The positions of marker proteins ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) and PP1γ are indicated.

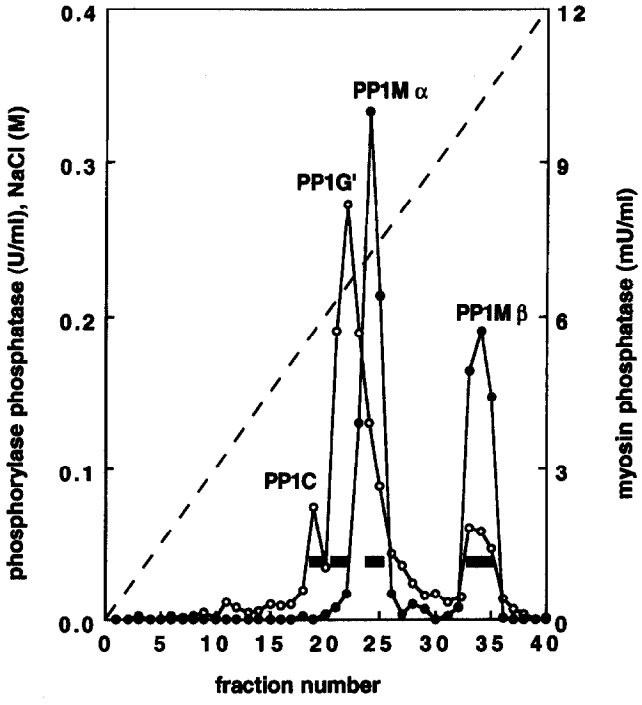

Fig. 3. Separation of myofibrillar forms of PP1 by chromatography on Mono-Q. The eluate from the MC-Sepharose column was applied to a 5 × 0.5 cm column of Mono-Q equilibrated in Buffer B. The column was then developed with a 40 ml linear gradient from 0 to 0.4 M NaCl in Buffer B at a flow rate of 1.0 ml/min. Fractions of 1.0 ml were collected and assayed for myosin phosphatase (●) and phosphorylase phosphatase (○) activities. The broken line denotes the NaCl gradient. The four peaks termed PP1C, PP1G′, PP1Mα and PP1Mβ were pooled as indicated by the solid bars.

The 37 kDa band in each peak was labelled specifically with ^{125}I MC-YR (Fig. 4) demonstrating that this protein was the catalytic subunit of PP1. The peak of PP1C (whose amount varied from preparation to preparation) eluted from Superose 12 with an apparent molecular mass of 35 kDa, demonstrating that it was the free catalytic subunit. The peak of PP1G′ from Mono Q contained a 43 kDa band which stained with similar intensity to the 37 kDa catalytic subunit, and a 63 kDa band which stained more strongly. The 43 kDa band continued to co-purify with the catalytic subunit on Superose 12 (Fig. 5) indicating that PP1G′ is a heterodimer. The 63 kDa band eluted earlier than PP1G′ (Fig. 5) and is therefore an impurity. Amino acid sequencing of several tryptic peptides from the 43 kDa subunit showed 100% identity with sequences found in the N-terminal domain of the glycogen binding (G)-subunit of PP1 [23].

The peak of PP1Mα from Mono Q contained a 35 kDa band which stained with similar intensity to the 37 kDa catalytic subunit, and continued to copurify with it on Superose 12 (Fig. 5). Similarly, PP1Mβ was also a heterodimer comprising a 50 kDa subunit complexed to PP1C (Fig. 5). Tryptic digestion and peptide sequencing revealed that several peptides from the 35 kDa subunit of PP1Mα were identical to peptides found in the

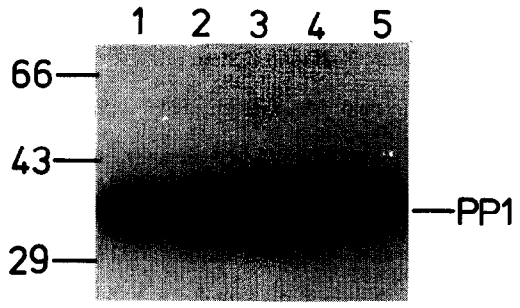

Fig. 4. Labelling of myofibrillar forms of PP1 with ^{125}I-microcystin YR. PP1C, PP1G′, PP1Mα and PP1Mβ from Fig. 3 and PP1M from chicken gizzard [27] were incubated with ^{125}I-MC-YR, denatured in SDS, heated at 100°C for 2 min, electrophoresed on SDS/10% polyacrylamide gels and autoradiographed. Lane 1, PP1C; Lane 2, PP1G′; Lane 3, PP1Mα, Lane 4, PP1Mβ; Lane 5 PP1M purified from chicken gizzard [27]. The positions of the PP1 catalytic subunit and the marker proteins bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) are indicated.

50 kDa subunit of PP1Mβ. Coupled with the high myosin phosphatase:phosphorylase phosphatase activity ratios of both species, this indicates that the 35 kDa and the 50 kDa subunits are likely to be derived from the same gene. Several peptides from the 35 and 50 kDa subunits also showed considerable homology to the ankyrin repeat region of the M_{110} subunit of smooth muscle PP1M, located near the N-terminus [24]. These results indicate that the N-terminal domain of the M_{110}-subunit contains the PP1-binding site and the site which enhances the dephosphorylation of myosin.

4. Discussion

In this paper we immobilised MC via a site that is not essential for the toxin-enzyme interaction to produce a powerful affinity column which is capable of producing 1,000-fold purification of either the catalytic subunit or native complexes of PP1 in a single step and in quantities sufficient for structural analysis. Indeed the capacity of the column (5 mg PP1 per mg MC) is 15% of the theoretical maximum. The key to the procedure was the use of a low molecular weight thiol which reacted with the Mdha sufficiently rapidly that coupling was complete before the MC was degraded under the alkali conditions used. The resulting thiol adduct was then resistant to alkali, probably because the susceptible peptide bond between the Mdha and Glu residues was stabilised once nucleophilic addition to Mdha had been achieved. The added thiol also contained an amino group which permitted linkage to *N*-hydroxysuccinimide-activated CH-Sepharose. In principle, MC could be linked to other matrices using the same chemistry.

Two other laboratories have previously described the use of MC affinity columns. Nishiwaki et al. [25] coupled MC-LR to ECH-Sepharose using carbodiimide and used it to purify $PP2A_2$ from a mouse brain extract. However, the capacity of the column was over 1000-fold lower than that reported here, only 3 μg PP2A being bound per mg MC. Moreover, no PP1 bound to the column. Beullens et al. [26] coupled MC-LR to epoxy-activated Sepharose (M. Bollen, personal communication) and used the resulting matrix to purify PP1C to a high specific activity. The PP1 was eluted from the MC-column with 3 M KSCN and we therefore adopted a thiocyanate elution procedure ourselves. The capacity of their column was not reported, but one of us (C.M.) found that columns made by linking to epoxy-activated Sepharose bound less than 10 μg PP1γ/mg of coupled MC.

The binding of MC to PP1 (Fig. 4) or PP2A (data not shown) is stable to heating at 100°C in 1% SDS, suggesting that MC binds covalently to these phosphatases. Indeed, one of us (R.W.M. unpublished work) has isolated a tryptic peptide con-

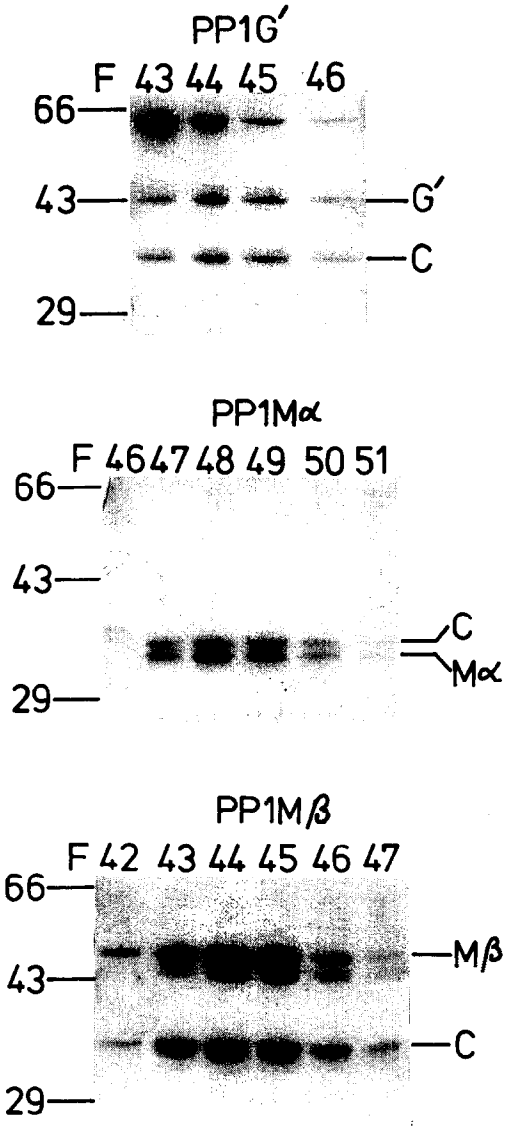

Fig. 5. Gel filtration of the myofibrillar forms of PP1. PP1G′, PP1Mα and PP1Mβ from Mono Q (Fig. 3) were each concentrated to 200 μl by ultrafiltration through Centricon 10 and applied to a 30 × 1 cm column of Superose 12 equilibrated in Buffer B containing 0.1 M NaCl. The flow rate was 0.5 ml/min and 0.25 ml fractions (F) were collected. The marker proteins were eluted from Superose 12 at fraction 41 (bovine serum albumin dimer, 132 kDa), fraction 45 (bovine serum albumin monomer, 66 kDa), fraction 49 (ovalbumin, 43 kDa) and fraction 54 (carbonic anhydrase, 29 kDa). Aliquots of the phosphorylase phosphatase-containing fractions were electrophoresed on SDS/10% polyacrylamide gels and stained with Coomassie blue. The positions of the catalytic subunit of PP1 (C), the glycogen binding subunit (G′) and the myosin phosphatase enhancing subunits (Mα, Mβ) are indicated and the marker proteins are as in Fig. 4.

taining the MC-binding site. This suggests that either the covalent linkage is labile in NaSCN, or that the immobilisation of MC prevents the covalent interaction with PP1.

It has been reported previously that the myofibrillar fraction of skeletal muscle contains two forms of PP1, one of which (PP1M) has a high activity towards myosin, and evidence has been provided that the high myosin phosphatase activity of PP1M is conferred by another (M)-subunit [14]. In this earlier study, a nine step procedure was needed to purify PP1M over 100,000-fold from the myofibrillar fraction, but the final product contained a 33 kDa proteolytic fragment of the PP1 catalytic subunit and the putative M-subunit could not be detected by SDS/PAGE. The MC-Sepharose affinity column allowed skeletal PP1M to be purified within two days and the final product contained the undegraded catalytic subunit complexed to M-subunits which were structurally related to the functionally distinct M_{110}-subunit from smooth muscle [24, 27]. It is possible that the failure to detect the M-subunit previously [14] resulted from co-migration of the 33 kDa fragment of the catalytic subunit with the 35 kDa fragment of the M-subunit (Fig. 5).

MC-Sepharose affinity chromatography also purified another myofibrillar form of PP1 to near homogeneity which contained a 43 kDa subunit bound to PP1C. Tryptic peptides from the 43 kDa species were all identical to sequences found near the N-terminus of the G-subunit which targets PP1 to both the glycogen particles and the membranes of the sarcoplasmic reticulum (SR). We therefore conclude that the 43 kDa protein is a fragment of the 124 kDa G-subunit [11,23]. This finding was somewhat unexpected since the myofibrils had been extracted several times with Triton X-100 which solubilises the SR and therefore the PP1 which is bound to these membranes. The results suggest that the myofibrils bind significant amounts of glycogen and its associated enzymes, which are not extracted at low ionic strength or with detergent, but released when the myofibrils are solubilised in 0.6 M NaCl.

The MC-Sepharose affinity column we have developed also has a high capacity for binding PP2A, but we have been unable thus far to recover significant PP2A activity. Nevertheless, the column should be useful for the isolation of regulatory subunits of PP2A as well as the regulatory and catalytic subunits of other microcystin-sensitive protein phosphatases.

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