Regulation of type-1 protein phosphatase in a model of metabolic arrest

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Type-1 phosphatase (PP-1) was assessed in foot muscle (FM) and hepatopancreas (HP) of estivating (EST) *Otala lactea*. Snail PP-1 displayed several conserved traits, including sensitivity to inhibitors, substrate affinity, and reduction in size to a 39 kDa catalytic subunit (PP-1c). During EST, PP-1 activity in FM and HP crude extracts was reduced, though kinetics and protein levels of purified PP-1c isoforms were not altered. PP-1c protein levels increased and decreased in nuclear and glycogen-associated fractions, respectively, during EST. Gel filtration determined that a 257 kDa low K_m PP-1α complex decreased during estivation whereas a 76 kDa high K_m complex increased in EST. Western blotting confirmed that the 76 kDa protein consisted of PP-1α and nuclear inhibitor of PP-1 (NIPP-1). A suppression of PP-1 activity factors in the overall metabolic rate depression in estivating snails and the mechanism is mediated through altered cellular localization and interaction with binding partners. [BMB reports 2009; 42(12): 817-822]

INTRODUCTION

During the dry season, the desert snail *Otala lactea* enters a state of dormancy (estivation, or EST) in response to water and/or food limitation. EST snails substantially depress metabolism to just 10-30% of the rate of active (ACT) snails (1-3). This transition requires coordinated suppression of both energy-consuming and energy-producing pathways which is achieved primarily by reversible protein phosphorylation (RPP) of key enzymes (3). RPP can have immediate, dramatic effects on enzyme properties (4, 5). RPP mediates EST-dependent adaptations in signal transduction, carbohydrate and protein metabolism, and ion pumping in *O. lactea* (3, 6-9). Thus, it is clear that protein kinases and protein phosphatases must have a regulatory role in *O. lactea*. The present study focuses on type-1

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protein phosphatase (PP1).

Several biochemical properties distinguish PP1 from type-2 serine/threonine phosphatases. PP1 is ion-independent, sensitive to okadaic acid, and retained by heparin and microcystin affinity columns (4). PP1 generally features a 33-40 kDa catalytic subunit (PP1c) that can form complexes with several types of regulatory proteins to modify and target phosphatase activity. Roles for PP1 have been defined in several forms of hypometabolism including cold-hardy insects (10-12), anoxia-tolerant turtles (13), freeze-tolerant wood frogs (14), anoxia-tolerant crayfish (15), and hibernating ground squirrels (16), though did not apparently have a major role in the estivating spadefoot toad (17). We predicted that PP1 would be differentially regulated as part of the transition to the EST state in *O. lactea*. We characterized both crude and purified PP1 activity to determine conserved, novel, and EST-dependent aspects of regulation in this EST model.

RESULTS AND DISCUSSION

Characterization of crude extract PP1

O. lactea PP1 activity in crude extracts showed multiple features of a classically defined PP1 activity. *O. lactea* PP1 was ion-independent and showed strong activity towards typical PP1 substrates phosphorylase a and Kemptide [LRRA(pS)LG]. In EST foot muscle and hepatopancreas crude extracts, PP1 V_{max} was decreased 35-45% when tested with Kemptide and phosphorylase a (Table 1). Consistent with a deactivated enzyme in EST, substrate affinities were significantly reduced for PP1 in dormancy, as indicated by 60-90% increases in substrate K_m values, and Arrhenius activation energy increased 49% in EST hepatopancreas (Table 1). PP1 suppression has been observed in animal tissues subjected to anoxia (13-15), yet EST had little effect on PP1 activity in the spadefoot toad (17). Thus, our data shows for the first time that PP1 activity is downregulated in aerobic EST.

PP1 activity in crude extracts also showed characteristic type-1 traits with respect to pharmacological and physiological inhibitors (Table 1). *O. lactea* PP1 was sensitive to okadaic acid within the typical range (4). PP1 sensitivity to okadaic acid increased in EST, whereas PP1 sensitivity to inhibition by ADP, AMP, and NaCl decreased in EST (Table 1). This latter

Table 1. Kinetic parameters for PP1-type activity in crude extracts from foot muscle (FM) and hepatopancreas (HP) of ACT and 10 d EST *O. lactea*

	Foot muscle		Hepatopancreas	
	ACT	Estivated	ACT	Estivated
V_{max} (mU/mg soluble protein) vs. Kemptide	1.16 ± 0.12	$0.76 \pm 0.08^*$	1.77 ± 0.22	$1.11 \pm 0.08^*$
V_{max} (mU/mg soluble protein) vs. phosphorylase a	1.33 ± 0.16	$0.81 \pm 0.09^*$	1.80 ± 0.18	$1.02 \pm 0.18^*$
K_m Kemptide (μM)	16.8 ± 3.3	$31.6 \pm 2.9^*$	27.0 ± 3.6	34.4 ± 6.0
E_a Arrhenius activation energy (kJ/mol)	31.2 ± 6.3	42.1 ± 5.1	26.8 ± 3.6	$39.9 \pm 4.1^*$
K_m phosphorylase a (μM)	19.6 ± 4.1	$33.8 \pm 5.3^*$	18.6 ± 2.9	$29.4 \pm 3.2^*$
I_{50} okadaic acid (nM)	99.0 ± 6.4	$72.7 \pm 4.3^*$	84.6 ± 5.8	$54.2 \pm 6.8^*$
I_{50} AMP (μM)	65 ± 8	$99 \pm 12^*$	119 ± 19	$175 \pm 29^*$
I_{50} ADP (μM)	73 ± 9	$99 \pm 8^*$	101 ± 13	$146 \pm 12^*$
I_{50} NaCl (mM)	211 ± 26	187 ± 29	275 ± 31	$415 \pm 43^*$

Assays were conducted at 22°C and data are means $\pm$ S.E.M. (n = 3-5). Kinetic determinations for I_{50} used phosphorylase a as the substrate. I_{50} is the concentration of inhibitor that reduces enzyme activity by 50%. Kinetic constants were determined under optimal assay conditions. Significance testing used the Student's t-test. *Significantly different from the corresponding value for ACT snails, P < 0.05.
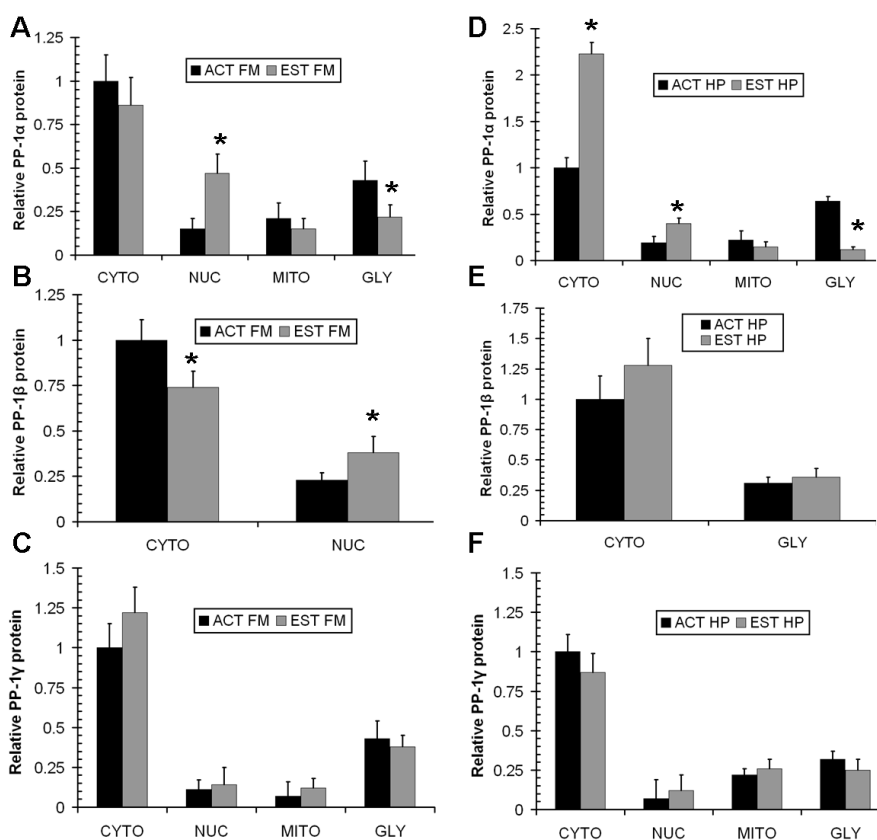

Fig. 1. Levels of different PP1c isoforms (α, β, and γ) in cytosolic (CYTO), nuclear (NUC), mitochondrial (MITO), and glycogen-associated (GLY) fractions in active (ACT) and estivating (EST) tissues. (A-C) PP1α, -1β, and -1γ in foot muscle (FM); (D-F) PP1α, -1β, and -1γ in hepatopancreas (HP). *Significantly different from corresponding protein level in ACT fraction, Student's t-test, P < 0.05.

observation may reflect an adaptation in PP1 functional stability specific to the estivated condition, since adenylate energy charge is maintained at a high level in this species during EST (18) and salt concentrations are known to increase in estivators as a consequence of dehydration.

Subcellular localization of PP1c isoforms

We assessed, via Western blotting, expression levels of different PP1c isoforms (α, β and γ, which cross-reacted at 39, 34, and 35 kDa, respectively). The α-isoform was expressed at ~10-fold higher levels of the β- and γ-isoforms in both tissues.

When assayed in whole cell extracts, total levels of PP1c iso-forms did not change in EST, nor did the levels of P-Thr320 PP1α (data not shown). Conversely, alterations in PP1c com-partmentalization were evident during EST (Fig. 1). In foot muscle, levels of PP1α increased 3-fold in the nucleus, but de-creased 50% in the glycogen fraction, during EST (Fig. 1A). PP1β decreased 26% in cytosol and increased 65% in nucleus in foot muscle during EST (Fig. 1B). Levels of PP1γ did not change in any fraction during EST (Fig. 1C). In hepatopancreas, cytosolic and nuclear PP1α levels increased 2.2- and 2.1-fold, respectively, whereas a substantial 81% reduction was ob-served in the glycogen fraction (Fig. 1D). No significant EST-dependent alterations in the localization of hepatopan-creas PP1β and PP1γ were evident (Fig. 1E, F). Thus, it was ap-parent that the significant changes in crude extract PP1 activity (that occurred in the absence of changes in total α-, β- and γ-protein) was related to changes in subcellular localization of the catalytic subunit (specifically, the highly expressed α-sub-unit).

The increase in EST nuclear PP1 activity could partially con-tribute to the observed enhanced phosphorylation state of tran-scription factors observed in dormant *O. lactea* (8), whereas the decrease in glycogen-associated PP1c fits with the overall suppression of metabolic rate and the reduction of cAMP pre-viously established in this model (6). In liver, PP1 and cAMP-dependent protein kinase (PKA) have opposing roles in glyco-gen metabolism, with PKA catalyzing the phosphorylation of glycogen phosphorylase (GP) and glycogen synthase (GS), fa-voring net glycogenolysis, and PP1 dephosphorylating GP and GS, resulting in net glycogen synthesis. EST-induced decreases in both GP (6) and GS (Storey, K. B., unpublished data) have been observed in *O. lactea*, consistent with a coordinated de-crease of PP1 and PKA. Thus, the overall metabolic rate sup-pression observed in *O. lactea* includes suppression of glyco-gen metabolism which facilitates survival throughout the dor-mant state, as these animals rely largely on carbohydrate re-serves during EST (19). Decreased glycogen-associated PP1c in EST snails is consistent with a similar finding in hibernating ground squirrels (16).

Purification and characterization of PP1

Foot muscle PP1 enzyme from ACT snails was purified 193-fold using a combination of chromatography steps that have been applied previously for PP1 purification, resulting in 11% yield with a final specific activity of 197 mU/mg protein (Suppl. Table 1). EST foot muscle PP1 was purified to essen-tially the same degree. *O. lactea* PP1 displayed characteristic type-1 properties during purification, including increased activ-ity with ethanol treatment and affinity for heparin and micro-cystin. In ethanol treated extracts, PP1 activity was associated with strictly with a protein of 38-40 kDa determined either by non-denaturing SDS-PAGE (Fig. 2) or Sephacryl size exclusion chromatography. Furthermore, ethanol treatment increased ac-tivity from ACT and EST snails to the same level. Taken togeth-
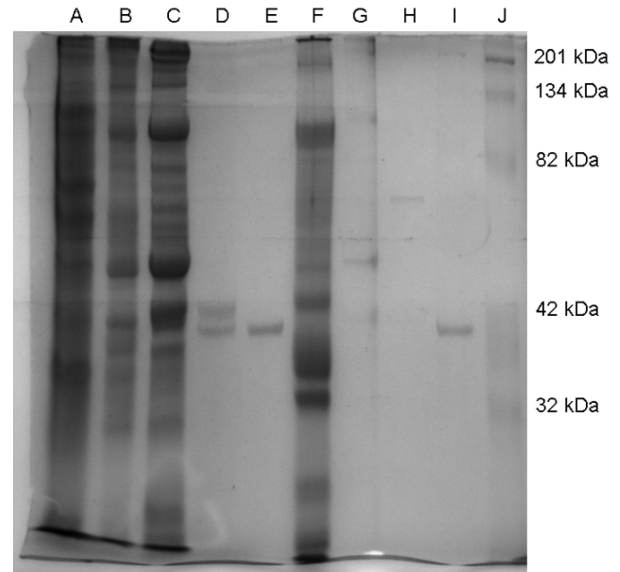

Fig. 2. Non-denaturing SDS-PAGE with Coomassie blue staining of samples taken at different steps in the purification and analysis of PP1 from foot muscle of ACT *O. lactea* snails. The purification of PP1 is illustrated in lanes A to E for the steps: (A) ethanol treat-ment of crude extract, (B) DEAE-ion exchange, (C) heparin-Sephar-ose, (D) Sephacryl S400 gel filtration, and (E) microcystin-agarose. Lanes F to I show an analysis of the peaks of PP1 activity recov-ered from gel filtration of untreated (ie. no ethanol step) crude extracts of foot muscle. Lanes are: (F) untreated crude extract, (G) peak I, (H) peak III, (I) peak IV. Lane (J) shows molecular weight standards as marked.

er, we interpret that ethanol treatment liberated the catalytic subunit (PP1c) from inhibitory complexes of higher molecular masses, as has been determined in vertebrates (20, 21) and ap-pears conserved in invertebrates (4, 22).

During initial heparin chromatography of snail extracts, a type-2A phosphatase also bound to the column. PP2A is typi-cally excluded from heparin columns and recovered entirely in the flow-through (23). *O. lactea* PP2A activity was recov-ered in both the heparin flow-through and off the heparin col-umn with a 0.1 M NaCl bump, similar to reports for solubi-lized-membrane phosphatases of *Paramecium tetraurelia* (24). Thus, inclusion of 0.1 M NaCl was necessary for washing the heparin column (to elute PP2A) prior to the 0.5 M NaCl bump that eluted the target PP1. This was a key step since *O. lactea* PP1 and PP2A eluted similarly from subsequent affinity steps. The microcystin-purified enzyme preparation showed only one band at 39 kDa which was confirmed (via Western blot-ting) to be PP1α, whereas β- and γ-isoforms were not detected at all. Although the kinetic parameters tested generally differed between ACT and estivated states for the crude enzyme (Table 1), there were no significant differences in these properties be-tween the two states when the purified enzymes were exam-ined (Suppl. Table 2).

Characterization of gel filtration PP1 peaks

Gel filtration chromatography of crude extracts (without prior ethanol treatment to remove regulatory subunits) resulted in
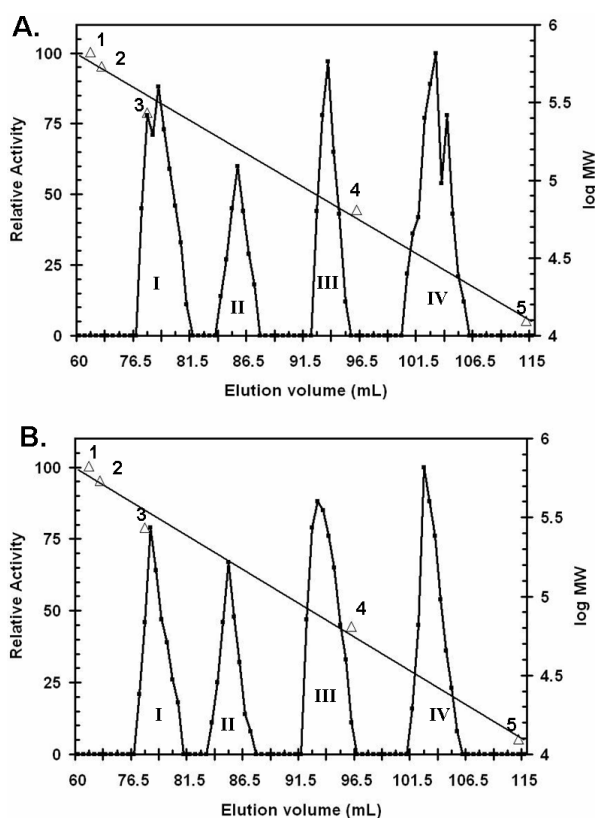

Fig. 3. Elution profiles of PP1 activity from Sephacryl S400 gel filtration for (A) ACT foot muscle and (B) EST foot muscle crude extracts. Crude extracts were not pre-treated with ethanol to remove regulatory subunits. Elution profiles are representative of n = 3 trials. Molecular weight standards are shown as open triangles and are as follows: (1) 670 kDa, (2) 545 kDa, (3) 272 kDa, (4) 64.5 kDa, and (5) 12.5 kDa.

four peaks of PP1 activity (Fig. 3). Peaks I, II, III, and IV were associated with protein sizes of 257 ± 8 kDa, 135 ± 4 kDa, 76 ± 2 kDa, and 40 ± 2 kDa, respectively (mean ± S.E.M., n = 3 independent determinations). Western blotting confirmed the presence of PP1α in these peaks of activity. PP1β was only weakly detected in the peak II fraction. The holoenzyme protein sizes associated with Peaks I, III, and IV of PP1 activity as determined by size exclusion chromatography was confirmed with non-denaturing electrophoresis (Fig. 2). On the other hand, the 135 kDa holoenzyme for Peak II was apparently unstable and only smaller proteins were consistently observed (data not shown).

The percentage of total PP1 activity that was present in peaks I, II, III, and IV was 30 ± 4%, 14 ± 3%, 20 ± 3%, and 35 ± 5 %, respectively, in extracts from ACT foot muscle, but shifted to 22 ± 2%, 16 ± 5%, 34 ± 4%, and 28 ± 5% in extracts from EST foot muscle (means ± S.E.M., n = 3 independent determinations). Thus, in EST, there was a significant decrease in the proportion of PP1 type activity associated with peak I, and a significant increase in PP1 activity in peak III, relative to the ACT condition. Selected kinetic parameters were determined for the isolated PP1 gel filtration peaks (Table 2). Peak III PP1 activity (76 kDa) differed in affinity for phosphorylase a from both peak I (257 kDa) and peak IV (40 kDa) with 3.7- and 2.2-fold higher K_m values, respectively. Type-1 phosphatase activity associated with Peak III also showed decreased affinity for Kemptide (3.0-fold increase in K_m value) and increased sensitivity to okadaic acid inhibition, relative to phosphatase activity from Peak I. Western blotting was performed to test for the presence for several well-known PP1c targeting subunits in the peaks representing larger holoenzymes. Antibodies raised against glycogen, myosin, and actin-targeting subunits failed to cross-react in this animal model, but the presence of the 38 kDa protein NIPP1 (nuclear inhibitor of protein phosphatase-1) was detected in Peak III. Thus, the previously observed increase in nuclear levels of PP1c isoforms could result in higher nuclear PP1 activity in EST, or may serve to sequester PP1c proteins in a relatively low activity form via proteins like NIPP1.

In summary, PP1 activity in crude *O. lactea* extracts was de-

Table 2. Kinetic parameters for ACT snail foot muscle PP1-type activity associated with different molecular weights recovered from Sephacryl S-400 gel filtration

	Peak I 257 kDa	Peak II 135 kDa	Peak III 76 kDa	Peak IV 40 kDa
K_m phosphorylase a (μM)	13.7 ± 2.6	35.7 ± 4.5	50.3 ± 8.1[a,b]	23.0 ± 3.1
K_m kemptide (μM)	11.0 ± 1.9	25.3 ± 5.5	33.1 ± 5.2[a]	19.2 ± 4.2
I_{50} okadaic acid (nM)	118 ± 9	80 ± 8	70 ± 9[a]	101 ± 7
I_{50} urea (M)	3.9 ± 0.6	2.6 ± 0.4	1.9 ± 0.5	2.8 ± 0.4

Fractions were pooled for each of the four peaks shown in Fig. 3. Data are means ± S.E.M. (n = 3). Significant differences among the groups were determined using the Tukey's test. [a]Significantly different from the corresponding value for peak I, P < 0.05, [b]significantly different from the corresponding peak IV value P < 0.05. Other information as in Table 1.

creased in EST in the absence of alterations of total PP1c protein levels and intrinsic kinetic properties of purified PP1α. The decreased PP1 activity was associated with a general increase in nuclear-associated PP1c protein and a decrease in glycogen-associated PP1c protein. Furthermore, differential association of PP1 holoenzymes contributes to the overall suppression of PP1 activity in EST, resulting in a decrease in a complex with high substrate affinity (Peak I, -260 kDa) and an increase in a complex with low substrate affinity (Peak III, 76 kDa). Western blotting confirmed that the Peak III holoenzyme contained PP1α complexed to NIPP1. Future studies will investigate the character and nature of the other PP1 targeting subunits present in Peaks I and II, and determine if these PP1 regulatory proteins are homologous to known proteins in mammals, or possibly unique invertebrate proteins. It is clear that PP1 activity is decreased in EST O. lactea, as a result of differential localization and association with binding partners, playing a key role in RPP that accompanies the transition to the dormant physiological state.

MATERIALS AND METHODS

Chemicals and animals
Centrifugal concentrating devices were purchased from Pall Life Sciences (Ann Arbor, MI). NIPP1 antibody was purchased from AbCam (Cambridge, MA). Antibodies detecting PP1α and P-Thr320 PP1α were purchased from Cell Signaling (Danvers, MA). Antibodies against PP1β and PP1γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Microcystin-agarose was purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were obtained from Sigma (St. Louis, MO). *Otala lactea* (Muller) snails were purchased from a retail source, raised at 22°C in plastic containers lined with damp paper towels and fed shredded carrots, cabbage, and crushed chalk every 2-3 days. After one month, EST was induced in a group of snails by placing them in a container with dry paper towels and no food, while ACT snails were maintained in fed conditions. After 10 days, ACT and EST snails were sacrificed and tissues were rapidly dissected out, immediately frozen in liquid nitrogen and stored at −70°C until use.

Preparation of crude extracts
Frozen tissue samples were homogenized 1 : 5 (w/v) in cold (4°C) buffer A containing 50 mM imidazole, pH 7.5, 10% v : v glycerol, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF and 10 mM 2-mercaptoethanol using a Polytron PT1000 homogenizer (Brinkmann Instruments, Rexdale, ON). Sigma Protease Inhibitor Cocktail (1 : 1000 v : v) was added at the time of homogenization. Homogenates were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was removed and either assayed immediately (crude extract activity) or subjected to further purification steps.

Purification of foot muscle PP1
Crude extracts were subjected to precipitation with 95% ethanol (5 : 1 v : v) for 1 h at 22°C to remove potential regulatory subunits (20, 21). After ethanol treatment, samples were centrifuged for 5 min at 10,000 g, ethanol was removed and pellets were resuspended in the original volume of buffer A. PP1 activity was then purified using modifications of established chromatography methods (10, 24). Our purification scheme also successfully separated PP1 from type-2 phosphatases. Briefly, ethanol-treated crude extract was purified by sequentially loading onto:

(i) DEAE-G25 Sephadex, which retained 100% of all measurable type-1 and type-2 phosphatase activity (eluted with 0.4-0.7 M NaCl in buffer A); (ii) heparin-Sepharose, which retained 100% of PP1 (eluted with 0.5 M NaCl in buffer A); (iii) Sephacryl s-400 equilibrated in buffer B (buffer A plus 150 mM NaCl, 20% v : v glycerol, and 0.04% w : v sodium azide; PP1 was eluted with buffer B); (iv) NanoSep centrifugal devices (to concentrate PP1 ~10-fold); and (v) microcystin- agarose equilibrated in buffer C (buffer A plus 0.5 M NaCl). PP1 activity was finally recovered by elution with buffer C plus 3 M sodium thiocyanate. Fractions were pooled, desalted and assayed immediately.

Gel filtration was also used to determine what protein sizes were correlated with PP1 activity in crude extracts that had not been treated with ethanol. Selected kinetic parameters were also determined for different protein sizes associated with PP1 type activity. Inclusion of protease inhibitors in purification steps ensured that the different peaks were not proteolysed forms of a single holoenzyme.

Electrophoresis and Western blotting
Electrophoresis and Western blotting was performed using standard methods (7-9). To assess purification, samples from various steps in our purification scheme were subjected to standard electrophoresis and Coomassie-gel staining as previously described (8).

PP1 assay
Before all assays, enzyme samples were desalted by passing through Sephadex G-25 equilibrated in buffer A. Parallel assays were run with 5 nM okadaic acid (abolishes PP2A activity, but PP1 is unaffected) and 1 μM okadaic acid (abolishes 100% of PP1 activity); the difference in activity was attributed to PP1. Assay conditions included 0-250 μM substrate, 25 mM imidazole, pH 7.2, 0.1% β-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, and 1 mM Na_3VO_4. Reaction time was 15 min. PP1 production of phosphate was detected using the ammonium molybdate/malachite green reagent that was prepared as described (7). Activity was reported as nmol phosphate released/min/mg soluble protein (mU/mg). Protein concentration was quantified using a bovine serum albumin standard curve and the Coomassie Blue dye binding method with prepared

protein reagent from Bio-Rad (Hercules, CA). Significance testing done using Mynova, Version 2.3.

Subcellular localization of PP1c protein

PP1c protein levels in were assayed in nuclear (NUC), cytosolic (CYTO), mitochondrial (MITO), and glycogen (GLY) subcellular fractions. Subcellular fractionation was performed using standard methodology (25). Each fraction was subjected to microcystin-agarose chromatography to enrich PP1c protein levels, and PP1c protein levels were analyzed via Western blotting as detailed above.

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