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The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1

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Abstract The interaction between protein phosphatase 1 (PP1) and microcystin (MC) was stable in 1% SDS or 70% formic acid indicative of a covalent interaction. Here we isolate the MC-binding peptide and demonstrate that Cys^{273} of PP1 binds covalently to the methyl-dehydroalanine (Mdha) residue of the toxin. Mutation of Cys^{273} to Ala, Ser or Leu abolished covalent binding to MC, as did reduction of the Mdha residue of the toxin with ethanethiol. The abolition of covalent binding increased the IC_{50} for toxin inhibition of PP1 by 5- to 20-fold. The covalent binding of MC to protein serine/threonine phosphatases explains the failure to detect this toxin post-mortem in suspected cases of MC poisoning.

Key words: Protein phosphatase; Microcystin; Protein phosphorylation; Signal transduction

1. Introduction

The family of protein phosphatases (PP) comprising PP1, PP2A and related enzymes [1,2] dephosphorylate serine, threonine and histidine [3] residues in proteins and are critical to the control of many biological processes [1]. These protein phosphatases are inhibited potently and specifically by a chemically diverse group of naturally occurring toxins that include okadaic acid, a polyketide produced by marine dinoflagellates and the major cause of diarrhetic shellfish poisoning [4]; tautomycin, a structurally related polyketide produced by the soil bacterium *Streptomyces* [5]; calyculin A, a phosphorylated polyketide isolated from the sea sponge *Discodermia calyx* [6]; cantharidin, a simple terpenoid produced as a defence compound by meloid blister beetles [7], and microcystins (MCs) [8] and nodularins [9], which are cyclic peptides produced by several species of cyanobacteria. It seems likely that the remarkable conservation in structure of these protein phosphatases throughout all eukaryotes [2], and their vital functions [1], has made these enzymes particularly susceptible as targets for the evolution of these toxins.

Although the toxins have diverse primary structures, kinetic analyses [5,8] and competition binding studies [7,10,11] suggest that they, and the specific heat stable protein inhibitors of PP1 (inhibitor-1 and inhibitor-2 [8]), may share common binding sites on protein serine/threonine phosphatases. Amino acid substitutions located towards the C-termini of PP1 and PP2A decrease their sensitivity to toxins and inhibitor-2 [12,13], but the precise binding sites have not yet been identified by direct structural analysis.

Okadaic acid, calyculin A and MC each have an acidic group, two potential hydrogen bonding sites and a hydrophobic side chain which can be superimposed by aligning molecular models of these three toxins, and have been proposed as the crucial common features for binding to protein phosphatases [14]. The importance of these carboxylates is supported by the finding that methyl esters of okadaic acid [15] and MC (C.M. unpublished) and the monopropyl esters of the glutamyl residue of MC and nodularin [16] do not inhibit protein phosphatases. Modifications in the hydrophobic tails of MC [16] and okadaic acid [17] also reduce enzyme inhibition.

The interactions between okadaic acid and PP1 [18], and between cantharidin and PP2A [7] are reversible, but the interaction of MC with PP1 and PP2A is stable to gel filtration, trichloroacetic acid, or heating at 100°C in 1% SDS [19,20], suggesting that MC binds covalently to these phosphatases. These findings have been exploited to radiolabel PP catalytic subunits using [^{3}H]MC-LR (tritiated on the glutamyl and β-methylaspartyl residues) [20,21] or [^{125}I]MC-YR (iodinated on the tyrosine residue of this MC variant) [19], and to detect these after SDS-PAGE and autoradiography. Here we demonstrate that Cys^{273} on PP1 binds covalently to the methyl-dehydroalanine residue on MC.

2. Materials and methods

2.1 Materials

Microcystin-LR (MC-LR) containing leucine and arginine at two positions in the ring (Fig. 1) was purified [8] from *Microcystis aeruginosa* harvested from Rutland Water (Leicestershire, UK). MC-YR (containing tyrosine and arginine at the equivalent positions in the ring) was a generous gift from Drs. P. Cruz and W. Carmichael (Wright State University, Dayton, OH, USA) or was purchased from Calbiochem. Both MCs were quantified by amino acid analysis [19]. The γ_1-isoform of human PP1 [22] expressed in *Escherichia coli* and purified [23] by Drs. A. Street and D. Alessi in this laboratory had a specific activity of 15 U/mg. PP1 was labelled with [^{125}I]MC-YR as in [19]. The enzyme was incubated for 30 min with 0.5% 2-mercaptoethanol prior to reaction with MC to eliminate dimeric forms of PP1 which accumulate during prolonged storage and which do not bind microcystin.

2.2. Preparation of iodinated MC-YR

MC-YR was labelled with Na^{125}I, freed of unreacted label on a Waters C_{18} Sep-pak cartridge [19] and chromatographed on a Vydac C_{18} column developed with a gradient of water/acetonitrile in 0.1% trifluoroacetic acid (TFA). Unlabelled MC-YR was eluted at 35.8% acetonitrile (peak A), while two peaks of radiolabelled MC were eluted at 36.7% (peak B) and 37.5% (peak C) acetonitrile, respectively. All three peaks inhibited PP1 and PP2A with equal potency Fast atom bombardment mass spectrometry (performed by Mr. F.B Caudwell,

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Abbreviations: IC_{50}, concentration giving 50% inhibition of enzyme activity

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MRC Protein Phosphorylation Unit) revealed that Peak B was mono-iodinated MC-YR while Peak C, was the di-iodinated derivative. The more stable mono-iodinated Peak B was used for studies where HPLC purification of labelled peptides was required. Ethanethiol-MC-YR was labelled with ^{125}I and freed from unreacted $Na^{125}I$ on a Waters C_{18} Sep-pak cartridge [19].

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

PP1 was assayed at 30°C by the dephosphorylation of phosphorylase *a* (10 μM, 10^6 cpm per nmol) in the presence of 5 mM caffeine as described [24]. When studying the inhibition of PP1 by MC, the enzyme was assayed for 30 min at the lowest possible concentration (0.025 mU/ml, 0.05 nM) for reasons discussed in section 4. One unit of activity (U) was the amount of enzyme that catalysed the release of 1 μmol of phosphate from substrate in one minute.

2.4 Cleavage of [^{125}I]MC-YR-labelled PP1 with CNBr and trypsin, HPLC purification of labelled peptides and amino acid sequencing

PP1 (45 μl, 3 nmol) was reacted with ^{125}I-labelled MC-YR (6 μl, 1.7×10^6 cpm/μl; 3.4×10^6 cpm/nmol) as in [19]. The solution was then made 70% in formic acid and a crystal of CNBr added. The mixture was incubated for 16 h at 4°C, then diluted to 55% (v/v) formic acid and chromatographed on a Vydac C_{18} column developed with a gradient of water/acetonitrile in 0.1% TFA. The labelled peptide, eluting at 49.6% acetonitrile was pooled, dried and redissolved in 40 μl of 100% methanol and 360 μl of 50 mM Tris-HCl (pH 8.0). Trypsin (4 μl, 1 $mg \cdot ml^{-1}$) was added, and after incubation for 16 h at 37°C, a further 4 μl of 1 $mg \cdot ml^{-1}$ trypsin was added. After a further 4 h, the solution was made 1% in TFA and the sample chromatographed on the Vydac C_{18} column as above. The ^{125}I-labelled peaks were then analysed on an Applied Biosystems 470A Sequencer

2.5 Site-directed mutagenesis and purification of mutant forms of PP1

pBS^+ vector containing a cDNA encoding human $PP1\gamma_1$ [22] was modified by PCR mutagenesis to give the following mutants; Ser^{268} to Ala, Tyr^{272} to Phe, Cys^{273} to Leu, Cys^{273} to Ala and Cys^{273} to Ser. The mutants were produced by substitution of the wild-type *Hin*dIII–*Bgl*II fragment of $pBS^+PP1\gamma_1$ encoding amino acids 179–323, with a *Hin*dIII–*Bgl*II cleaved PCR fragment containing the mutation. The mutated PCR fragments were created by two step recombinant PCR [25]. In the first PCR, two overlapping fragments, termed here A and B were prepared using the $pBS^+PP1\gamma_1$ plasmid as template. Fragment A was amplified with outer forward primer (5′-GTCACTCTGTTTGCCGC-GCCCAAT-3′) covering a unique *Bgl*II site and an inner reverse primer. Fragment B was amplified with an inner forward primer and an outer primer (5′-CAGTGAGACAAACGGCGCGGGTTA-3′) covering a unique *Hin*dIII site (in the pBS^+ linker). The inner forward and reverse primers, used to generate the individual mutations, were: (a) Ser^{268} to Ala, 5′-GCCCAATTATTCCGGAGAGTTTG-3′ and 5′-CAAACTCTCCGGAATAATTGGGC-3′; (b) Tyr^{272} to Phe, 5′-GCGCCCAATTTTTGCGGAGAGTTT3′ and 5′-CAAACTCTCCG-CAAAAATTGGGCG-3′; (c) Cys^{273} to Leu, 5′-GCCCAATATCTC-GGAGAGTTTG-3′ and 5′-CAAACTCTCCGAGATAATTG-GGC-3′; (d) Cys^{273} to Ala, 5′-GCCCAATTATGCCGGAGAGTTTG-3′ and 5′-CAAACTCTCCGGCATAATTGGGC-3′ and (e) Cys^{273} to Ser, 5′-GCCCAATTATTCCGGAGAGTTTG-3′ and 5′-CAAACTC-TCCGGAATAATTGGGC-3′ The reactions (0.1 ml) contained 20 mM Tris- HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM of each deoxynucleoside triphosphate, 50 pmol of each of the appropriate pairs of oligonucleotides, 10 ng of $pBS^+PP1\gamma_1$ as template, and 5 units of *Taq* polymerase (Cetus). Conditions were: 94°C for 4 min to denature the DNA; followed by 25 cycles of 55°C for 1 min, 72°C for 2 min and 94°C for 1 min, then 55°C for 2 min; and 72°C for 20 min. The reactions were extracted with an equal volume of phenol/chloroform (1:1, by vol), followed by chloroform extraction and the DNA was purified by gel electrophoresis. Single ethidium bromide stained fragments of the expected size were excised from a 2% gel and purified using a Mermaid kit (Stratech, Luton, UK). Fragments A and B were annealed and used as a template (10 ng) for second-round PCR amplification with the outer forward and reverse primers under the same conditions used for the amplification at the first round. The reactions were extracted as before and precipitated with ethanol. The DNA was digested with *Hin*dIII and *Bgl*II, excised after electrophoresis on a 1% gel, purified using Geneclean (Stratech, Luton, UK) and subcloned into the $pBS^+PP1\gamma$ construct cleaved previously with *Hin*dIII and *Bgl*II to remove the corresponding wild-type *Hin*dIII–*Bgl*II fragment. The sequences of the $PP1\gamma_1$ mutants were checked on an Applied Biosystems 373A DNA sequencer using specific oligonucleotide primers.

The mutant $PP1\gamma_1$ DNAs were expressed by subcloning from the pBS^+ vector into the pCW vector using unique *Nde*I and *Hin*dIII sites. The pCW $PP1\gamma_1$ plasmids were transformed into *E. coli.* DH5α and the mutant proteins expressed as described previously [23]

3. Results

3.1. Identification of a specific [^{125}I]MC-YR labelled peptide from PP1

The complex formed by incubating [^{125}I]MC-YR for 2 h with $PP1\gamma_1$ ran on SDS-PAGE as a radioactive band of 38 kDa, equivalent to the molecular mass of $PP1\gamma_1$ (37 kDa) plus toxin (1 kDa) [19]. No $PP1\gamma_1$–MC complex was formed if the phosphatases were denatured in 1% SDS prior to the addition of MC. After cleavage with CNBr and SDS-PAGE of the toxin–enzyme complex, a single radiolabelled band was seen with an apparent molecular mass of 11 kDa (10 kDa + 1 kDa for the toxin), pinpointing the toxin binding site to a 10.15 kDa CNBr peptide which lies towards the C-terminus of PP1. The N-terminal sequence of this peptide was found to be RPTDVPDQGLL, establishing that it represents the peptide comprising residues 191 to 282 [22].

The PP1 catalytic subunit was radiolabelled with [^{125}I]MC-

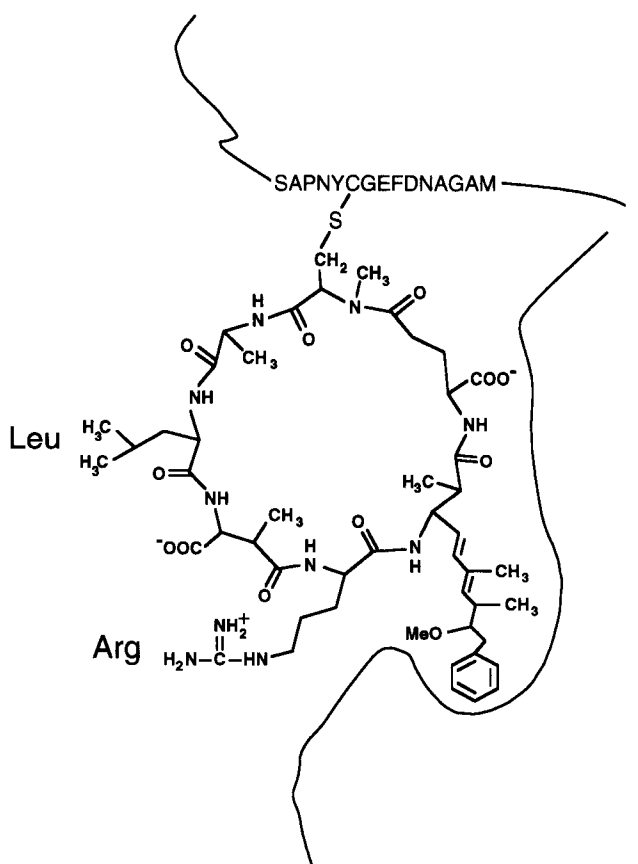

Fig. 1. Model for the interaction of PP1 with microcystin. Cys^{273} of PP1 binds covalently to the methyl-dehydroalanine residue of MC, and the ADDA moiety and the glutamyl carboxylate group of MC are also critical for inhibition of PP1.

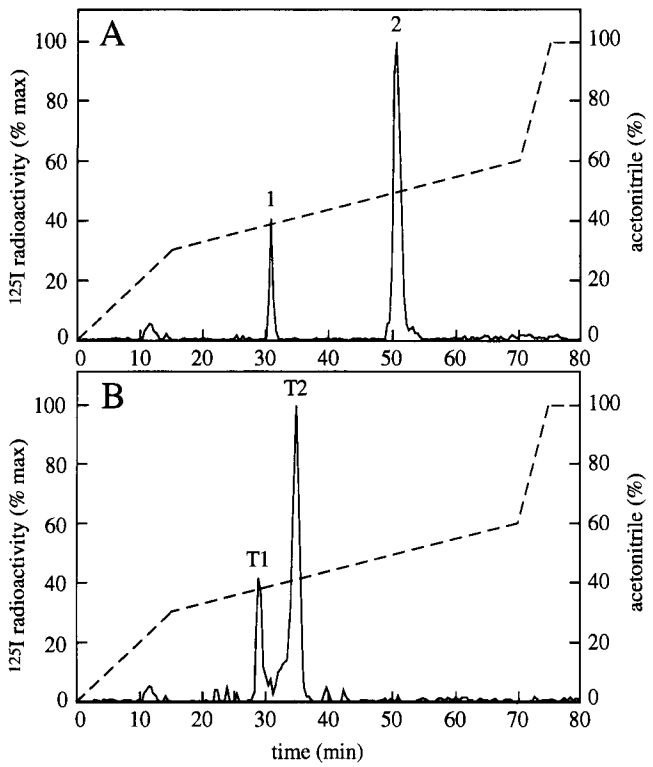

Fig. 2. Purification of microcystin-binding peptides from PP1γ. (a) [^{125}I]MC-YR-labelled PP1γ was digested with CNBr and chromatographed on a Vydac C_{18} column (section 2.4). The acetonitrile gradient is shown by the broken line Fractions of 0.5 ml were collected and counted for ^{125}I radioactivity. Peak 1, eluting at 36.7% acetonitrile, is unreacted [^{125}I]MC-YR, while peak 2, eluting at 49.6% acetonitrile, is the [^{125}I]MC-YR-labelled peptide. (b) Peak 2 from (a) was digested with trypsin (section 2.4) and re-chromatographed on the Vydac C_{18} column as in (a) to generate peptides T1 and T2.

YR, digested with CNBr and chromatographed on a C_{18} column. The minor peak (peptide 1) eluting at 36.7% acetonitrile was free [^{125}I]MC-YR, while the major peak (peptide 2) eluting at 49.6% acetonitrile was the peptide-MC complex (Fig. 2). Peptide 2 was pooled, digested with trypsin and rechromatographed on the C_{18} column. Two radiolabelled peaks were obtained eluting at 37.2% (T1) and 40.3% (T2) acetonitrile and their N-terminal sequences were SAPNYCGEFDNAGA and XXVTLFSAPNYCGE, respectively (where X represents a residue which could not be assigned unambiguously). These results demonstrated that peptides T1 and T2 commenced at residues 268 and 262 of PP1, respectively, and that T1 was derived from T2 by a chymotryptic-like cleavage of the Phe–Ser bond between residues 267 and 268.

Peptides T1 and T2 were subjected to solid phase sequencing to locate the residue which had bound to [^{125}I]MC. However, this proved to be impossible because opening of the MC ring during Edman degradation led to the sequencing of the [^{125}I]MC molecule itself, resulting in the release of ^{125}I radioactivity at the third cycle for both peptides T1 and T2. Site-directed mutagenesis was therefore adopted to identify the residue which binds to MC.

3.2. Site-directed mutagenesis of PP1

Peptide T1, comprising residues 268 to 282, contains only three residues (Ser^{268}, Tyr^{272}, Cys^{273} that have the potential to form a covalent bond and which are conserved among all protein phosphatases known to be potently inhibited by MC. These three residues were therefore mutated to amino acids with side chains unable to form a covalent bond. Mutation of Ser^{268} to Ala or Tyr^{272} to Phe had no effect on the inhibition of PP1 by MC (Table 1) and the toxin still bound covalently to the enzyme (Fig. 3). However, mutation of Cys^{273} to Leu, Ala or Ser increased the IC_{50} for toxin 10-fold or more (Table 1) and abolished covalent binding (Fig. 3). All the PP1 mutants showed a sensitivity to inhibitor-2 similar to that of the wild-type enzyme (Table 1).

3.3. The ethanethiol derivative of MC-YR does not bind covalently to PP1

A likely mechanism for covalent binding of MC to Cys^{273} was Michael addition to the methyl-dehydroalanine (Mdha) residue of MC [19]. Consistent with this mechanism, ethanethiol addition to the Mdha residue of MC-YR prevented covalent binding to PP1 as judged by SDS-PAGE and autoradiography (Fig. 3). The loss of covalent binding was accompanied by a fivefold increase in the IC_{50} for inhibition of wild-type PP1 without a significant difference in the IC_{50} for inhibition of the Leu^{273} mutant (Table 1).

4. Discussion

MC-YR labelled with ^{125}I was used previously as an affinity tag to identify protein phosphatase catalytic subunits after SDS-PAGE. Here, the [^{125}I]MC probe has been exploited to identify Cys^{273} of PP1γ_1 as the residue which binds covalently to the toxin. The sequence SAPNYC, including Cys^{273}, is conserved in virtually all known members of the PP1/PP2A family [26], and [^{125}I]MC should therefore be a useful affinity tag for many protein serine/threonine phosphatases; indeed, we have shown that it binds covalently to PP2A. An exception is the Ca^{2+}/calmodulin-dependent protein phosphatase PP2B (also termed calcineurin) which contains Leu in place of Cys and fails to bind [^{125}I]MC covalently (R.W.M., unpublished results).

Table 1
Inhibition of wild-type and mutant forms of the protein phosphatase-1 catalytic subunit by MC and inhibitor 2

Form of PP1	IC_{50} (nM) with	
	MC-LR	Inhibitor-2
Wild type	0.2	1.2
Ser^{268} to Ala	0.1	1.0
Tyr^{272} to Phe	0.2	3.0
Cys^{273} to Ala	4.0	4.5
Cys^{273} to Leu	4.0	2.0
Cys^{273} to Ser	2.2	3.0
Form of PP1	IC_{50} (nM) with	
	MC-YR	ET-MC-YR
Wild type	0.2	0.9
Cys^{273} to Leu	3.0	4.0

The concentration of PP1 in each assay was 0.05 nM. IC_{50} is the concentration required for 50% inhibition. Ethanethiol-MC-YR (ET-MC-YR) was prepared as described for aminoethanethiol-MC-LR [19]. The derivatized toxin was chromatographed on a Vydac C_{18} column as in section 2.2 and the peak of ethanethiol-MC-YR eluting at 37.2% acetonitrile (and which comprised 95% of the material) was collected, dried and redissolved in 10% methanol to give a concentration of 1 mM.

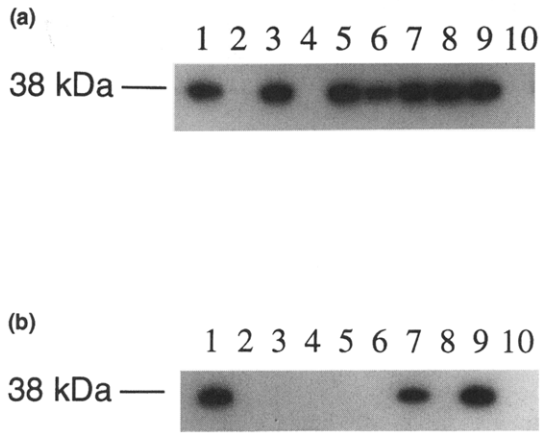

Fig. 3. Covalent binding of [^{125}I]MC-YR to wild-type and mutant forms of PP-1γ. (a) Samples of PP1γ (10 μg) were incubated for 2 h with [^{125}I]MC-YR as in [19], then denatured in SDS and subjected to electrophoresis on a 12% SDS polyacrylamide gel, and autoradiographed to reveal the MC-YR-PP1 covalent complex (38 kDa). Lanes 1, 3, 5, 7 and 9, wild-type PP1; lane 2, PP1 Cys^{273} to Ala; lane 4, PP1 Cys^{273} to Leu; lane 6, PP1 Ser^{268} to Ala; lane 8, PP1 Tyr^{272} to Phe; lane 10, PP1 Cys^{273} to Ser. (b) The experiment was carried out as in (a), except that [^{125}I]ethanethiol-MC-YR (ET-MC) was used instead of [^{125}I]MC-YR (MC) where indicated. Lane 1, wild-type PP1 labelled with MC; lane 2, wild-type PP1 with ET-MC; lane 3, PP1 Cys^{273} to Ala with MC, lane 4, PP1 Cys^{273} to Ala with ET-MC; lane 5, PP1 Cys^{273} to Leu with MC; lane 6, PP1 Cys^{273} to Leu with ET-MC; lane 7, PP1 Ser^{268} to Ala with MC, lane 8. PP1 Ser^{268} to Ala with ET-MC, lane 9, PP1 Tyr^{272} to Phe with MC, lane 10, PP1 Tyr^{272} to Phe with ET-MC.

MC binds to PP1 with extremely high affinity and the concentration required for 50% inhibition (IC_{50}) is similar to the concentration of PP1 in the assays, indicating a stoichiometric interaction. For this reason the IC_{50} value overestimates the true K_i value [8]. In the present work, the effects of MC on PP1 activity were carried out at the lowest possible enzyme concentration (0.05 nM) but, even so, the IC_{50} for inhibition of wild-type PP1 by unmodified MC (0.1–0.2 nM, Table 1) was still similar to the PP1 concentration. The observed 10- to 20-fold increase in the IC_{50} values for inhibition of the Cys^{273}-mutants by MC, and the 4- to 5-fold increase in the IC_{50} value for inhibition of wild-type PP1 by ethanethiol-modified-MC, are therefore likely to underestimate the decrease in affinity resulting from loss of interaction with Cys^{273}. If the assays were carried out at the usual PP1 concentrations, which are 10- to 20-fold higher, almost no difference in the IC_{50} values for inhibition by MC could be detected after mutation of Cys^{273}. This may explain the failure of another laboratory to detect any change in inhibition by MC when Cys^{273} of PP1α was mutated to Ser [27].

Taken together, all the available data relating to modifications of MC supports and extends a model proposed earlier for the structure of the toxin binding pocket on protein phosphatases [14]. According to this model, binding of the toxin and resultant inhibition of the protein phosphatase occurs through interaction of the hydrophobic ADDA side-chain and the glutamyl carboxyl of MC. Modification of either of these groups abolishes inhibition. The results presented in this paper lead us to propose that a further interaction is important, namely the covalent linkage of the Mhda residue of MC to Cys^{273} (Fig. 1). The double bond of Mdha is chemically the only group of MC to which cysteine could link by a Michael-type addition, and its reduction with ethanethiol abolished covalent binding to PP1. There are many naturally occurring toxic microcystins in which a variety of residues replace the leucine and/or the arginine in the peptide ring (Fig. 1), indicating that these residues are not critical for the inhibition of PP1. In summary, the generation of toxin-insensitive phosphatases may require the mutation of Cys^{273}, as well the residues that coordinate with the glutamyl carboxylate and the ADDA chain.

We have previously described an MC-affinity matrix which is exceptionally useful for the purification of different forms of PP1 from mammalian tissues [19,28]. In this matrix, aminoethanethiol is added to reduce the Mdha residue before coupling to NHS-activated CH-Sepharose. The present results not only explain why this matrix still binds PP1 with high affinity, but also why PP1 can be recovered from the matrix in an active form after elution with high concentrations of chaotropic agents.

MC is a potent hepatotoxin and liver carcinogen [29], which has become a potential threat to the health of man and his domestic livestock worldwide, because the cyanobacteria which produce it are found in many reservoirs, and because their growth has increased in recent years due to the enrichment of lakes with nitrates from fertilisers used in modern agriculture and with phosphates from detergents. The finding of a covalent linkage between PP1 and the Mdha residue of MC is therefore additionally important because it explains why free toxin cannot be found in postmortem samples from animals that have died from suspected MC poisoning (personal communications from George Gunn, Scottish Agricultural College, Inverness and Wayne Carmichel, Wright State University, Dayton, OH, USA).

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