A Molecular Basis for Different Interactions of Marine Toxins with Protein Phosphatase-1

MOLECULAR MODELS FOR BOUND MOTUPORIN, MICROCYSTINS, OKADAIC ACID, AND CALYCLININ A

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The hepatotoxic cyclic heptapeptide microcystins and cyclic pentapeptide nodularins are powerful liver tumor promoters and potent inhibitors of the catalytic subunits of protein phosphatase-1 and -2A (PP-1c and PP-2Ac). In marked contrast to microcystins, which interact covalently with PP-1 and PP-2A, the nodularins do not bind covalently to PP-1 and PP-2A and may additionally possess unique carcinogenic properties. The conformation of microcystin-LR has been determined in solution and bound to PP-1c. We show here that the free NMR solution structures of two distinct microcystin structural congeners (microcystin-LR and -LL) are remarkably similar to the bound crystal structure of microcystin-LR. We have exploited this finding by using Metropolis Monte Carlo modeling to dock the solution structures of microcystin-LL and the marine toxin motuporin (nodularin-V) onto the crystal structure of PP-1c. Both of these toxins occupy a position similar to that of microcystin-LR when bound to PP-1c. However, although there are relatively minor differences in the structural orientation of microcystin-LL compared with microcystin-LR, there is a striking difference in the position of the N-methyldehydrobutyryne residue in motuporin relative to the comparable N-methyldehydroalanine residue in microcystin-LR. We propose that this difference in orientation provides a molecular explanation for why nodularins are incapable of forming a covalent linkage with PP-1c. Furthermore, the predicted position of N-methyldehydrobutyryne in motuporin is at the surface of the PP-1c-toxin complex, which may thus facilitate chemical interaction with a further macromolecule(s) possibly relating to its carcinogenic properties. PP-1c and PP-2Ac are also targets for other marine toxins such as okadaic acid and calyculin A. It was therefore of interest to use Metropolis Monte Carlo modeling to dock the known free crystal structures of okadaic acid and calyculin A to the crystal structure of PP-1c. These experiments predict that both okadaic acid and calyculin A are strikingly similar to microcystins and motuporin in their tertiary structure and relative PP-1c binding position.

The micr0cystin and nodularin classes of peptide hepatotoxins (see summary and Refs. 1–4) are metabolites of cyanobacteria in the genera Microcystis, Anabaena, and Nodularia which grow worldwide in fresh and brackish water (5). It has been shown that microcystin-LR is a potent inhibitor of the catalytic subunits of protein phosphatase-1 and -2A (PP-1c and PP-2Ac) as well as a powerful tumor promoter (6–10). Inhibition of these enzymes in the liver is apparently associated with hepatoxyte deformation due to reorganization of microfilaments (11). Liver tumor promotion may be linked to the ability of this cyclic peptide to promote hyperphosphorylation of cytolkeratins associated with morphological changes in rat hepatocytes (12).

To date more than 40 different microcystins have been characterized (13, 14). Within the microcystin family, members differ from each other in the nature of two variable L-amino acids indicated by suffix letters (e.g. L = Leu, R = Arg) and in the presence or absence of methyl groups on the α-erythro-β-methylaspartic acid (Masp) and/or N-methyldehydroalanine (Mdha) residues (Fig. 1). Despite these differences no loss of PPase inhibition or tumor promotion has been observed. Nodularins are structurally related cyclic pentapeptides (Fig. 1) that inhibit PP-1c and PP-2Ac with potency similar to that of microcystins and are also powerful tumor promoters (15). The relatively hydrophobic cyclic pentapeptide motuporin (also termed nodularin-V in Fig. 1), which was isolated from the marine sponge Theonella swinhoei, differs from nodularin by substitution of a valine residue for an arginine residue (16).

Although sharing similar biological properties, important functional differences between the microcystins and nodularins have been identified. One difference is in the interaction with PP-1c and PP-2Ac. Although both toxins initially bind noncovalently and inhibit these enzymes, microcystin-LR, -LA, and -LL undergo a secondary time-dependent interaction with the phosphatase (3, 4, 17). This secondary interaction results in a covalent linkage causing irreversible modification of PP-1c/PP-2Ac. In contrast, nodularin or motuporin does not bind covalently to PP-1c/PP-2Ac after inhibiting it. A second difference is that in addition to acting as a tumor promoter, nodularin may also act as a carcinogen/tumor initiator (15).

Okadaic acid (OA) and related congeners are polyether-like compounds (Fig. 1), potent inhibitors of PP-1c/PP-2Ac, and powerful tumor promoters (for review, see Ref. 10). OA is produced by unicellular dinoflagellates such as Dinophysis and...
Prorocentrum, causes diarrhetic shellfish poisoning in humans, and has also been used extensively as a research tool in studying reversible protein phosphorylation in many cellular processes (18). Calyculin A (Fig. 1) and its analogs isolated from the marine sponge Discodermia calyx are also effective inhibitors of PP-1c and PP-2Ac (19). Paradoxically, although microcystin/nodularin, OA, and calyculin A possess strikingly different linear structures (Fig. 1), they are all potent PP-1c/PP-2Ac inhibitors and appear to bind to a similar region on PP-1c (discussed in Ref. 20 and references therein).

Previously, the solution structures of microcystin-LR and motuporin (nodularin-V) were compared with each other to give insights into how their residues and tertiary structure were important for binding to PP-1c (1). Comparison of these solution structures revealed how the Mdhb residue in motuporin was relatively significantly displaced from the Mdha residue in microcystin-LR, thus providing a preliminary explanation for why microcystins are capable of binding covalently to the PPases, whereas motuporin lacks this ability.

Elucidation of the crystal structure of microcystin-LR bound covalently to PP-1c (2) provided a detailed view of key interactions between microcystin-LR and PP-1c and facilitated a detailed comparison of the structures of unbound and bound toxin. Accordingly, in this paper we show that the published free solution NMR structure of microcystin-LR does not change significantly in conformation upon inhibiting and binding covalently to PP-1c. This may be a factor contributing to the high affinity of microcystin-LR for PP-1c.

Given the remarkable similarity between the free solution structure and bound crystal structure of microcystin-LR, we predict that the other microcystins will have similar free and bound structures. In accordance with this hypothesis, we also determined the solution structure of the hydrophobic microcystin congener microcystin-LL and docked this structure (using Metropolis Monte Carlo modeling) onto the crystal structure of PP-1c. Furthermore, because nodularins are structurally related to microcystins, we also assume that the nodularins will largely retain their solution structure upon binding to PP-1c and have docked motuporin onto this enzyme. A comparison of the complexes of microcystin-LR, microcystin-LL, and motuporin with PP-1c sheds light upon the different molecular mechanisms underlying their interaction with the phosphatase. These comparisons strongly suggest why the microcystins react covalently, whereas the nodularins do not, and provide a preliminary hypothesis for explaining the carcinogenic properties of the nodularins.

### EXPERIMENTAL PROCEDURES

Microcystin-LL and motuporin were purified as described previously (14, 16). Samples used for NMR studies were reconstituted at 1 mM in potassium phosphate (10 mM), sodium chloride (50 mM) buffer with 80–90% H_2O, 10–20% D_2O at pH 7. 2,2-Dimethyl-2-silapentane-5-sulfonic acid (0.1 mM) was added as a 1H NMR chemical shift standard.

| Toxin         | RMSD Å |
|---------------|--------|
| Microcystin-LR| 0.68   |
| Microcystin-LL| 1.56   |
| Motuporin     | 1.96   |
| Okadaic acid  | 2.36   |
| Calyculin A   | 1.26   |

Microcystin-LL and motuporin were made using standard sequential assignment methods with double quantum filtered COSY, TOCSY, and NOESY two-dimensional 1H NMR spectra and were similar to microcystin-LR and motuporin assignments (1). 1H-1H internuclear distance restraints were obtained.
from two-dimensional $^1$H NOESY taken with a mixing time of 150 ms so as to minimize spin diffusion. $^3J_{\text{NH-CH}}$ and $^3J_{\text{CH-CH}}$ coupling constants were obtained by curve fitting one-dimensional $^1$H NMR spectra. Assignments were made at 25 and 5°C, with structural information recorded at 5°C. Calibration of the NOEs was accomplished using the known distance of 1.8 Å between the HB protons of the sp2 carbon of the Mda side chain to scale the experimental cross-peak intensity. Sixty structures were generated using the distance geometry program DGII in the program Insight II version 2.3 (Biosym Technologies Inc.) with 58 of the 60 structures converging into one fold and the other 2 discarded because of violations. For this cyclic peptide the vicinal angle restraints were very important in restraining the structure of the ring. Distance and angle violations were analyzed using the NMR refine module in Insight II. There were no consistently violated distance restraints over 0.1 Å, and angles fell within the defined restraint range in almost all cases for the 58 converging structures. An average structure was then

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**Fig. 2.** Stereo view of comparison between the free solution microcystin-LR and bound crystal microcystin-LR. The free average minimized NMR structure of microcystin-LR is red, and the bound crystal structure is in blue. For clarity, neither structure has hydrogen atoms. The RMSD of the backbones superimposed on each other is 0.65 Å.

**Fig. 3.** Panel A, solution structure of microcystin-LL in stereo. The 58 calculated DGII structures are shown in red; the average minimized structure is in green. The backbone RMSD is $0.57 \pm 0.10$ Å of the calculated structures relative to the average structure. Hydrogen atoms are not shown. Panel B, superimposition of free solution microcystin-LR and free solution microcystin-LL in stereo. The average minimized free NMR structure of microcystin-LR is shown in red, with the green structure being the averaged minimized free NMR structure of microcystin-LL. The difference in sequence is that microcystin-LR has an arginine (label in red) where microcystin-LL has a leucine (label in green). The backbone RMSD with respect to the two structures is 0.77 Å. No hydrogen atoms are shown.
generated from the 58 structures and was subjected to constrained minimization to correct bond distances that had been distorted through averaging.

Metropolis Monte Carlo docking of microcystin-LL and motuporin was accomplished using the Monte Carlo macro in Insight II version 2.3. For microcystin-LL, the minimized average structure was used for docking with the PP-1c crystal structure (2). The PP-1c crystal structure was modified to facilitate docking by removing the bound microcystin-LL and adding protons. The starting position for placing microcystin-LL relative to PP-1c was obtained by superimposing microcystin-LL onto the bound x-ray crystal structure of microcystin-LL. This was accomplished by superimposing the identical backbone
atoms of the docking PPase structure onto the original PPase structure that still had microcystin-LR attached, superimposing microcystin-LL onto microcystin-LR in the crystal structure, then removing original PPase + microcystin-LR, thus leaving docking PPase with microcystin-LL in its starting position. From this single starting point, the Monte Carlo macro then performed docking calculations for 2,000 trials at a temperature of 50 K. Other temperatures (1 and 100 K) were tried, but varying the temperature did not make any difference in positioning the final docked structure of microcystin-LL (provided the number of trials was equivalent). Furthermore, different numbers of trials were used (100, 1,000, and 2,000) at 50 K with all three amounts of trials resulting in a similarly docked microcystin-LL. Because 2,000 trials resulted in the lowest energy for the final structure, this was chosen as the optimum number of trials to be performed. For motuporin, the minimized average solution structure was again used for docking with PP-1c (the latter structure used exhibited protons but lacked bound microcystin-LR). Superimposition of motuporin onto bound microcystin-LR was accomplished in a manner similar to that of microcystin-LL. Docking of motuporin using the Monte Carlo macro was at 50 K with 2,000 trials.

To carry out molecular modeling studies with OA (3) and calyculin A (4), the free crystal structures of these toxins were retrieved from the Cambridge Structural Database (21). In the case of OA, the bromobenzyl region of the crystal structure was removed so as to use native OA in the docking process. Both OA and calyculin A were docked in the same manner as microcystin-LL and motuporin. The starting point used for OA and calyculin A was from visual superimposition of the individual toxin onto bound microcystin-LR.

As a rigorous test of our procedures, the minimized average solution structure of microcystin-LR was docked onto the crystal structure of PP-1c in the same manner and starting point as microcystin-LL and motuporin. The docked structure of microcystin-LR hardly moved from its starting position, indicating that the Metropolis Monte Carlo macro used was successful in keeping the free solution structure of microcystin-LR at the same binding position observed in the PP-1c-microcystin-LR crystal structure (data not shown). In addition, we calculated the root mean square deviation (RMSD) change in atomic positions from the original manually positioned toxins to the final model after docking. These data (Table I) show that the RMSD for all atoms between starting and docked structures for each toxin varied between 0.68 Å for microcystin-LR and 2.36 Å for OA.

As a further test of the robustness of our docking methods, we employed our Metropolis Monte Carlo procedures for the converging 58 NMR structures of microcystin-LL. Although most of these structures do indeed dock in a manner similar to that of the average structure, to be objective we focused particularly on the six most disparate microcystin-LL NMR structures. The disparate nature of these distorted structures was predicated largely by the flexibility of their Adda side chains in solution. Therefore, even though the main ring of these toxins might have to move an unrealistic amount to compensate for an unusually positioned Adda side chain, these six most distorted toxin structures still fitted well, wherein the backbone RMSD (comparing docked NMR calculated structures with the docked average solution structure for microcystin-LL) averaged 1.28 Å (± 0.54 S.D.).

**RESULTS**

Comparison of Free Microcystins (NMR Solution Structures) with Microcystin-LR Bound to PP-1c (X-ray Crystal Structure (2))—We compared extensively (Fig. 2) the minimized average free solution structure of microcystin-LR (determined by NMR spectroscopy) with that of microcystin-LR bound to PP-1c (determined by x-ray crystallography). The RMSD of all backbone atoms superimposed on each other was 0.65 Å. The two structures are strikingly similar, indicating that microcystin-LR retains its free conformation while inhibiting and binding covalently to PP-1c. The minor differences in orientation of Arg and Mdha side chains between the two structures may be accounted for by the flexibility of the Arg side chain in solution and the change in the position of the Mdha side chain (where it points upward from the saddle in the free solution structure and downward in the bound crystal structure; Fig. 2) following covalent bonding between this residue and Cys-273 of PP-1c. In solution the Adda side chain is flexible, with the position adopted in Fig. 2 representing that of the average structure. In the crystal structure the Adda side chain is inflexible and clearly defined.
The family of solution structures of microcystin-LL determined by 1HNMR is shown in Fig. 3A. The RMSD of backbone atoms relative to the average structure is 0.57 ± 0.10 Å. The cyclic backbone of microcystin-LL forms a relatively rigid saddle, whereas the Adda side chain is highly flexible. The Glu and Masp carboxyl groups are positioned such that their negative charges are situated below the saddle. Mdha is located at the top, front part of the saddle in the perspective shown in Fig. 3A. The minimized average structure of microcystin-LL is highly comparable to the minimized average solution structure of microcystin-LR (Fig. 3B), with the RMSD of the two cyclic backbones being 0.77 Å. Clearly, substitution of positively charged Arg with hydrophobic Leu in microcystin-LL results in no overall structural change. Given that microcystin-LR retains its solution structure upon binding to PP-1c, we postulated that microcystin-LL would do likewise. The minimized average free solution structure of microcystin-LL was docked onto the crystal structure of PP-1c using Metropolis Monte Carlo procedures. These data show that microcystin-LR retains its solution structure upon binding to PP-1c, as we postulated that microcystin-LL would do likewise. The minimized average free solution structure of microcystin-LL was docked onto the crystal structure of microcystin-LR (Fig. 3B), with the RMSD of the two cyclic backbones being 0.77 Å. Clearly, substitution of positively charged Arg with hydrophobic Leu in microcystin-LL results in no overall structural change. Given that microcystin-LR retains its solution structure upon binding to PP-1c, we postulated that microcystin-LL would do likewise. The minimized average free solution structure of microcystin-LL was docked onto the crystal structure of PP-1c using Metropolis Monte Carlo procedures (Fig. 3B). In our model the distance between the β carbon of the Mdha residue and the sulfur atom on the Cys-273 side chain is 10 Å. Motuporin differs only slightly in position relative to PP-1c-bound microcystin-LR. This model predicts similar PPase residues to be within 4 Å of the toxin when compared with bound microcystin-LR (Fig. 5A) and docked microcystin-LL, but it lacks some proximity connections (within 4 Å) particularly with respect to PP-1c residues 274–276.

The overall crystal structure of microcystin-LR bound to PP-1c (Fig. 6A) was compared with our structural model for motuporin docked onto the crystal structure of microcystin-LR. We identified a remarkable difference between the two PPase-toxin complexes such that whereas Mdha in microcystin-LR is bonded covalently to Cys-273 and relatively buried, the equiv-
alent Mdhb residue in motuporin should be highly accessible and actually protrude from the molecular surface of the PP-1c-motuporin complex.

Superimposition of the Free X-ray Crystal Structures of OA and Calyculin A onto the Bound PP-1c-Microcystin-LR Complex—Fig. 7A depicts the PP-1c-docked free crystal structure of OA (see “Experimental Procedures”) compared with the bound crystal structure of microcystin-LR (blue). The two toxins are overlapped in the same relative position to PP-1c, with the superimposed phosphatases not shown. The red atoms represent oxygen (O14, O12, and O13, top to bottom, respectively, for the three oxygens near the Arg residue; the remaining three nonphosphate group oxygens, left to right, are O1, O2, and O9). The phosphate group has P in purple with the four oxygens attached to it in red. The dark blue atom at the hydrophobic end is the nitrogen that is triple bonded to the first carbon atom. The white labels are the microcystin-LR residues. Panel B, same position for calyculin A as in panel A, except now showing the surrounding PP-1c residues within 4 Å. Color and label scheme are the same as in panel A. Panels A and B are both in stereo, and hydrogen atoms are not shown.

Determination of the Predicted Molecular Surfaces of Free and PP-1c-bound Marine Toxins—The nature of the molecular surfaces of microcystin-LR, motuporin, OA, and calyculin A in both their unbound (Fig. 9) and PP-1c bound/docked (Fig. 10) conformations was calculated using the GRASP procedure. The surface of each toxin was examined according to its electrostatic surface potential (in the unbound form) and its distance from the surface of PP-1c in the bound/docked form. These experiments revealed a strong similarity in the tertiary structures of the toxins and prompted a further GRASP analysis of the molecular surfaces of PP-1c involved in toxin binding...
Interestingly, the predicted molecular surfaces of PP-1c closely involved in binding microcystin-LR (Fig. 11A) and OA (Fig. 11C) were the most strikingly similar. These include the region on PP-1c (represented in white, 0–1.5 Å distance) comprising Tyr-272 and Cys-273. Notably, this region (represented in red in Fig. 11B) is not predicted to be close to Mdhb in motuporin and appears to be less important in interacting with this toxin. These data are again consistent with the idea that motuporin does not interact covalently with PP-1c.

**DISCUSSION**

Microcystin-LR is a potent inhibitor of PP-1c, with a high affinity for the enzyme (K_i in the low nanomolar range). With the exception of minor differences noted under “Results,” the free NMR solution structure of microcystin-LR matches its crystal structure when complexed with PP-1c (Fig. 2). Can we understand the reason why microcystin-LR does not change its structure significantly upon binding to PP-1c? Retaining the cyclic backbone of microcystin-LR would reduce the unfavorable conformational entropy lost upon binding (2). The rigid saddle-shaped backbone provides the proper framework for the hydrophobic, ionic, and covalent interactions between microcystin-LR and the phosphatase. These interactions involve the Adda and Masp/Glu residues, which are essential for inhibition (9, 13, 22), and the Mdh residue, which is required for secondary covalent linkage.

The rare, hydrophobic Adda amino acid located behind the rigid saddle of microcystin-LR is flexible in solution and therefore able to adjust to fit into a hydrophobic groove of PP-1c. Because hydrophobicity may be the initial driving force behind the binding of microcystin-LR to PP-1c, the Adda residue could be responsible for anchoring the cyclic backbone ring into its bound position. With respect to the Adda residue, hydrophobic interactions probably play a more than make up for the conformational entropy cost that is required to stabilize the Adda side chain into one conformation in the bound state. The other contributing residues for inhibition, Masp and Glu, have d-oriented, negatively charged carboxyl groups located underneath the saddle which are both oriented to interact with positively charged Arg-96 of PP-1c. Finally, the secondary covalent linkage that forms after inhibition (3, 4, 17) is dependent on the modified amino acid Mdhb being located at the front and top of the toxin saddle to link covalently to Cys-273.

There are other examples of peptides, either flexible or rigid by nature, which bind to their target proteins with little change in conformation as we have observed for microcystin-LR. The free solution structure in water of disulfide bond-linked desmopressin (23) and cyclic cyclosporin A (24–26) were found to be similar to that of their bound forms (with desmopressin one of the five families of the free-water structure matches the bound form, whereas only one free form of cyclosporin A exists in water). In the case of hirudin, a linear peptide inhibitor of the interaction of thrombin with fibrinogen, the COOH-terminal region that binds to thrombin was found to have the same global fold free and bound to thrombin (27). Oxytocin, a disulfide linked hormonal peptide, has been compared free and bound with its carrier protein neurophysin (28). The two most important residues for binding, Cys-1 and Tyr-2, do not change when complexed to neurophysin (28). The rest of the ring undergoes a conformational change, but this is thought to occur after binding takes place. Taken together these examples, coupled with our microcystin-LR data, indicate that determination of free solution structures in water of many biologically active peptides may provide useful insights into their function, since these structures may be retained upon binding to their target(s).

The high degree of similarity between microcystin-LR and the more hydrophobic microcystin-LL (Fig. 3, A and B) suggests that the microcystin class of inhibitors probably has a basic
saddle-shaped backbone with the Adda side chain protruding behind it. This would account for the similar properties of microcystins in binding to PP-1c. It should be noted that other published microcystin structures have been carried out in the presence of dimethyl sulfoxide solvent. The published microcystin-LR structure in dimethyl sulfoxide (29) was found to have three different conformational families with highly compact ring structures that do not appear to match the saddle-shaped ring of the free solution structure in water or bound crystal structure. The structure of microcystin-LY in dimethyl sulfoxide was described as being a boat-like ring with the Adda residue protruding form the concave side, which is more in line with our microcystin-LR and microcystin-LL structures. Recently, a paper was published comparing the structure of microcystin-LR in water and in dimethyl sulfoxide (30). In this study, microcystin-LR was found to have the same structure in water and dimethyl sulfoxide, and both structures were similar to our previously published free solution structure (1). Thus, the conformation of microcystin-LR seems to be unclear in dimethyl sulfoxide but is confirmed in water (1, 2, 30).

Accurate docking of the unbound structures of microcystin-LL, motuporin (Fig. 5B), OA (Fig. 7), and calyculin A (Fig. 8) relies upon the assumption that like microcystin-LR each of the free structures of these toxins would not change dramatically upon binding. Of course, it is also dependent on the assumption that PP-1c itself does not differ greatly in conformation when microcystin-LR or the other inhibitors are bound. The latter assumption is supported by x-ray crystallographic determination of free PP-1c (i.e. without microcystin-LR) which was found to be in overall agreement with the previously published PP-1c crystal structure bound to microcystin-LR (2, 31). This indicates that the inhibitors described in this study probably do not have a significant impact on the overall tertiary structure of PP-1c when bound to the enzyme. Because microcystin-LL and motuporin are similar to microcystin-LR in terms of structure and function, the best possible starting point for docking of both inhibitors is in the position relative to PP-1c where they are superimposed onto the bound crystal structure of microcystin-LR. We employed a similar approach for OA and calyculin A, whose free crystal structures are very similar to the tertiary structure of microcystin-LR.

As mentioned under “Experimental Procedures,” we applied a rigorous test for the Monte Carlo docking macro procedure used in this study, wherein the minimized average free solution structure of microcystin-LR was docked in the same manner as microcystin-LL and motuporin. The final position of the docked microcystin-LR was only slightly shifted from its starting point (data not shown), suggesting that Metropolis Monte Carlo docking was successful in placing the free solution structure of microcystin-LR very near the bound structure determined by x-ray crystallography.

The similarities in the backbone atoms of Masp, Arg/Val, and Adda, positioning of the Adda side chain, and the Glu and Masp carboxyls between microcystin-LR and motuporin are probably responsible for both cyclic peptides being specific, high affinity and tight binding inhibitors of PP-1c (Fig. 4). Although they are functionally and structurally similar, there are differences between the microcystin and nodularin class inhibitors. Microcystins bind covalently to PP-1c, whereas nodularins are unable to link covalently to this enzyme. We proposed that the basis for this functional difference was found in the structural displacement of the Mdha residue (involved in the covalent linkage with PP-1c) in microcystin-LR when compared with its counterpart residue Mdhb in motuporin (1). By comparing the
bound crystal structure of microcystin-LR with the free solution structure of motuporin superimposed in the same manner (Fig. 4) we substantiate our previous hypothesis that a large displacement (7.13 Å as seen in Fig. 4) occurs between the β carbon of the Mdha residue located at the front, top of the saddle in bound microcystin-LR and the β carbon of the Mdhb residue in motuporin. The Mdhb residue is predicted to be located far below the Mdha residue near the Leu position of microcystin-LR.

Docking of the motuporin solution structure onto the crystal structure of PP-1c by Metropolis Monte Carlo procedures resulted in only a minor displacement from the starting point of motuporin superimposed onto bound microcystin (Fig. 5, A versus B). With docked motuporin, the distance between the β carbon of the Mdhb residue located at the front, top of the saddle in bound microcystin-LR and the β carbon of the Mdhb residue in motuporin. The Mdhb residue is predicted to be located far below the Mdha residue near the Leu position of microcystin-LR.

Docking of the motuporin solution structure onto the crystal structure of PP-1c by Metropolis Monte Carlo procedures resulted in only a minor displacement from the starting point of motuporin superimposed onto bound microcystin (Fig. 5, A versus B). With docked motuporin, the distance between the β carbon of the Mdhb residue located at the front, top of the saddle in bound microcystin-LR and the β carbon of the Mdhb residue in motuporin. The Mdhb residue is predicted to be located far below the Mdha residue near the Leu position of microcystin-LR.

PP-2Ac may ultimately explain why the nodularins may be able to function as carcinogens. Since these toxins form no covalent linkage to PPases, their electrophilic Mdhb residues may be free to form direct adducts with nucleophilic groups on other informational macromolecules either when bound to protein phosphatase (as viewed in Fig. 6B) or following a dissociation event from these enzymes. It is conceivable that this may ultimately explain why microcystin-LR appears to be devoid of carcinogenic properties, since it would be expected to remain covalently attached to PP-1c/PP-2Ac and therefore unable to form an adduct with other macromolecules via the Mdha residue.

The model for docked OA has distinct similarities to the bound tertiary structure and position for microcystin-LR (Fig. 7, A and B). This would explain how these structurally diverse toxins are both able to inhibit PP-1c and agrees with competition studies between OA and microcystin as well as recent extensive PP-1c mutagenesis studies that suggest that these toxins bind in a mutually exclusive manner (2, 20, 32). Our model is also in agreement with the previous biochemical studies that examined the interaction between OA and PP-1c (33, 34). For example, O5 of OA has been altered by the additions of bulky groups that do not affect the binding of the toxin. In our structural model, this oxygen would be predicted to be highly solvent accessible and is not near the phosphatase-toxin interface. Therefore, chemical additions to this oxygen should not hinder OA binding to PP-1c. The oxygen atoms at the C1 carboxyl end of OA (O1, O2, and O3), especially O1, are predicted to be situated near Tyr-272, Val-250, Arg-96, and Arg.
221. Methylation of O1 has been shown to affect OA binding detrimentally (33), which in the model would be explained by disruption of the O1 interaction with one of the above PP-1 residues, most probably Tyr-272. Mutations of PP-1c residues 271–277 (20), which affect the sensitivity of PP-1c to OA, can be explained by the association of the C1 carboxyl terminus of OA with Tyr-272 and that other regions of OA are also near this stretch of amino acids (Fig. 7B) and may interact with these PPase residues (e.g. Phe-276). In our OA binding model, the O10 (C24) hydroxyl group is very near (less than 3 Å) Arg-221. Finally, the C14-C15 double bond, which needs to be in the E conformation for OA to be active (34), is to some extent close to Glu-275, but it is conceivable that a change in the configuration of this double bond could alter the overall structure of OA, thus influencing its binding. The model for calycin A, like OA, shares many features with bound microcystin-LR (Fig. 8, A and B). In calycin A, the unusual phosphate group is predicted to contribute to binding, possibly by interacting with Arg-96 and/or Arg-221.

Previously, Quinn et al. (34) recognized common structural components (namely an acidic group and hydrophobic region) that microcystin-LR, OA, and calycin A share. With the current availability of the free solution structures of microcystin-LR, microcystin-LL, and motuporin, as well as the crystal structure of microcystin-LR bound to PP-1, we have extended these preliminary studies further by taking the previously published free crystal structures of OA and calcin A and docking them to PP-1 in the same manner as microcystin-LL and motuporin. These docked models of OA and calycin A (when compared with microcyclins and motuporin) now provide a compelling hypothesis to account for how these diverse toxins are able to inhibit PP-1c.

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