

METAL IONS MEDIATE THE BINDING OF CYANOBACTERIAL TOXINS TO HUMAN PROTEIN PHOSPHATASE I

A Computational Study

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OULU 2000



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Manuscript received 8 June 2000
Accepted 12 September 2000

ISBN 951-42-5769-3

ISBN 951-42-5768-5
ISSN 0355-3191 (URL: <http://herkules.oulu.fi/issn03553191/>)

ALSO AVAILABLE IN PRINTED FORMAT

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Kimmo Mattila, Arto Annala and Tapio T. Rantala, Metal Ions Mediate the Binding of Cyanobacterial Toxins to Human Protein Phosphatase 1, A Computational Study

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2000

Oulu, Finland

Abstract

The binding mechanisms of two cyanobacterial toxins, Microcystin-LR and Nodularin, to Protein Phosphatase 1 (PP1) were studied with molecular modeling and molecular dynamics simulations. The models of the two toxin-phosphatase complexes are based on the structure of PP1-microcystin complex determined by X-ray crystallography, and on the conformation of nodularin in water determined by NMR. The binding site of PP1 including the ligated toxin and the surrounding water molecules were simulated for 600 ps using CHARMM software. Conformations, dynamics and intermolecular interactions were analyzed from the simulations. The binding was found to be quite similar for both toxins. The characteristic feature in the binding was the interaction of IGlu amino acid carboxyl group of the toxins with the metal ions in the PP1 catalytic site. A number of other interactions in the enzyme and toxins, which participated in the binding, was found as well. The two models of the PP1-inhibitor complexes, presented here, are mostly in agreement with the experimental data, and explain why certain modifications in cyanobacterial hepatotoxins and in protein phosphatases affect the toxin binding.

Keywords: nodularin, microcystin, protein phosphatase, simulation, molecular dynamics.

Acknowledgments

The present work has been supported by a grant from the Emil Aaltonen Foundation and by the Academy of Finland. The Center of Scientific Computing (CSC, Espoo, Finland) is acknowledged for providing the computational resources. We also wish to thank Dr. J. Kuriyan and his group for providing the crystal structure of PP1-microcystin complex and Dr. Sabine Rudolph-Böhner for comments while preparing the manuscript.

Abbreviations

PP1:	Protein Phosphatase 1
PPs:	Protein Phosphatases
NMR:	Nuclear Magnetic Resonance spectroscopy
MD:	Molecular Dynamics
SD:	steepest descents
ABNR:	adopted basis Newton-Raphson
RMS:	Root Means Square
RMSD:	Root Mean Square Deviation
Sdev:	Standard Deviation;
Adda:	(2S, 3S, 8S, 9S)-3-amino-9-methoxyl-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Masp:	δ -erythro- β -methyl-aspartic acid
MDHB:	2-(methylamino)-2-dehydrobutyric acid
MDHA:	N-methyl-dehydroalanine

Contents

Abstract

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1 Introduction

Reversible protein phosphorylation plays a vital role in several intracellular regulation and signaling mechanisms (Johnson and Barford, 1993). Adding and removing phosphate groups in Ser, Thr or Tyr side chains provide means for controlling biochemical pathways, e.g., hormonal response, gene transcription or cell division (Mellgren et al., 1993). Phosphorylation, in turn, is controlled by protein kinases (phosphorylation) and protein phosphatases (dephosphorylation). Several protein kinases and phosphatases have been discovered from eucaryotic and procaryotic cells, among them Protein Phosphatase 1 (PP1), which regulates the glycogen metabolism response to insulin and adrenaline (Dent et al., 1990). It also has an effect on the activity of sarcoplasmic reticulum Ca^{2+} -ATPase (MacDougall et al., 1991), smooth muscle contraction and protein synthesis (Cohen, 1989; MacKintosh and MacKintosh, 1994).

So far, at atomic resolution, comparatively little is known about the cellular regulation mechanisms of these enzymes. Several natural occurring toxins are found to inhibit Protein Phosphatases (PPs). Consequently, understanding of the interactions which cause the toxicity of these peptides would be advantageous in investigations of functions regulated by PPs.

Cyanobacterial hepatotoxins, microcystins and nodularins (Fig. 1) (Carmichael et al., 1988b; Eriksson et al., 1988; Rinehart et al., 1988; Sivonen et al., 1989), form a group of toxins, which strongly inhibit PP1 and other serine/threonine specific protein phosphatases. In cells, these toxins suppress cell transformation, induce apoptosis and are also tumor promoters. A high level of inhibition of PPs by the toxins leads to a collapse of the cytoskeleton (Eriksson et al., 1992; Eriksson and Goldman, 1993), while smaller doses may perturb the cell's regulation system to the extent that a cell will escape to a state of uncontrolled growth (Nishiwaki-Matsushima et al., 1992).

During the last few years, several structural NMR studies about the free conformations of microcystins and nodularins have been published. The toxins have well-defined cyclic backbones that share a common structural motif around the characteristic ADDA-amino acid, found in these toxins. (Rudolph-Böhner et al., 1994; Bagu et al., 1995; Annala et al., 1996; Trogen et al., 1996)

Recently, structural information has become available also for serine/threonine-specific protein phosphatases as the structures of PP1 in complex with tungstate (Egloff et al., 1995) and in complex with microcystin-LR (Goldberg et al., 1995) have been determined by X-ray crystallography. These structures provide considerable insight into the catalytic function of PP1 and into the underlying structural motifs of the binding of toxins. The structural features of binding were further studied by Bagu et. al. (Bagu et. al. 1997). In that study, molecular models on microcystin-LR and -LL, motuporin, Ocadaic acid and calyculin were docked to the active site of PP1 using Metropolis Monte Carlo simulations. Crystal structure and docking studies show that Microcystin-LR occupies a cleft in the vicinity of the catalytically active metal ions of PP1. Remarkably, the conformation of the bound resembles closely that of a free microcystin-LR and certain regions of free nodularin. This implies that only minor conformational changes occur in the toxins upon the binding. In the complex, microcystin-LR is covalently bound from N-methyl dehydroalanine (MDHA) to Cys-273 (Goldberg et al., 1995; MacKintosh et al., 1995). Covalent binding is apparently a secondary reaction, unnecessary for the binding to PP1 (Zhang et al., 1992; Moorhead et al., 1994). Nodularins, which are chemically quite similar to microcystins (Fig. 1), and equally toxic, do not bind covalently to PP1 (MacKintosh and MacKintosh, 1994; Rinehart et al., 1994).

In the present study, further insight into the binding mechanism of the cyanobacterial toxins to PPs has been acquired by molecular modeling and Molecular Dynamics (MD) simulations. We present here molecular models, in which we have combined the structural data of PP1 from X-ray crystallography (Egloff et al., 1995; Goldberg et al., 1995) and the structural NMR-data of nodularin and microcystin-LR (Annala et al., 1996; Trogen et al., 1996). The value of molecular dynamics simulations arises from the fact that the crystal structures present the proteins as rather rigid structures, with only little dynamical features. Also the conditions in the crystal differ remarkably from the *in vivo* environment. By using molecular dynamics simulations with stochastic boundary conditions we have been able to simulate the active site of the protein together with the inhibiting peptide and surrounding solvent at room temperature and thus add information about the dynamics to the structural model. Compared to the earlier Monte Carlo docking studies, the present models give a more accurate approach to the enzyme-peptide interactions as dynamical aspects of the active site of PP1 and surrounding water molecules are also taken into account. The results presented here are overall in agreement with the docking studies, but also contradicting features were found for example in the case of interactions of toxins with metal ions in the active site and the orientations of Arg side chains of the toxins.

The simulations of the active site of PP1 together with the toxins give an atom level model of the binding interactions, which can be utilized in design of novel PP1 binding compounds. We could also recognize similarities and differences between the binding mechanisms of the two toxins by replacing the bound microcystin-LR in the model with a nodularin molecule, and comparing the conformational states of the bound and free nodularin.

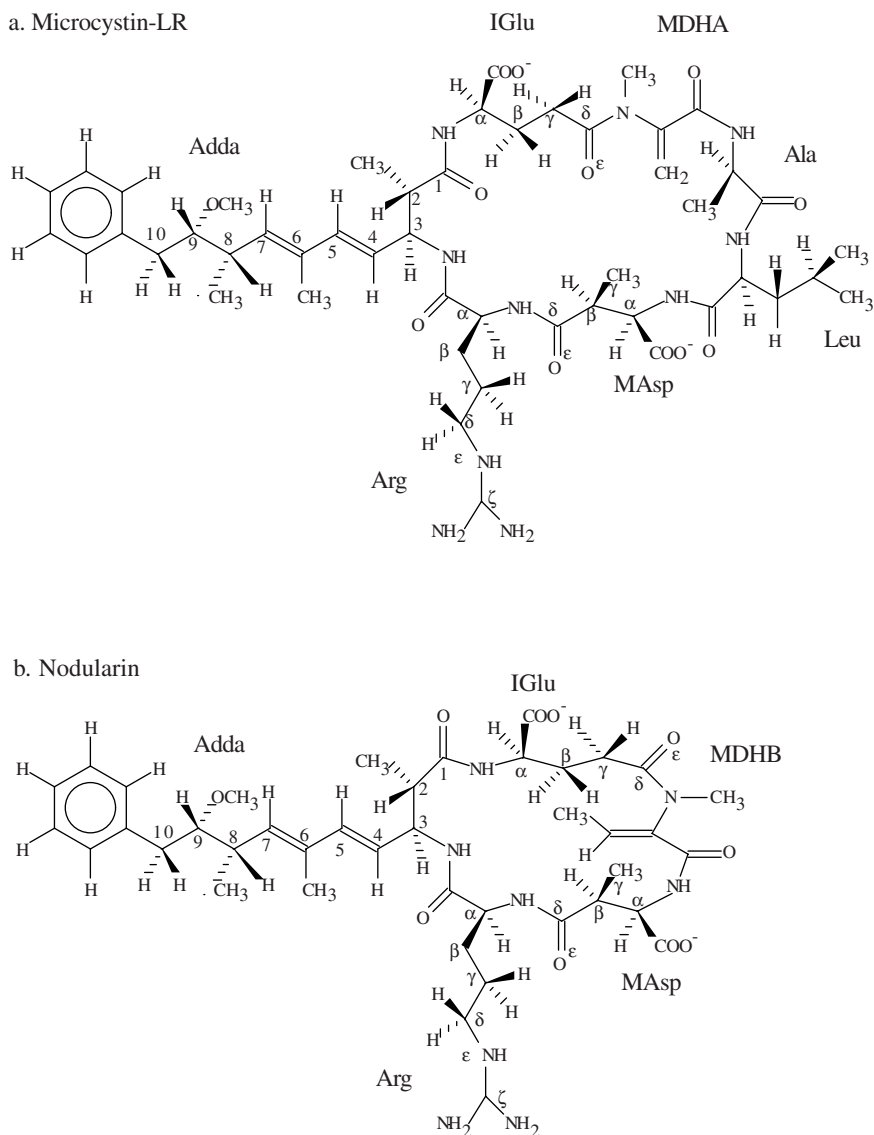


Fig. 1. Chemical structures of microcystin-LR (a) and nodularin-R (b). Both of these toxins form a ring structure which share two common (Arg and IGlu) and two unusual amino acids: (2S, 3S, 8S, 9S)-3-amino-9-methoxyl-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and δ -erythro- β -methyl-aspartic acid (MAsp). In nodularin, the ring structure is completed by 2-(methylamino)-2-dehydrobutyric acid (MDHB), which form a cis peptide bond to IGlu and trans peptide bond to MAsp. In microcystin there are three residues N-methyl-dehydroalanine (MDHA), Ala and D-Leu which form the other half of the ring structure. Different forms of microcystin and nodularin vary usually in the L-amino acid units (Arg and Leu) or the degree of methylation and stereo chemistry. For example motuporin, an analog of nodularin, has L-valine instead of Arg. Furthermore, non-toxic nodularin and microcystin isomers have been found in which the double bond C₆-C₇ of Adda is in cis configuration.

2 Materials and methods

2.1 PP1-toxin models

The simulated PP1-toxin models were based on the crystal structure of PP1-microcystin complex, determined by Goldberg et. al. (Fig. 2). For a detailed description of the PP1 structure, we refer to the articles of Goldberg et. al. and Egloff et al. The modeling and simulations were carried out using QUANTA/CHARMm software (Brooks et al., 1983; Molecular Simulations Incorporated 1992/1994). The more completely defined conformation of the two in the asymmetric unit was selected. It included 294 amino acids (from 7 to 300) of the total 324 amino acids of the catalytic subunit of PP1. Furthermore, the model contained microcystin-LR, 127 crystal water molecules, one β -mercaptoethanol, which was bound to Cys291, and two metal ions. In the original crystal structure, both metal ions were assumed to be Mn^{2+} but, according to the more recent X-ray emission spectroscopic measurements (Egloff et al., 1995), one Mn^{2+} was replaced by Fe^{2+} .

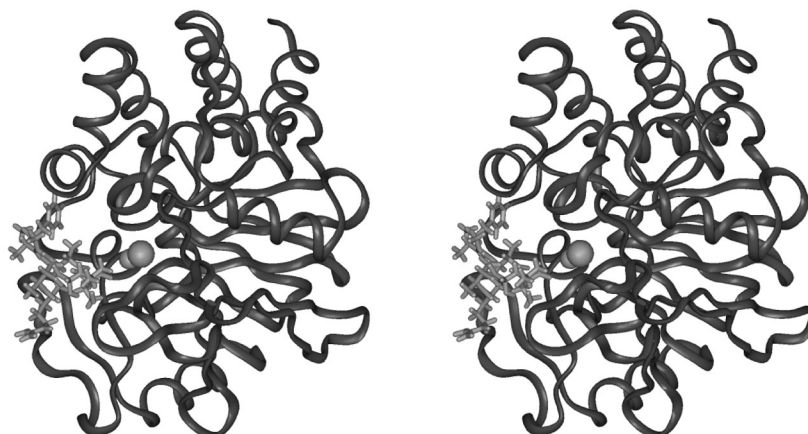


Fig. 2. The basic structure of the catalytic subunit of PP1. The backbone of PP1 is presented as a ribbon model. The reservoir region is presented in gray and the simulated region in black. The heavy atoms of microcystin are displayed as a stick model and the catalytic metal ions as spheres. The catalytic subunit forms an ellipsoidal structure, with dimensions of 50 Å * 35 Å * 35 Å. The 330 amino acids of the subunit are arranged into nine α -helixes and fourteen β -strands, which form three β sheets. The catalytic site, with two metal ions, lies in a shallow pocket in the center of a Y-shaped groove on the surface of the molecule. Microcystins bind in the groove and cover the two metal ions. The carboxyl group of IGlu is in the vicinity of the metal binding site, pointing towards the PP1, MDHA binds covalently to Cys273 and the long hydrophobic side chain of Adda packs into the hydrophobic branch of the groove of PP1.

In the case of 16 amino acids, located on the surface of the protein, the side chains were entirely or partly unidentified. These undefined heavy atoms, and all the hydrogen atoms which were not described in the crystal structure, were first set according to the standard values of CHARMM. The positions of undefined atoms were then minimized with 100 steps of steepest descents (SD) followed by 50 steps of adopted basis Newton-Raphson (ABNR), while the rest of the molecule was kept fixed. In the case of water molecules in the crystal, the positions of the hydrogens were first set randomly to the neighborhood of the water oxygens, after which the hydrogen coordinates were minimized (150 SD and 100 ABNR), while the oxygen positions were kept fixed.

For the amino acids of PP1, the standard CHARMM parameters and partial charges were applied. Arg and Lys side chains were set to carry a unit positive charge (+e), while carboxyl groups and Asp and Glu side chains carried a unit negative charge (-e). For the unusual amino acids of microcystin and nodularin, the applied partial charges were obtained from previous *ab initio* calculations (Annala et al., 1996) of unbound models of these peptides.

In the PP1-nodularin model, the conformation of PP1 and crystal water molecules were first set as in the PP1-microcystin model. After that, microcystin was replaced by nodularin using the analogous region (Arg-Adda-IGlu) of microcystin-LR and the solution conformation of nodularin (Annala et al., 1996). The conformation of PP1 and the

side chain of Adda were placed as in the PP1-microcystin model. The rest of the nodularin was set according to the internal coordinates of the solution conformation, except for the side chain of Arg, where the torsion angles were set equal to the corresponding values of the bound microcystin-LR. Finally, the whole structure was minimized in two steps, first only PP1 (200 SD and 100 ABNR) and then nodularin together with the closest side chains of PP1 (2000 SD). The validity of the conformation was checked with a 100 ps vacuum simulation in 500 K, where only nodularin and those amino acids of PP1 or water molecules which had atoms closer than 3 Å to nodularin, were allowed to move. The remote part of Adda was also fixed, since in vacuum there were no hydrophobic forces present to keep the side chain of Adda in the hydrophobic groove of PP1. During this high-temperature simulation, nodularin fluctuated around the initial conformation, but no major changes occurred. From the last 50 ps, a minimized average conformation was computed (200 SD and 500 ABNR), this served as an initial conformation for the actual simulations.

2.2 Solvent simulations with stochastic boundary conditions

Stochastic boundary conditions and partitioning of the complex into reaction, buffer and reservoir regions (Brooks and Karplus, 1989) were applied in the simulations with solvent. In this approach, only a sphere of 16 Å radius around the binding site of the toxins was explicitly simulated. Outside this reaction region were a 2 Å thick buffer region where the normal molecular dynamics was replaced by Langevin dynamics (Brooks et al., 1988; Allen and Tildesley, 1990). The purpose of the buffer region is to smooth the boundary effects between the reaction region and the completely fixed reservoir region, and to provide a temperature stabilizing heat bath for the system.

The preparatory steps, adding the solvent and partitioning of the system, were performed according to the standard CHARMM procedures (Molecular Simulations Incorporated, 1992). For water molecules a TIP3P type water model (Jorgensen, 1982) was used. The crystal structure was solvated by placing a 19 Å water sphere over the PP1-toxin complex and by removing the overlapping solvent molecules. After that, the protein atoms were kept fixed and the remaining solvent molecules were simulated for a few ps. Residual holes in the solvent sphere were filled by subsequently overlaying several randomly rotated extra water spheres over the previous model and by removing the water molecules which overlapped with the original ones. Before the final partitioning the solvent molecules were simulated for 10 ps. The reaction region was co-centered with the center of mass of nodularin, which was located on the surface of the PP1-complex, near C₃ of Adda. Reaction and buffer regions consisted of 98 amino acids of PP1 (Table 1), the two metal ions in the metal binding site, 33 crystal waters, microcystin or nodularin and 437 or 481 solvent molecules. In the microcystin model, the MDHA side chain was covalently bound to Cys273 while there were no covalent bonds between nodularin and PP1.

Table 1: The amino acid sequence of PP1 inside the reaction and buffer regions. The sequences of the simulated 15 short fragments are listed below, separated with spaces. The numbering above is according to the original sequence. The amino acids with the most negative interaction energy with respect to microcystin are in bold face and the residues, which coordinate metal ions, are underlined.

63				68		90						98		100
GLY	<u>ASP</u>	ILE	<u>HIS</u>	GLY	GLN	LEU	GLY	<u>ASP</u>	TYR	VAL	ASP	ARG	GLY	SER
122														139
ARG	GLY	<u>ASN</u>	HIS	GLU	CYS	ALA	SER	ILE	ASN	ARG	ILE	TYR	GLY	GLU
149		153		156		172					178		194	
TRP		THR		PHE		CYS	<u>HIS</u>	GLY	GLY	LEU	SER	PRO	ASP	ASP
													213	
GLN	GLY	LEU	LEU	CYS	ASP	LEU	LEU	TRP	SER	ASP	PRO	ASP	LYS	VAL
216											227		246	
TRP	GLY	GLU	ASN	ASP	ARG	GLY	VAL	SER	PHE	THR	PHE	ARG	ALA	VAL
	252		256		263		265							
VAL	GLU		GLU		LEU		THR	LEU	PHE	SER	ALA	PRO	ASN	GLU
276		278												
PHE		ASN												

After setting constraints for the buffer region atoms and a boundary potential for the water, both models were simulated for 600 ps at 300 K. Standard algorithms for molecular dynamics were employed for atoms in the reaction region. Langevin dynamics were utilized in the buffer region, where the effect of random force and friction was stepwise increased from the inner boundary to the outer one. Since 1 fs time step was used, the bond lengths of hydrogens were constrained by the SHAKE algorithm (van Gunsteren and Berendsen, 1977). The van der Waals interactions were truncated by the SWITCH function at 10 Å distance, and the electrostatic interactions by the SHIFT (Molecular Simulations Incorporated, 1992) function with an 11 Å cut-off radius. The updating frequency of the nonbonded interactions was controlled using the heuristic method (Molecular Simulations Incorporated, 1992).

The MD simulations were carried out on Silicon Graphics Power Onyx and Cray C90 computers at CSC (The Finnish National Supercomputer Center). The coordinate trajectories were recorded with a 50 fs time step interval and energies in a 100 fs interval. Trajectories were analyzed by the QUANTA/CHARMm package and SCARECROW software (Laaksonen, 1992).

3 Results

3.1 PP1-microcystin complex

The model of the active site of PP1-microcystin complex was simulated for 600 ps. The heat bath of the buffer region quickly equilibrated the system close to 295.5 K (Fig. 3). The fluctuations of total energy were larger than normally observed in the case of periodic boundary conditions (Table 2), because the heat bath allowed the system to exchange energy with its environment. However, the total energy did not have a tendency to increase or decrease after the first 10 ps.

Table 2: Statistics from the last 500 ps of the simulations.

		PP1-microcyst.	PP1-nodularin
Temp. (K)	average	295.5	295.7
	RMSD	5.0	4.9
E _{pot} (kcal/mol)	average	2031	2091
	SDev	34	35
RMS fluctuations of heavy atoms (Å)			
PP1, all	average	0.53	0.54
PP1, backbone	average	0.41	0.44
	max.	1.26	1.91
		(Asp194 O)	(Glu275 O)
toxin, all	average	0.61	0.67
toxin, backbone	average	0.47	0.46
	max.	0.56	0.56
		(Ala C)	(MDHB N)
	min.	0.39	0.34
		(IGlu C _α)	(IGlu C)

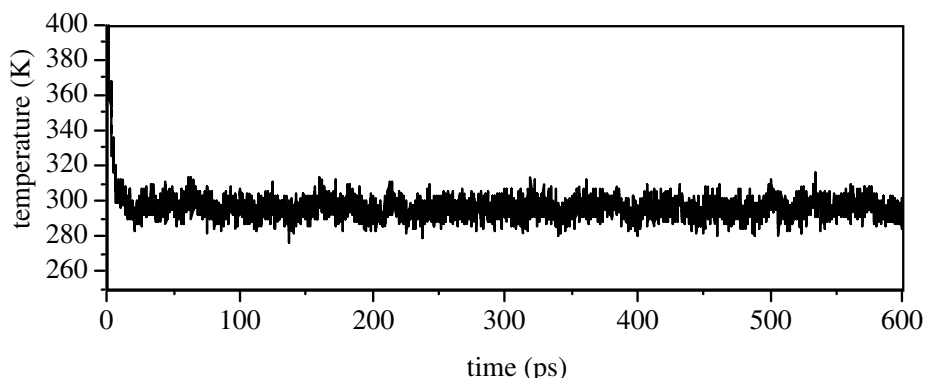


Fig. 3. Temperature trajectory of the microcystin-PP1 simulation.

During the simulation microcystin drifted away from the crystal conformation to a slightly different one. Several backbone dihedral angles changed more than 30 degrees (Fig. 4, Table 3). The largest changes occurred for the IGlu side chain and for the ϕ - and ψ -angles between Arg and Adda. The conformational change of the IGlu side chain took place rapidly during the first pico seconds, while in the other parts of microcystin the changes were slower. The drift ceased after 100 ps, and during the subsequent 500 ps larger backbone fluctuations were observed only in the α - γ region of IGlu. The structural changes were coupled to the movement of the IGlu carboxyl group towards the metal ions. The distance between the IGlu COO⁻ group and Fe²⁺ ion reduced from 4.4 Å to 2.1 Å during the first 100 ps of the simulation. However, the positions of the two metal ions and the side chains of PP1, which coordinate the metal ions, remained close to those of the initial structure.

Table 3: Average values and RMS fluctuations of backbone dihedrals of microcystin and nodularin. The first two columns contain the information of the two centermost atoms defining the dihedral angle. For microcystin, the values are from the crystal structure (Goldberg et al., 1995) and from the last 500 ps of the PP1-microcystin simulation. For the unbound nodularin, the values are obtained from a 400 ps solvent simulation (Annala et al., 1996) and for the bound conformation from a total 600 ps of the PP1-nodularin simulation

Residue	microcystin				nodularin			
	crystal		simulation		unbound		bound	
	atoms	value(°)	mean (°)	fluct. (°)	mean (°)	fluct. (°)	mean(°)	fluct. (°)
Masp	N-C α	-142	-103	15	-129	32	-174	9
	C α -C β	-59	-60	5	-60	7	-68	6
	C β -C δ	-123	-126	9	-115	14	-96	9
	C δ -N	174	169	7	-174	7	179	6
Arg	N-C α	-112	-121	10	-103	19	-130	11
	C α -C	-12	42	9	25	24	17	15
	C-N	181	-154	7	177	9	-179	7
Adda	N-C3	-98	-153	9	-136	24	-134	14
	C3-C2	75	55	6	63	8	68	6
	C2-C1	-100	-81	7	-140	21	-91	8
	C1-N	173	176	7	-178	9	167	6
Iglu	N-C α	-159	-171	8	-104	28	-155	8
	C α -C β	174	57	15	-55	10	-73	17
	C β -C γ	142	177	7	-177	9	-177	8
	C γ -C δ	155	-129	19	175	14	-155	19
	C δ -N	174	169	8	4	9	-3	9
MDHA/B	N-C α	78	58	9	93	11	76	8
	C α -C	9	41	8	-45	35	14	8
	C-N	-167	168	7	173	9	-177	8
Ala	N-C α	77	80	10				
	C α -C	8	-6	18				
	C-N	-173	180	7				
Leu	N-C α	-77	-86	16				
	C α -C	-31	-17	14				
	C-N	-171	166	7				

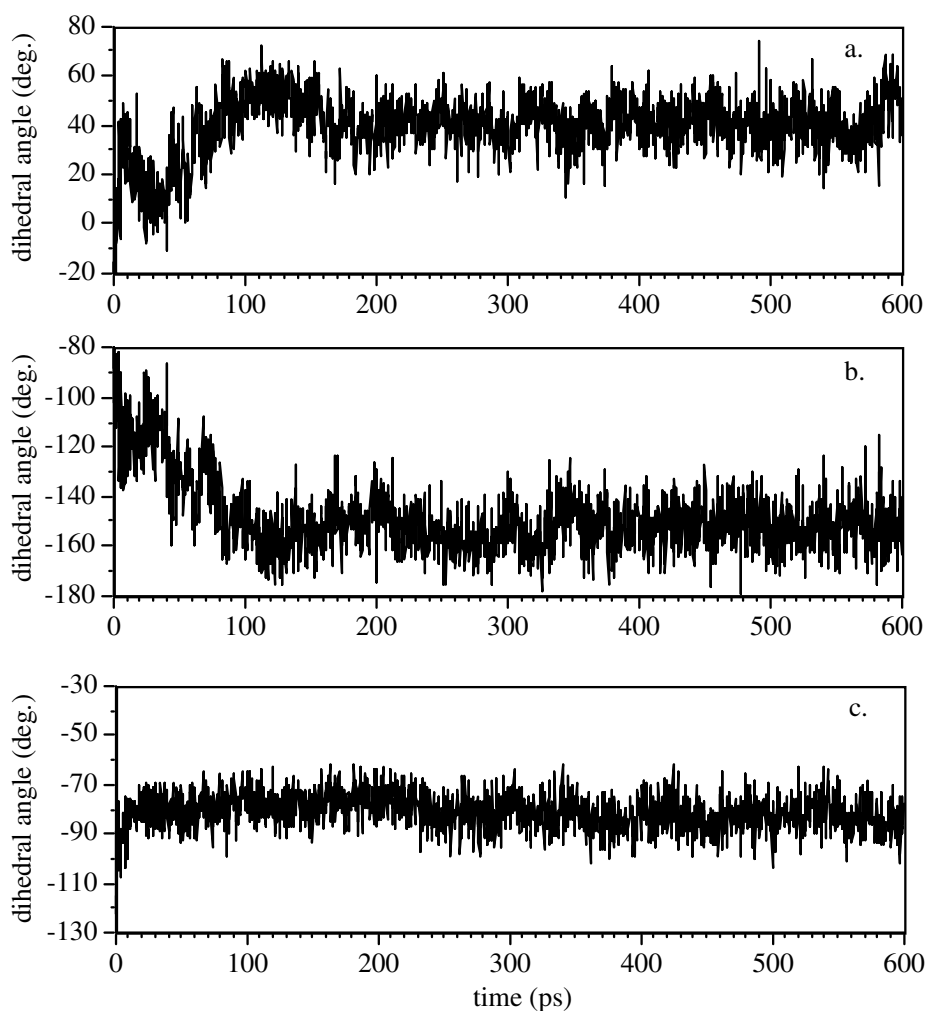


Fig. 4. Trajectories of three dihedrals of microcystin during the simulation. The values are plotted using 0.3 ps intervals. a: Arg N - Arg C $_{\alpha}$ - Arg C - Adda N, b: Arg C - Adda N - Adda C $_3$ - Adda C $_2$, c: Adda C $_2$ - Adda C $_1$ - I Glu N - I Glu C $_{\alpha}$

In microcystin, four internal hydrogen bonds were frequently formed. Stable hydrogen bonds were formed from one of the carboxyl oxygens of MAsp to the amide groups of Adda and I Glu. The other carboxyl oxygen formed a hydrogen bond to the side chain of Arg96*, which stabilized the orientation of the MAsp COO⁻ group further. The carbonyl group of MAsp formed intramolecular hydrogen bonds to amide groups of MAsp and Ala. These bonds were present only during about 30 % and 50 % of the simulation time, due to the unfavorable angle between the donor and acceptor groups. Occasionally, a

*. The amino acids specified with a number refer to PP1 and its sequence. For the amino acids of the toxins, plain amino acid names are used.

hydrogen bond was also formed between IGlu O_ε and Leu HN. Three similar hydrogen bonds were found in the crystal structure of microcystin, one between the MAsp carboxyl group and IGlu HN, the second between the carbonyl and amide groups of MAsp and the third between IGlu O_ε and Leu HN.

The overall structure of PP1 remained stable. All C_α RMS fluctuations were less than 0.9 Å. The mean RMS fluctuations during the last 500 ps were 0.4 Å for backbone atoms and 0.5 Å for all heavy atoms of PP1. The reason for the stability is obvious, since some of the first and the last backbone atoms of each of the 15 PP1 fragments (Table 1) were in the buffer region and thus were constrained to their initial positions. However, certain changes occurred, especially in the residues next to the glycines. The largest changes took place for Tyr134-Gly135 where, a local backbone change occurred together with the movement of the phenyl ring of Tyr134. This was probably necessary to allow IGlu to approach the metal binding site. However, the RMS fluctuations of C_α atoms during the simulation were less than 0.6 Å in this region which implied that after the relocation, the backbone conformation remained stable. Other larger fluctuations occurred at the surface of the protein, and they had only minor effects on the metal binding site or the residues around the microcystin.

3.2 PP1-microcystin interaction

The interaction between the microcystin and PP1 was analyzed by calculating electrostatic and van der Waals forces between the two molecules. An overview of the locations and relative strengths of these interactions was obtained from the averages of the nonbonded interactions during the last 500 ps of simulations (Table 4). After the first 100 ps, the interaction energies had only fluctuations of a few kcal/mol. In the minimized average structure from period the 500 ps - 500.5 ps the interactions had analogous distribution and strengths compared to the 500 ps average.

In general, the strongest interactions between microcystin and PP1 were located in the same places as in the crystal structure. The results revealed three sites which mostly contribute to the attraction between microcystin and PP1. The strongest attraction, i.e., the lowest nonbonded interaction energies, was between the carboxyl group of IGlu and the two metal ions of PP1. The carboxyl group had moved close (2.2 Å) to Fe²⁺ ion coordinated by Asp64, His66 and Asp92. The distance to Mn²⁺ ion was 0.5 Å longer, but the attraction forces were still strong (Fig. 5). As the negatively charged carboxyl group approached the metal ions, also the repulsive forces began to increase towards negatively charged side chains of Asp64 and Asp92 at the metal coordination site. In the equilibrium conformation, the IGlu carboxyl group was interacting directly with the two metal ions, without mediating water molecules which were present in the crystal structure. The change of the location of IGlu affected also Adda and increased the interaction of the Adda carbonyl group with the Mn²⁺ ion.

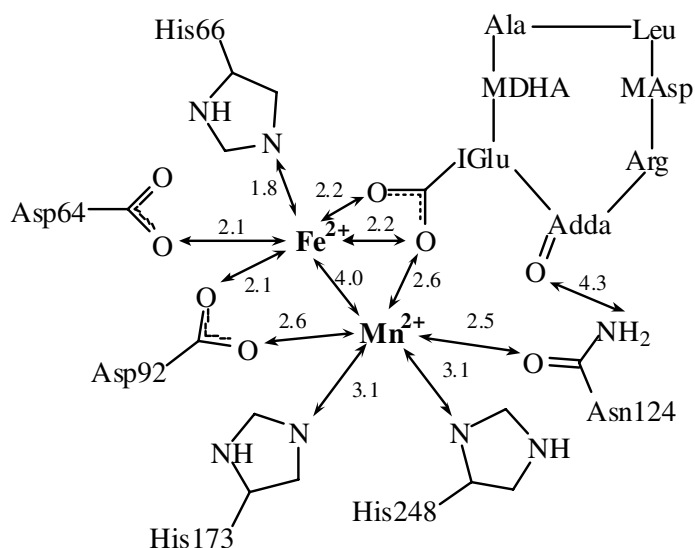
The carboxyl group of MAsp also took part in the binding. It formed stable hydrogen bonds to the side chains of Arg96 and Tyr134. In the beginning of the simulation, one hydrogen bond was formed between Arg and Asp220. As the simulation proceeded, the remote part of the Arg side chain flipped 180°, which turned Arg H_ε atom towards the

Asp220 and allowed Arg to form two hydrogen bonds with Asp220. Also the carbonyl group of MDHA and the amide and carbonyl groups of Arg, which formed hydrogen bonds to Gly274 and Arg221, had over 5 kcal/mol interaction energies with PP1. Ala and Leu residues of microcystin did not experience significant interaction energies (strengths from 5 kcal/mol to - 5 kcal/mol) and also the side chain of Adda seemed to be quite passive in this sense.

Table 4: The highest and the lowest inter residual interaction energies. All those microcystin-PP1 and nodularin-PP1 residue pairs are listed which have a total interaction energy larger than 5 kcal/mol or under -5 kcal/mol. The energies are average values computed at 5 ps intervals from the last 500 ps of the simulation.

Microcystin	PP1	Energy (kcal/mol)	Nodularin	PP1	Energy (kcal/mol)
IGlu	Fe	-281.6	IGlu	Fe	-285.7
IGlu	Mn	-177.5	Arg	Asp220	-83.3
MAsp	Arg96	-91.1	MAsp	Arg96	-62.6
Arg	Asp220	-78.5	IGlu	Arg96	-46.1
Adda	Mn	-40.9	IGlu	Mn	-40.2
IGlu	Arg96	-33.5	MAsp	Fe	-17.3
MAsp	Mn	-23.0	MDHB	Arg96	-15.7
MAsp	Fe	-19.9	MAsp	Tyr134	-12.5
MDHA	Glu275	-13.7	IGlu	Arg221	-11.3
Adda	Arg221	-7.5	Adda	Fe	-10.5
Arg	Glu275	-5.5	Adda	Mn	-7.44
MDHA	Gly274	-5.5	MDHB	Glu275	-5.0
Adda	His248	5.2	IGlu	Asp95	6.9
MAsp	Asp92	5.9	IGlu	His125	8.7
IGlu	Asn124	6.3	Adda	Arg96	10.2
IGlu	His125	7.0	IGlu	His66	10.9
Adda	Arg96	9.7	IGlu	Tyr272	11.4
IGlu	His66	14.1	IGlu	Asp64	17.6
IGlu	His248	16.9	IGlu	Asp92	20.0
IGlu	Asp64	62.4			
IGlu	Asp92	78.9			

a. PP1/microcystin



b. PP1/nodularin

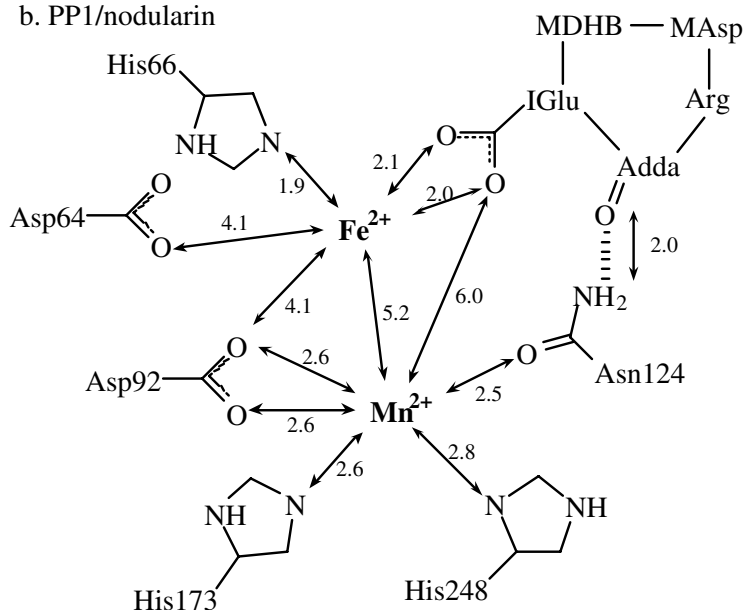


Fig. 5. Interatomic distances (Å) at the metal coordination site of PP1-microcystin complex (a) and PP1-nodularin complex (b). The values are averages from the last 500 ps of the simulations. During that period, the RMS fluctuations of all displayed distances were less than 0.25 Å.

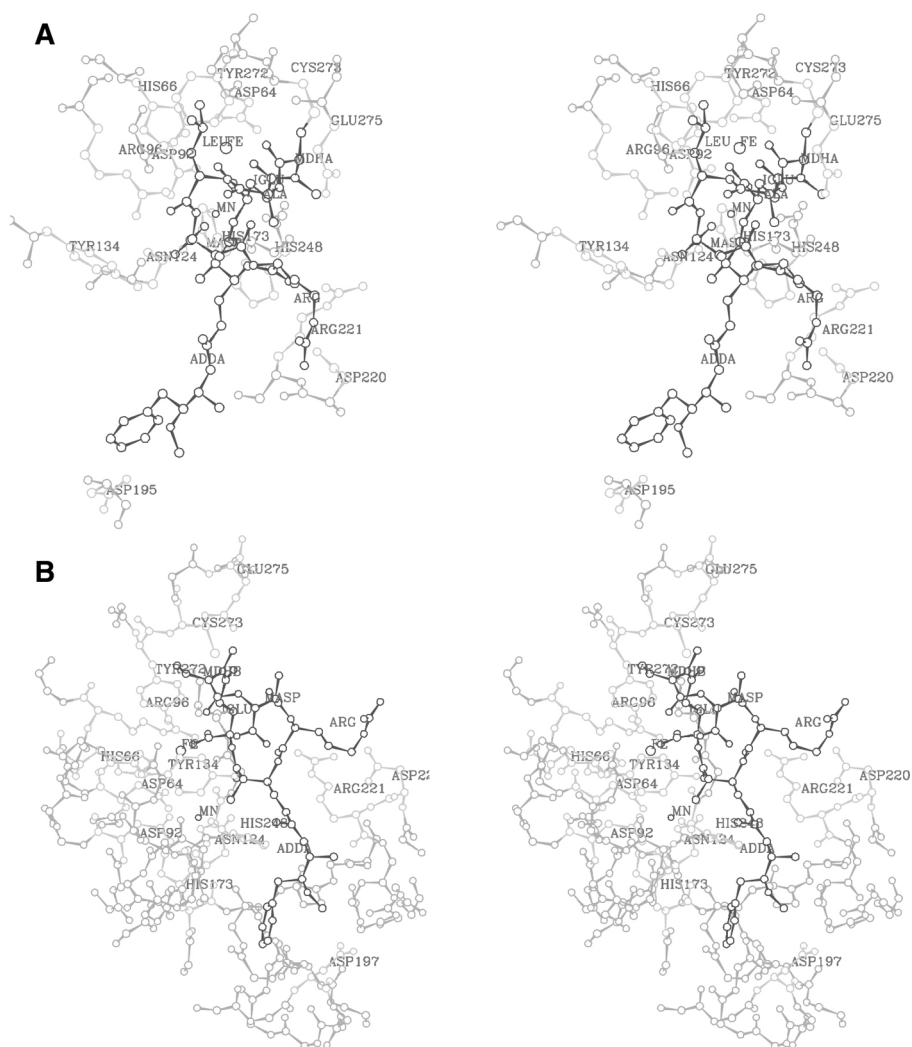


Fig. 6. a, b. Conformations of the bound microcystin and nodularin. The stereo images present slightly minimized average conformations of microcystin (a) and nodularin (b) from the time interval 500 - 500.5 ps. The heavy atoms of the toxin and the two metal ions are in black. The side chains that coordinate metal ions and the side chains that interact with the toxins are in dark gray. Some fractions of the PP1 backbone are displayed in light gray. Large parts of the simulated region of PP1, as well as the water molecules are, not shown.

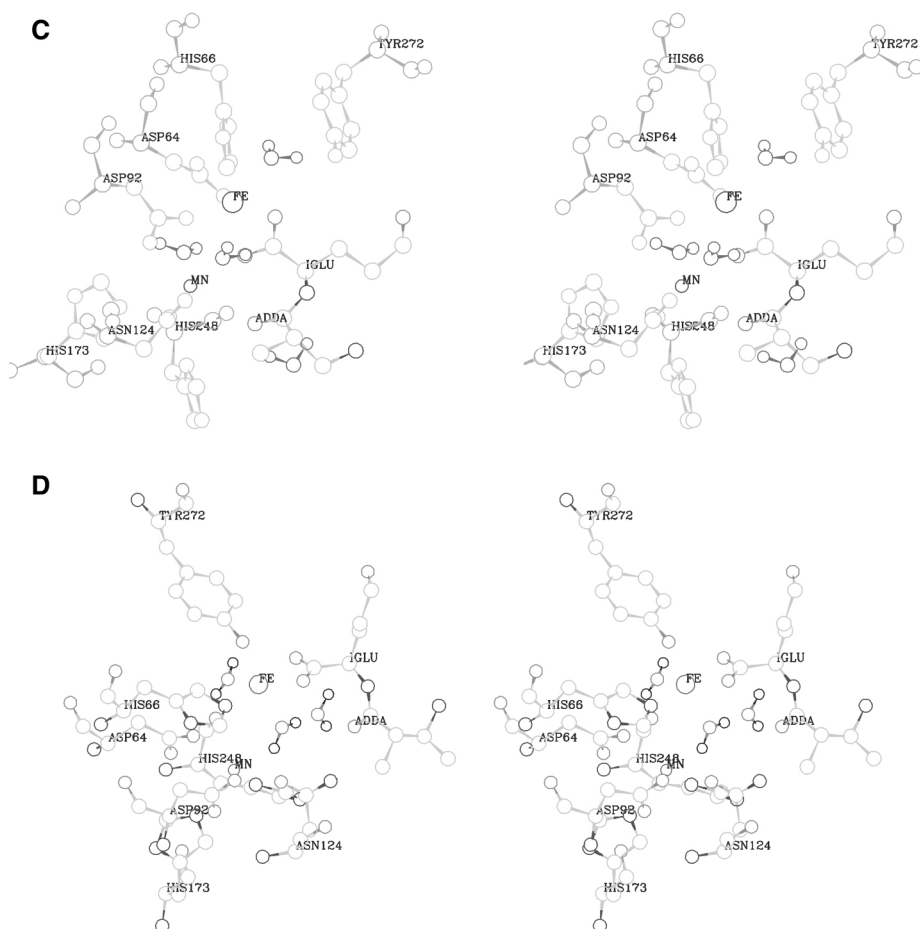


Fig. 6. c,d. Conformations of the bound microcystin and nodularin. Figures c and d present more detailed structures of the metal coordination region with the IGLU and ADDA main chains for microcystin (c) and nodularin (d). The water molecules around the metal ions are also included. Note that the view angles, as well as the amount of displayed atoms, alter to some extent between the images.

3.3 PP1-nodularin complex

The simulation and analysis of PP1-nodularin complex was carried out in a similar manner as for the microcystin complex. During the simulation, the system evolved steadily with an average temperature of 295.7 K. Conformation and dynamics of the bound nodularin were also compared with the previous solvent simulations of unbound nodularin (Annala et al., 1996).

Nodularin experienced certain conformational changes during the initial minimizations and during the short high-temperature simulation which was carried out before the actual solvent simulation. However, interactions with PP1 imposed a strong stabilizing effect, so that even at a high temperature (500 K), the backbone of nodularin generally fluctuated less than in the simulation of an unbound molecule at 300 K. The dihedral angles remained mainly within values which were found also in the simulation of the unbound toxin. The largest differences between the average values (Table 3), arise from the fact that, for some regions of the nodularin backbone, local conformational fluctuations between two states had occurred during the simulation of the free molecule. In these cases, the comparison of angle histograms of the free and bound nodularins (Fig. 7) revealed that binding had reduced the fluctuations of these angles, and selected one of the subconformations of the free molecule.

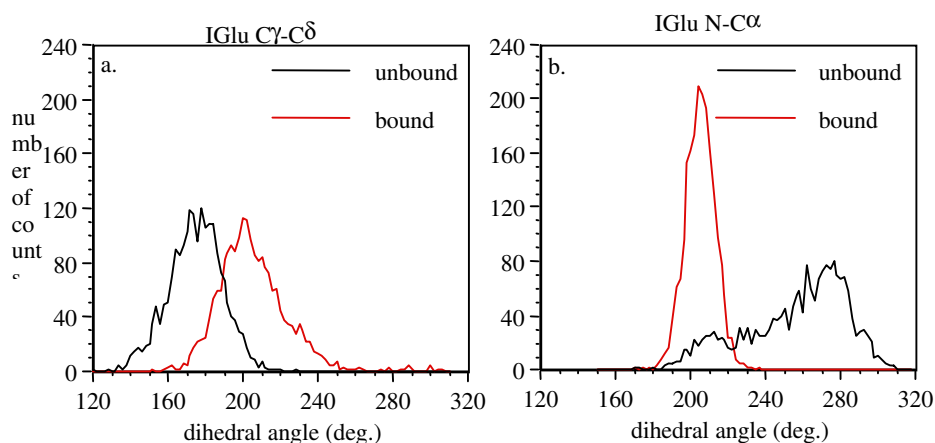


Fig. 7. Two backbone dihedral histograms. Two examples of the value histograms of backbone dihedral angles of nodularin. (a) Histograms of dihedral IGlu C_β - IGlu C_γ - IGlu C_δ - MDHB N as unbound in solvent and when bound to PP1. This was the case of the largest observed deviation between the bound conformation histogram and the corresponding solvent simulation histogram. (b) Histograms of dihedral Adda C_1 - IGlu N - IGlu C_α - IGlu C_β as unbound in solvent and bound to PP1. It is also illustrated how PP1 holds the backbone conformation in one of the subconformations found in solvent. The histograms were generated with a 2° interval from sets of 2000 sample points.

During the high-temperature simulation of PP1-nodularin complex, the C_α - C_δ region of IGlu fluctuated between two conformations, but in the actual PP1-nodularin simulation at 300 K, IGlu remained in the free-like conformation with only one brief jump to the other conformation during the 600 ps period. The ϕ - and ψ -angles between Arg and Adda also adopted different conformation during the high temperature simulation, but the angles returned to the free-like conformation during the actual simulation. Interestingly, these were the two sites where the largest changes took place, also during the simulation of the PP1-microcystin complex.

The conformation of the proximal part of the side chain of Adda remained rigid and close to its initial values which were obtained from the crystal structure of the PP1-microcystin complex. The remote part of the side chain varied its conformation, but only the phenyl ring at the end of the side chain fluctuated more strongly. The Arg side chain underwent conformational changes, but as in microcystin, the hydrogen bonds to Asp220 damped the fluctuations.

3.4 PP1-nodularin interaction

The basic binding mechanism of nodularin was found to be analogous to microcystin. However, for some reason, nodularin did not approach the metal binding site to the same extent as microcystin did. Even though IGlu remained further away from the metal coordination site, the strongest interactions were still found in this region. Unlike microcystin, nodularin seemed to perturb the metal coordination site. Mn^{2+} ion remained at its original site about 6 Å away from IGlu, but the Fe^{2+} ion moved into a new location between His66 and the carboxyl group of IGlu, about 2 Å further away from the other two Fe^{2+} coordinating side chains: Asp92 and Asp64 (Fig. 5.). The longer distance from Adda to Mn^{2+} ion was partly compensated because this conformation allowed the carbonyl oxygen of Adda to form a hydrogen bond to the side chain of Asn124, which coordinates Mn^{2+} .

The side chain of Arg96 was pointing towards the center of the backbone ring of nodularin. In this conformation, the Arg96 side chain was able to form stable hydrogen bonds with three amino acids of nodularin (IGlu, MAsp and MDHB), while only one stable hydrogen bond between Arg96 and microcystin was observed. Nevertheless, the locations and orientations of the Arg96 side chains in the two simulations were quite similar. Tyr134, which was located next to Arg96, further stabilized the position of nodularin backbone ring by interacting with the carbonyl group of MDHB and the carboxyl group of MAsp. Strong interactions were involved also between nodularin and Asp220, which was now capable of forming two or three hydrogen bonds to the Arg of nodularin.

The structure of PP1 remained generally close to its initial one, as in the PP1-microcystin simulation. The fluctuations of PP1 were to an equal extent compared to the PP1-microcystin simulation (Table 2). Nodularin did not approach the metal binding site as close as microcystin-LR and, consequently, Tyr134 did not change its conformation either. Because the backbone ring of nodularin is smaller than that of microcystin-LR, a water filled cleft was left between the toxin and PP1. This allowed both main and side chains of Gly274-Phe276 to move to a new location, closer to MDHB. Nevertheless, the side chain of Cys273 was still located about 8 Å away from MDHB.

4 Discussion

When discussing the results from PP1-toxin complex simulations, it should be noticed that the simulations and the analysis performed include a number of significant simplifications and possible sources of errors, and thus the results should be interpreted cautiously. In the simulations, only a small fraction of the enzyme was presented explicitly, and the simulated sequence fragments were handled in a way which prevented large-scale conformational changes. In addition, the simulated region was not neutral, but carried a significant negative total charge; this is known to cause artifacts and spurious effects in the simulations. Further, the simulations were very short compared with the time scale of toxin binding processes. The stochastic boundary conditions were parametrized according to previous lysozyme simulations (Molecular Simulations Incorporated, 1992), since suitable data was not available for an evaluation of the parameters for PP1. At the moment, there is only little experimental data about the dynamics of the PP1-toxin complexes, so that a direct verification of the results remains to be made later.

The analysis of the non-bonded interactions that was performed, does not give information about the binding free energies, since solvation effects were not included in the analysis. Thus, the values should not be considered to give direct information about the binding process and affinity, but to describe the interactions only after the binding process. In that context, the two simulations presented here are able to give a consistent model of the PP1-toxin interactions. The general binding characteristics of the two toxins are similar, even though the conformations were partly different. The results support the previous hypothesis that the homologous region between the carboxyl groups of MAsp and IGlu primarily cause the affinity (Bagu, 1995; Goldberg, 1995; Annala, 1996), since the strongest interactions are present in this region. The model of binding interactions presented here is mainly similar to the results obtained by Bagu et. al. from the docking calculations of microcystins and motuporin. However, the strong interaction of the IGlu carboxyl group with the metal binding site was not observed in the docking study, where the main interaction of IGlu was predicted to occur with Arg96. Also in the present models, the IGlu and MAsp carboxyl groups interact with positively charged side chains of Arg96 and Tyr134 at the surface of PP1, but these interactions are significantly weaker com-

pared to the interactions between IGlu and metal ions. In the bound conformations, Adda does not significantly interact with PP1, but the long side chain of Adda may ensure the right orientation of the toxins as the side chain settles to the hydrophobic groove of PP1.

The result that the major contribution to the binding comes from the IGlu carboxyl group is well in accordance with the experimental results, which have proved that modification of the carboxyl group of microcystin to $-OCH_2CH_3$ leads to a nonbinding variant (Stotts et al., 1993). According to the models presented here, the result seems quite evident, as this modification discharges the carboxyl group IGlu and thus removes the strongest interactions between the toxin and PP1. The importance of the MAsp carboxyl group is also in agreement with the toxicity. In this case, the neutralization of the carboxyl group was observed only to decrease the toxicity (Stotts et al., 1993), which can be considered to be due to the absence of hydrogen bonds to Arg96 and Tyr134. The replacement of the Arg of microcystin by nonpolar Ala or by Val in nodularin does not affect the toxicity (Rinehart et al., 1994), however the models suggest hydrogen bonds between Arg and Asp220 (Table 4.). It is also worth noting that Arg96, and Tyr134 are conserved in different PPs, but Asp220 is not (Goldberg et al., 1995). The side chains Arg96 and Tyr134 in PP1 seem to stabilize of the ring structure of the peptides, especially in the case of nodularin, and Asp220 stabilizes the Arg side chain.

Mutation studies of PP1 have demonstrated, that substitution of Phe276 with Cys, as well as mutations of Tyr272, reduce the binding of cyanobacterial toxins (Zhang et al., 1992; Egloff et al., 1995). In the present models, Tyr272 is clearly involved in the binding, but in two different ways for the two toxins. In both cases, removing the polar hydroxyl group of the Tyr272 would reduce the attractive forces between PP1 and the toxins. In the PP1-microcystin model, Tyr272 forms hydrogen bonds to the side chain of Arg96 and therefore further stabilizes the position of this amino acid, which has notable interactions with the toxins. In the nodularin model, the Tyr272 side chain has turned closer to the catalytic site and coordinates the metal ions. In the average conformation, the distance from Tyr272 O_H to Fe^{2+} was only about 2.1 Å, which indicated that Tyr272 participates to hold the Fe^{2+} ion in its altered position between His64 and IGlu of nodularin. In the simulations, Phe276 did not interact strongly with the toxins, but one could postulate that Cys in position 276 could form a disulfide bond to Cys273 and cause a local conformational change.

In comparison with solution conformations of the toxins, no major structural changes occurred in the binding. The exception to this is IGlu of microcystin, which has a conformation different from the ones observed in solvent (Troger et al., 1996). The altered conformation may be caused by the strong attraction of the IGlu carboxyl group towards the metal ions, which draws the β , γ and δ methylenes against the adjacent PP1 side chains. On the other hand, in the solvent simulation of nodularin, the largest fluctuations were found at the same region. The interactions between nodularin and PP1 were found to stabilize the ring structure, which is well illustrated by comparing the RMS fluctuations of backbone dihedrals between the bound and unbound conformations (table 3. fig. 7). In the solvent simulations of nodularin, local backbone fluctuations were detected. Such fluctuations were not observed in the bound conformation and nodularin backbone was found to remain mainly in one conformation, which could be considered to be a certain combination of different subconformations found for free nodularin.

The differences between the crystal structure of microcystin and the prevailing conformation during the simulation, may arise from several reasons. First, the model itself may cause artifacts. The partial charges of the toxin atoms were evaluated for the solvent environment. The binding to the PP1 affects the charge distribution, and thus the observed movement of IGlu towards the metal ions may be caused by inaccurate partial charges which overemphasize the Coulombic forces. The translation of Fe^{2+} in the nodularin simulation may arise from the same reasons. However, from the modeling point of view, the conformations of both toxins seem possible and in the crystal structure of the PP1-tungstate complex, the location of two tungstate oxygen atoms is analogous to the carboxyl group of IGlu of microcystin (Egloff et al., 1995). Finding some differences between the structures determined by X-ray crystallography and the structures in simulations is also expected, since the crystal environment in 100 K significantly differs from the simulated solvent environment at 300 K.

In conclusion, the simulations suggest that the binding of nodularins and microcystins to PP1 is mainly caused by the interactions of the carboxyl group of IGlu and the metal ions associated to the PP1. The MAsp carboxyl group and the long side chain of Adda further determine the orientation of bound peptides, so that in the case of nodularins the MDHB side chain is located too far from the Cys273 and microcystin-like covalent bond to PP1 cannot be formed. The binding does not cause major changes to the conformations of the peptides or PP1 but stabilizes the peptide structures and can perhaps cause some local perturbations in the metal binding site of the enzyme.

On the basis of the results, several hypotheses, which could be studied experimentally, can be formed. For example, single amino acid substitution of Arg96, Tyr134 and Asp220 could give interesting results.

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