The three-dimensional solution structure of nodularin was studied by NMR and molecular dynamics simulations. The conformation in water was determined from the distance and dihedral data by distance geometry and refined by iterative relaxation matrix analysis. The cyclic backbone adopts a well defined conformation but the remote parts of the side chains of arginine as well as the amino acid derivative Adda have a large spatial dispersion. For the unusual amino acids the partial charges were calculated and nodularin was subjected to molecular dynamic simulations in water. A good agreement was found between experimental and computational data with hydrogen bonds, solvent accessibility, molecular motion, and conformational exchange. The three-dimensional structure resembles very closely that of microcystin-LR in the chemically equivalent segment. Therefore, it is expected that the binding of both microcystins and nodularins to serine/threonine-specific protein phosphatases is similar on an atomic level.

Reversible protein phosphorylation plays a pivotal role in various signaling pathways that control numerous physiological processes from light responses in plants to muscle contraction in animals (1, 2). The reversibility of protein phosphorylation and the presence of protein phosphatase activities in cells and tissues were established almost as early as the initial characterization of protein kinases. However, the true importance of protein phosphatases in regulating protein phosphorylation was fully understood only when highly specific inhibitors of these enzymes were discovered. A number of compounds, among them okadaic acid (3), a diarrhetic shellfish poison, as well as microcystins and nodularins, specific peptide hepatotoxins (4, 5) from cyanobacteria, have been found to act on the major serine/threonine-specific protein phosphatases PP-2A and PP-1. These phosphatases are of crucial importance in maintaining cellular homeostasis as they participate in carbohydrate and lipid metabolism, signal transduction (1), maintenance of cytoskeletal structure (6, 7), suppression of cell transformation (8), regulation of apoptosis (9), and cell division rates. Consequently, the inhibitors can, depending on the dose and the duration of exposure, induce severe cellular effects ranging from cytoskeletal contortion to tumor promotion or apoptosis. Acute microcystin poisoning is characterized by specific liver damage. Due to a selective uptake mechanism present in parenchymal liver cells (10, 11), microcystin inhibits PP5 (2) in liver cells. This leads to a complete disruption of cytoskeletal structure (6, 12) and consequent loss of cell morphology and cell interactions. As a result there is an extremely rapid and complete loss of sinusoidal architecture, leading to intraorgan bleeding and rapid hypovolemic shock (7, 13). Lower doses of microcystins, for example, as contaminants of drinking water, may perturb cell division control mechanisms and cause tumor promotion when suitable tumor initiators are present (4, 5, 15).

Microcystins (16, 17), the cyclic cyanobacteria heptapeptides from Microcystis aeruginosa and nodularins (18–21), similar pentapeptides from Nodularia spumigena, are particularly advantageous in investigations of functions regulated by PP5s. The association to protein phosphatases is extremely tight (IC50 < nM) (22). The peptides are stable and can easily be derivatized for the purposes of detection, immobilization, or addition of fluorochromes to be used as probes for PP5s (23). There are also naturally occurring non-toxic variants for control experiments. Microcystins and nodularins have become more readily available as the expertise on culturing cyanobacteria has progressed.

A detailed knowledge on a molecular level of the interactions underlying the biological effects of microcystins is emerging. Recently the three-dimensional structures of microcystin-LR as free in solution have been determined by NMR in dimethyl sulfoxide (25), dimethyl sulfoxide/water mixtures (26), and in water (27, 26). Conformations of two other microcystin variants in dimethyl sulfoxide (25) and motuporin (28), a homologue to nodularin, in water have been obtained as well (27). The conformations are markedly well defined for the cyclic backbone and similar among this group of peptides. Recently microcystin-LR and PP-1 were co-crystallized (29, 30). The three-dimensional structure of the complex (31) reveals how microcystin-LR occupies a cleft near the catalytically active metal ions (32). The bound conformation resembles closely that of free microcystin-LR. However, in this complex microcystin-LR is covalently bound to Cys-273 (31) via N-methyldehydroalanine

The abbreviations used are: PP, protein phosphatase; MDHA, N-methyldehydroalanine; MeAsp, methyl-β-aspartic acid; Adda, (25, 35, 85, 95)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenylenecac-4,6-dienolic acid.

Härmälä, A. S., Mikhailor, A., Meriluoto, J. A. O., and Eriksson, J. E., unpublished results.
(MDHA), a result which has been shown by biochemical means earlier (33).

Nodularin which is chemically quite similar to microcystin-LR (Fig. 1) and equally toxic does not bind covalently to PP-1. The present study focuses on the three-dimensional solution structure of nodularin in an attempt to find clues for the similarities and differences found between the binding of nodularins and microcystins. The conformation in water was studied by nuclear magnetic resonance spectroscopy and molecular dynamics simulations.

MATERIALS AND METHODS

Organism, Culturing, and toxin Isolation—N. spumigena strain BY1 was isolated from the hepatotoxic water bloom sample in 1986 from the southern Baltic Sea (21) and purified axenic (34). The strain was mass cultivated in the inorganic nutrient medium ZB, without nitrogen, in 3.5‰ salinity at about 22°C with continuous illumination.

BY1 was isolated from the hepatotoxic water bloom sample in 1986. CH3CO2NH4) at a flow rate of 5 ml/min. The pure nodularin was equally toxic does not bind covalently to PP-1. The present study focuses on the three-dimensional solution structure of nodularin (Fig. 1) and assigns independently (Table I). Arg and γ-glutamic acid (IGlu) had typical chemical shifts (45) for their methylene protons. Methyl-γ-amino acid (Me-Asp) and (23,35,85,95)-3-amino-9-methoxy-2,6,8-tri-methyl-10-phenyldeca-4,6-dienoic acid (Adda) have similar δ-amino acid backbones, however, Adda showed more cross-peaks due to protons in its long side chain. CH and CH3 of MDHB were recognized from their mutual cross-peak.

Structure Generation and Refinement—Distance restraints were extracted from a second-order polynomial fitted to integrated and normalized cross-peak volumes (I) of the NOE series with the initial condition I(t=0) = 0. The intra methylene NOEs of C9H and C10H of IGl serves for the calibration (1.78 Å). Distances were given as ± 20% uncertainty. When a distance could not be extracted by isolated spin pair approximation, owing to a poor signal-to-noise ratio or disturbances, it was only required that the distance was at most 6.0 Å. The upper bounds were extended by 1.0 Å for each pseudo atom. Coupling constants (J) were obtained by the J-doubling method (46) from fine structures of COSY cross-peaks or from line splittings in one-dimensional spectra. Dihe-drals characterized by intermediate J were not constrained but small and large J (37, 38) were related to staggered conformers (± 30°) on the basis of Karplus functions and NOEs (47).

Structures were generated by distance geometry followed by simulated annealing (force field cff91) (48, 49). The redundant NOE-derived restraints were consistent with the covalently implied limits from tetragonal bound smoothing for group of pairs which indicated that the calibration of distances was reasonable. Distance restraints were refined by an iterative relaxation matrix method (50) based on a family of structures to avoid biasing to a particular structure. This was imperative at least for the long side chains. In the calculation, 1.0-ns correlation time (τc) was used based on the molecular weight (824 g/mol) compared to microcystin-LR (994 g/mol) (18) for which there was an estimate of τc based on our earlier relaxation measurements at 1 ºC. Bounds were kept at least ± 15% of the exact distance given by iterative relaxation matrix method to avoid bias for a particular conformation and to take into account an uncertainty in τc. Restraints were computed also when τc = 0.4 and 2 ns. The correlation time could not be much smaller because at τc = 2 × 600 ns −1 for τc = √(2/τc) = 0.3 ns NOEs vanish (37). On the other hand when assuming τc = 2 ns the computation lead to unphysical negative rates for many local geometries.

MD-simulations and Force Field Parameters—The standard CHARMM force field was supplemented with parameters for the unusual amino acids Adda, MDHB, and Me-Asp. The atom and bond types were defined by ChemNote of Quanta software (51). Consistent partial charges were calculated with a self-consistent ab initio method using DMd software (52-54). The computation with a minimum basis set and 1-s orbitals frozen (except for H) converged within 44 iterations so that
the occupation of one-electron levels corresponded to room temperature. The HOMO-LUMO difference was 0.004 eV. The charge distribution was evaluated with the Hirschfeld population analysis (55). The distribution remained essentially the same during the 20 last iterations. The ab initio charges computed for vacuum require at least a scaling to serve as force field parameters in a solution. The scaling factor (1.2) was determined by comparing the calculated charges of Arg and IGlu with the corresponding parameters of the standard CHARMM. Certain charges were adjusted and balanced (0.01–0.02) to obtain a smooth and uniform distribution (Table II). The charges and their distribution were quite similar to those of CHARMM in general. Only for the phenyl ring of Adda the obtained values were clearly smaller than the CHARMM values for Phe.

CHARMM versions 2.2 and 2.3 (56, 57) with standard algorithms for molecular mechanics and dynamics were employed in the simulation. Bond lengths of hydrogens were constrained by SHAKE algorithm (58). The van der Waals interactions were truncated by SWITCH and electrostatic interactions by SHIFT functions at 13 Å (53). The NMR-derived initial conformation was embedded to a 30 × 30 × 30 Å box of about 1000 TIP3P type water molecules (59). The kinetic energy was increased stepwise during 10 ps to 274.15 K (1 °C) where the NMR experiments had been carried out. During a 30-ps equilibration phase, atom velocities were scaled toward the reference value if temperature departed more than 10°. Then the system was allowed to evolve without interference for 400 ps. Trajectories of atom coordinates in 25 fs and energies in 50 fs interval were analyzed by Quanta/CHARMM and SCARECROW (60) programs. The calculations and simulations were carried out on Silicon Graphics Power Onyx and Cray C90.

RESULTS

NMR—A one-dimensional spectrum of nodularin shows well resolved lines over a range of 9 ppm (Fig. 2). In addition, there are weak resonances probably from a minor conformation or isomer but their intensity is at most 2% of the main lines (Fig. 2). The assignment of 1H-shifts is complete (Table I) and consistent with earlier results (28, 43, 44). The stereospecific assignments were deduced except for the methylenes of IGlu for which there was no reliable means when considering only the intraresidue data. Many NOEs were sequential around the peptide backbone or along the side chains. There remained few unambiguous NOEs involving degenerate Cg backbone or along the side chains. There remained few NOEs with Adda and Me-Asp. Following traces showed the decreasing IGlu HN line and during the time course of 12 h it had disappeared whereas, HN of Adda and Me-Asp had decreased comparatively little (Fig. 4).

By model building it was concluded that the IGlu-MDHB peptide bond is cis because NCH3 of MDHB was not close in space either to CgHs or CgH2 of Arg. According to ROESY there were no mutually slowly exchanging conformations.

By model building it was concluded that the IGlu-MDHB peptide bond is cis because NCH3 of MDHB was not close in space either to CgHs or CgH2 of IGlu. In contrast, in microcystin-LR the peptide bond was found to be trans because strong cross-peaks were seen between NCH3 of MDHA and CgHs of IGlu (Fig. 3). All other peptide bonds were trans. The stereospecificity problem of the IGlu γ-backbone methylenes remained even though there were unequal NOEs between CgHs of MDHB and CgHs of IGlu, because the orientation of the MDHB side chain was not known. Consequently, the two possibilities for the stereospecificity were considered separately in the structure generation.

The variable temperature data revealed that HN of Arg shifted most with temperature (−1.8 ppm/°C) whereas HN of Adda (0.0 ppm/°C) and Me-Asp (−0.3 ppm/°C) remained practically independent of temperature and IGlu depended only weakly (−1.3 ppm/°C). At higher temperatures the intensity of the Arg line was suppressed owing to the fast exchange with the presaturated solvent. In the first spectrum from the freeze-dried sample dissolved in D2O, HN of Arg had completely disappeared and HN of IGlu had reduced compared to HN of Adda and Me-Asp. Following traces showed the decreasing IGlu HN line and during the time course of 12 h it had disappeared completely whereas, HN of Adda and Me-Asp had decreased comparatively little (Fig. 4).

Conformations—The distance geometry was computed with an initial set of 92 distance and 10 dihedral restraints excluding those that were more accurately determined by the covalent structure. Two families of conformations, that differed by the orientation of the MDHB side chain with respect to the plane of the peptide bond, were considered. The stereochemistry of the IGlu γ-backbone methylenes remained even though there were unequal NOEs between CgHs of MDHB and CgHs of IGlu, because the orientation of the MDHB side chain was not known. Consequently, the two possibilities for the stereospecificity were considered separately in the structure generation.
Iglu-MDHB peptide bond, emerged for each stereospecificity of the methylenes of Iglu. That is, there were four families of models. It was concluded that the methyls of MDHB and Me-Asp are on the opposite sides with respect to the plane of the Iglu-MDHB peptide bond because otherwise there should have been cross-peaks for the protons in the side chain of MDHB and C₃H₃ of Me-Asp. The stereospecificity was then deduced relying on the unequal NOEs between CH₃ of MDHB and CgH₃ of Iglu (Fig. 3). This family of conformations also had fewer restraint violations than the three other sets.

A new set of 100 structures was computed in which ϕ of MDHB was restrained between 0 and 180° and the pseudo atoms of the methyls of MDHB and Me-Asp were forced to be further apart than 4 Å. A set of 25 structures that had at most three distance restraint violations below 0.3 Å and no dihedral violations above 10° had similar backbone folds but the remote parts of Adda and Arg had large spatial dispersions. For this family the refined distances computed by iterative relaxation matrix method (τc = 1.0 ns) included in their ±15% uncertainty interval also, apart from few minor (<0.1 Å) exceptions, distances computed with the estimated extreme values of τc. Therefore the accuracy should not have been sacrificed for the precision. Based on the refined restraint set 100 structures were computed. There were 47 structures free of restraint violations apart from minor, below 0.2 Å, in the remote part of Adda and Arg. These were tolerated because the presumably mobile termini of Adda and Arg might generate non-simultaneous NOEs. There was no sign of a mutual interaction for these side chains. For this ensemble R-factors did not reduce significantly because of the large spatial dispersion of the long side chains.

The result is only one well defined family of the cyclic saddle-shaped backbone conformations (Fig. 5). The side chains protrude from the otherwise fairly globular backbone structure. Even though the van der Waals radii of the atoms do not completely fill the interior encircled by the backbone there is no room in the Connolly surface for water to pass through the compact backbone ring. The heavy atom root mean square deviation in the cyclic backbone was 0.16 ± 0.05 Å. There was no marked deviation per residue and the HN line widths did not imply differential mobility in the cyclic backbone. The long side chains of Adda and Arg and more specifically their remote parts contributed mostly to the all atom root mean square deviation 2.6 ± 0.4 Å. The computed energies were within ±15% from the average −115 kcal/mol.

Molecular Dynamics Simulations—A representative NMR structure was selected to act as an initial conformation for the simulation. Short simulations were run also for two other initial conformations, but results were mostly similar to the main simulation, analyzed below. The energy drift remained during the 400 ps simulation with 10 fs nonbonded interaction updating frequency rather small (9.3 · 10⁻⁷ kcal/mol) and the RMSEₛₑₒ₉/RMSEₖᵣ₉ ratio (0.006) was acceptable (below 0.01) (53). There was one prevailing backbone conformation which satisfied the NMR-derived restraints well (Fig. 6). Temporary and small, under 0.5 Å or 10°, violations were observed for nearly half of the restraints, but the average values remained mainly within the restraints. Only for methylenes of Iglu, four distances had average values, which were 0.1-0.4 Å out of range. Most of the other temporary violations were caused by movements of remote parts of Adda and the side chain of MDHB, which was, despite of the double bond, found to move quite readily. The two double bonds made the proximal part of Adda the most rigid part of the structure but the remote part moved readily. The angles (ϕ and ψ) adjacent to the trans peptide bonds between MDHB-Me-Asp, Arg-Adda, and Adda-Iglu fluctuated most up to 90° and caused local flip-flop movements of
the peptide bond plane (Fig. 7). This affected the hydrogen bonding, especially from HN of Iglu. The fluctuations were not correlated and the changes of $\phi$ and $\psi$ mostly compensated each other so that root mean square fluctuation of the other backbone dihedrals remained approximately 10°. The side chain of Arg adopted multiple conformations, but this did not exert an effect on the cyclic backbone fold. Also all NMR restraints of Arg remained satisfied.

The simulations were carried out without stabilizing hydrogen bond potentials which allowed alternatives to explore for hydrogen bonding. The hydrogen bonds frequently broke and reformed but the bonds from HN of Adda to the rotating COO$^-$ of Me-Asp were prevailing (80% of the time). The hydrogen bonds from HN of Iglu to COO$^-$ of Me-Asp formed during a short period when $\psi$ of the Arg and Adda-Iglu peptide bond had suitable orientations. The third frequently formed hydrogen bond was between HN and C$_5$O of Me-Asp but the unfavorable O–H–N angle, 120–140°, made it unstable (50% of the time). The hydrogen bonding pattern is in a good agreement with the NMR data.

The solvent accessibility of the amides was probed by the radial distributions (Fig. 8). HN of Me-Asp and Adda were found to be buried with very low water density at distances below 3 Å. Iglu has a small peaked density at 2.6 Å which probably corresponds to the small hydrogen exchange with water. There is a small but distinctive density for Me-Asp and Iglu at larger distances (6–7 Å) which most likely results from the order of water created by the COO$^-$ groups. HN of Arg was exposed with high water density already at 2.5 Å distance.

DISCUSSION

The conformation of nodularin bears a remarkable similarity to the three-dimensional structure of microcystin-LR. Both molecules have a saddle-shaped backbone conformation but microcystin-LR is more buckled than nodularin (Fig. 9). In water the saddle-shaped conformation is preferred over a planar arrangement probably due to a smaller wetted surface. The molecules are comparatively hydrophobic. For example, earlier we had observed that the commencing protonation of the carboxylic groups of microcystin-LR leads to a precipitation. The backbone fold is apparently stable because the structures in water, dimethyl sulfoxide, and chloroform appear quite similar. The MD simulations, nevertheless, reveal a certain degree of sway for the trans peptide bonds. This may be characteristic of small constrained cyclic peptides.

In particular the backbone fold in the conserved region Me-Asp-Arg-Adda-Iglu is almost identical among nodularin and microcystins. Also the proximal part of the Adda's side chain is alike in the two structures. The precision in these parts of the molecules is sufficient to allow a detailed comparison. Root mean square deviation of the heavy atoms between microcystin-LR and nodularin is less than 0.5 Å in the segment from the C$_a$ of Me-Asp to C$_a$ of Iglu and from C$_a$ to C$_7$ of Adda. The lower root mean square deviation for nodularin backbone results most likely from the compact ring structure which allows fewer degrees of freedom compared to microcystin-LR. Our structure is based on a larger restraint set but not necessarily as precise as those used in the other studies (25–27). The remote parts of

![Fig. 4. Exchange of amide protons to deuterium at 1 °C.](image)

![Fig. 5. Family of 47 nodularin conformations.](image)
Adda and Arg are lacking structural definitions in the same way and they are also mobile according to the MD simulations. Considering the large spatial dispersion of the remote parts of Arg and Adda side chains according to NMR data and on the short time scale motion observed during the simulation, the remote parts are indeed flexible in the solution.

The HN groups are accessible to the solvent in the same way in nodularin and microcystin-LR which also proves the similarity of the backbone structures. The exchange rates are in a qualitative agreement with the variable temperature data. HN of Arg that has the largest temperature coefficients also exchanges most rapidly, whereas HN of Adda and Me-Asp that have the smallest coefficients exchange most slowly. HN of IGlu falls in between when considering temperature coefficients or exchange rates. This observation further strengthens the conclusion that HN of Me-Asp and Adda and also HN of IGlu to some extent participate in intramolecular hydrogen bonds or are not readily accessible to the solvent, whereas the amide of Arg is exposed. There are presently no means for NMR to identify acceptor oxygens (24) but according to the structure the most favorable hydrogen bond acceptors are COO$^-$ of Me-Asp for HN of Adda and IGlu. The best candidate for HN of Me-Asp is C=O of Me-Asp but the angle is about 120°. The exchange might be merely hindered because of the buried HN of Me-Asp. In this case the slow exchange rate implies a stable backbone fold. In the three-dimensional model HN of Arg is exposed to solvent in an agreement with the exchange and variable temperature data.

The results of the molecular dynamics simulations, hydrogen bonds, and radial distribution functions of water are self-consistent and in a good agreement with the experimental data. The same hydrogen bond donors, that is, HN of Adda and Me-Asp were buried and most frequently hydrogen bonded. Furthermore, HN of IGlu is more accessible to solvent but still also hydrogen bonded from time to time, which makes the hydrogen exchange slower than in the case of most solvent-exposed HN of Arg. There is very little water in the vicinity of the buried HN of Me-Asp but the exposed amide of Arg creates order to the water.

The respective regions, MDHA-DAIa-Leu in microcystin-LR and MDHB in nodularin, are significantly different from each other. The IGlu-MDHA peptide bond in microcystin-LR is trans, whereas the corresponding bond is cis in nodularin. Consequently, the methylene of IGlu are in alternate orientations for microcystin-LR and nodularin. The isomerism of the IGlu-MDHA peptide bond allows the Me-Asp-Arg-Adda-IGlu fragment to adopt the same fold in these two cyclic peptides.

For nodularins the $N$-methylation of MDHB certainly makes the cis-bond more favorable whereas there are also demethylated toxic microcystin variants.

Similarity in the three-dimensional structures implies that...
nodularin would bind to PP-1 in the same way as microcystin-LR, i.e. without any major conformational change in the cyclic backbone. A comparison of nodularin with the bound microcystin-LR (31) also shows that the segment from Me-Asp Cα through Arg and Adda to IGlu Cα is homologous. During simulations the backbone dihedrals of nodularin in this region occasionally adopted precisely the bound microcystin-like values, although one single dihedral could at some moment differ significantly from the reference value. The largest, over 60°, momentary differences were caused by the flip-flop movement of the peptide bond planes. The proximal part of the side chain of Adda was also very similar. The space accessible to the remote part of Adda in the solution accommodates also the extended conformation found in the structure of the complex (31). In the bound microcystin three intramolecular hydrogen bonds were found. Like in the solution structure of nodularin one hydrogen bond was between Me-Asp C=O and HN, but the COO− group of Me-Asp was bonded to HN of IGlu instead of Adda. However, as observed during the simulation, the Me-Asp COO−→IGlu HN hydrogen bond formed when the plane of Adda→IGlu peptide bond tilted. The third hydrogen bond of microcystin-LR was between IGlu C=O and HN. The side chain of MDHA of microcystin-LR points to a different direction than that of MDHB of nodularin. MDHB occupies partly the same region of space as Leu of microcystin-LR. Therefore, MDHB of bound nodularin should not reach to Cys-273 of PP-1 and consequently not form a covalent bond with the SH group. This implies that the covalent binding of microcystins is not mandatory for the inhibition but rather a secondary effect, as suggested earlier (23). The structurally conserved epitope is primarily responsible for the tight binding to the protein phosphatases. Chemical modifications of various groups in this region have rendered the molecules non-toxic. In particular COO− of IGlu and the diene system of Adda have been found to be detrimental.

It has been shown that MDHA of microcystin-LR can be reduced with NaBH4 to enable tritiation (10, 11) or modified through nucleophilic addition with thiol compounds to enable attachment of, e.g. a primary amine which can then be used for further modifications.6 This approach is useful for production of radiola beled or fluorescent microcystin-derivatives for detection of protein phosphatases in tissues or for production of immobilized microcystin for affinity purification of protein phosphatases (23). Provided that nodularin in an analogy to microcystin-LR does not undergo a major conformation change upon binding the MDHB side chain will point away from the binding site. Therefore, nodularin might be an even more suitable probe at least for PP-1 because chemical modifications to MDHB should at least perturb the binding.

As a conclusion the well defined backbone conformations of microcystin-LR and nodularin are very similar to each other in the very same region that has been inferred to be essential for the toxicity. We expect that the relevant interactions responsible for the binding of nodularin to the serine/threonine-specific protein phosphatases are similar to those for the binding of microcystin-LR. Therefore the structure of PP-1 complexed with microcystin-LR (31) provides much understanding of the binding of nodularin as well.

Acknowledgments—We thank Dr. John Kuriyan for providing us with the coordinates of the PP-1-microcystin-LR complex. We have relied on the computational resources given by the Center of Scientific Computing (CSC) in Espoo, Finland.

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