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Computational study of the covalent bonding of microcystins to cysteine residues – a reaction involved in the inhibition of the PPP family of protein phosphatases

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Microcystins (MCs) are cyclic peptides, produced by cyanobacteria, that are hepatotoxic to mammals. The toxicity mechanism involves the potent inhibition of protein phosphatases, as the toxins bind the catalytic subunits of five enzymes of the phosphoprotein phosphatase (PPP) family of serine/threonine-specific phosphatases: Ppp1 (aka PP1), Ppp2 (aka PP2A), Ppp4, Ppp5 and Ppp6. The interaction with the proteins includes the formation of a covalent bond with a cysteine residue. Although this reaction seems to be accessory for the inhibition of PPP enzymes, it has been suggested to play an important part in the biological role of MCs and furthermore is involved in their nonenzymatic conjugation to glutathione. In this study, the molecular interaction of microcystins with their targeted PPP catalytic subunits is reviewed, including the relevance of the covalent bond for overall inhibition. The chemical reaction that leads to the formation of the covalent bond was evaluated *in silico*, both thermodynamically and kinetically, using quantum mechanical-based methods. As a result, it was confirmed to be a Michael-type addition, with simultaneous abstraction of the thiol hydrogen by a water molecule, transfer of hydrogen from the water to the α,β-unsaturated carbonyl group of the microcystin and addition of the sulfur to the β-carbon of the microcystin moiety. The calculated kinetics are in agreement with previous experimental results that had indicated the reaction to occur in a second step after a fast noncovalent interaction that inhibited the enzymes per se.

Introduction

Microcystins (MCs) are cyclic heptapeptides that are produced by several genera of cyanobacteria and are hepatotoxic to mammals. MCs cannot move across cell membranes [1], but are able to leave the digestive tract and enter hepatocytes through active uptake by a non-specific organic anion-transport system [2]. In the mammalian liver, MCs can be conjugated with reduced glutathione (GSH), a general detoxification mechanism that enhances the water solubility of xenobiotic compounds in order to aid their renal excretion. GSH-conjugated MCs have been identified in several aquatic organisms (fish, mussels, daphnids and macrophytes) and in rodents [3,4]. They are somewhat less toxic than nonconjugated MCs [5,6].

The cellular mechanism of MC toxicity involves the potent inhibition of protein phosphatases present in

Abbreviations
Adda, 2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; GSH, reduced glutathione; Masp, D-methyl-aspartate; MC, microcystin; Mdha, *N*-methyl-dehydro-alanine; PPP, phosphoprotein phosphatase.

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the cytoplasm [7]. More explicitly, the toxins are known to interact with the catalytic subunits of five enzymes of the phosphoprotein phosphatase (PPP) family of serine/threonine-specific phosphatases: Ppp1 (aka PP1), Ppp2 (aka PP2A), Ppp4, Ppp5 and Ppp6. The metazoan PPP family is composed of seven enzymes, but the activity of the remaining two – Ppp3 (aka PP2B) and Ppp7 (aka PPEF) – is not affected by MCs [8].

Over one-third of all proteins undergo reversible phosphorylation at one or more residues, a chemical modification that modulates their conformation, activity, localization and/or stability [9,10]. As protein phosphatases catalyze the removal of the phosphate group from those residues, they are key players for maintaining an adequate level of protein phosphorylation at every moment of the cellular cycle and it would not be an overstatement to say that they control virtually every physiological process and every cell-signaling pathway. The PPP family of serine/threonine-specific phosphatases has the additional particularity of being the physiological target of a number of natural toxins, of which MCs are a classic example [8]. The understanding of the chemical interaction between PPP enzymes and the toxic compounds will help to predict possible harmful effects and to explore their pharmacological potential in greater detail.

In this study, the molecular interaction of MCs with their targeted PPP catalytic subunits was characterized in detail, and particular emphasis was given to a covalent bond that is known to be formed between a PPP cysteine residue and the methylene group of an MC. The reaction mechanism of the covalent addition was clarified through *in silico* calculations using quantum mechanical-based methods. The small size of the models used in these calculations allows the generalization of the results to other biological systems that involve this specific type of reaction. This may prove interesting because the addition of cysteine residues to the methylene group of MCs seems to have a more general occurrence, having been suggested to be involved in the biological role of the toxins [11]. Moreover, the same chemical reaction is responsible for the nonenzymatic conjugation of MCs with GSH.

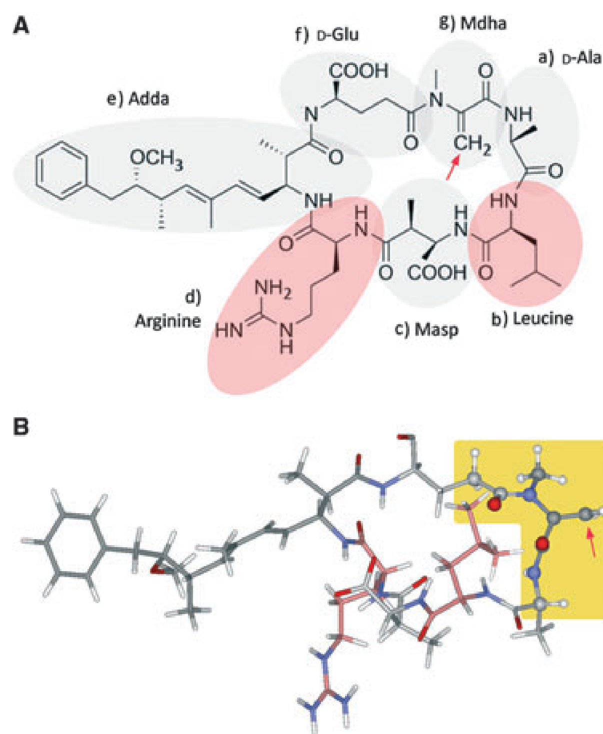


Fig. 1. Chemical structure of MCs. MCs are cyclic heptapeptides with five common and two variable amino acid residues. (A) Chemical structure of MCLR, where the variable residues, highlighted in pink, correspond to a leucine and an arginine. (B) 3D structure of MCLR; the leucine and arginine carbon atoms are represented in pink; and the atoms shown as a ball-and-stick model with the yellow background correspond to those included in the computational study. The red arrow indicates the methylene group where the cysteine residue binds covalently.

Microcystins

MCs are synthesized by nonribosomal enzyme complexes [12,13] and contain unusual, nonproteinogenic amino acids (see Fig. 1). The seven constituent amino acids are (a) D-alanine (D-Ala), (b) a variable **X** residue, (c) D-methyl-aspartate (Masp), (d) a variable **Z** residue, (e) (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), (f) D-glutamic acid (D-Glu) and (g) *N*-methyl-dehydro-alanine (Mdha).

Over 80 MCs have been identified so far, presenting diverse **X** and/or **Z** residues, and/or minor differences in the other amino acids. The variable **X** and **Z** residues are always L-amino acids that are indicated in the name of the molecule by their one-letter code (e.g. MC**LR** has leucine and arginine in positions 2 and 4, respectively). The modifications in the other amino-acid residues include demethylation of Masp and Mdha, and methylesterification of D-Glu. The different MCs have different toxicity profiles, the most toxic being MCLR [14]. MCLR has similar potency in the inhibition of Ppp1, Ppp2, Ppp4, Ppp5 and Ppp6, presenting a half maximal inhibitory concentration (IC_{50}) of around 0.1–1 nM [15–18]. It is 1000-fold less potent towards Ppp3 (IC_{50} of ~ 1 μM) and seems to have no effect on the activity of Ppp7 [18,19].

Interaction of MCs with PPP catalytic subunits

There are three available crystallographic structures of PPP catalytic subunits with bound MCs: Ppp1cα with MCLR [20], Ppp1cγ with 2H-MCLA [21] and Ppp2cα with MCLR [22]. 2H-MCLA differs from MCLR in that it presents an alanine in the place of arginine as the variable residue **Z**, and an *N*-methyl-alanine instead of Mdha, given the saturation of the double bond provided by the two extra hydrogen atoms.

From these structures, it is evident that MCs bind directly in the catalytic center of the PPP enzymes, completely blocking the access of the substrate to the active site. The area of the catalytic subunits where the toxins bind presents three surface grooves – the hydrophobic groove, the acidic groove and the C-terminal groove – which occur in a Y-shape with the active site situated at the bifurcation point (see Fig. 2).

The Adda side-chain of MCs is accommodated closely in the hydrophobic groove and accounts for a significant portion of the toxin's binding potential – modifications in the configuration of the Adda side-chain that produce a bent orientation unable to accommodate as tightly reduce the potency of the toxin [23,24]. The D-Glu (carboxyl group) of MCs, as well as the adjacent carbonyl group, make hydrogen bonds to metal-bound water molecules, which are important interactions as D-Glu was found to be essential for the toxicity of MC [24,25]. Additionally, the carboxyl group of Masp is hydrogen-bonded to two PPP conserved residues: an arginine and a tyrosine (Arg96 and Tyr134 of Ppp1c; and Arg89 and Tyr127 of Ppp2c).

A final interaction site consists of the PPP β12–β13 loop, where the Sγ atom of a cysteine residue (Cys273 in Ppp1c and Cys269 in Ppp2c) bonds covalently to the Mdha of MCLR, and the aromatic ring of a conserved tyrosine (Tyr272 of Ppp1c; and Tyr265 of Ppp2c) packs closely to the leucine residue of MCs, suggesting the presence of hydrophobic interactions.

Comparing the three structures, there is one clear structural change in the toxin-binding area, which only occurs in the case of Ppp1c bound to MCLR. It consists of a shift in the β12–β13 loop that results from the formation of the covalent bond with Cys273 (see Fig. 2). This shift is not observed in the structure of 2H-MCLA-bound Ppp1c because the hydrogenation of the Mdha residue precludes the formation of the covalent bond. In Ppp2c bound to MCLR the loop is not shifted because the cysteine residue involved in the bond is Cys269 (Phe276 in Ppp1c) and not Cys266 (corresponding to Ppp1c Cys273).

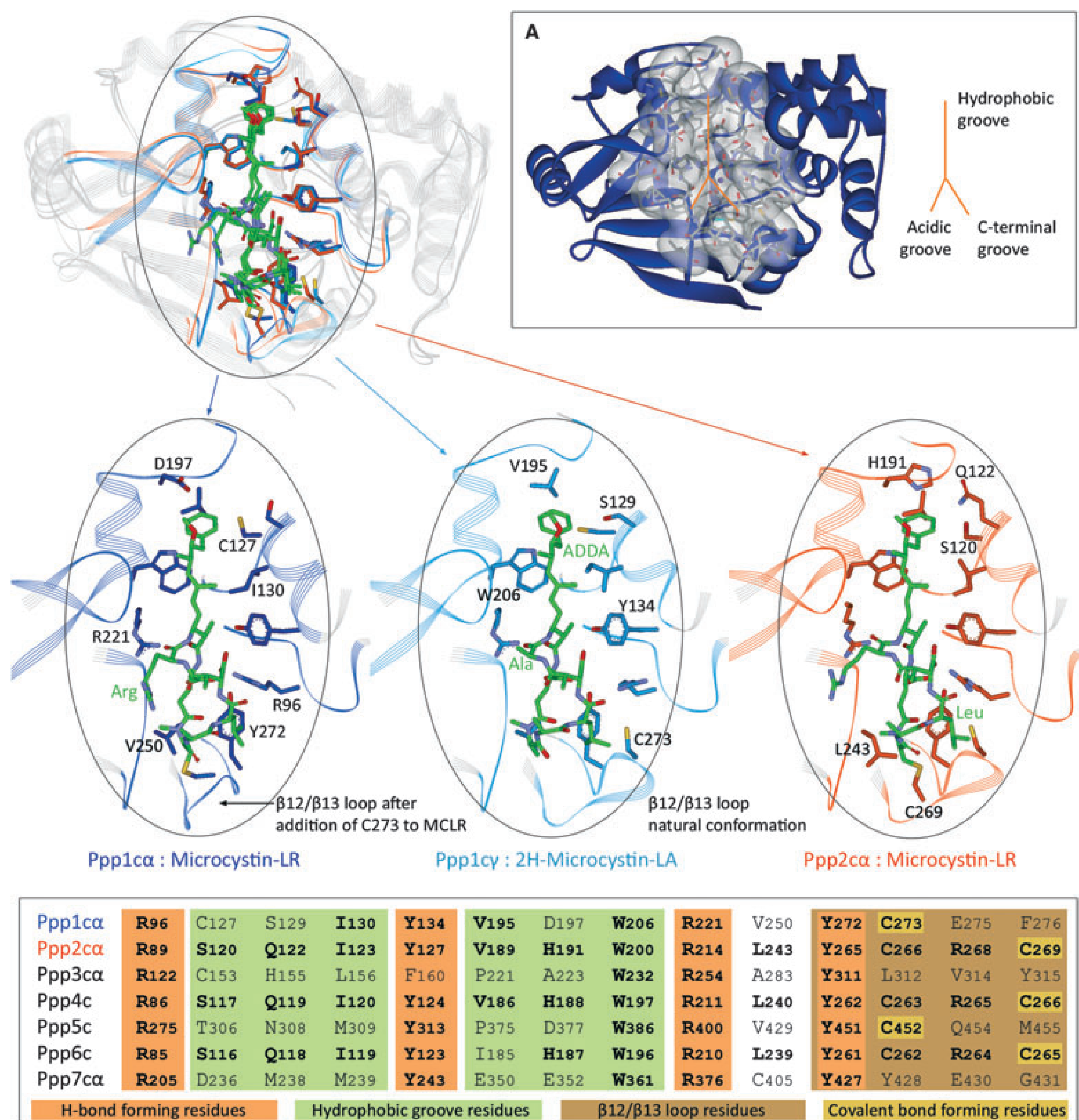
The covalent bond of MCs to cysteine residues

Interestingly, whereas all the PPP catalytic subunits that are inhibited by MCs have at least one cysteine residue in the β12–β13 loop in position to form the covalent bond with the Mdha methylene group, those that are resistant to MC (Ppp3 and Ppp7) do not have a cysteine residue available at this position (see Fig. 2). Ppp4c and Ppp6c have two cysteine residues corresponding to Cys266 and Cys269 in Ppp2c; Ppp5 has only one cysteine residue, corresponding to the Cys273 residue of Ppp1c (Ppp2c Cys266), indicating that its interaction with MCs will also result in the shift of the β12–β13 loop.

Although the previous observation is unlikely to be just a coincidence, the formation of the covalent bond is not considered essential for the inactivation of the enzymes by MCs [26]. Several findings support this conclusion: the existence of equally potent PPP-inhibiting MCs that cannot form the covalent bond (e.g. Asp, ADMAdda, Dhb-microcystin-HtyR) [27]; the existence of other naturally occurring, PPP-inhibiting toxins that do not bind them covalently (e.g. nodularins, which are cyanobacteria-produced pentapeptidic toxins structurally related to MCs); the finding that MCLR inhibits Ppp1 and Ppp2 in a two-step mechanism, and, while the noncovalent interaction completely inactivates the enzymes within minutes, the formation of the covalent bond between the cysteine and the Mdha residues takes hours [28]; and the fact that glutathione-conjugated MCs (or cysteine-conjugated MCs) still have the capacity to inhibit PPP activity, although with less potency [5].

Nevertheless, even if the formation of the covalent bond is accepted as an accessory event in the context of PPP inhibition, it should no doubt contribute to the stabilization of the enzyme–toxin complex. Additionally, the reaction appears to have relevance outside this context, as it has been proposed to be involved in the, so far elusive, biological role of MCs [11] and is responsible for the nonenzymatic conjugation of MCs to GSH. In fact, whereas conjugation with GSH is usually catalyzed by a glutathione *S*-transferase it can also occur spontaneously given strong alkaline conditions [3,4].

The nature of the chemical reaction itself is generally believed to consist of a Michael-type addition of the nucleophilic sulfur atom of the cysteine residue to the α,β-unsaturated carbonyl group of the Mdha residue, but so far this has not been confirmed. In this study, quantum mechanical-based methods were used to clarify the chemical mechanism of the formation of the



Ppp1cα	R96	C127	S129	I130	Y134	V195	D197	W206	R221	V250	Y272	C273	E275	F276
Ppp2cα	R89	S120	Q122	I123	Y127	V189	H191	W200	R214	L243	Y265	C266	R268	C269
Ppp3cα	R122	C153	H155	L156	F160	P221	A223	W232	R254	A283	Y311	L312	V314	Y315
Ppp4c	R86	S117	Q119	I120	Y124	V186	H188	W197	R211	L240	Y262	C263	R265	C266
Ppp5c	R275	T306	N308	M309	Y313	P375	D377	W386	R400	V429	Y451	C452	Q454	M455
Ppp6c	R85	S116	Q118	I119	Y123	I185	H187	W196	R210	L239	Y261	C262	R264	C265
Ppp7cα	R205	D236	M238	M239	Y243	E350	E352	W361	R376	C405	Y427	Y428	E430	G431

H-bond forming residues; Hydrophobic groove residues; β12/β13 loop residues; Covalent bond forming residues

Fig. 2. Global and detailed view of the interaction of MCs with PPP catalytic subunits. Three crystallographic structures of microcystin-bound PPP catalytic subunits are represented superimposed and in individual close-ups of the toxin-binding site (PDB ID: 1fjm, 2bdx and 2ie3). (A) The PPP surface area where the toxins bind have three grooves arranged in a Y configuration, with the active site situated at its bifurcation. The partial sequence alignment shown at the bottom of the figure includes the Ppp1c and Ppp2c amino acid residues that interact with MCs – as observed in the referred crystallographic structures – and the corresponding residues in the other PPP enzymes. Ppp3c and Ppp7c are the only PPP catalytic subunits with no covalent bond-forming cysteine residues in the β12–β13 loop; coincidentally, they also offer higher resistance to inhibition by MCs.

covalent bond and to ascertain the energetics involved in the reaction.

The three optimized geometries of the stationary points corresponding to the reaction that was found to be more favorable, both thermodynamically and kinetically, are represented in Fig. 3. This reaction represents the addition of the cysteine sulfur atom to the β-carbon of the α,β-unsaturated carbonyl moiety of

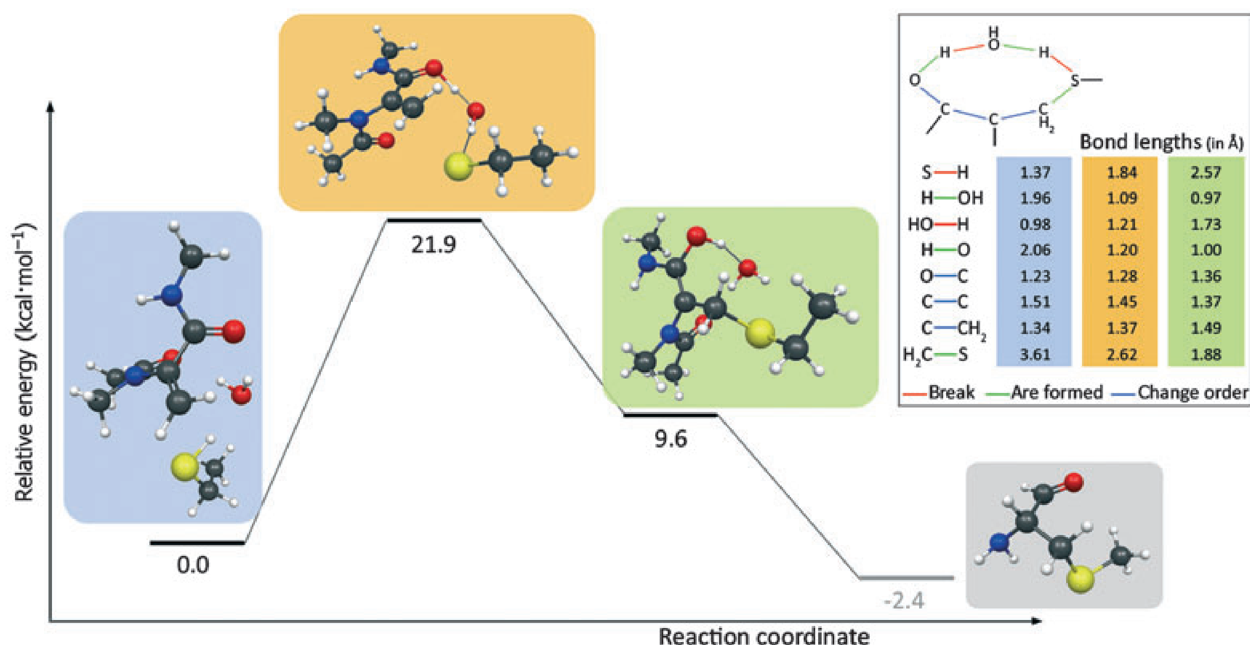


Fig. 3. Stationary points of the modeled reaction, representing the addition of a cysteine residue to the methylene group of MCs. The modeled system includes part of an MC, the side-chain of the cysteine residue and a water molecule. The reaction coordinate was found to be complex: with the addition of the sulfur atom to the carbon of the MC, the hydrogen of the thiol group passes to the water, which releases one of its hydrogen atoms to form a covalent bond with the oxygen of the Mdha carbonyl group. As the inserted panel shows, there are a total of eight covalent bonds undergoing prominent changes: two that break, three that are formed, and three that change order. The relative energy of the ketonic form of the product has been extrapolated from calculations with smaller models.

the Mdha residue, with the assistance of a water molecule. The presence of the water as a catalyst was found to be necessary, as calculations with alternative reaction coordinates, where no water was present, involved prohibitive energies and/or resulted in thermodynamically unstable products (data not shown).

The reaction coordinate is complex: with the addition of the sulfur atom to the carbon, the hydrogen of the thiol group passes to the water, which releases one of its hydrogen atoms to form a covalent bond with the oxygen of the Mdha carbonyl group. There are a total of eight covalent bonds involved in the reaction, which undergo prominent changes: two that break; three that are formed; and three that change order (see the inset in Fig. 3). In the end-product, the sulfur is covalently bonded to the carbon of an enolic compound, which should decay to the corresponding ketone as this form seems to be thermodynamically more favorable.

The classic Michael reaction refers to the addition of a carbanion to the β-carbon of an α,β-unsaturated carbonyl compound, but the concept has been generalized to include nucleophiles such as sulfur, nitrogen, phosphorous or oxygen. The assistance of a base is usually required to activate the nucleophilic attack, which is consistent both with the alkaline medium needed to trigger the nonenzymatic conjugation of GSH with MCs and with the role played by the water molecule in these calculations.

The calculated activation energy for this reaction is 21.9 $kcal \cdot mol^{-1}$ (see Fig. 3), which, using transition state theory, corresponds to a rate constant of around $10^{-3}\ s^{-1}$ (2.4E $10^{-3}\ s^{-1}$ for $T = 310.15$ K and 1.0E $10^{-3}\ s^{-1}$ for $T = 303.15$ K). If the reaction is regarded as pseudo first-order, by considering the MC concentration constant in relation to the protein, the corresponding half-life for $T = 303.15$ K is approximately 2 h. This value is in agreement with the previous finding that the formation of the covalent bond between the cysteine and the Mdha residues was the second step of the interaction of MCLR with Ppp1 and Ppp2, which took place a couple of hours after a first noncovalent interaction completely inactivated the enzymes [28].

These calculations do not contribute to the understanding of the differences in the toxicity of diverse MC isoforms, or their toxicity towards diverse PPP enzymes. It would be interesting to compare MCs with different variable residues but we believe that the influence of those residues is more likely to reside in the

noncovalent part of the toxin–enzyme interaction [8] and would not have an impact on the results obtained in this study.

In conclusion, the computational study of the reaction mechanism that is responsible for the formation of a covalent bond between the Mdha residue of MCs and a cysteine residue, confirmed the reaction to be a Michael-type addition, with simultaneous abstraction of the thiol hydrogen by a water molecule, addition of the sulfur to the Mdha methylene carbon and transfer of the hydrogen from the water to the Mdha carbonyl oxygen. The calculated kinetics are in agreement with previous experimental results.

The importance of characterizing this reaction is primarily related to the toxicological properties of MCs, which involve interaction with protein phosphatases of the PPP family. However, the insights obtained can also be applied both to the ecological role of these toxins, proposed to include binding of a variety of proteins in response to oxidative stress, and to the nonenzymatic conjugation of the toxin to GSH, which is thought to constitute the first step in the biotransformation of the molecule.

Methods

Density functional theory (DFT), at the B3LYP/6-31G(d) level of theory [29,30], was used within the General Atomic and Molecular Electronic Structure System (GAMESS) software package [31] to perform all the calculations. The model system had a total of 35 atoms: those from MCLR are represented as a ball-and-stick model in Fig. 1B, plus the side-chain of the cysteine residue added, plus a water molecule.

The computational study of each reaction consisted of the following steps. First, the transition state was found by optimizing a close geometry, obtained from a scan of the reaction coordinate, and was confirmed through the identification of the respective imaginary frequency. Second, intrinsic reaction coordinate calculations were performed to identify the two minima connected by that transition state. MacMolPlt graphical user interface [32] was used to visualize the geometric and electronic features of the different stationary points.

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