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Review

Plant thionins – the structural perspective

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Abstract. Thionins belong to a rapidly growing family of biologically active peptides in the plant kingdom. Thionins are small (~5 kDA), cysteine-rich peptides with toxic and antimicrobial properties. They show a broad cellular toxicity against wide range of organisms and eukaryotic cell lines; while possessing some selectivity. Thionins are believed to be involved in protection against plant pathogens, including bacteria and fungi, by working directly at the membrane. The direct mechanism of action is still surrounded by controversy. Here the results of structural studies are reviewed and confronted with recent results of biophysical studies aimed at defining the function of thionins. The proposed toxicity mechanisms are reviewed and the attempt to reconcile competing hypotheses with a wealth of structural and functional studies is made.

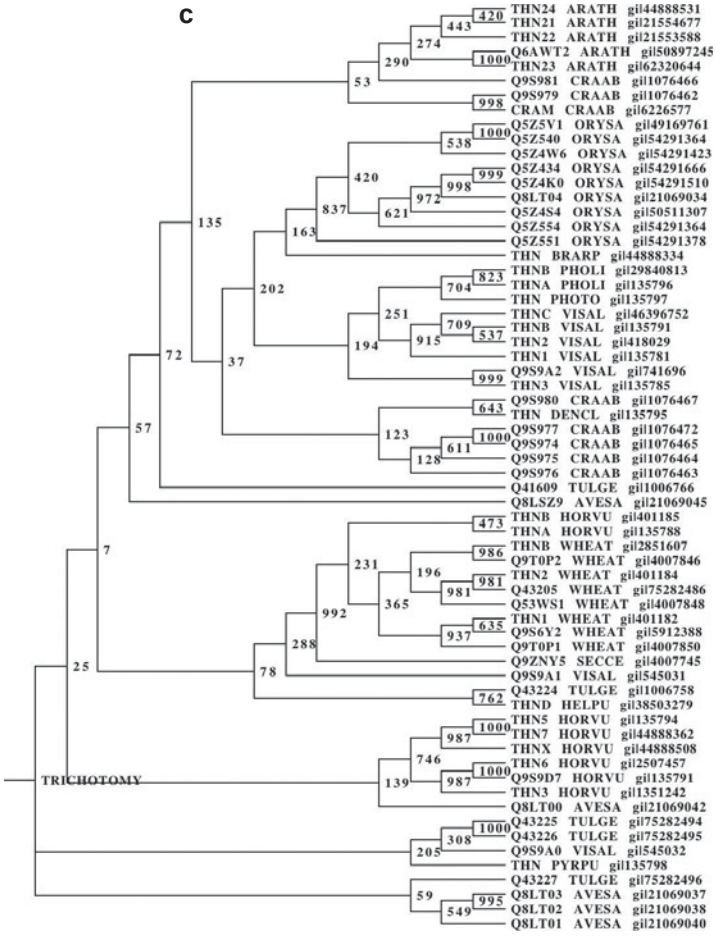
Keywords. Plant toxins, thionins, structure, mechanism of toxicity, membrane lysis.

Introduction

Plants produce a bewildering variety of biologically active compounds, including small peptides (for recent reviews see [1–4]). Peptides such as lipid transfer proteins, puroindolines, α/β-thionins, γ-thionins, plant defensins, hevein-like peptides, knottin-like peptides, glycine-rich peptides, and the homologs of MBP-1, snakins, play an important role in fending off bacterial and fungal invaders [2].

Thionins [5] are small (~5 kDa), cysteine-rich, usually basic proteins found in monocots (grains), eudicots including dicotyledonous plants (different species of mistletoe and *Pyrularia pubera*), and rosids (crambin). Around 100 individual thionin sequences have been identified in more than 15 different plant species (Fig. 1a, b). For taxonomic details please refer to Figure 1a. The name thionins is used for two distinct but well characterized groups of plant peptides, α/β-thionins and γ-thionins. Despite common name and probable very distant common origin, they have quite distinct three-dimensional architectures (Figs 2–4). As such they have to be considered separate protein families, and γ-thionins should more appropriately be called plant defensins.

Traditionally, α/β-thionins were subdivided into five different classes (I, II, III, IV, and V) [5], and many researchers distinguish an additional class, γ-thionins (see above). Type I thionins are present in the endosperm of grains (the family Poaceae) [6], are highly basic, and consist of 45 amino acids, 8 of which are cysteines. Type II thionins were isolated from leaves and nuts of the parasitic plant Pyrularia pubera [7] and from the leaves of barley Hordeum vulgare [8, 9]. They are slightly less basic than type I thionins (reduced positive charge from +10 to +7) and consist of 46–47 amino acids. Both type I and II thionins have four disulfide bonds. Type III thionins have been extracted from leaves and stems of mistletoe species, such as *Viscum album, Phoradendron tomentosum, Phoradendron liga,* and *Dendrophthora clavata* [10–13], and consist of 45–46 amino acids. These thionins, which have three disulfide bridges conserved with respect to types I and II, are as basic as type II thionins. Type IV thionins, which consist of 46 amino acids with three disulfide bonds and are neutral in charge, have been extracted from



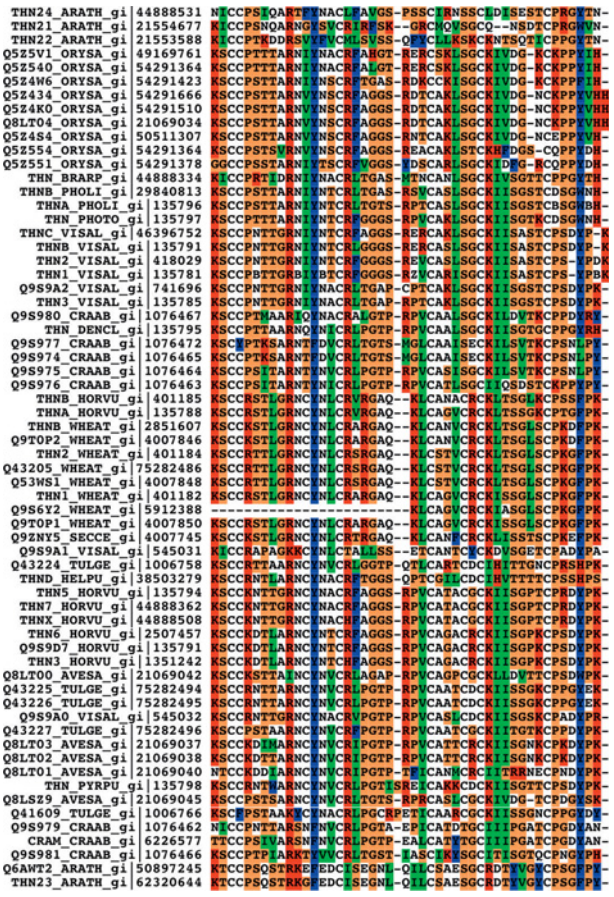


Figure 1. (*a*) The taxonomy report of the *α/β*-thionin family as characterized by PFAM. The number in parenthesis (next to the species name) represents the number of *α/β*-thionins sequences detected in a particular species. (*b*) Sequence alignment of selected representatives (64 sequences presented in the PFAM web-site) of the *α/β*-thionin family. Some sequences from *Crambe abyssinica* lack the defining components of toxicity (Tyr13 and Lys1) and this renders them inactive (*e.g.* crambin). (*c*) The alignment produced in ClustalX [60] was used to produced a bootstrapped (1000 iterations) phylogenetic tree (visualized by TreeView [115]).

seeds of Abyssinian cabbage [14, 15]. Type V thionins are truncated forms of regular thionins found in some grains like wheat [16]. Since they have no demonstrated toxic activities they will not be reviewed here.

All five types of *α/β*–thionins appear to be highly homologous at the amino acid level. Modern structural work has shown that all five classes exhibit the same structural motif, and past classifications appear less supported by three-dimensional structures. *γ*-Thionins have much more in common with a large family of membrane active peptides, called defensins, found in plants and animals [17, 18], as noted above. To underscore the structural differences between both classes, and because the name *γ*-thionins is still quite popular, the structural results for *γ*-thio-

nins are briefly reviewed. However, for their biological activities the reader is referred to the defensin literature [19, 20].

History

Knowledge of the presence of unusual substances with medicinal properties in mistletoe can be traced back several millennia. Even five centuries BC ancient peoples used its extracts for several sicknesses. This ancient knowledge was passed on to medieval times when mistletoe ointments were widely used for sores and festering wounds. Additionally, some blood circulation problems were treated with mistletoe. In modern times extracts of mistletoe are potential candidates for selective treatment of cancers [21, 22].

The first written reports describing inhibition of fermentation of bread or beer by unknown grain compounds appeared at the end of the 19th century. Those observations were linked with use of some batches of wheat flour. For instance, in 1895, it was suggested that there was a substance in wheat flour that was lethal to bread yeast (cited in [23]). This suggestion was made after observing a decrease in the rate of fermentation measured by carbon dioxide release. The first record of a successful isolation of the active ingredient inhibiting yeast growth was in 1942 by Balls and collaborators [24]. They isolated and crystallized a toxic substance from wheat endosperm (*Triticum aestivum*), later called purothionin (from Greek *puro*, wheat, and *thio*, sulfur) [25, 26].

In recent years, many other small basic peptides have been isolated from different grains like wheat [24] barley [9, 27], oats [28], rice [28], and rye [30]. Actually, some grain species such as wheat or rice have multiple variants of thionins differentially expressed in several tissues. Thionins have also been found in multiple species of mistletoe [10–13]. Over the years, Samuelsson isolated thionins from other members of the Viscacea family, such as phoratoxins A and B, from *Phoradendron tomentosum,* denclatoxin B from *Dendrophtora clavata*, and ligatoxins A and B from *P. liga* [10–13]. The main thrust of those studies was the potential application of mistletoe extracts for medical purposes. Those extracts showed several toxic effects, especially against cancer cells [31].

Thionins were also extracted from oil nut (*Pyrularia pubera*) [7] and Abyssinian cabbage (*Crambe abyssinica*). Crambin initially extracted by van Etten et al. [14] showed no toxic effects, had slightly hydrophobic characteristics, and was the first thionin crystallized and solved by X-ray crystallography. Later, it was shown by Schrade-Fisher and Apel [15] that those hydrophobic and non-toxic variants were representative of a highly diverse and large group of thionins expressed in *C. abyssinica* (Fig. 1b).

In most species in which careful genetic characterization has been made, thionins appear to be expressed as non-toxic propeptides activated upon pathogenic attack [32–34]. Analysis of thionin cDNA showed that thionins have a common gene structure with an approximately 20-amino acid-long leader peptide and an approximately 60-amino acid-long trailing acidic peptide, which neutralizes the basic toxin [15]. Cleavage of the leader peptide is necessary for toxin activation [32]. The toxin domain is less conserved than the two flanking peptides in the proprotein.

One of the more interesting facts about the thionin family is that these proteins are usually extracted from natural sources by acid wash or organic solvent extraction as proteolipid complexes [24]. The identity of the tightly bound lipid has not always been determined, but it was indicated to be a derivative of diacyl-glycerol. More recent studies of *Pyrularia* toxin implicated negatively charged phospholipids, such as phosphatidic acid and phosphatidyl serine [35], but studies with artificial membranes showed that thionins most likely interact with a much wider class of phospholipids [36].

There are several independently proposed functions for these small cysteine-rich basic peptides. The function of most interest to this review is the defense function. Several experiments suggest an important role in defense against pathogenic invaders [37–39]. Thionin synthesis in response to bacterial invasion [40–42], its accumulation in vulnerable tissues [15, 43–45], its toxicity to different organisms and cell lines [37, 46–49], and the improved resistance observed when expressed transgenetically [50–52], all strongly support this function. However, their high concentration in the endosperm (in some species up to 10% of the seed mass) and high cysteine content suggest that they also serve as storage proteins. Purothionins have also been shown to influence the redox potential of other proteins (thioredoxins), and are known to activate chloroplast fructose-1,6-bisphosphatase by promoting disulfide bond formation [53, 54]. Therefore, purothionins are patented and sold as a biological oxidizing/reducing agent (Corystein™ TaKaRa Co). An intense effort is also being put into discovering medicinal uses for thionins [55], as well as into improving crop resistance by engineering more potent versions [56] of thionins or translating them to species that do not express them [57].

Structural studies

Despite being crystallized in the 1940s, the first structural characterization of thionins was carried out in the 1980s, when Hendrickson and Teeter [58] determined the structure of the neutral thionin crambin. In subsequent years, several studies made crambin one of the best structurally

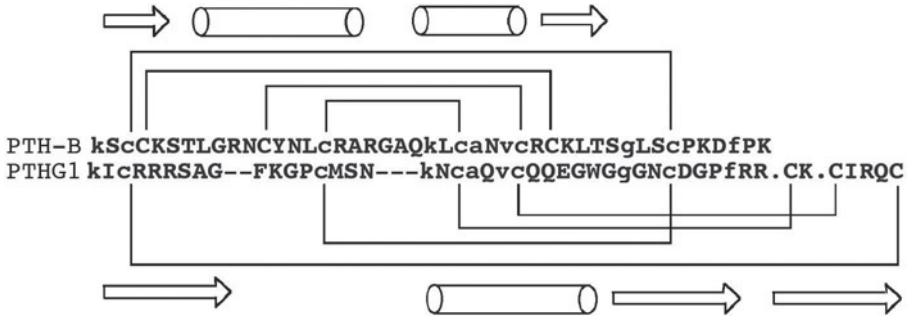


Figure 2. Sequence alignment of the β-purothionin with γ-purothionin. The presented sequences were aligned by ALIGN as implemented on the BiologyWorkbench web site (SDSC). The identities are represented by lower case letters. The disulfides are presented in brackets joining the cysteine residues. The secondary structure is marked with arrows and cylinders above and below the sequences for β and γ-hordothionins, respectively.

studied proteins. A number of X-ray structure determinations of different thionins, as well as determinations by NMR, followed these initial studies. The major results of those studies are described in the next chapter.

Structural studies clearly showed distinct architectural features for these small toxins. Despite small variations in length (45–48 amino acids), α- and β-thionins share the same three-dimensional architecture. Prior to structural studies, γ-thionins were recognized by their small molecular weight, the presence of disulfides, and residual sequence similarity (~25% identity, Fig. 2) to α- and β-thionins. Clearly, γ-thionins must be derived from a distant but common ancestor. However, unlike many proteins that have a similar level of sequence homology (~30%), they evolved a novel fold by rearranging the disulfides bridges and the β-sheet, which resulted in different architectures. All thionins have a broad distribution and are present in almost every crucial plant tissue from endosperm to leaves. Their toxic effect was postulated to arise from lysis of the membranes of attacking cells [37–39]. However, the precise mechanism underlying toxicity remains unknown.

All the members of the thionin family represented in the PFAM database [59] are aligned in Figure 1b, and the phylogenetic tree produced in ClustalX [60] is also presented (Fig. 1c). A striking feature is that many thionins of the same species belong to different branches of the tree, suggesting that even though they have the same origin they also have a different evolutionary history for different family members. Evolution has also produced non-toxic representatives of the family, as evidenced by loss of crucial elements of toxicity such as Tyr13 [61] or Lys1 [62] in a few thionins from several species (for instance, crambin, Fig. 1b, c). The residue Trp8 has also been implicated in the mechanism of toxicity [63]. Trp8 is not well conserved, but other amino acids at this position might serve a similar function (Fig. 4).

Structural characterization

Representative proteins with known three-dimensional structures determined by X-ray crystallography are crambin (mixed, PL and SI variants), α_1- and β-purothionins, β-hordothionin, and viscotoxin-A3 (Figs 1, 4, and Table 1). The first to be determined was the mixed form of crambin [61, 64]. It showed a distinct capital Γ letter shape with the N terminus forming the first strand in a β-sheet. The architecture of this sheet is additionally strengthened by two disulfides. After a short stretch of extended conformation, there is a helix-turn-helix motif. In crambin, there is a single disulfide involved in stabilizing the helix-to-helix contacts. At the center of this motif there is a crucial Arg10 that forms five hydrogen bonds to tie in together the first strand, the first helix and the C terminus. Mutation of this residue makes the architecture unstable and difficult to fold [56]. However, three disulfides in a very compact structure render crambin very stable and difficult to denature or cleave.

Subsequently, the X-ray structures for purothionins were determined. The structures turned out very similar to crambin with small exceptions. *Gramineae* purothionins are 45 amino acids long with four disulfide bonds. In purothionins an additional disulfide connects the helices, as compared with crambin, making this toxin even more stable than crambin [65, 66]. The structural determination for crambin and purothionins were followed with those of viscotoxin A3 [67] and β-hordothionin [68]. Common feature of those structures was a very well conserved molecular architecture and the presence of additional solutes that highlighted the importance of several residues in the mechanism of toxicity. The details are discussed below.

The structures of several members of the thionin family were determined by NMR (Table 2), *i.e.* phoratoxin-A [69], crambin [70], viscotoxin A3 and C1 [71, 72], α-hordothionin [73], purothionin [74], and hellethionin [75]. These structures showed high similarity with those determined by X-ray crystallography.

Table 1. Data collection and refinement statistics for crambin, α_1-and β-purothionin, A3-viscotoxin, and β-hordothionin.

Protein	PDB code	Space group	Contents asymm. unit	Resolution (Å)	R_{merg} [a]	Unit cell (Å)	Solute contents of asym. unit	Final R [b]	Final R_{free}
Crambin mixed form	1EJG	$P2_1$	Monomer	0.54	0.055	a = 40.82 b = 18.49 c = 22.31	1 ethanol	0.090	0.094
Crambin PL form	1CNR	$P2_1$	Monomer	1.05	0.056	a = 40.62 b = 18.34 c = 22.14	1 ethanol	0.105	0.112
Crambin SI form	1AB1	$P2_1$	Monomer	0.89	0.056	a = 40.76 b = 18.40 c = 22.27	1 ethanol	0.147	0.168
α_1-Purothionin	2PHN	I422	Monomer	2.8	0.12	a = b = 53.59 c = 69.79	3 acetate 2 *sec*-butanol 2 glycerol 1 PO_4	0.155	0.24
β-Purothionin	1BHP	I422	Monomer	1.7	0.056	a = b = 53.94 c = 72.75	2 acetate 2 glycerol 1 PO_4	0.198	0.281
Viscotoxin A3	1OKH	$P2_12_12$	Dimer	1.75	0.089	a = 48.00 b = 68.77 c = 25.35	2 PO_4	0.167	0.239
β-Hordothionin	1WUW	$P4_12_12$	Dimer	1.9	0.078	a = b = 68.66 c = 47.47	2 serine 2 *p*-toluene sulfate	0.170	0.212

[a] $R_{merg} = \Sigma |I_i - \langle I \rangle| / \Sigma \langle I \rangle$.
[b] $R = \Sigma |F_o - F_c| / \Sigma F_o$.

γ-Thionins

In the introduction both families were classified as separate. To underline their structural differences, the determinations for γ-thionins are briefly reviewed. The structures of several members of the defensin family (γ-thionins) were determined by NMR (Table 2). The most representative of these are γ-purothinon, γ-hordothionin [76] and NaD1 thionin [77]. Numerous studies have indicated the similarities to representative molecules from the animal defensin family. The similarities are also exhibited on the level of the distribution of charged residues on the surface and the resulting electrostatic potential generated by those molecules (Fig. 3b). Several γ-thionins structures are available in the PDB. Steady progress in NMR techniques makes those structures quite reliable, making verification of functional hypotheses possible. Nevertheless, lack of the X-ray structure makes it somewhat difficult to compare them in more detail, especially when it comes to the solvent molecules. As was the case for α/β-thionins described below, the important functional insight usually comes with the analysis of the solvent molecules in crystal structures.

The structures of the most representative for γ-thionins from wheat and barley [76] can be seen in Table 2 and Figure 3. They show small RMSD variations in the family of representative models. Other available structures are of those from *Petunia hybrida* [78], *Nicotiana alata* NaD1 [77], and *Pisum sativum* [79]. Despite small sequence changes, they superimpose very well, revealing a common underlying structural motif shared by both γ-thionins and animal defensins.

There is a basic difference in structural features when γ-thionins are compared with that of α-thionins. The characteristic motif of the α-thionin β–α–α–β-coil is replaced by the motif β-coil-α–β–β. In this new motif, the second strand of the β-sheet is in the opposite direction compared with the α/β-thionin architecture, and an additional strand is inserted between the first and the second strand. Nevertheless, the first cysteine residue in both architectures is conserved and remains crucial for structural integrity, tying up the N- and C-terminal segments together.

The toxins share some common features. They are quite basic (Figs 3b, 4c, 4d) and stabilized by extensive network of disulfide bridges. They are also implicated in direct interactions with the membrane even though γ-thionins were shown to inhibit several enzymes [20].

α/β-Thionins

The X-ray structure of crambin was determined at high resolution [58], and in time gradually improved to a very high resolution. At present, the structures are available at 0.83 Å [64], 0.67 Å [80] and 0.54 Å [81], so that protein structure is known with unprecedented atomic detail. Additionally, two pure forms of crambin

Table 2. Summary of experimental data and model quality for α/β-thionins and for γ-thionins determined by the NMR spectroscopy [a].

Protein	PDB code	Number of NOES	Number of J couplings	Number of restraints	Number of models deposited	RMSD backbone all atoms
Crambin mixed form	1CCM	320r	12	342	8	1.5 2.0
α_1-Purothionin	Not deposited	310	0	310	9	1.5 2.0
Hellethionin-D	1NBL	594	0	594	20	0.47 0.82
Viscotoxin A3	1ED0	734	32	782	20	0.33 0.81
Viscotoxin C	1JMN	631	18	649	20	0.7 1.3
Viscotoxin B	1JMP	531	16	547	20	0.87 1.56
α-Hordothionin	Not deposited	292	36	336	15	0.62 1.16
Phoratoxin	Not deposited	250	19	269	15	1.6 2.6
γ-1-H-hordothionin (*Hordeum vulgare*)	1GPT	299	0	320	8	1.3 1.7
γ-1-P-purothionin (*Triticum turgidum*)	1GPS	285	0	331	8	1.4 1.8
NaD1- (*Nicotiana alata*)	1MR4	527	38	877	20	1.5 2.0
Antifungal protein *Raphanus sativus*	1AYJ	533	132	665	20	0.33 0.81
Antimicrobial protein *Aesculus hippocastanum*)	1BK8	962	42	1004	25	0.7 1.18
Defensin *Pisum sativum*	1JKZ	675	51	726	20	0.7 1.9
Defensin *Petunia hybrida*	1N4N	407	26	433	20	1.22 1.64

[a] RMSD, root mean square deviation, NOE, nuclear Overhauser enhancement.

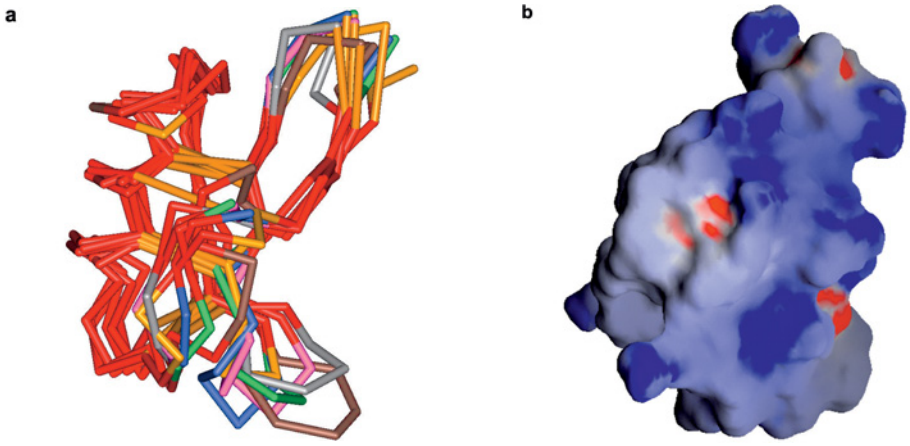


Figure 3. (*a*) Carbon alpha model of representative γ-thionins from *Hordeum vulgare* (barley), 1GPT [76]; *Triticum turgidum* (wheat), 1GPS [76]; *Pisum sativum* (pea) [79]; *Petunia hybrida*, 1N4N [78]; *Raphanus sativum* (radish), 1AYJ (not published); and *Nicotiana alata*, 1MR4 [77]. (*b*) Molecular surface representation of γ-thionins from *Hordeum vulgare* (barley) with electrostatic potential mapped. Blue represents potential 10 kT/e, red –8 kT/e. The back-bone figures were made using Cn3D molecular visualization software [116], while the electrostatic surfaces were made using GRASP [117].

were crystallized where at positions 22 and 25 there are either Pro/Leu or Ser/Ile present [82, 83]. Three disulfides render this protein very stable and heat resistant [84]. Crambin has no charge at neutral pH, and its helices have a significant hydrophobic character. It is insoluble in water and was crystallized by lowering the percentage of ethanol in aqueous solution. Despite crystallization from a high concentration of ethanol, there is only a single molecule of ethanol present in the solvent located close to the C-terminal Asn. Despite overall hydrophobic character (neutral charge), crambin is amphipathic with two Arg residues (10, 17) facing the groove between the β-sheet and α-helices, while two Asp residues are located on the other side of the molecule. The residue at position 13 [60], which has been implicated in toxicity, is occupied by a Phe residue in crambin. In contrast to other representatives of this family, crambin is non-toxic and does not possess lytic powers. It was suggested that crambin has some anesthetic properties.

In contrast to crambin, which is neutral and hydrophobic, the toxins are all highly basic with amphipathic character greater than crambin. Some reports attempted to correlate the increase in charge with potency. Both α_1- and β-purothionins obtained from wheat have been crystallized and their X-ray structures determined [65, 66] (Table 1). The resolution of this study was less than that of crambin (1.7 Å for β-purothionin). The overall architecture of α_1- and β-purothionins is very similar to that of crambin. The structure is compact and has a flat polar face. Despite the small size, the structure is comprised of a two-stranded β-sheet, two antiparallel helices, and a C-terminal coil region [64] (Figs 3, 4). As in crambin, there is a groove between the α and β segments. The central residue of this groove is Tyr13, and iodination of this residue abolishes toxicity [61, 63, 85]. Sequence alignment of diverse thionins indicates that the 5 amino acids (9–14) that line the cleft are the most strongly conserved fragment of the molecule besides the cysteine residues (Fig. 4). The crystal lattice contains several solute molecules. Close to Ser2 and Tyr13, a glycerol molecule was detected and secondary butanol or glycerol was detected close to residue Leu8. The general packing of purothionins is in layers with α-helices packing against each other. This is reminiscent of crambin packing, where the main structural motif of the lattice was packing of helices.

Recently, two new structures have become available. A German group headed by Dr. Sheldrick determined viscotoxin A3 (VT A3) [67]. The sequence of viscotoxins is highly conserved: the majority of the sequence is identical amongst different isoforms. The protein has the structural motif of the capital Greek Γ letter, as the other thionins, with very few modifications. At the site at which glycerol was detected in purothionin, inorganic phosphate was found in both molecules present in the asymmetric unit. In this study two independent molecules were packed with helices in direct hydrophobic contact. The crystal packing of VT A3 in layers is similar to that of α- and β-purothionin [65, 66]. Hydrophobic molecular associations are reinforced by hydrophilic contacts in the lattice. Around the crystallographic twofold axis, a strong interaction involving the binding of a sulfate anion leads to tight packing of four molecules. A sulfate ion coordinates molecules in such a fashion that they form a hydrophilic layer. It is bound to one of the most conserved residues in the phosphate binding Tyr13, which was previously identified as a key residue for toxicity. Residues of this region either have structure-stabilizing functions or are directly involved in phosphate binding.

Recently, a new structure of β-hordothionin was published [68]. In this structure there is also an independent dimer present in the asymmetric unit. This dimer is different from that of viscotoxin, but reminiscent of crambin and purothionin packing. Actually, the arrangement of the dimer is similar, in the direction of the helices, to that of purothionin, but the direction of helices is reversed, resulting in hydrophilic binding surfaces being on the opposite sites in hordothionin. The hordothionin dimer is reinforced with the bound solute *p*-toluene sulfate. The position analogous to that was occupied by sec-butanol in the α-purothionin structure. A serine molecule is bound at sites analogous to the site of glycerol binding in purothionin and phosphate binding in viscotoxin. Serine glycerol and phosphate form a hydrogen bond with the Tyr13 OH group. The fact that an important solute molecule is localized in every thionin structure, bound to Tyr13, acquires real importance in conjunction with biochemical modification studies. As can be seen from direct superposition of the structures in Figure 4, solutes have the tendency to cluster, mapping the important sites on the surface of the toxin. One of those most important is the site defined by bound serine (Fig. 4d).

Biological effects

Cellular effect

In 1972 Fernandez de Caleya et al. [37] suggested that thionins might play a role in the defense mechanism of plants against external pathogens. He studied susceptibility of Gram-positive or Gram-negative bacteria such as *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Erwinia*, and *Corynebacterium* to wheat endosperm thionins. Thionins (exception for non-toxic members such as crambin) are toxic to different biological systems such as bacteria [37], fungi, [39], cultured mammalian cells [38], whole animals [87], and insect larvae [49]). Mammalian cell lines show substantial differences in sensitiv-

ity to different thionins. These observations support the suggestion that thionins are defense proteins [37]. Additional evidence comes from observations that expression of several thionins is inducible by phytopathogenic fungi [39, 40].

Primary effect

Antimicrobial activity has been observed for several thionins [37–39, 88], demonstrated by an increase in cell membrane permeability. It has been suggested that most of the observed biological activities result from the interaction of thionins with the target cell membrane [38]. Details of interactions with phospholipid bilayers and natural membranes *in vitro* and *in vivo* for several thionins have been presented [34, 89–95]. The effect of application of thionins on the membrane has been investigated using many techniques and clearly demonstrated by electron microscopy [96] (Fig. 5). The effect is dose dependent, and an average time for the destruction of the membrane is around 1 h. The critical dose directly associated with toxicity and membrane lysis is ~1 µM (~5 µg/ml) [34, 89].

Numerous studies suggest that the primary interaction of thionins is an electrostatic one with negatively charged membrane phospholipids [93, 95]. This primary binding event is followed by either pore formation [95], or a specific interaction with a given domain in the membrane and formation of patches or rafts (carpet model) [34, 88]. The first step, the purely electrostatic interaction of thionins with membranes, can be inhibited by divalent cations such as calcium and phosphates [88, 97]. Cytotoxicity

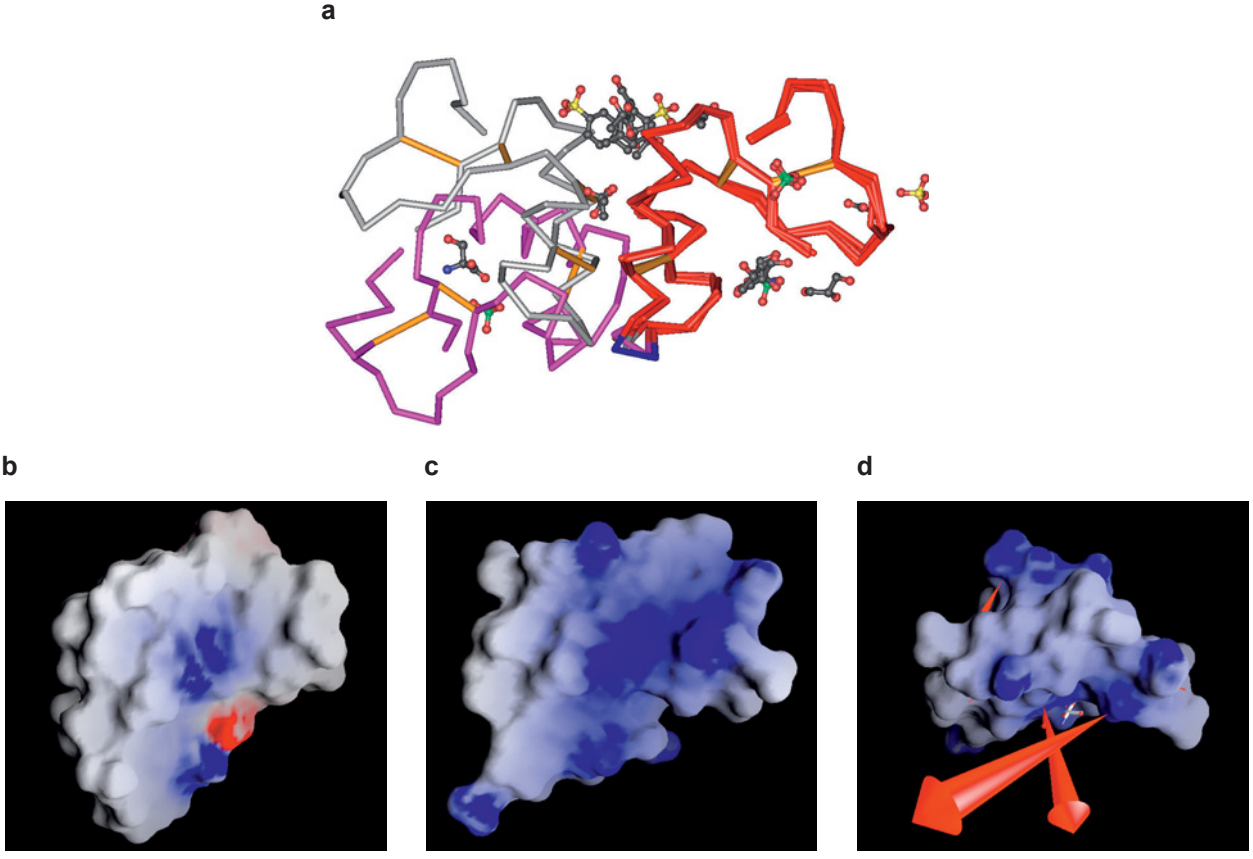


Figure 4. (*a*) Carbon alpha model of all known *α/β*-thionin structures obtained by X-ray crystallography (crambin, 1AB1 [83]; purothionin, 1BHP [66]; viscotoxin, 1OKH [67]; hordothionin, 1WUW [68]). The common aligned motif is represented in red, while non-conserved loops are in different colors. Crystals of viscotoxin and hodothionin contain a dimer in an asymmetric unit. The second subunit is represented in magenta and silver, respectively. Note clustering of auxiliary solutes refined in the crystal structures. In all three models (excluding nontoxic crambin) the postulated phospholipid binding site was occupied by glycerol, phosphate group and serine (purothionin, viscotoxin, hordothionin). Besides those solutes, others such as secondary butanol, glycerol and toluene sulfonate are present in the lattice. Those solutes are in close proximity to the residues implicated in the mechanism of toxicity (1, 8, 10, 13, 17). (*b*) The surface representation of crambin (no effective charge) with electrostatic potential mapped. Blue represents the positive potential mapped on the 5 kT/e level and red the negative potential mapped at –5 kT/e. (*c*) The surface representation of hordothionin (effective charge +10) with contouring level 10 kT/e in blue. (*d*) The view of the 90° rotated towards the viewer hordothion with the vectors representing the strength of the electromagnetic field radiating from the binding site at which the serine is docked, as observed in the crystal structure. The vectors point away from the positive charge radiating from the flat surface of the molecule, which suggests that it is a membrane binding surface. The electrostatic surfaces of other thionins look very similar to that shown above.

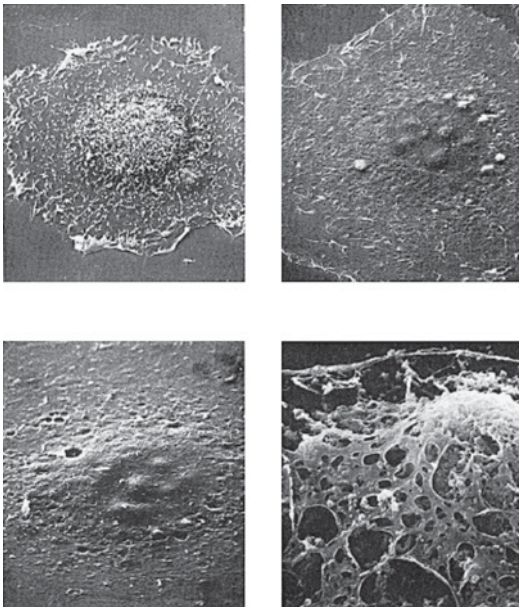


Figure 5. An electron microscopy image of a mouse A-31 cell suspended in Eagle's media supplemented with 5% fetal calf serum upon application of 10 µg/ml purothionin. Upper left: untreated cells (×1400); upper right: 0.5 h after application (×1800), lower left: 1 h (×2000), and lower right: 2 h after application (×4800). The figure shows the changes in the membrane upon application of purothionin, the disappearance of microvillia, then appearance of blebs and eventually total membrane lysis. The figure is reproduced from Oka et al. [96] with permission by the Oxford Press.

correlates well with the applied dose of thionins. In fact, Hughes et al. [95] proposed that the primary mode of action for thionin toxicity is their ability to form ion channels in cell membranes; they proposed that the passage of ions through the channel probably involves Tyr13. Upon thionin treatment of mammalian cells, the specific interaction of thionins with phospholipids, which mediates transduction of cellular signals, can explain the release of specific compounds along with the activation of calcium channels and specific enzymes. Those secondary effects are discussed below.

Secondary effects

Several other effects accompany cell lysis triggered by thionins. Upon application of toxin, there is a depolarization of the membrane and Ca^{2+} ion permeability increases [85]. Endogenous phospholipase A2 (PLA2) [85, 98, 99] and adenylate cyclase are activated [92]. In direct enzymatic assays, it was shown that β-purothionin can inhibit protein kinase C (A. Newton, personal communication) and bind to calmodulin. High positive charge was suggested to be linked to the direct interaction of viscotoxins with DNA and RNA [100, 101]. Thionins can also inhibit protein synthesis in a cell-free system [17], probably through direct interaction with mRNA or at the initial translation level [100]. The activity of the enzyme ribonucleotide reductase [53] is inhibited in the presence of purothionins, interfering with DNA synthesis [66], and irreversible inhibition of β-glucuronidase was also observed [102].

It was also shown that viscotoxins exert strong immunomodulatory effects [103, 104]. There are indications that thionins may function as regulatory proteins, like thioredoxin [54]. Thus, thionins could act as thiol secondary messengers in the redox regulation of enzymes [53, 54]. All these events that follow disruption of the cell membrane magnify the initial toxic effects, and lead to disruption of many crucial cell processes and may lead ultimately to cell death [105, 106]. However, these diverse 'targets' [102], make analysis of the primary mechanism of toxicity more complicated. Here in this review, the focus is on the primary effect that thionins exert on the membrane of the invading cell, leading to the secondary effects described above.

Proposed mechanisms of toxicity

Several experiments carried out on artificial membranes as well as on different cell lines clearly suggest that the toxins target membranes directly, and do not utilize a specific protein receptor on the cell surface. Experiments with *Pyrularia pubera* thionin suggest that negatively charged lipids are the prime target of the toxin [93]. However, other experiments suggest that in the absence of negatively charged phospholipids, any membrane with neutral or modestly cationic phospholipids is also lysed [36, 90]. Several hypotheses were put forward to explain the lytic activity of those toxins. A schematic of the different lytic mechanisms of thionins is presented in Figure 6. Before presenting a detailed review of those proposals, the general principles utilized by any of the membrane-active peptides are introduced.

General principles of membrane activity

Membrane-active antimicrobial peptides share a common set of properties. These properties, despite a small molecular mass, allow them to function in a very demanding environment at the cell membrane quite efficiently. A recent review defined the properties that endow antimicrobial peptides with affinity for one or more microbial features common to a broad spectrum of pathogens [107]. Some of these determinants, which include biophysical and structural elements, are: stable molecular conformation (c), charge (Q), amphipathicity (A), hydrophobic moment (M_H), general hydrophobicity (H), and polar angle (q). Those features are considered crucial to antimicrobial activity (potency) and selectivity. Those properties are in-

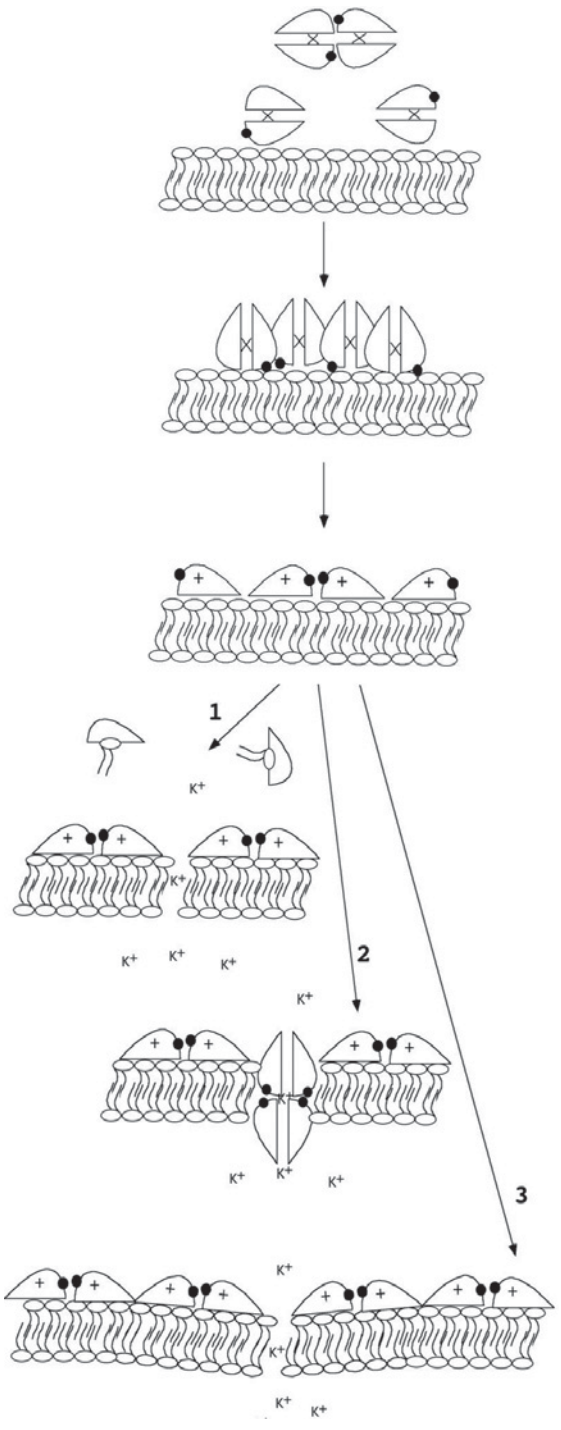


Figure 6. A schematic representation of the action of thionins on the cell membrane. The three panels at the top represent common stages for all proposed mechanisms. The oligomeric species of thionins present in solution dissociate and adsorb on the surface forming protein patches. The lower panels represent three possible scenarios for membrane lysis proposed in the literature. Three different mechanisms are: (1) the dissolution of the membrane by individual toxin molecules, leakiness and destabilization of the membrane at the edges of the patches; (2) the ion channel formation; and (3) the 'carpet model' where the instability introduced by thionin patches causes leakiness at the edges and eventual the destruction of the membrane.

terdependent; therefore, modification of one parameter often leads to compensatory changes in others.

Despite conformational differences, α-helix- and β-sheet-dominated structures are present in this highly diverse group. These peptides share common features, including amphipathic composition, with distinct hydrophilic and hydrophobic surfaces. A dominating motif is the presence in a relatively stable context of basic groups responsible for the initial contact with the membrane. Charge is the second most important feature. The presence of a large number of Lys and Arg residues results in highly positively charged molecules with $2 < Q < 10$. The increased positive charge is in full agreement with the transmembrane potential $\Delta\Xi$ that in prokaryotes is on average 50% higher than in mammalian cells. There is a high correlation between peptide basicity and antimicrobial activity [108].

The presence of numerous positively charged residues leads also to high amphipathicity and to a high hydrophobic moment. Those features allow the peptides to have an electrostatically driven approach to the membrane and uniquely defined docking partners. Prokaryotic membranes are relatively rich in acidic phospholipids. Once the hydrophobic moment allows for directional interaction with the membrane, the general hydrophobicity is important for partitioning peptides into the membrane. Finally, the polar angle (the angle between the membrane and the vector pointing from the hydrophobic to the hydrophilic face of the molecule) plays an important role in the efficiency of antimicrobial activity. Both hydrophobicity and polar angle must be optimized for the particular target because an increase beyond a certain value of both leads to a decrease in activity [109].

Analysis of all those determinants clearly suggests general principles of interactions of antimicrobial peptides. The initial approach must be electrostatically driven, taking advantage of high charge and a dipole moment of the molecules. Subsequently, the appropriate interaction with individual phospholipids or patches of phospholipids must take place. In this interaction some conformational or oligomeric rearrangement is expected. Additionally, depending on the detailed mechanism of lysis, the surface density must reach a critical value. This critical threshold concentration leads either to pore formation or to phase transition of the membrane matrix itself. However, it must be noted that permeabilization or depolarization of the membrane does not necessarily lead directly to cell death [110]. Secondary events or triggered apoptosis must occur to directly trigger cell death.

Experimental evidence and three mechanisms

Much indirect experimental evidence has accumulated indicating membrane disintegration upon thionin appli-

cation at the membrane [95–97]. Nevertheless, only few direct studies in well-controlled conditions have been carried out, and the precise mechanism of permeabilization remains unknown. Nonetheless, *in vivo* and *in vitro* studies suggest three different mechanisms (not mutually exclusive) to explain the primary lytic effect of toxin action on the membrane. An undisputed common result is that in approximately 1 h after toxin application the membrane is damaged beyond repair and the cell collapses (Fig. 5) [96]. This result has been confirmed *in vitro* using artificial membranes [95].

The first mechanism proposes that toxins arrange themselves at the surface of a bilayer and form an ion-selective channel [91, 95]. This proposal is advocated by at least two different groups and is supported by detection of ionic currents in patch clamp experiments. The interpretation of this evidence, however, does not directly support the view that the formation of ion channels is the only or direct reason for cell lysis. The second proposal contends that toxins modify the surface of the bilayer by forming patches or carpets of toxins that leads to membrane rigidity and simultaneous increase of fluidity at the edges of those patches [36, 89]. Massive binding would then cause membrane instability and, ultimately, collapse. This hypothesis is supported by circumstantial evidence and is less detailed. The third mechanism states that upon membrane binding of toxins, the membrane instability caused by the patch formation is attenuated by phospholipid withdrawal from the edges of the patches. Stabilization (rigidification) of the membrane in patches and liquefaction of membrane at the edges promotes loss of phospholipids [62]. A combined effect of the rigidification of the membrane and withdrawal of the phospholipids leads to final destabilization of the membrane. Experimental details and arguments for and against individual hypotheses are reviewed below.

Ion channel formation. There is an emerging consensus that the major action of thionin is directly at the cell membrane. Several groups have concluded that toxins form cation-selective ion channels with some rectifying properties [91, 95]. The ion channel formation upon application of α/β-thionin to natural membranes was inferred from experiments with artificial membranes. Ion channel properties are observed in the low nanomolar to the submicromolar range. Usually, in the range of ~1 μM, the artificial membranes start to disintegrate, corresponding well to observed toxicity on cells [89] These findings correlate well with multiple studies suggesting that the damage to the membrane is dose and time dependent.

Experiments with artificial membranes show that membrane destruction occurs within 1 h of treatment, which corresponds well with *in vivo* studies (Fig. 5) [89, 96]. The solid evidence provided by these experiments suggests ion-selective channel formation. However, some questions remain. The foremost question is that the artificial membrane is destroyed (*i.e.* ion channel activity disappears) in the same time frame as reported for *in vivo* toxicity. Therefore, the proposition that, even with artificial membranes, membrane integrity is invariably lost at similar concentration and time thresholds argues against stable ion channel formation. The carpet or membrane solubilization models can easily incorporate a transient leakage of ions at the initial stage of toxins attack on the membrane.

Additionally, the structural constraints provided by the size of a thionin molecule excludes the construction of a simple but detailed model of a selective-ion channel. The membrane thickness, polarity in combination with observed crystal packing is more suggestive of a planar association of the toxins with the membrane. For instance, Thevissen et al. [89] excluded selective channel formation as a model, arguing that compounds with sizes exceeding those allowed through the selective ion channel, such as antibiotics and fluorescent dyes, passed through the membrane. There is no significant hydrophobic surface on any of the thionins to explain the partitioning of the toxins to the lipidic phase. Thionins such as purothionins are highly soluble up to 300 mg/ml, which in combination with mostly charged surface makes such a model very difficult to accept.

Carpet model. According to a second model, the carpet model for membrane permeabilization, a high density of peptide accumulates on the target membrane surface and causes phospholipid strain and changes in membrane fluidity [111]. The change in physical properties of the membrane, specifically a reduction in membrane fluidity leading to changes in barrier properties, eventually leads to membrane disruption [89]. Thus, when a threshold peptide density or concentration in solution is reached, the membrane is subjected to unfavorable energetics, and its integrity is lost. From this perspective, membrane disruption occurs in a dispersion-like manner that does not lead to regulated ion channel formation.

One of the peptides known to function by a carpet mechanism is Cecropin, a peptide derived from moth haemolymph [112]. Magainin also appears to perturb microorganisms through this type of mechanism [113]. However, magainin likely undergoes an intermediate step that includes formation of transient pores or channel aggregates [113]. Thus, the channel-pore and carpet mechanisms may be two facets of the permeabilization process active at different toxin concentrations and different times.

Several studies carried out with thionins provide arguments supporting this type of model. The crystal packing of all four thionins clearly suggests potential arrangements that would promote patch formation. Particularly illustrative in this regard is the report by Debreczeni et al. [67] in which they show a hydrophilic motif of inor-

ganic phosphate ions that organizes viscotoxin molecules around a crystallographic axis. This sheet-like arrangement can serve as a model of a patch formation. Additionally, the observation by Vernon and Rogers [114] that toxins promote growth of the patch supports this mechanism.

Dissolution of membrane. Recently we proposed a modification of the carpet model of toxicity [62]. We observed the existence of a common binding site present in all known structures of thionins solved by X-ray crystallography. We proposed that this site is a phospholipid binding site. By small angle X-ray scattering and fluorescence anisotropy measurement, we confirmed an old observation that thionins in solution have dimeric or tetrameric organization [6, 75]. Subsequently, using solubilization studies, we confirmed that thionins can form proteolipid complexes, and that the toxins are capable of solubilizing individual phospholipids.

Finally, we proposed a molecular mechanism for the toxic effects of thionins at the phospholipid membrane that implicates not only residues Tyr13, Arg10 and Trp8 but also the highly conserved Lys1. In the native, inactive state, toxins are organized in oligomers (dimers or tetramers) bridged by a bound inorganic phosphate ion that renders them inactive. The positively charged protein aggregates, driven by electrostatic interactions, approach a negatively charged membrane. In the first phase, the oligomer dissociates and the helical hydrophobic face initially associates with the membrane. This initial mode of association is suggested by NMR experiments [63] carried out on *Pyrularia* toxin. Those experiments showed that when *Pyrularia* thionin interacts with a cell membrane, Trp8 located at the hydrophobic face inserts into the membrane. This process was also investigated by spectroscopic studies suggesting a 30° angle between the normal to the membrane and the helices and a 60° angle with the β-sheet [36]. While those angles can be easily explained by known structural features of the toxins, the model presented in the study exposed Tyr13 to the solution, which contradicts the subsequent study by the same group [90]. The latest study confirms that Tyr13 is directed towards the membrane, which agrees with our proposed model [62].

In the second stage, binding of highly positively charged toxins to the membrane promotes phospholipid segregation and formation of negatively charged patches of phospholipids [63]. The patches reorganize the membrane, and drive the physical behavior so that its melting temperature changes. The membrane undergoes a series of gel-liquid transitions, as suggested by Giudici et al. [111]. There are two potential sources of this membrane behavior: rigidification of significant part of the membrane and liquefaction of the intermediate boundaries. Such behavior agrees with the conclusion that organization of negatively charged patches lowers the free energy for withdrawal of individual phospholipids from the edges of rigid fragments of the membrane. Chemical modifications of Trp8 led to much less extensive organization of negative patches and to lowered toxicity [63].

At the membrane, dissociated monomers could reorient to expose Tyr13 to the phosphate head groups of phospholipids. The acyl chains would be tightly bound in the toxin groove (Fig. 4). The process of toxin binding to phospholipids increases the rigidity of the membrane locally at the area of binding and, at the same time, liquefies the membrane in areas between the patches. That effect is sufficient to explain variable leakage and final collapse of the membrane in the hypothesis proposed by Coulon et al. [94]. The withdrawal of phospholipids by the toxins would create a significant change in the natural composition of the membrane, increasing its fluidity and affecting its curvature. All these processes would result in formation of irregular membrane pores causing significant leakage and would finally result in membrane lysis. The membrane destruction process would be accelerated by the initial depolarization of the membrane, influx of exogenous Ca^{2+}, and finally activation of PLA2.

Summary

Here the recent structural and functional studies carried out on plant toxins – the thionins – are reviewed. Competing hypotheses relating to the primary effect of those toxins at the cell membrane are also presented. A direct comparison of the presented results allows the formulation of a unified hypothesis of their primary lytic activity. The literature provides unequivocal evidence of the time- and dose-dependent physical disruption of the membrane [96].

In light of this evidence, ion channel formation must be considered a transient phenomenon. Nevertheless, it is an interesting phenomenon that needs to be studied more carefully in the context of the temporal development of defects. The same evidence also does not fully support of the raft model, in which binding of toxins to the surface of the bilayer is sufficient for pore formation and physical destruction of the membrane. The most likely molecular mechanism of action of thionins on the phospholipid membrane is a combination of all three mechanisms presented in this review. Initially, highly positively-charged toxins remain aggregated in solution. That stage is fully relevant to the model of activation of the toxins.

Invading organisms usually secrete extracellular factors that promote invasion, such as proteases, cellulases or glycosidases. An aqueous excretion allows for solubilization and activation of toxins, even in a relatively dry environment like that found in seeds. Once activated, toxins

bind to the membrane where the oligomers dissociate and monomers cover the specific target patches.

Creation of patches is responsible for the dose-dependent action of toxins and their relative selectivity. The differential composition of the membrane as well as selective binding for individual phospholipids offers an attractive explanation for selectivity of closely related toxins. Binding of toxins to individual phospholipid head groups in a reciprocal manner promotes formation of negatively charged patches of phospholipid molecules and promotes their segregation. Creation of the patches of bound toxins accentuates existing rafts, and is responsible for physical changes in the membrane as reported here.

Protein patches have two effects on the membrane. First, they make the parts of the membrane covered with the toxins more rigid and, secondly, they simultaneously make the membranes at the edges of rafts more fluid. Both phenomena facilitate phospholipid withdrawal from the membrane by lowering the energy penalty for phospholipid-membrane separation. Both phenomena are responsible for significant membrane instability and lead to irreparable lysis. The organization of larger rafts and withdrawal of a sufficient number of phospholipid molecules must be a relatively slow process limited by local fluctuations. During this initial process transient ion leakage must occur, which has been recognized as temporary ion channel formation.

The initial lysis event is followed by massive depolarization and release of components of the cytoplasm. This stage is characterized by extensive multidirectional damage done by toxins to cytoplasmic and nuclear components of the cell (DNA and RNA) and other agents (like phospholipases and proteases), leading to necrosis or apoptosis. Therefore, this unified mechanism would promote a rapid and complete destruction of the invading pathogen. However, the selective nature of toxin binding at the membrane is responsible for the limited range of targets for this defense mechanism.

Some organisms (*e.g. C. abyssinica* or *O. sativa*) produce a wide range of toxins exhibiting variable toxicity. More detailed studies are needed to explain this variable potency and elucidate the details of the mechanism presented here. More insight is needed to highlight the determinants of specificity and design more effective artificial toxins that can be used in crop protection.

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