Thionins

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1. CHARACTERISTICS

1.1 A single peptide family

The general designation of thionins has been proposed for a family of homologous peptides that includes purothionins, which were first isolated from wheat seeds (Balls *et al.*, 1942) and their homologues from various taxa that have been named viscotoxins and crambins (see García-Olmedo *et al.*, 1989). The crystalline protein material obtained from lipid extracts of wheat endosperm was designated "purothionins" because of its high sulphur content (Balls *et al.*, 1942). This material was found to have bactericidal and fungicidal properties (Stuart and Harris, 1942), to inhibit fermentation of wheat mashes (Balls and Harris, 1944), and to be toxic to laboratory animals (Coulson *et al.*, 1942). The toxic properties of mistletoes were ascribed to the viscumin lectin and to a mixture of small basic proteins, designated "viscotoxins" (Samuelsson, 1974). In a study of the seeds of the Abyssinian cabbage (*Crambe abyssinica*), a high-sulphur crystalline protein was obtained from the aqueous acetone extracts and designated "crambin" (Van Etten *et al.*, 1965).

The thionins have become the subject of intensive structural studies, both in crystalline form and in solution, as they are excellent model systems in the development and refinement of novel methods for the elucidation of the three-dimensional structure of macromolecules. Recombinant DNA and other techniques have been applied to the study of relevant aspects of their molecular biology, such as synthesis and deposition, genetic control and gene structure, *in vitro* activities and possible physiological function.

1.2 Distribution

The original purothionin from hexaploid wheat was later found to be heterogenous, as it was fractionated into two closely-migrating bands by electrophoresis (Nimmo *et al.*, 1968; Fisher *et al.*, 1968; García-Olmedo *et al.*, 1968). In a survey of 22 diploid, tetraploid and hexaploid species of the *Aegilops-Triticum* group, the presence of thionins was demonstrated in the endosperms of all of them, and their electrophoretic mobilities in diploid and tetraploid species suggested that at least one variant per diploid genome complement should be present in hexaploid wheat (Carbonero and García-Olmedo, 1969). Genetic analysis and the determination of amino acid sequences confirmed the existence of three different thionins in the endosperm of hexaploid wheat (reviewed in García-Olmedo *et al.*, 1989). Two thionin components, designated α and β-hordothionin, were found in the endosperm of barley (*Hordeum vulgare*) by Redman and Fisher (1969). The nucleotide sequences of the corresponding cDNA and genomic DNA clones are in agreement with the presence of more than one thionin in this barley tissue (Hernandez-Lucas *et al.*, 1986; Ponz *et al.*, 1986; Rodriguez-Palenzuela *et al.*, 1988). Two thionin variants from oats endosperm (*Avena sativa*) have been also sequenced by Bekes and Lasztity (1981).

The viscotoxin from leaves and stems of European mistletoe (*Viscum album*, Loranthaceae) was found to be a mixture of closely related components. Similar toxins, such as phoratoxins A and B from *Phoradendron tomentosum*, denclatoxin B from *Dendrophtora clavata* and ligatoxin A from *Phoradendron liga*, were also characterized within the Loranthaceae (reviewed in García-Olmedo *et al.*, 1989). Additional leaf thionins have been identified in *Pyrularia pubera*, a parasitic plant from the Santalaceae (Vernon *et al.*, 1985), and from barley (Böhlmann and Apel, 1987; Gausing, 1987).

The crambin reported by Van Etten *et al.* (1965) was also found to be a mixture of two variants, whose primary structures were homologous to the thionins and the viscotoxins (Teeter *et al.*, 1981; Vermeulen *et al.*, 1987).

1.3 Thionin types

The mature thionin peptides are generally 45 to 47 amino acids in length. An unrooted phylogenetic tree allowed the classification of the available amino acid sequences from the thionins (either directly determined or deduced from cDNAs) into at least five types (I-V), one of which (type V) lacks the C-terminal nonapeptide (García-Olmedo *et al.*, 1989; Castagnaro *et al.*, 1992).

The original purothionin type isolated from wheat endosperm (Balls *et al.*, 1942) has four disulphide bridges and is highly basic, with no negatively

charged residues. The sequences that are known of this type comprise 45 amino-acid residues, 8 of which are in the central disulphide loop. The second type has been isolated from the leaves of *Pyrularia pubera* (Vernon *et al.*, 1985) and barley (Gausing, 1987; Bohlmann and Apel, 1987). This type has four disulphide bridges at the same positions as those of the type I thionin, but the molecules are less basic, with some negatively-charged residues, and their central disulphide loop contains one or two more amino acid residues. The third type includes the viscotoxins and phoratoxins from mistletoes (Loranthaceae), and has the following distinctive features: three disulphide bridges that are conserved with respect to the previous types; fewer basic amino acid residues; a sequence with 46 residues, 9 of which are in the central disulphide loop. The crambins isolated from the Abyssinian cabbage (Cruciferae) represent the fourth type, which has the same sequence length and disulphide-bridge arrangement as type II thionins, but the molecules are neutral, with a low proportion of charged amino acid residues.

The fifth type is quite divergent: the 2nd and 8th cysteines of type I thionins are missing through point mutation and deletion, respectively, thus disrupting the first and second disulphide bridges and potentially allowing the formation of a new bridge between the unpaired cysteines. This new type, which is also neutral, has been identified in a cDNA library derived from developing wheat kernels and is also present in related *Aegilops* species (Castagnaro *et al.* 1992, 1995).

It can be noted that type I and II thionins are closer to each other than to the other types, not only in their gross architecture but also at the level of their amino acid sequences, and that the same is true for types III and IV. Although the different types have been mostly investigated in particular tissues and organs of certain species or groups of related species, the observed divergence between the types cannot be correlated with the evolutionary relationships among the taxa because the types defined here do not represent equivalent subsets of the protein family. Thus, type I, II and III thionins may coexist in a single species, and the thionin isolated from *Pyrularia pubera*, a parasitic plant closely related to the mistletoes, is closer to the barley-leaf thionins than to the viscotoxins and phoratoxins. In *Viscum album* there are thionin genes that are expressed in seeds and in leaves, and in *Crambe abyssinica*, thionin variants distinct from the seed-specific crambin are found that are ubiquitously expressed (Schrader-Fischer and Apel, 1993, 1994). In *Arabidopsis thaliana* there is a thionin gene that is expressed in flowers, siliques and rosette leaves, and a second one that is only expressed at basal levels in seedlings and rosette leaves (Epple *et al.* 1995).

If only the disulphide-bound structure is considered, all known thionins could be classified into three groups: a group with 4 disulphide bonds, which would include types I and II, a group with only 3 of the above disulphide bonds (types III and IV) and a group which presumably has only 2 of the

above bonds, plus a novel bond that would involve one cysteine from each of the two above bonds (Figs. 1,2).

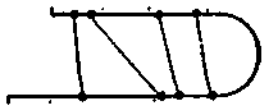
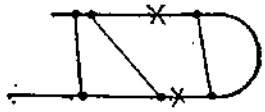
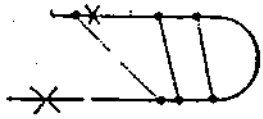
DISULPHIDE STRUCTURE	CHARGES +/-	RESIDUES Total/Loop	TYPE
1 	10/0	45/8	I
	9/3	47/10	II
	7/1	46/9	
2 	6/0-2	46/9	III
	5/1-2		
	2/2		IV
3 	2/2	38/9	V

Fig. 1. Structural features of thionins.

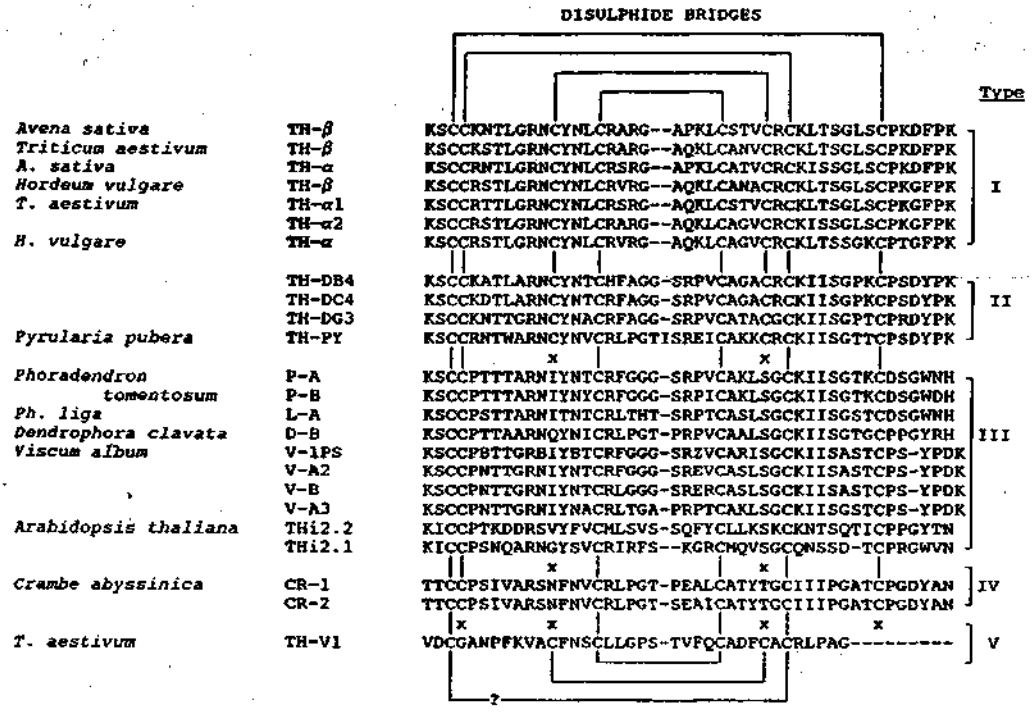


Fig. 2. Amino acid sequence alignment of representative examples of thionin types.

1.4 Solubility

Because of their solubility in petroleum-ether, thionins from wheat endosperm were first thought to be lipoproteins in which the protein moiety was associated with a lecithin-like lipoid (Balls *et al.*, 1942). The protein could be precipitated from the lipid extract as a hydrochloride that was no longer soluble in organic solvents and was soluble in water and aqueous alcohols. Lipid-thionin complexes were converted to a petroleum-ether-insoluble, chloroform-soluble form by precipitation with acetone. Digalactosyl diglyceride (DGDG), a component of the acetone extract, was able to restore petroleum-ether solubility when added back to the chloroform-soluble form (Hernandez-Lucas *et al.*, 1977). The chloroform-soluble thionin preparation obtained by acetone precipitation contained phosphatidylethanolamine, phosphatidylcholine, and other polar lipids. The apoprotein was not soluble in chloroform by itself, so it was assumed that at least some of these lipids were required for chloroform solubility (Hernandez-Lucas *et al.*, 1977). When flour from the tetraploid wheat *T. turgidum* was extracted with petroleum ether supplemented with DGDG, the yield of thionin was increased significantly, suggesting that the differences in yield between tetraploid and hexaploid wheats reported previously were probably due to interspecific differences in the levels of DGDG (García-Olmedo *et al.* 1968; Hernandez-Lucas *et al.*, 1977). Petroleum ether treatment of rye flour had repeatedly failed to extract any thionin, but when the solvent was complemented with acetone-extracted wheat lipids, a good yield of rye thionin was obtained (Hernandez-Lucas *et al.*, 1978). The idea that DGDG was limiting the yield of thionin extracted with petroleum ether was also consistent with the fact that extraction with aqueous solvents, such as 0.05N H_2SO_4 or 1M NaCl, was more efficient than with the organic solvents (Fernandez de Caleya *et al.*, 1976).

1.5 Biosynthesis and subcellular location.

Barley endosperm thionins are synthesized by membrane-bound polysomes as much larger precursors that undergo at least two pocessing steps (Ponz *et al.*, 1983). Using monospecific antibodies raised against the mature protein, two types of precursors were identified: one was detected as an *in vitro* translation product that could not be detected *in vivo*, and the other was detected by *in vivo* labelling (Fig. 3). Pulse-labelling experiments showed conversion of the second precursor into the mature protein (Ponz *et al.* 1983). As predicted from the study of their biosynthesis, the nucleotide sequences of the cDNAs corresponding to α and β thionins from barley endosperm were found to encode precursors that were much larger than the mature protein

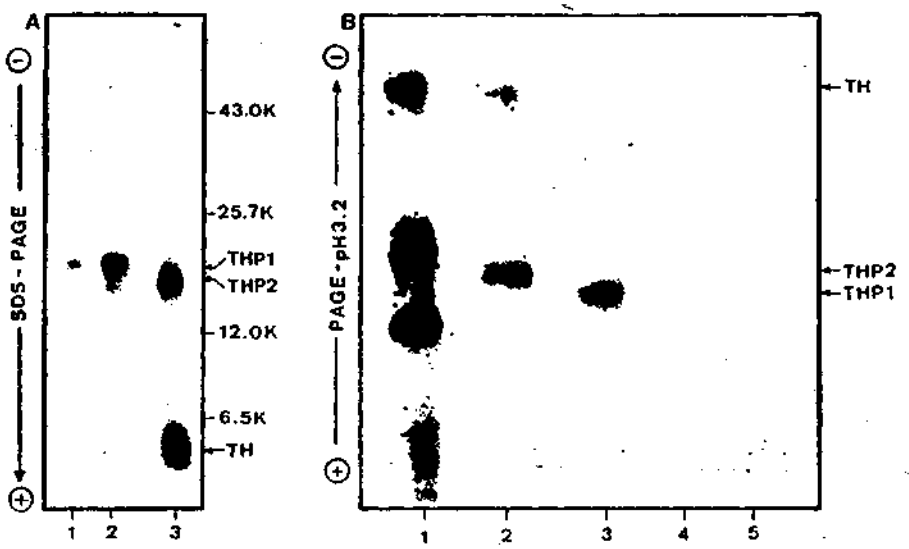


Fig. 3. Thionin biosynthesis. Comparison of *in vivo* and *in vitro* products selected with monospecific antibodies. **A**, Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) of alkylated products: 1, *in vitro* precursor THP1 labelled with [^{35}S]methionine; 2, as in 1 but labelled with [^{35}S]cysteine; 3, *in vivo* precursor (THP2) and mature thionin (TH). **B**, Polyacrylamide gel electrophoresis (PAGE) at pH 3.2 of reduced, non-alkylated products: 1, total *in vivo* extract; 2, *in vivo* products, THP2 and TH; 3, *in vitro* precursor THP1; 4,5 displacement of precursors by non-radioactive thionin (5 µg). (From Ponz *et al.*, 1983, with permission)

(Ponz *et al.*, 1986; Hernandez-Lucas *et al.*, 1986). The deduced structures of these precursors consisted of an *N*-terminal signal peptide, followed by the mature protein and a *C*-terminal acidic protein, as shown in Fig. 4. The same precursor structure was later found for thionins of the other types (Gausing, 1987; Bohlmann and Apel, 1987; Castagnaro *et al.*, 1992; Schrader-Fischer and Apel, 1993, 1994), which strongly suggests that all types of thionins have the same biosynthetic pathway as that of type I thionins.

Cellular fractionation studies of developing barley endosperm by Ponz *et al.* (1983) indicated that type I thionins were intracellular and in a labile association with the particulate fraction. Subsequent studies, using immunogold detection by electron microscopy, have shown that type I thionins are in the periphery of the protein bodies in electron-dense ovoidal structures (Carmona *et al.*, 1993a).

A recent report (Romero *et al.*, 1997) has shown that in barley leaves, mature thionins accumulated in the vacuoles, while the acidic peptide was not detected in any cell fraction. Both purified vacuoles and an acid (pH 5.5) extract from leaves liberated the acidic peptide. An *Mr* 70,000 proteinase that effected this cleavage was purified from the acid extract. Processing by both

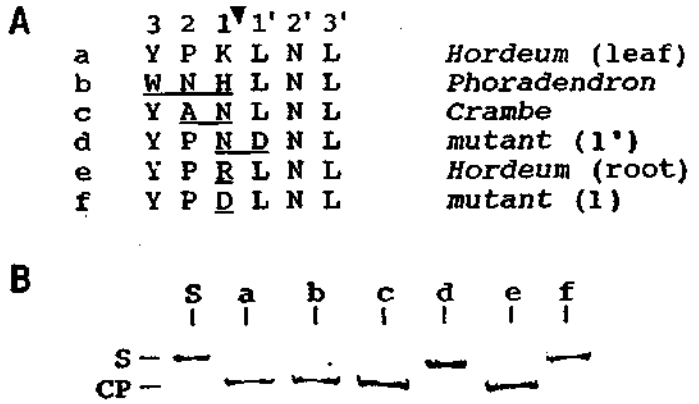


Fig. 4. Structure of the thionin precursor (Ponz *et al.*, 1986) and of the α-thionin gene from barley endosperm. (From Rodriguez-Palenzuela *et al.*, 1988, with permission).

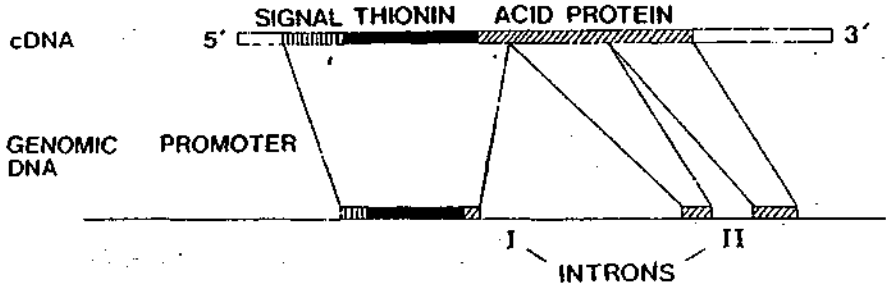


Fig. 5. Processing of cleavage-site sequences from thionin precursors. **A**, amino acid sequences near the cleavage site (arrowhead) of the indicated thionin precursors and mutants. A complete precursor coding sequence was expressed in *Escherichia coli* as a fusion with the MalE protein. **B**, SDS-PAGE of the proteins listed in **A** with the purified processing proteinase from barley. (From Romero *et al.*, 1997; with permission)

lysed vacuoles and by the purified proteinase was inhibited by Zn^{2+} and by Cu^{2+}, but not by inhibitors of previously described vacuolar processing thiol or aspartic proteinases. *In vivo* processing of the thionin precursor in leaf sections was also inhibited by Zn^{2+} and Cu^{2+}. Variants of a fusion protein with altered processing sites that represented those of thionin precursors from different taxa were readily processed by the proteinase, whereas changing the polarity of either the *C*-terminal or *N*-terminal residues of the processing site prevented cleavage by the enzyme (Fig. 5).

2. GENETICS

2.1 Chromosomal locations

Using wheat aneuploids (nulli-tetrasomic and ditelosomic lines), as well as wheat-rye and wheat-barley chromosomal addition lines, genes encoding thionins have been associated with specific chromosomes of these species (García-Olmedo *et al.*, 1982). Three genes (*Pur-A1*, *Pur-B1* and *Pur-D1*), which corresponded to the β, α1, and α2 thionin variants from wheat endosperm (type I), respectively, were identified in the long arms of chromosomes 1A, 1B and 1D, through the electrophoretic analysis of the appropriate aneuploids and the characterization of the isolated proteins (Fernandez de Caleya *et al.*, 1976). In a similar manner, a gene for an endosperm thionin was located in the long arm of chromosome 1R of rye (Sanchez-Monge *et al.*, 1979). Southern-blot analyses of genomic DNAs of wheat and barley, using cDNA probes for type I thionins, were consistent with the presence of 1-2 gene copies per haploid genome (Rodriguez-Palenzuela *et al.*, 1988). Type II genes have been located in chromosome 6H of barley by Southern-blot analysis of DNAs from wheat-barley addition lines (Bohlmann *et al.*, 1988); but there are discrepancies as to the number of copies of this type present: while Bohlmann *et al.* (1988) estimated about 100 genes/haploid genome, Gausing (1987) gave a lower estimate of 9-11 genes. More recently, type V genes have been located within a few kb of the type I genes in wheat, with 1 or 2 copies per haploid genome. Although the amino acid sequence of the mature type V thionin is quite different from those of the other types, the *C*-terminal acidic peptide of the corresponding precursor is less divergent than the mature protein and closer to the type I than to the type II peptide (Castagnaro *et al.*, 1992, 1995).

The gene for α-hordothionin, a type I thionin from barley endosperm, has two introns, 420 and 91 nucleotides long, that interrupt the sequence encoding the *C*-terminal, acidic peptide of the precursor (Rodriguez-Palenzuela *et al.*, 1988)(Fig. 3). Genomic clones of type II thionins have two introns in similar positions as those of type I clones (Bohlmann *et al.*, 1988).

2.2 Gene expression

In barley endosperm, a maximum steady state concentration of thionin mRNA occurred between 13 and 16 days after anthesis. Thus, synthesis of these proteins seemed to take place during the cell-proliferation phase of endosperm development and to cease at the beginning of the cell-enlargement phase. When a fusion of the cauliflower mosaic virus 35S RNA gene promoter with the α-hordothionin gene (comprising coding regions and

introns) was expressed in transgenic tobacco, introns from this monocot gene were properly spliced in the dicot species (Carmona *et al.*, 1993b).

The expression of type-II thionin genes has been investigated in barley leaves and a number of interesting responses of these genes to external stimuli have been described. Large amounts of messenger for type II thionins were detected in dark-grown barley seedlings (Gausing, 1987; Bohlmann and Apel, 1987). Steady state messenger levels seemed to be higher in the lower 1/3 of the leaf (younger cells) than in the upper 2/3 (older cells) and to decline sharply upon illumination (Gausing, 1987). The effect of light has been further investigated by Reimann-Philipp *et al.* (1989a), who have postulated the mediation of two photoreceptors, phytochrome and a blue-light-absorbing photoreceptor. Synthesis of thionins concomitantly ceased upon illumination, but the previously accumulated thionin was rather stable (Reimann-Philipp *et al.*, 1989a). The inhibitory effect of light can be overcome by stress- and pathogen-induced signals, as it has been shown that fungal infection induces a transient expression of the thionin genes in the leaves (Bohlmann *et al.*, 1988); Ebrahim-Nesbat *et al.*, 1989) and that the chlorides of divalent cations (Mg^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}) elicit a more permanent response (Fisher *et al.*, 1989).

3. BIOLOGICAL PROPERTIES

3.1 Redox properties

Thionin from wheat endosperm can substitute for thioredoxin *f* from spinach chloroplasts in the dithiothreitol-linked activation of chloroplast fructose-1,6-bisphosphatase (Wada and Buchanan, 1981). Under the standard assay conditions, the thionin was only 2% as active as authentic thioredoxin *f*. Nevertheless, activity could be improved by increasing the time of preincubation and the concentration of reductant, suggesting that the thionin could be effectively reduced by thioredoxin *f* (Wada and Buchanan, 1981). This led to experiments which implicate thionins in plant redox metabolism. Johnson *et al.* (1987) have reported a thioredoxin system, consisting of a homogeneous preparation of thioredoxin *h* and partially purified thioredoxin reductase (NADPH), which effectively reduced thionin with NADPH as the hydrogen donor. The reduced thionin, in turn, was capable of activating fructose-1,6-bisphosphatase, suggesting a possible role of thionins as secondary thiol messengers in the redox regulation of enzymes. In the opinion of these authors, the redox properties of thionins could also explain their toxicity.

A property of thionins that might be related to its redox properties is their ability to form selective disulphide bridges with other proteins (Diaz *et al*, 1992; Piñeiro *et al.*, 1995). The enzymes β-glucuronidase and neomycin phosphotransferase II were inhibited by thionins through the formation of disulphide-linked adducts and the inhibition was reversed by DTT.

3.2 Interactions with artificial and biological membranes

The ability of thionins to induce leakage of intracellular material was first demonstrated in bacteria and in yeast. The effect could be reversed by certain divalent cations, such as Ca^{2+}, Zn^{2+}, or Fe^{2+} (Reviewed in García-Olmedo *et al.*, 1989). The effects of thionins of types I and IV on cultured mammalian cells occurred at the minimum cytotoxic concentration that caused leakage of Rb^{1+} and of uridine. Concentrations of thionins that had no detectable effects on the cultured cells lead to inhibition of translation by antibiotics such as hygromycin B that do not normally cross the plasma membrane (Carrasco *et al.*, 1981). As in the case of yeast, Ca^{2+} and Mg^{2+} could revert the action of thionin. The effect of thionins on fungal membranes has been investigated in *Neurospora crassa*, where a similarity in the minimum concentrations required to cause leakage and growth inhibition was observed (Guihard *et al.*, 1993; Thevissen *et al.*, 1996).

The effects of thionins on smooth-muscle contraction and on insect flight muscle, as well as the sensitivity to thionins of A31 cells infected with the Moloney strain of murine leukemia virus, are all probably related to interactions of thionins with the cell membrane (reviewed by García-Olmedo *et al.*, 1989). It has been recently reported that thionins induce leakage and aggregation of artifitial, negatively-charged membranes under conditions in which other plant toxic peptides have no effect (Caaveiro *et al.*, 1997).

3.3 Antimicrobial properties and other *in vitro* activities

The toxicity of thionins to different kinds of organisms and to cells in culture has been investigated for several decades. Gram-positive bacteria and, to a lesser extent, Gram-negative bacteria, bakers yeast, and some human pathogenic fungi, were found to be sensitive to a crystalline mixture of type I α-thionins from wheat endosperm, whereas the mycelial fungi tested were found to be insensitive (Stuart and Harris, 1942). After these initial findings, the toxicity to bacteria (Fernandez de Caleya *et al.*, 1972; Cammue *et al.*, 1992; Florack *et al.*, 1993; Molina *et al.*, 1993), to yeast (Balls and Harris, 1944; Nose and Ichikawa, 1968; Okada and Yoshizumi, 1970, 1973; Hernandez-Lucas *et al.*, 1974), and to fungi (Bohlmann *et al.*, 1988; Reimann-Philipp *et al.*, 1989b; Terras *et al.*, 1992; Molina *et al.* 1993) has

been further demonstrated for different thionins. The antifungal activity of thionins is inhibited by Ca^{2+} at concentrations that other divalent and monovalents cations had no effect (Okada and Yoshizumi, 1970; Terras *et al.*, 1992; Cammue *et al.*, 1992).

Type-I thionins were also found to be toxic to mice, guinea-pigs and rabbits when injected intravenously or intra-peritoneally, but not upon oral administration (Coulson *et al.*, 1942). Type-III thionins, isolated from the leaves of the mistletoes and related species, were also found to be toxic on parenteral administration to mice and cats (see Samuelsson, 1974). At sublethal doses they produced hypotension, bradycardia and a negative ionotropic effect on the heart muscle. Intraarterial administration, in higher doses, produced vasoconstriction in arteries of skin and skeletal muscle (Samuelsson, 1974). Cytotoxic effects on cultured mammalian cells have been reported for different thionin types (Carrasco *et al.*, 1981; Vernon *et al.*, 1985).

3.4 Possible role in plant defence

The hypothesis that thionins might play a role in the protection of plants against pathogens was proposed by Fernandez de Caleya *et al.* (1972), who investigated the susceptibility to wheat endosperm thionins of phytopathogenic bacteria in the genera *Pseudomonas, Xanthomonas, Agrobacterium, Erwinia*, and *Corynebacterium*. Purified genetic variants of these thionins differed in activity and showed some degree of specificity. Bohlmann *et al.* (1988) have shown that both endosperm (type I) and leaf (type II) thionins from barley inhibit the fungi *Thielaviopsis paradoxa*, a pathogen of sugar cane, and *Drechslera teres*, a pathogen of barley, at concentrations of 5×10^{-4}M. Fungal and bacterial pathogens included in a recent survey were inhibited by thionins at concentrations in the 10^{-6}-10^{-5}M range, which are similar to those found in certain plant tissues (Molina *et al.* 1993).

Direct evidence of a defense role for the thionins is lacking at present. Although thionin mRNA is transiently induced in barley upon infection with *Erysiphe graminis* (Bohlmann *et al.*, 1988) and slight differences in the localization of thionins seems to occcur in the cell walls of susceptible and resistant barley cultivars (Ebrahim-Nesbat *et al.*, 1989), the resistance gene and the thionin genes are located in different chromosomes and the thionin mRNA is induced to similar levels in both susceptible and resistant cultivars. Furthermore, the pre-induction levels of thionins seem to be quite high, due to their low turn-over (Reimann-Philipp *et al.*, 1989a). Transgenic expression in tobacco plants of a barley thionin gene showed reduced lession size when the plants were challenged with two strains of *Pseudomonas syringae* (Carmona

et al., 1993b), whereas other strains did not seem to be affected (Florack, 1993). More recently, overexpression of an endogenous thionin has been reported to enhance resistance of *Arabidopsis thaliana* against *Fusarium oxysporum* (Epple *et al.*, 1997) and thionin-sensitive mutants of *P. solanacearum* were found to be avirulent (Titarenko *et al.*, 1997).

4. 3D STRUCTURE

The thionins are among the best characterized proteins with respect to their three-dimensional structure, both in crystals and in solution. Because of their peculiar features they have become model molecules in the development and refinement of novel methods for the elucidation of three-dimensional structures. Thus, an X-ray diffraction method based on the anomalous scattering of sulphur was specifically developed to solve the structure of crambin (Hendrickson and Teeter, 1981). This molecule was also used to test the utility of molecular dynamics with interproton distance restraints for structure determination (Brünger *et al.*, 1987; Clore *et al.* , 1986), and to show that structures obtained for a protein in solution with NMR data can be used to solve the crystal structure of the same protein by molecular replacement (Brünger *et al.*, 1987). This wealth of information indicates that type I, III, and IV thionins, in spite of their extreme divergence, have essentially the same three-dimensional shape, which resembles the Greek capital letter gamma (Γ). The molecules are amphipathic, quite rigid and present very similar three-dimensional structures in solution and in crystal form (Fig. 6). The long arm is formed by two antiparallel α-helices and the short arm by a β-sheet consisting of two short antiparallel β-strands. The hydrophobic residues are clustered at the outer surface of the long arm of theΓ, whereas hydrophilic residues mainly occur at the inner surface of it and at the outer surface of the corner of the Γ (Clore *et al.*; 1986; Hendrickson and Teeter, 1981). It has been proposed that type I thionins have a binding site for phospholipids, which may be implicated in their toxic activity (Rao *et al.*, 1995; Stec *et al.*, 1995).

5. EVOLUTION

The evolution of thionins, particularly that leading to changes in the numbers of disulphide bridges, merits special attention because it represents a general evolutionary problem. Thus, divergence between type I and V thionins has occurred through a putative process of accelerated evolution that has affected the amino acid sequence of the mature thionin but not the precursor

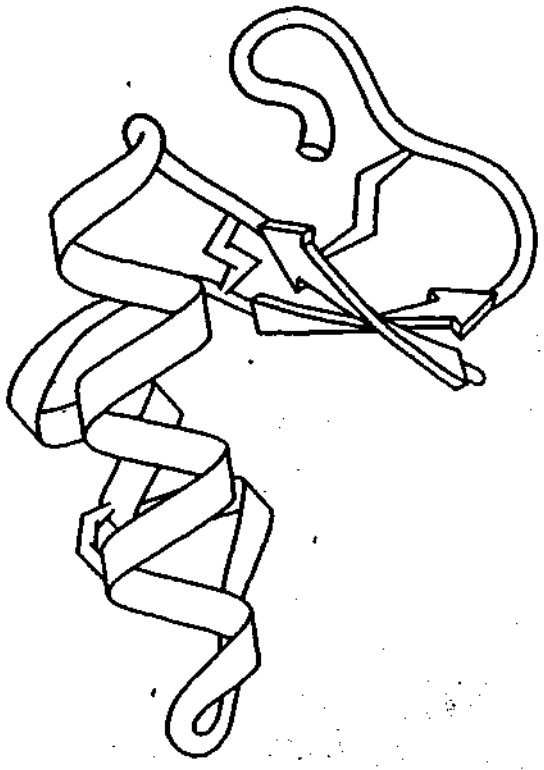


Fig. 6. Structure of crambin (Whitlow and Teeter, 1985)

domains corresponding to the *N*-terminal signal peptide and the long *C*-terminal acidic peptide (Castagnaro *et al.*, 1992, 1995). This process involved a deletion and a non-synonymous nucleotide substitution rate equal to the synonymous rate in the thionin sequence.

Percentages of sequence divergence for binary comparisons of equivalent domains within and between types I and V have been calculated (Castagnaro *et al.*, 1992, 1995). The coding sequences of two type-V thionins have been absolutely conserved during the approximately 10,000 years that the D genomes of the diploid and the alloploid *Triticum* and *Aegilops* species have been evolving separately, whereas the introns have diverged, especially the larger one, which has suffered one major and several minor deletions. Percentages of divergence between type I and V thionins in the mature-protein domain (59-69%) are about twice as great than those occurring in the other domains, namely signal peptide, *C*-terminal acidic peptide, and introns (21-36%). In contrast, divergence in the mature protein domain of type V thionins is equal to or lower than within this domain of type I thionins, and is certainly much lower than in the corresponding introns.

Type I thionins have four disulphide bridges, whereas type V thionins have only three. It is possible that a temporary loss of function due to mutation of one cysteine (gain or loss) in the duplicated gene might have resulted in a period of accelerated evolution. Mutation of a second cysteine (loss or gain)

would have then led to a mature thionin with an even number of cysteines (a common feature of all known thionins) and to a recovery of function that would in turn impose a slower rate of evolution. The extreme divergence in the proportion of charged residues suggests that the functions of the two types, although similar, might be exerted in different contexts or in different cellular environments.

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