Structure of $\beta$-cinnamomin, a protein toxic to some plant species

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Phytophthora and Pythium species are among the most aggressive plant pathogens, as they invade many economically important crops and forest trees. They secrete large amounts of 10 kDa proteins called elicitors that can act as elicitors of plant defence mechanisms. These proteins may also induce a hypersensitive response (HR) including plant cell necrosis, with different levels of toxicity depending on their pI. Recent studies showed that elicitors function as sterol carrier proteins. The crystallographic structure of the highly necrotic recombinant \( \beta \)-cinnamomin (\( \beta \)-CIN) from Phytophthora cinnamomi has been determined at 1.8 \( \AA \) resolution using the molecular-replacement method. \( \beta \)-CIN has the same overall structure as \( \beta \)-cryptogein (\( \beta \)-CRY), an elicitor secreted by Phytophthora cryptogea, although it shows a different surface electrostatic potential distribution. The protein was expressed in Pichia pastoris and crystallized in the triclinic space group with two monomers in the asymmetric unit. The interface formed by these two monomers resembles that from \( \beta \)-CRY dimer, although with fewer interactions.

1. Introduction

Plant diseases caused by the oomycetous fungi, actually phylogenetically classified with the heterokont algae (Kumar & Rzhetsky, 1996), of Phytophthora (\( P. \)) species involve thousands of plant species and are spread worldwide. The genus comprises approximately 60 species, almost all destructive pathogens that cause rot in roots, stems, leaves and fruits of a large range of agricultural and ornamentally important plant hosts. Examples of outbreaks leading to crop and forest devastation of catastrophic economical and social consequences abound (Bourke, 1991; Large, 1940; Gregory, 1983; Podger et al., 1965; Podger, 1972; Shea et al., 1983; McBlain, Hacker et al., 1991; McBlain, Zimmerly et al., 1991; Evans & Prior, 1987). Recently, the decline of Quercus suber (\( Q. \) suber) and \( Q. \) ilet in Portugal and Spain has been associated with infection by \( P. \) cinnamoni (Brasier, 1992; Brasier et al., 1993; Sánchez et al., 2002) causing widespread deaths of cork oak and cork holm, and threatening oak ecosystems. Most Phytophthora species studied secrete large amounts of elicitors, a group of highly conserved proteins. Elicitin-like proteins have also been detected in some Pythium (\( P. \)) species such as \( P. \) vexan or \( P. \) oligandrum (Huet et al., 1995; Panabières et al., 1997).

Since the isolation and primary structure determination of the first elements of this novel class of proteins (Huet & Pernollet, 1989; Ricci et al., 1989), a great research effort has been directed towards the study of their chemistry and physiological effects (for reviews, see Grant et al., 1996; Ponchet et al., 1999). These polypeptides are holoproteins of
98 residues ($M_r \simeq 10\,300$) whose amino-acid composition shows some particular features, namely six Cys residues participating in three S–S bridges at conserved positions, an absence of Arg, His and Trp, and a high content of Ser and Thr. They are classified as acidic, $\alpha$, or basic, $\beta$, proteins according to their $pI$ ($\alpha$, $pI < 5.0$; $\beta$, $pI > 7.5$), mainly determined by the number of Lys residues. The physiology of elicitin activity was extensively studied in tobacco plants, where they were shown to induce a systemic acquired resistance (SAR) against fungal and bacterial pathogens, accompanied by limited leaf necrosis (Ricci et al., 1989; Yu, 1995). Moreover, elicitins can stimulate natural defences in some other plant species, such as tomato, which upon inoculation with oligandrin (from Py. oligandrum) becomes resistant to $P$. parasitica (Picard et al., 2000). Transgenic tobacco plants that produce cryptogeytin upon challenge with virulent $P$. parasitica var. nicotianae were shown to develop an HR and to be resistant to disease caused by fungal pathogens unrelated to Phytophthora species (Keller et al., 1999).

The necrotic activity of $\alpha$- and $\beta$-elicitin isofoms has been studied on leaves of tobacco plants and suggested that the degree of necroticity is related to the nature of the amino acid at position 13: a hydrophilic residue in $\beta$-elicitins, usually a lysine, and a hydrophobic residue in $\alpha$-elicitins, a valine (Ricci et al., 1989; Huet et al., 1992, 1994; Nespoulous et al., 1992; Kamoun et al., 1993; Pernollet et al., 1993). These studies show that $\beta$-elicitins are more necrotic than $\alpha$-elicitins, $\beta$-CIN being the most necrotic (Pernollet et al., 1993).

Further potential elicitor and/or necrotic sites were identified, namely at positions 2, 22, 25, 28, 39, 61, 70, 72, 93, 94 and 96, either by sequence comparisons, site-directed mutagenesis (Perez et al., 1992, 1994; Nespoulous et al., 1999; Kamoun et al., 1993; Pernollet et al., 1993). These studies show that $\beta$-elicitins are more necrotic than $\alpha$-elicitins, $\beta$-CIN being the most necrotic (Pernollet et al., 1993).

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The three-dimensional crystal structure of wild-type cryptogeytin was determined, revealing a polypeptide chain with a novel folding type (Boissy et al., 1996). Subsequently, the three-dimensional structure of a recombinant K13H-mutated cryptogeytin revealed a molecule of ergosterol enclosed in a hydrophobic cavity of the polypeptide (Boissy et al., 1999). This finding is in accordance with previous studies suggesting that elicitins are a new class of sterol carrier proteins (Mikes et al., 1997, 1998) and are able to sequester sterols from plant plasma membranes and transfer them to and from lipid micelles (Vauhterin et al., 1999). Elicitins can also bind fatty acids, although with significantly lower affinity (Osman, Mikes et al., 2001). In an attempt to elucidate the recognition of these elicitor proteins by the plant host, putative high-affinity binding sites were identified in tobacco plasma membranes (Wendehenne et al., 1995) and later characterized as glycoproteins (Bourque et al., 1999).

Recent studies have further suggested that the sterol carrier capacity and elicitor activity are correlated (Ponchet et al., 1999) and the formation of a sterol–elicitin complex is a prerequisite step before elicitins bind to their putative receptors and induce cell-defence responses (Osman, Vauhterin et al., 2001).

As part of a programme to investigate $P$. cinnamomi–$Q$. suber interactions at the molecular level, namely to investigate the role of $\beta$-cinnamomin in the pathogenesis process and to contribute to the elucidation of the biological functions of these holoproteins, we have recently identified an elicitin gene cluster in $P$. cinnamomin (Duclos, Fauconnier et al., 1999), constructed a synthetic gene on the basis of the amino-acid sequence of $\beta$-cinnamomin, expressed it (Duclos, Trincao Aurelio et al., 1998) and crystallized the recombinant protein (Archer et al., 2000).

We report here the crystal structure of $\beta$-cinnamomin ($\beta$-CIN). The three-dimensional structure of $\beta$-CIN is similar to the structure of $\beta$-cryptogeytin ($\beta$-CRY). Owing to the high amino-acid sequence homology among elicitins, it is expected that the overall fold should be conserved within this family of proteins. More structural information is important to contribute to a better understanding of the interactions between plants and this type of pathogen.

2. Experimental

2.1. Purification, crystallization and data collection

$\beta$-CIN was expressed in the methylotrophic yeast Pichia pastoris as described in Archer et al. (2000). The purified recombinant protein was crystallized using either polyethylene glycol (PEG) or ammonium sulfate as precipitants at a pH around 7. Both conditions gave isomorphous crystals
that belong to the triclinic space group (Archer et al., 2000). X-ray diffraction of a frozen crystal was measured at 1.8 Å resolution using a copper rotating-anode generator.

The crystal has unit-cell parameters \( a = 31.85, b = 36.91, c = 43.93 \text{ Å}, \alpha = 77.64, \beta = 86.67, \gamma = 79.55^\circ \). The reflections were integrated and scaled with the program suite HKL (Otwinowski & Minor, 1997). The asymmetric unit contains two molecules of \( \beta\)-CIN, corresponding to a solvent content of about 49%. Relevant processing statistics are presented in Table 1.

### 2.2. Structure solution and model refinement

The structure of \( \beta\)-CIN was determined by molecular replacement with the program AMoRe (Navaza, 1994), using the coordinates of \( \beta\)-cryptogein (PDB code 1beo) as a search model. Calculations were performed over the resolution range 15–3.5 Å. Rigid-body refinement within AMoRe gave a correlation coefficient of 49.4% and an \( R \) factor of 41.0% for the correct solution (values for the second ranked solution were 15.8% for the correlation coefficient and 52.8% for the \( R \) factor).

Structure refinement was carried out with CNS (Brünger et al., 1998) using the maximum-likelihood ‘mlf’ target function. For cross-validation, 5% of randomly selected reflections were kept apart. The graphic program TURBO (Roussel & Cambillau, 1989) was used for map inspections and manual model building.

Initial steps of refinement included the WarpNTrace option of the ARPLwARP program (Perrakis et al., 1999) to optimize the position of a region on the \( \Omega \)-loop (residues 33–39) and simulated annealing with CNS using strict non-crystallographic symmetry (NCS) constraints. Energy minimization proceeded first with restrained NCS of successively decreasing restraint strength and finally with total release of the restraints, monitored by evolution of \( R \) and \( R_{\text{free}} \) values. All refinement calculations were performed using diffraction data to 1.8 Å. No cutoff on the low-resolution data was applied in the bulk-solvent correction. After individual \( B \)-factor refinement, water molecules were introduced with the WATER-PICK routine of CNS.

The final model includes all the 98 residues for both monomers and 201 water molecules, corresponding to a total of 1629 non-H atoms. The \( R \) factor and \( R_{\text{free}} \) values converged to 19.8 and 21.9%, respectively. Refinement statistics are summarized in Table 1.

### 3. Results and discussion

#### 3.1. Analysis of elicitin sequences

A scan of the PIR-NREF database (Barker et al., 2001) against the \( \beta\)-cinnamomin sequence matched 32 sequences with significant homology scores, 26 of which are unique. The sequences were aligned with the multiple alignment program CLUSTALX (Thompson et al., 1994) and a cluster analysis based on the identity matrix was performed with the MODELLER program (Sali & Blundell, 1993). Almost all primary sequences show pairwise identities above 65% and the sequence alignment reveals several highly conserved regions (residues 7–9, 15–20, 31–38, 40–43, 50–51, 53–56, 69–85 and 95–98; Fig. 1). This high level of identity suggests a very similar three-dimensional structure and makes the elicitins excellent candidates for homology modelling (Marti-Renom et al., 2000).

Three sequences stand out from the set, oligandrin (from \( Py. \) oligandrum) and infestins 2A and 2B (from \( P. \) infestans) and therefore are not included in the alignment. Infestins display identities in the range 50–60% when compared with the remaining sequences (however, the alignment corresponds

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**Figure 1**

Amino-acid sequences alignment of the 23 elicitins, from \( Pythium (Py.) \) and \( Phytophthora (P.) \) species that share pairwise identities above 65%. *, identity; :, strongly similar; ., weakly similar. The elicitin sequences were determined from the following organisms: Vex1 and Vex2, \( Py. \) vexans; B-Cin, A-Cin, HA-Cin and A-Cin2, \( P. \) cinnamomi; B-Meg and A-Meg, \( P. \) megasperma; B-Cry, A-Cry_A1, HA-Cryp20 and HA-Cryp26, \( P. \) cryptogea; B-Cap (fragment) and A-Cap, \( P. \) capsici; A-Dre and B-Dre, \( P. \) drescheleri; Soj1, Soj2, Soj3 and Soj4, \( P. \) sojae; Par_310, \( P. \) parasitica; A-Inf, \( P. \) infestans; Cac, \( P. \) cactorum (A, acidic elicitin; B, basic elicitin; HA, highly acidic). Sequence lengths are indicated in the far right column.

1316 Rodrigues et al. · \( \beta\)-Cinnamomin Acta Cryst. (2002). D58, 1314–1321
to a small fraction of the total length of these 195 and 198 amino-acid sequences), while oligandrin is a more distant relative (identities between 36 and 43%).

3.2. Structure analysis

The final model of $\beta$-CIN comprises all 98 residues for each monomer ($A$ and $B$) in the asymmetric unit. The electron density is well defined for most of the protein, with some exceptions: Thr1 in both monomers, Ser34 and Met35 of molecule $B$ and a few surface polar residues.

In general, the two monomers are very similar (r.m.s. deviations of 0.32 Å for main-chain and 0.63 Å for all protein atoms), differing essentially through residues 34–39 (with a largest main-chain r.m.s.d. of 1.4 Å for Ser34 and a side-chain r.m.s.d. of 4.4 Å for Met35). Not surprisingly, this region is expected to have a higher flexibility since it belongs to the loop that forms a lid over the ligand cavity of elicitors (Boissy et al., 1999). This mobility is also observed in the NMR solution structure of cryptogelin (Fefeu et al., 1997).

Inspection of the Ramachandran plot (Ramachandran & Sasisekharan, 1968) as defined by the program PROCHECK (Laskowski et al., 1993) shows six residues (Asn67$A$/B, Asp70$A$/B, Ser34 and Ser78$A$) in the additionally allowed regions, corresponding to 3% of the total structure.

3.3. Overall structure

The overall structure of $\beta$-CIN is rather similar to that of $\beta$-CRY (Boissy et al., 1996) and is probably representative of the elicitin family. A ribbon representation of the secondary structure of $\beta$-CIN is shown in Fig. 2. It comprises six $\alpha$-helices, a short two-stranded $\beta$-sheet and a large $\Omega$-loop (Tyr33–Pro42). Three disulfide bonds (Cys3–Cys71, Cys27–Cys56 and Cys51–Cys95) strengthen this fold. Two ionic interactions, N-terminus–Asp72 and Asp21–Lys62, are thought to be important in the stabilization of the $\beta$-CRY structure (Gooley et al., 1998). In $\beta$-CIN, the first interaction is conserved, while the second is mediated by a water molecule between Glu21 and Lys62, although the main-chain atom positions of both residues remain almost the same in the two structures.

The so-called ‘beak-like motif’ formed by the $\Omega$-loop, the $\beta$-sheet and its associated hairpin loop borders a large internal hydrophobic cavity. In the structure of the mutant K13H of $\beta$-CRY this cavity accommodates ergosterol, a small ligand molecule (Boissy et al., 1999). All residues in the beak-like motif region (residues 33–42 and 72–82) are strictly conserved among elicins, except for residue 39, which in most elicins, including $\beta$-CIN, is a threonine instead of a lysine (Fig. 1).

As previously noted (Boissy et al., 1996), elicins are structurally unrelated to any known protein family, thus displaying a new fold. Submission of the $\beta$-CIN coordinates to the DALI fold comparison software (Holm & Sander, 1993) matched $\beta$-CRY with a $Z$ score of 19.2. The second hit, cole1 primer repressor, whose structure consists of two $\alpha$-helices, matched $\beta$-CIN with a low $Z$ score (3.4) and has no biological or functional analogy with elicins.

3.4. Comparison with other elicitin structures

$\beta$-CIN is the second elicitin to be structurally characterized. With the objective of analyzing the structural details that may account for the small differences in biological activity between
$\beta$-CIN and $\beta$-CRY and that result from ligand binding, we shall compare the structures of the two native proteins with that of the mutated K13H $\beta$-CRY bound with ergosterol (Boissy et al., 1999). The superposition of the 98 C$^\alpha$ atoms of chains A and B of $\beta$-CIN with $\beta$-CRY and $\beta$-CRY–ergosterol is shown in Fig. 3. As illustrated in the figure, the three molecules are very similar. The overall structural differences are somewhat higher between $\beta$-CIN and the complex (r.m.s.d. for C$^\alpha$ atoms of 0.89 Å for molecule A and 0.92 Å for molecule B) than between $\beta$-CIN and $\beta$-CRY (r.m.s.d. for C$^\alpha$ atoms of 0.62 Å for molecule A and 0.54 Å for molecule B). The higher discrepancy between $\beta$-CIN and $\beta$-CRY–ergosterol complex mainly arises from the deformation of helix $\alpha_1$ in the complex structure. Bending of helix $\alpha_1$ is one of the major conformational changes that occur upon ligand binding (Boissy et al., 1999).

On one hand, the $\Omega$-loop region of monomer A shows a lower structural similarity with the native cryptogein (average difference of 1.3 Å for the C$^\alpha$ atoms for residues 33–39, with a maximum value of 2.5 Å for Ser34) than with the $\beta$-CRY–ergosterol complex (average difference of 0.5 Å for the same C$^\alpha$ atoms) (Fig. 3). On the other hand, the $\Omega$-loop region of monomer B is slightly closer to the native cryptogein than to the complex structure (average differences of 0.7 Å and of 0.9 Å, respectively).

The comparison of both CRY structures (free and bound) shows that Leu15, Met35 and Tyr87 are the residues that undergo major conformational changes upon ligand binding. It is worthwhile to note that in $\beta$-CIN the conformation adopted by Met35 in monomer A is equivalent to that observed in the complex structure (Fig. 3), facilitating an eventual access to the internal cavity, whereas in monomer B this residue is in a similar position to that of the native $\beta$-CRY structure. Fig. 4 represents a section through the $\beta$-CIN A molecule, showing the size and shape of this internal cavity. The alternative conformation of the Met35 side chain, as observed in molecule B and in the native $\beta$-CRY structure, limits the entrance to that cavity and significantly reduces its size. In both $\beta$-CIN molecules the side chains of Leu15 and Tyr87 have similar conformations to those of the native cryptogein structure, limiting access and binding of a ligand (Fig. 3). Therefore, bending of helix $\alpha_1$ and alterations in the side-chain conformations of Leu15 and Tyr87 may be solely correlated with ligand binding, while conformational changes in some residues of the $\Omega$-loop, in particular Met35, may be related to the flexibility of this region, although a conformational change is certainly needed to allow the ligand entry into the hydrophobic cavity.

Another residue involved in ligand binding is Tyr47, since it establishes a hydrogen bond with the hydroxyl group of ergosterol as shown in the $\beta$-CRY complex structure (Boissy et al., 1999). In order to assess the relationship between sterol loading and elicitor properties, mutagenesis studies and binding experiments were performed on Tyr47 and Tyr87 in $\beta$-CRY (Y47F/G and Y87F), since they were thought to be important in sterol binding (Osman, Vauthrin et al., 2001). The mutation Y47F was found to slightly affect the dissociation constant ($K_d$) of elicitin–sterol complexes, whereas
the Y47G mutation strongly increases this constant, meaning that the hydrogen bond and especially the van der Waals interactions are important for ligand stabilization. This residue is in a very similar conformation in the three crystal structures represented in Fig. 3 and is therefore ready to accept, in the native proteins, a sterol molecule in the interior cavity. An even more drastic increase in $K_d$ is observed with the Y87F mutation (Osman, Vauthrin et al., 2001). Phenylalanines are normally located in hydrophobic interior regions of protein structures, avoiding any contact with the solvent. Therefore, it is presumable that this aromatic side chain on the Y87F mutant may keep the same orientation as those of the equivalent residues in the two native elicitin structures β-CIN and β-CRY, thus preventing adaptation of a ligand into the internal cavity of the mutated protein.

3.5. Homodimer interactions

The two molecules present in the asymmetric unit of β-CIN are related by a pseudo-twofold axis and thus form a crystallographic dimer. However, this homodimer is different from that observed in the crystal (Boissy et al., 1996) and solution (Gooley et al., 1998) structures of β-CRY. Fig. 5 compares the crystal dimers of both elicits.

In β-CIN the dimer interface is based on a central core of hydrophobic interactions between residues located on helix α1 and on the beak-like motif, and one hydrogen bond between residues Thr77A and Ser20B. In the case of β-CRY, the dimer has more hydrophobic interactions in the interface core and is further flanked by hydrophilic interactions through the beginning of helix α1. Table 2 lists residues and distances (below a cutoff value of 5.0 Å) involved in dimer interactions in both elicitin structures. The central hydrophobic set of contacts is similar in both dimers, with two conserved pairs, Val16-Pro76 and Leu19-Leu19. Since the residues that constitute this hydrophobic core are highly conserved (Leu15, Val16, Leu19, Leu36, Pro76) (see Fig. 1) one may suggest an eventual relevance of dimerization to the elicitin biological activity. On the other hand, upon ligand binding most of these residues are involved in contacts with the ligand molecule. Therefore, this may be an alternative explanation for the conserved region from residue 15 to residue 20 observed within the elicitin family.

It was previously shown that the elicitin β-CRY is predominantly a dimer in concentrated aqueous solutions (in the millimolar range: Gooley et al., 1998). The solution structure of this dimer (Gooley et al., 1998) is essentially the same as that observed in the crystalline structure, where a crystallographic twofold axis correlates the two molecules. In order to assess the role of the elicitin dimerization on their necrotic activity, further investigation is needed since it is known that elicits are monomeric at physiological nanomolar concentrations.

The comparison between the crystallographic homodimers of β-CIN and β-CRY shows some differences (Fig. 5). Analysis of the hydrophobic contacts in the β-CIN and β-CRY dimers indicates a stronger stabilization for the β-CRY dimer in terms of excluded surface area, interface gap volume and atomic solvation energy (calculated with the program ASC; Eisenhaber et al., 1995). These results, together with the aforementioned similarity between solution and crystalline β-CRY dimers, suggest that the distinct conformation of the β-CIN dimer may just be an artifact of crystal packing rather than having any biological relevance. In both monomers, residue Lys13 located in helix α1 forms a salt bridge with the C-terminus of a symmetry-related molecule, modifying the position of the helix so as to prevent an extensive interaction between them as in the case of the β-CRY dimer.

However, it is worthwhile to point out that upon dimerization the access to the ligand-binding pocket located between helix α1 and the beak-like motif is blocked. Since the recognition
Table 2

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<th>Residues involved in dimer interactions in β-CIN and β-CRY X-ray structures.</th>
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† The equivalent interactions between the reverse residues from monomer B to monomer A have the distances quoted in parentheses. 1 Equivalent interactions are observed between the reverse residues from monomer B to monomer A owing to the twofold crystallographic axis.

and eventually the penetration of a sterol molecule into that cavity occurs through this face of the protein molecule, one can speculate that the dimerization and ligand binding may be correlated. Dimerization may also be associated in some as yet unknown way with the possible existence of two elicitin localizations or domains, as extracellular and cell-wall-associated proteins (Ponchet et al., 1999).

3.6. Surface electrostatic potential calculations

The calculated molecular electrostatic potentials at the surfaces of β-CIN and β-CRY calculated with the GRASP software (Nicholls et al., 1993) are depicted in Fig. 6. The presence of two additional aspartate residues (at positions 70 and 83) in β-CIN compared with one (Asp72) in β-CRY creates a larger patch of negative charges, visible in the front representations, thus making the electrostatic potential at this side of both molecules very different. Two separate regions of negative and positively charged residues are seen in the front side of both molecules very different. Two separate regions include some of the 11 residues shown to be important for biological activity (Perez et al., 1997): Ala2, Asp70 and Asp72 are located on the negative side, while Ser93, Lys94 and Ala96 correspond to the positive region. On the opposite side of the molecule, the potentials of β-CIN and β-CRY are much more similar, although the bottom region where Lys13 is located is more positive in β-CRY than in β-CIN. This side of the molecule also contains other putative active residues besides Lys13, namely three solvent-exposed serine residues (22, 25 and 28), and thus may be involved in a molecular interaction (Perez et al., 1999).

It has been shown that in their respective classes, β-cinnamomin and α-cinnamomin are the most toxic elicitins against tobacco leaves (Pernollet et al., 1993). Amino-acid sequence comparison and site-directed mutagenesis studies pointed out the important role of Ser25 in the high necrogenicity of both cinnamomin (Perez et al., 1999). From analysis of the electrostatic potential, it is difficult to interpret this fact, since replacement of Ser25 by Asn (as in β-CRY) should not significantly change the molecular surface potential. On the other hand, the number of basic residues seems to correlate with the necrotic power of elicitins, as evidenced by basic elicitins being much more necrotic than the acidic elicitins. However, since β-CIN is less basic than β-CRY, its higher necrogenicity might be a consequence of a higher charge-distribution asymmetry at the molecular surface.

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