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Crystallization and preliminary X-ray studies of oligandrin, a sterol-carrier elicitor from *Pythium oligandrum*

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Oligandrin is a 10 kDa acidic protein produced by the fungus micromycete *Pythium oligandrum* and is a member of the α-elicitin group, with sterol- and lipid-carrier properties. Oligandrin has been crystallized at 290 K using PEG 4000 as a precipitant. A cholesterol complex was obtained under the same conditions. The space group of the crystals at low temperature (100 K) is *C*222, with unit-cell parameters $a = 94.0$, $b = 171.1$, $c = 55.3$ Å. Four molecules are present in the asymmetric unit. Data from the free and cholesterol-complexed forms were recorded at synchrotron sources to resolutions of 2.4 (uncomplexed) and 1.9 Å (complexed), respectively.

1. Introduction

Phytophtora and *Phytium* spp. fungi are micromycetes, parasites of many economically important crops. They produce proteins that induce leaf necrosis and further a systemic acquired resistance (SAR) against a wide range of other pathogens, including fungal and bacterial species. The main component responsible for the necrosis is a small 10 kDa extracellular protein elicitor belonging to a new class of proteins termed elicitins (Ricci *et al.*, 1989; Kamoun *et al.*, 1994; Ponchet *et al.*, 1999).

Depending on the *Phytophtora* strains, elicitins can be divided into two classes depending on their pIs: the basic proteins (β-elicitins) display a twofold increase in their necrotic power compared with the acidic class (termed α-elicitins). In addition, elicitins are sterol-carrier proteins and the role of this function in biological activities is being investigated (Mikes *et al.*, 1998; Vauthrin *et al.*, 1999). Recently, the structure of cryptogein, a β-elicitor isolated from *Ph. cryptogea*, has been solved (Boissy *et al.*, 1996), as well as that of a natural ergosterol complex of its K13H mutant (Boissy *et al.*, 1999). The sterol is located in a large hydrophobic pocket delimited by the arrangement of five α-helices and two short β-bends. The present paper concerns the first crystallization of an α-elicitin (acid elicitin), oligandrin, produced by the fungus *Py. oligandrum*. In contrast to cryptogein elicitins, oligandrin displays very interesting properties. As its necrotic effect is very low, it does not induce any necrosis and it triggers a high level of protection against other pathogenic agents.

2. Cultures and purification

The protein was obtained from liquid fungal cultures (*Py. oligandrum* Drechsler, strain Py 7 from the collection of INRA, Antibes). Culture filtrates were obtained by growing the fungus in a previously described liquid medium (Bonnet *et al.*, 1996). 1 l flasks containing 100 ml of medium were inoculated and incubated in the dark (8 d, 297 K). *Py. oligandrum* culture filtrate was recovered after mycelium removal on a GF/C filter (Whatman) under vacuum.

The crude culture filtrate (5 l) was concentrated and extensively dialyzed against deionized water for 24 h at 313 K. The pH was adjusted to 3.5 with 10% aqueous trifluoroacetic acid and loaded onto a 20 ml Macroprep sulfopropyl High S (BioRad) column previously equilibrated with 10 m*M* sodium acetate pH 3.5. The eluted fraction was adjusted to pH 7.0 before being subjected to reversed-phase liquid chromatography (Synchroprep C4 column). Elution was performed at room temperature using a discontinuous gradient of acetonitrile (CH_3CN) [20, 30, 40%(*v*/*v*)] in 50 m*M* aqueous sodium formate. The fraction containing oligandrin was evaporated until the CH_3CN was completely removed and was then extensively dialyzed against ultrapure water and freeze-dried.

The purity of the protein was further assessed by SDS–PAGE on 15.4% polyacrylamide–SDS gels (20 mA per gel in 0.25 m*M* Tris, 1.92 *M* glycine, 0.1% SDS).

3. Crystallizations

Preliminary screening experiments were performed with Hampton Research Crystal Screen kits. Of all the combinations tested, only a small number were able to produce crystalline precipitates, mainly in the presence of PEG crystallizing agents at acidic pH. These

Table 1
Data-collection statistics.

Values for the last resolution shell are given in parentheses.

Crystal	Uncomplexed, T = 277 K†	Uncomplexed, T = 100 K	Complexed (cholesterol), T = 100 K
Space group	$C222_1$	$C222$	$C222$
Unit-cell parameters (Å)	a = 94.9	a = 94.0	a = 93.25
	b = 177.2	b = 171.1	b = 171.9
	c = 113.9	c = 55.3	c = 54.81
No. of measured reflections	112616	190195	337731
No. of unique reflections	34349	17461	34651
Resolution limits (Å)	31–2.45 (2.63–2.45)	27–2.39 (2.68–2.39)	19.2–1.9 (1.97–1.90)
Data completeness (%)	98 (67)	94 (56)	88.5 (45)
$I/\sigma(I)$	13.5 (3.8)	11.1 (4.1)	13.0 (3.5)
$R_{\rm merge}$‡ (%)	8.4 (20.1)	5.5 (18.6)	7.2 (22.0)

† Merging of eight randomly oriented crystals. $R_{\rm merge}$ calculated after scaling to a common data set. ‡ $R_{\rm merge}$ is defined as $\sum_{\mathbf{h}}\sum_i|\langle I(\mathbf{h})\rangle - I_i(\mathbf{h})|/\sum_{\mathbf{h}}\sum_i I_i(\mathbf{h})$, where $I_i(\mathbf{h})$ is the ith observation of reflection $\mathbf{h}$.

conditions were further optimized to yield repeatedly large plates (more than 1 mm long but only 50 µm thick) using the hanging-drop technique at room temperature.

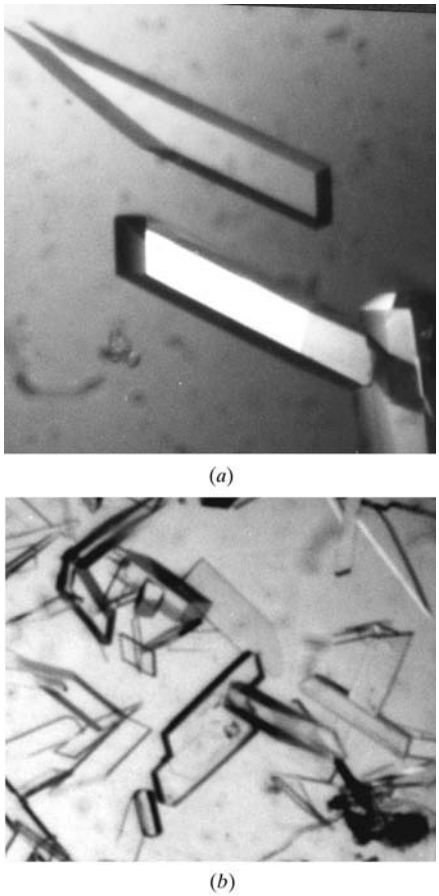

Figure 1
Crystals of (*a*) oligandrin and (*b*) its cholesterol complex grown in the presence of PEG 4000 as a precipitant. The approximate dimensions of the uncomplexed crystals may reach ∼2 mm. In the case of the complex, dimensions are never greater than 0.2 mm.

3.1. Native crystals

The reservoir was set up with 1 ml 8% PEG 4000 pH 4.5 (0.015 *M* acetate buffer) and 5 m*M* $ZnSO_4$. Drops were composed of 4 µl of the reservoir and 4 µl of a 20 mg ml^{-1} protein solution in 0.015 *M* acetate buffer. Crystals appear in several days and develop to their maximum size in about two weeks (Fig. 1*a*).

3.2. Cholesterol complex

The previous protein solution was first incubated at room temperature in the presence of a 1:1(*w*/*w*) ratio of solid cholesterol in acetate buffer at pH 4.5 for 24 h. The excess of cholesterol was eliminated by filtering the solution through a Pasteur pipette with a cotton-wool tip or by centrifugation. The resulting solution was set to crystallize under nearly the same conditions as for the native. Very small prismatic crystals developed rapidly with a strong tendency towards twinning. The best crystals were obtained with a reservoir concentration of 7–8% PEG 4000 (Fig. 1*b*).

In both cases, Zn^{2+} cations were found to be essential for the crystallization and are expected to participate to the crystal packing as observed in many other cases (see, for example, Radhakrishnan *et al.*, 1996).

3.3. Cryo-cooling conditions

Crystals were subjected to liquid-nitrogen freezing for synchrotron data collections. Because of a dramatic change in the unit cell (the *c* parameter was halved and the space group changed from $C222_1$ to $C222$), only a small number of crystals survived the process. The resolution of native crystals was about the same (2.4–2.5 Å), but the resolution of the complex increased from 2.5 to 1.9 Å (at ESRF beamline ID-14-1).

4. Data collection and processing

Data were recorded from crystals in wet sealed glass capillaries at the wiggler line W32, LURE facility, Orsay (Fourme *et al.*, 1992) at 278 K. The lifetime of the crystals was rather short (less than 2 h with 2 min exposure time per degree). Data from the frozen crystals were recorded at the ID-14-1 beamline, ESRF, Grenoble (Wakatsuki *et al.*, 1998).

The data were processed with the *MOSFLM* program (Leslie, 1994) interfaced with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Data sets are summarized in Table 1.

In the case of the low-temperature form, the unit-cell parameters are still indicative of a multimeric protein in the crystal. Self-Patterson investigations clearly show the presence of twofold non-crystallographic axes in the polarographic projection $\kappa = 180^\circ$. With a volume of 8.89×10^5 Å^3 and an estimated Matthews coefficient of 2.2 Å^3 Da^{-1}, four molecules are expected in the asymmetric unit (corresponding to ∼45% solvent).

The structure solution is under investigation using the multibody molecular-replacement method (Navaza *et al.*, 1993, 1998).

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