NMR Solution Structure of the Antifungal Protein from *Aspergillus giganteus*: Evidence for Cysteine Pairing Isomerism[†,‡]

Ramón Campos-Olivas,[§] Marta Bruix,[§] Jorge Santoro,[§] Javier Lacadena,[||] Alvaro Martinez del Pozo,[||] José G. Gavilanes,[||] and Manuel Rico*[,§]

Instituto de Estructura de la Materia, Consejo Superior de Investigaciones Cientificas, Serrano 119, 28006 Madrid, Spain, and Departamento de Bioquimica y Biologia Molecular I, Facultad de Quimica, Universidad Complutense, 28040 Madrid, Spain

Received November 8, 1994; Revised Manuscript Received December 21, 1994[⊗]

ABSTRACT: The solution structure of the antifungal protein (AFP, 51 residues, 4 disulfide bridges) from *Aspergillus giganteus* has been determined by using experimentally derived interproton distance constraints from nuclear magnetic resonance (NMR) spectroscopy. Complete sequence-specific proton assignments were obtained at pH 5.0 and 35 °C. A set of 834 upper limit distance constraints from nuclear Overhauser effect measurements was used as input for the calculation of structures with the program DIANA. An initial family of 40 structures calculated with no disulfide constraints was used to obtain information about the disulfide connectivities, which could not be determined by standard biochemical methods. Three possible disulfide patterns were selected and the corresponding disulfide constraints applied to generate a family of 20 DIANA conformers for each pattern. Following energy minimization, the average pairwise RMSD of the 20 conformers of each family is 1.01, 0.89, and 1.01 Å for backbone atoms and 1.82, 1.74, and 1.81 Å for all heavy atoms. One of these three families contains the disulfide bridge arrangement actually present in the solution structure of AFP. Although the three families fulfill the NMR constraints, one of the disulfide patterns considered (cysteine pairs 7−33, 14−40, 26−49, 28−51) is favored among the others on the basis of previous chemical studies. It thus probably corresponds to the actual pattern of disulfide bridges present in the protein, and the corresponding family represents the solution structure of AFP. The folding of AFP consists of five antiparallel β strands connected in a −1, −1, +3, +1 topology and highly twisted, defining a small and compact β barrel stabilized by four internal disulfide bridges. A cationic site formed by up to three lysine side chains adjacent to a hydrophobic stretch, both at the protein surface, may constitute a potential binding site for phospholipids which would be the basis of its biological function. On the other hand, a second, minor form of AFP has been detected. NMR data, together with results from mass spectrometry, chemical analysis, and sedimentation equilibrium, suggest that this species differs from the major form in the pairs of cysteines involved in the four disulfide bridges.

Several small proteins with antifungal activity have been isolated from plants and are believed to be involved in a defense mechanism against phytopathogenic fungi by inhibiting fungal growth through diverse molecular modes, such as binding to chitin or permeabilizing fungal membranes or cell walls. They include the barley thionins (Bohlmann et al., 1988), stinging nettle lectin (Broekart et al., 1989), maize zeamatin (Roberts & Seletrennikoff, 1990), and rubber-tree hevein (Parijs et al., 1990). However, antifungal proteins are not exclusively produced by plants. A small, very basic protein with antifungal activity has been purified from the extracellular medium of the imperfect ascomycete *Aspergillus giganteus* (Olson & Goerner, 1965). The so-called antifungal protein (AFP)[1] displays an inhibitory activity against the growth of a variety of filamentous fungi, having no effect on the growth of mammalian cells, yeast, and eubacteria. It consists of 51 amino acids with a high content (12) of lysine residues, and eight cysteine residues which are all involved in disulfide bridges (Nakaya et al., 1990). The structural organization of the gene encoding AFP and its transfer into another filamentous fungi, *Aspergillus niger*, has also been described (Wnendt et al., 1994).

[†] This work was supported by the Direccion General de Investigacion Cientifica y Tecnica (Spain) (PB90/0120 and PB93/0090). R.C.O. was recipient of a predoctoral grant (FP92-02882770) from the Ministerio de Educacion y Ciencia (Spain).

[‡] The atomic coordinates for the 40 "R" DIANA conformers have been deposited in the Brookhaven Protein Data Bank under the ident code 1AFP.

* Author to whom correspondence should be addressed (Fax: 34-1-5642431).

[§] Instituto de Estructura de la Materia.

[||] Universidad Complutense.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1995.

[1] Abbreviations: AFP, antifungal protein from *Aspergillus giganteus*; CITY, computer-improved total correlation spectroscopy; COSY, correlated spectroscopy; 2D, two dimensional; $d_{\alpha\alpha}(i,j)$, NOE connectivity between the $C_\alpha H$ proton on residue i and the $C_\alpha H$ proton on residue j; $d_{\alpha N}(i,j)$, NOE connectivity between the $C_\alpha H$ proton on residue i and the NH proton on residue j; $d_{\beta N}(i,j)$ NOE connectivity between the $C_\beta H$ proton on residue i and the NH proton on residue j; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; $d_{NN}(i,j)$, NOE connectivity between the NH proton on residue i and the NH proton on residue j; HPLC, high performance liquid chromatography; M_r, relative molecular mass; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; PAGE, polyacrylamide gel electrophoresis; ppm, parts per million; REM, restrained energy minimization; RMSD, root mean square deviation; ROESY, rotating frame Overhauser effect spectroscopy; SDS, sodium dodecyl sulfate; SNS, nuclease from *Staphylococcus aureus*; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)-aminomethane; TSP, sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate; WATERGATE, water suppression by gradient-tailored excitation.

0006-2960/95/0434-3009$09.00/0