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ARTICLE

^{1}H, ^{13}C and ^{15}N resonance assignments of the RodA hydrophobin from the opportunistic pathogen *Aspergillus fumigatus*

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Abstract Hydrophobins are fungal proteins characterised by their amphipathic properties and an idiosyncratic pattern of eight cysteine residues involved in four disulphide bridges. The soluble form of these proteins spontaneously self-assembles at hydrophobic/hydrophilic interfaces to form an amphipathic monolayer. The RodA hydrophobin of the opportunistic pathogen *Aspergillus fumigatus* forms an amyloid layer with a rodlet morphology that covers the surface of fungal spores. This rodlet layer bestows hydrophobicity to the spores facilitating their dispersal in the air and rendering the conidia inert relative to the human immune system. As a first step in the analysis of the solution structure and self-association of RodA, we report the ^{1}H, ^{13}C and ^{15}N resonance assignments of the soluble monomeric form of RodA.

Keywords Hydrophobin · Functional amyloids · Rodlets · Cell wall · *Aspergillus fumigatus* · NMR

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Biological context

Hydrophobins are small (<20 kDa) amphipathic proteins produced by most filamentous fungi. These proteins with remarkable physicochemical properties are defined by a pattern of eight cysteine (Cys) residues that form four disulphide bonds (C1–C6, C2–C5, C3–C4, C7–C8) (Kwan et al. 2006). They are produced in a soluble form that spontaneously self-assembles at hydrophobic/hydrophilic or air/water interfaces to form amphipathic monolayers with high surfactant activity. Hydrophobins are divided in two classes based on their sequence, hydropathy and on the physicochemical characteristics of the monolayers (Wosten et al. 1994). Class I hydrophobins show very little conservation in their amino acid sequence and vary in size, with different spacings between cysteine residues; these hydrophobins associate into very robust monolayers that are resistant to detergents even at boiling temperatures and require concentrated acids to dissociate. Class I hydrophobin monolayers show a very well ordered rodlet morphology and display the hallmarks of amyloid fibres (Wosten and de Vocht 2000). The amino acid sequences of class II hydrophobins are more conserved, they show less size and inter-cysteine-spacing variability; the corresponding monolayers do not contain amyloid fibres and can be disaggregated with detergents and hot alcohol solutions.

Although some degree of order has been observed for hydrophobins HFBI (Szilvay et al. 2007) and NC2 monolayers (Ren et al. 2013a), class II hydrophobins seem to form less well ordered monolayers. The structures of the soluble forms of hydrophobins from both classes that self-associate into monolayers are quite different but show a common central β-barrel stabilised by the four disulphide bridges and surrounding loops of different length and secondary structure (Sunde et al. 2008). The biological functions of hydrophobins rely on the formation of the amphipathic monolayers. Hydrophobins are used by fungi to breach the air/water barrier and develop aerial hyphae by lowering the surface tension of the aqueous environment, to prevent water-logging, to facilitate aerial growth and spore dispersion, to participate in the extracellular matrix and biofilms as well as to form a protective layer during fruiting body development. Hydrophobins can also participate in host-fungi interactions and have been described as pathogenicity factors in plant- or entomo-pathogenic fungi. The surfactant activity of hydrophobins and the capacity to reverse the wettability of surfaces offers the possibility to use these proteins for different biotechnological applications such as drug delivery, cell attachment, surface functional modification and protein purification. The biological functions, structural knowledge and biotechnical applications of hydrophobins have been reviewed in (Wosten 2001; Scholtmeijer et al. 2001; Tucker and Talbot 2001; Linder et al. 2005; Bayry et al. 2012; Ren et al. 2013b).

Aspergillus fumigatus is an opportunistic pathogen than can cause a variety of diseases in immunocompromised individuals that range from allergies to threatful invasive aspergillosis. This filamentous fungus has become the major aerial fungal pathogen of humans and represents a major burden in terms of morbidity, mortality and cost for hospitals (Hayes and Denning 2013). The conidia, which are the infective form of the fungus, are covered by a rodlet layer formed by the class I hydrophobin RodA (Paris et al. 2003). This proteinaceous coat bestows hydrophobicity to the spores facilitating their dispersal in the air and masks pathogen-associated molecular patterns (PAMPs) thus rendering the conidia inert relative to the innate and adaptive immune systems (Aimanianda et al. 2009). With the aim of understanding the mechanism of rodlet formation and the rodlet structure, as well as shedding light on the possible links between the structure of RodA and its immunologic characteristics, we are analysing the solution structure of RodA, its self-assembly, the structure of the rodlets and the immunological characteristics of RodA mutants. Here we describe the ^{1}H, ^{15}N, ^{13}C resonance assignments of the soluble form of RodA and the comparison of its secondary structure and dynamics with that of other hydrophobins.

Materials and methods

Protein preparation

The RodA gene sequence (AAB07707) was cloned into a pHUE expression vector used for other hydrophobins (Kwan et al. 2006) to generate the plasmid pHUE-RodA. The resulting plasmid encodes for a fusion protein (H_6-Ub-RodA) of N-terminal hexa-histidine-tagged ubiquitin (H_6-Ub) coupled to RodA and contains a deubiquinating UBP41 enzyme cleavage site between the ubiquitin and RodA proteins. The plasmid also codes for ampicillin resistance.

The protein H_6-Ub-RodA was expressed using the *Escherichia coli* BL21 (DE3) strain. Bacteria were grown at 37 °C in minimal M9 media supplemented with 0.43 g/L of yeast extract without amino acids and without ammonium sulphate (DIFCO), and containing $^{15}NH_4Cl$ (0.52 g/L, ≥98 %) and $^{13}C_6$ glucose (2 g/L, ≥98 %) as sole sources of nitrogen and carbon, respectively. When cultures reached an absorbance of 0.6 at 600 nm, expression was induced with 0.5 mM isopropyl-β-thio-galactopyranoside. Cells were harvested by centrifugation at 6700 g and 4 °C after 3 h of induction, resuspended in 150 mM Tris–HCl, 300 mM NaCl pH 8, re-pelleted by centrifugation and frozen at −80 °C.

The fusion protein was obtained simultaneously from both the soluble and insoluble fractions under denaturing and reducing conditions, purified by affinity chromatography and folded under oxidative conditions. To extract the protein, the cell pellet was thawed and solubilised with buffer A (10 mM TrisHCl, 100 mM NaH_2PO_4, 4 mM β-mercaptoethanol [βME], 8 M urea pH 8). After eliminating the cell debris by centrifugation, H_6-Ub-RodA was loaded into a nickel column (Hitrap™, GE Healthcare) pre-equilibrated with buffer A. The column was successively washed with the equilibrating buffer prepared at pH 8 and at pH 6.3. The fusion protein was then eluted with the same buffer prepared at pH 4.3. Oxidative refolding was performed by dialysis of the eluted protein at 4 °C against the refolding buffer (50 mM sodium acetate, 100 mM NaCl, pH 5) supplemented with a redox glutathione couple (10 mM reduced, 1 mM oxidised).

RodA was obtained from the fusion protein by cleavage with UBP41 and further purification by affinity chromatography and reverse-phase high-pressure liquid chromatography (HPLC). After the refolding step, the H_6-Ub-RodA containing samples were dialysed against the cleavage buffer (50 mM TrisHCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8) and incubated at 37 °C for 2.5 h in the presence of the deubiquinating enzyme UBP41 (20:1 w:w protein:protease ratio). The UBP41 protease, which contains a hexa-histidine tag and cleaves at a specific C-terminal site on ubiquitin fusions, was previously prepared as described in (Kwan et al. 2006). H_6-ubiquitin, uncleaved

H_6-Ub-RodA and the histidine-tagged UBP41 protease were removed by affinity chromatography on a nickel HiTrap™ column. The column was pre-equilibrated with 50 mM TrisHCl, 300 mM NaCl, 2 mM $CaCl_2$, 20 mM imidazole, pH 8. The cleaved mixture was loaded on the column after adjusting the NaCl and imidazole concentrations to 300 and 20 mM, respectively. RodA was eluted with the equilibrating buffer. A final HPLC purification step using a semi-preparative reverse phase C18 column (PepRPC 15 µm HR 10/10, Amersham Biosciences) was performed to separate the folded from the unfolded/partially folded forms of RodA, as well as to further purify the protein. The HPLC column was equilibrated with 10 % methanol, 0.1 % TFA. After adjusting the pH to ca. 4 with HCl, the RodA sample was loaded on the column and a linear gradient from 0 to 100 % of acetonitrile (90 min) in equilibrating buffer was applied. Folded RodA-containing samples were pooled and lyophilised. Protein concentrations were determined by the bicinchoninic acid assay (BCA, Pierce Thermo Scientific) and/or absorption spectrophotometry, protein identity and integrity were monitored by SELDI-TOF (surface enhanced laser desorption/ionization time-of-flight) mass spectrometry and N-terminal microsequencing (Protein Analysis and Microsequencing Facility, Institut Pasteur), and purity was assessed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis under reducing conditions and NMR (^{15}N–^{1}H HSQC spectra). In addition, ^{15}N–^{1}H HSQC spectra served to establish the foldedness of the HPLC eluted fractions.

The recombinant protein obtained in this way consists of residues 19–159 of RodA and an additional N-terminal Ser residue that remains after cleavage of the fusion. The 18 N-terminal residues coded by the *RodA* gene, which correspond to the secretion signal and are expected to be cleaved by *A. fumigatus* upon secretion, were not cloned.

Doubly labelled RodA was also extracted by acid treatment from *A. fumigatus* spores obtained from minimal media containing ^{15}N NH_4Cl and $^{13}C_6$ glucose as described (Aimanianda et al. 2009) and purified by reverse-phase C18 HPLC as for the recombinant protein.

NMR spectroscopy

Doubly labelled (^{15}N, ^{13}C) recombinant RodA samples were prepared from lyophilised protein in 20 mM deuterated sodium acetate (CD_3COONa) buffer pH 4.3, 10 % D_2O at a protein concentration of 0.36 mM.

NMR experiments were recorded on an Agilent DirectDrive 600 spectrometer (Agilent Technologies, Santa Clara) with a proton resonating frequency of 599.4 MHz. The spectrometer was equipped with a triple resonance cryogenic probe. Experiments were run at 25 °C and referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate following IUPAC recommendations. Data were collected using VnmrJ 3.2A (Agilent Technologies), processed with NMRPipe (Delaglio et al. 1995) and analysed with CCPNMR Analysis (Vranken et al. 2005).

Standard two- and three-dimensional experiments were recorded to assign chemical shifts to the protein ^{1}H, ^{13}C and ^{15}N nuclei. Backbone and CB resonances were assigned using [^{1}H-^{13}C/^{15}N] HSQC, HNCO, HNCACB, CBCA(CO)NH, HNCA and HNHA spectra. Side chains signals were assigned through H(CC-TOCSY)NNH, C(CC-TOCSY)NNH, HCCH-TOCSY, (HB)CB(CGCD)HD and (HB)CB(CGCDCE)HE experiments.

Typical spectral widths were 12.6 ppm for ^{1}H, 31.5 ppm for ^{15}N and 80 (aliphatic carbon), 14 (carbonyl), 30 (aromatic carbon), 36 (α carbon), or 22 ppm (β carbon) for ^{13}C spectral regions.

Assignments and secondary structure

Doubly labelled (^{15}N, ^{13}C) recombinant RodA was produced in *E. coli* as a fusion protein with His_6-tagged ubiquitin. The fusion protein was purified under denaturing conditions, refolded in vitro with a glutathione redox couple, cleaved and further purified by affinity chromatography and reverse-phase HPLC. ^{1}H–^{15}N HSQC spectra (Fig. 1) showed a chemical shift dispersion typical of folded proteins.

The ^{1}H, ^{13}C and ^{15}N resonances of RodA were identified following a standard strategy (see Methods) that led to nearly complete assignments. Only side chain exchangeable groups from 4 Asn residues, 8 Lys and one Arg residue, some non-exchangeable side chain resonances (K33 Cδ, K111 CεHε$_2$, F23 Cδ, F85 Cε and CζHζ) as well as 8 backbone carbonyls from residues preceding Pro residues and from the C-terminal residue, were not identified. All assigned chemical shifts have been deposited to the BMRB (accession code: 19782).

The redox state of the eight Cys residues of recombinant RodA was analysed using the Cys Cβ and Cα chemical shifts as described by Sharma and co-workers (Sharma and Rajarathnam 2000). The data is consistent with all the Cys residues being involved in disulphide bonds. Importantly, preliminary analysis of the pattern of ^{1}H-^{1}H nuclear Overhauser effects (nOes) observed in ^{13}C and ^{15}N edited NOESY spectra (not shown), unambiguously indicated that the disulphide bonds are formed by cysteines C1–C6 (39–116), C2–C5 (47–110), C3–C4 (48–88) and C7–C8 (117–135). The latter pattern is in accordance with the one established by chemical methods for other hydrophobins isolated from fungal sources (Kwan et al. 2006) or by X-ray crystallography from recombinant proteins (Hakanpaa et al. 2006a, b), supporting that this pairing is a

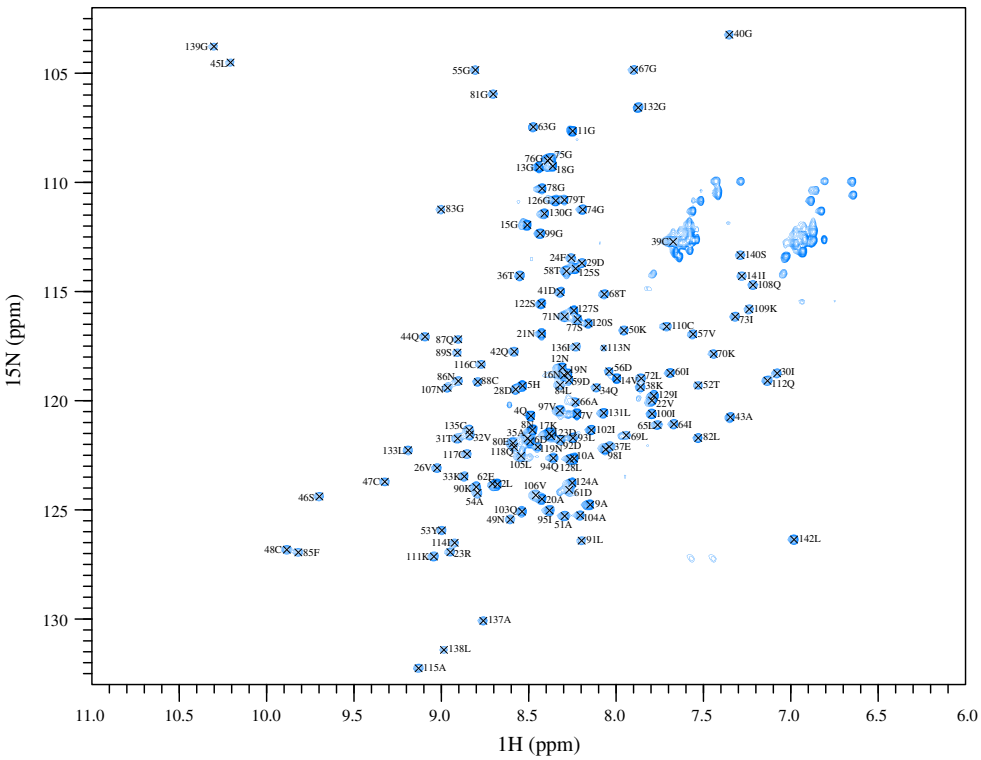

Fig. 1 $^{1}H–^{15}N$ HSQC spectrum of RodA. The spectrum was recorded at 25 °C in buffer CD3COONa 20 mM, 10 % D2O, pH 4.3. Assignments are shown only for backbone amide correlations. Chemical shifts (δ) are expressed in ppm relative to DSS (sodium 4,4-dimethyl-4-silapentane-1-sulphonate)

characteristic feature of hydrophobins. Furthermore, the superposition of $^{15}N–^{1}H$ HSQC spectra of the recombinant protein and the protein extracted from *A. fumigatus* spores (not shown) indicated that the recombinant and *A. fumigatus* proteins show the same structure and disulphide bond pattern. Therefore, the recombinant protein is natively folded and contains the native S–S bond topology.

The secondary structure of RodA was determined from the backbone and Cβ chemical shifts using the PECAN software (Eghbalnia et al. 2005). It consists of four β-strands (S1: Q44–T52, S2: L82–C88, S3: I114–C117, S4: L133–C135) and two α-helices (H1: V32–K38, H2: G63–L72). Except for helix H1, the secondary structure elements are located in the vicinity of cysteine residues (Fig. 2a).

The dynamics of RodA on the nano-pico second time scale were analysed using the RCI method (Berjanskii and Wishart 2005). RCI values, which are a quantitative measure of how close the backbone and CB chemical shifts of a given residue are to their corresponding "random coil" values, are positively correlated with the amplitude of motions on the nanosecond to picosecond time scale. Hence, the higher the RCI value, the higher the flexibility. The RCI values calculated for RodA indicated that the N-terminal region (S1–N19) is disordered and that the loops between cysteine residues C4–C5 and C7–C8, and to a lesser extent a region (N71–T79) of the long C3–C4 loop, are highly flexible (Fig. 2b).

Comparison with other class I hydrophobins

The comparison of the secondary structure and dynamics (RCI values) of RodA, and the class I hydrophobins EAS from *Neurospora crassa* (Kwan et al. 2006), DewA from *Aspergillus nidulans* (Morris et al. 2012, 2013) and MPG1 from M*agnaporthe oryzae* (Rey et al. 2013) is shown in Fig. 2. The limited sequence similarity and varying length of these hydrophobins is translated into different secondary structures. Nevertheless, the proteins display common features centred on the four conserved disulphide bridges. Indeed, the four hydrophobins show β-strands in the vicinity of all the S–S bonds. This observation strongly suggests that the RodA (and MPG1) structure will show the characteristic β-barrel topology organised around the S–S bridges observed for EAS and DewA, as well as for the class II hydrophobins HFB1 and HFBII (Hakanpaa et al. 2006a, b). Besides the β-barrel secondary structure, two common features can be highlighted for these class I hydrophobins. The first is a C7–C8 loop devoid of secondary structures and the second is, for hydrophobins with long N-terminal regions preceding the first cysteine (C1), a disordered N-terminal sequence of varying length (high RCI values, Fig. 2b) followed by an α-helix close to the first disulphide bridge (C1–C6). In the case of DewA, the latter α-helix packs against the β-barrel and the alignment suggests that this might hold true for RodA and MPG1. The secondary structure content in other regions of the proteins, i.e. in the C3–C4 and C4–C5

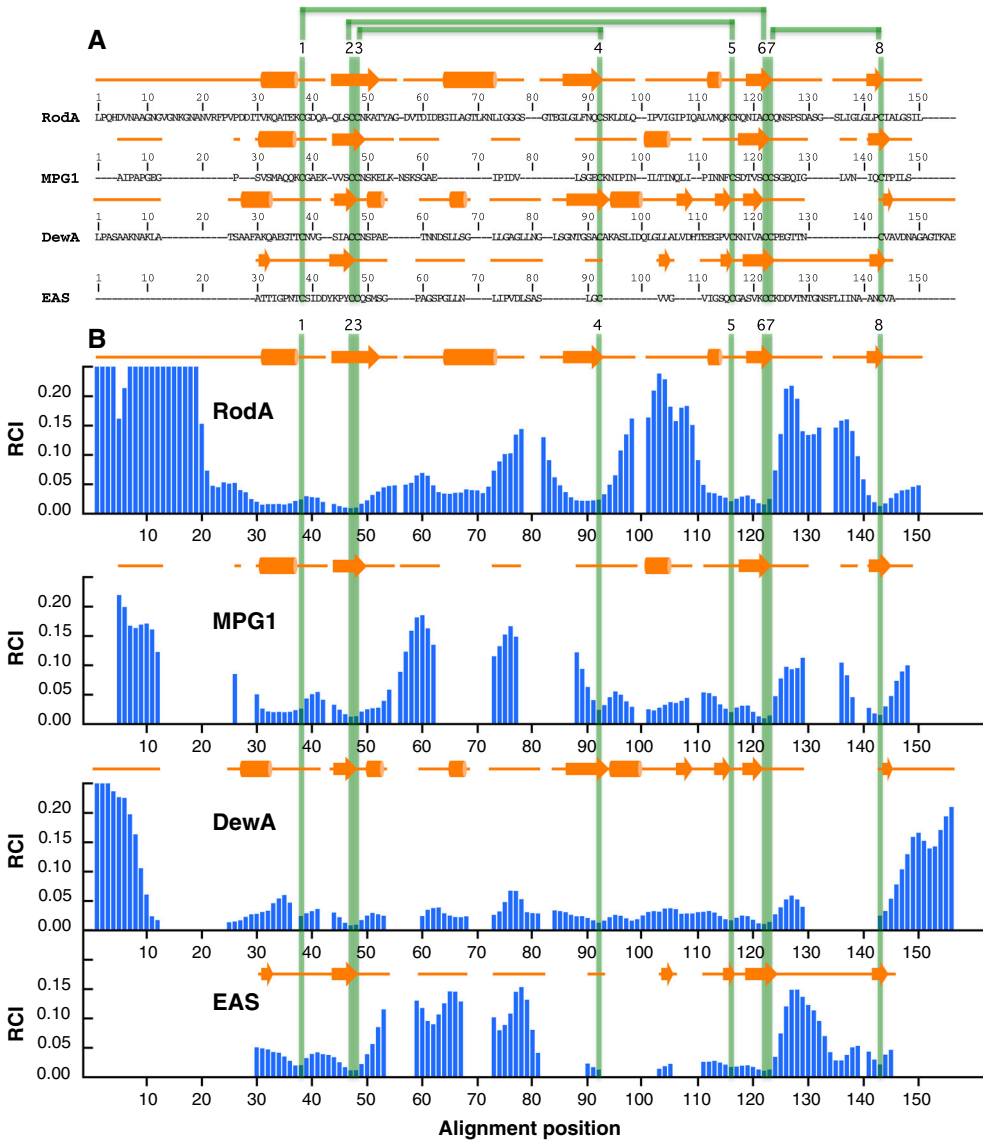

Fig. 2 **a** Sequence and secondary structure alignment of class I hydrophobins. Sequence alignment of class I hydrophobins with known structure and/or published chemical shifts was obtained by manual modification of a ClustalW alignment (Thompson et al. 1994) with constrained cysteine residue positions. Secondary structure was determined from backbone (N, C, Cα, Hα) and Cβ chemical shifts using the PECAN software (RodA and MPG1) or from structural ensembles (DewA and EAS) by means of the DSSP algorithm. α-helices are represented by cylinders and β-strands by arrows. Positions of Cys residues are numbered from 1 to 8 and are highlighted by horizontal green lines, while the disulphide topology is indicated by horizontal green lines. **b** Random coil index (RCI) as a function of alignment position. RCI values were calculated from chemical shifts deposited at the BMRB or in the case of EAS with unpublished chemical shifts, using the RCI software (Berjanskii and Wishart 2005). The RCI of the N-terminal residues of RodA and MPG1 are truncated for visualisation purposes. Secondary structure and Cys positions are displayed as in **a**. In **b**, blank spaces represent gap positions in the sequence alignment. Alignment position for RodA starts at residue 2, i.e., at the first residue belonging to RodA in the recombinant protein used in this work

regions, differs in the four proteins. In these regions, RodA shows two secondary structure elements that are not observed in EAS, DewA or MPG1: a long α-helix in the C3–C4 loop and a short α-helix in the C4–C5 loop. Regarding the nanosecond to picosecond dynamics, the RCI data indicates that the β-barrel region is rather rigid for the four proteins, while the C7–C8 and C3–C4 regions are flexible at least in some segment(s), except for DewA. The C4–C5 region of RodA also shows significant dynamics, a feature not observed in the corresponding region in other hydrophobins.

In summary, our data strongly suggest that RodA adopts the characteristic β-barrel topology observed in other hydrophobin structures, and indicates that it displays novel secondary structure elements, as well as a highly flexible N-terminal region and C4–C5 and C7–C8 loops. Interestingly, the highly flexible C7–C8 loop of EAS contains an

amyloidogenic sequence (FLIIN) that has been shown by mutagenesis and peptide experiments to be implicated in the core cross β-structure of the EAS amyloid fibres (Macindoe et al. 2012). Flexibility of regions containing amyloidogenic sequences seems to be an important factor to allow the conformational changes that take place at the interface between hydrophobic and hydrophilic interfaces to form rodlets (Morris et al. 2011). Amyloid prediction algorithms such as AMYLPRED (http://aias.biol.uoa.gr/AMYLPRED) predict an amyloidogenic sequence in the C7–C8 loop of RodA (data not shown). The assignments presented in this worked open the way for determining the structure of the soluble monomeric form of RodA, which will be valuable for the characterisation of the association mechanism into rodlets and for the study of RodA rodlets, which render the spores of the fungal pathogen *A. fumigatus* inert to the human immune system.

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